Investigating red pigment production by *Penicillium purpurogenum* DSM 62866

Caryn Ashleigh Horn

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Supervisor:

Prof Sue TL Harrison

Department of Chemical Engineering
Faculty of Engineering and the Built Environment
University of Cape Town

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Abstract

The production of a diverse range of pigments with variable properties and application potential highlights filamentous fungi as potential sources of pigments in the consumer-driven move toward natural colourant alternatives in food, cosmetic and nutraceutical products. The majority of natural pigments currently in use are obtained from sources such as insects and plants, including fruit and vegetables. These sources are inherently affected by natural variation and seasonal availability. Fungal pigments can, however, be produced in large-scale processes, under optimised and controlled conditions, with minimal dependence on weather and seasonal raw materials. *Penicillium purpurogenum* was selected for investigation in this study on the basis of reported pigment production without the co-production of mycotoxins, and the ability to adjust pigmentation through modifying cultivation conditions. Red pigments produced by this organism are of particular interest given the demand for this colour in food and cosmetic applications.

Red pigment production by *P. purpurogenum* DSM 62866 was confirmed on a medium composed of 30 $g.L^{-1}$ malt extract and 3 $g.L^{-1}$ soya peptone (MESP medium), following which the impact of varying cultivation conditions was investigated. Factors considered included cultivation temperature, pH and the application of buffers, and shaking speed during incubation of flask cultures. Conditions identified as beneficial for pigment production and, therefore, applied to 5 L cultivation in a benchtop bioreactor, were a temperature of 30 °C and a culture pH of 5.0 maintained through the application of a 50 mM citrate buffer. The pH, growth and pigment production trends were consistent upon scaling up the cultivation from 100 mL shake flask to 5 L bioreactor scale. The volumetric biomass concentration achieved under these base case conditions in the bioreactor system was approximately 8.4 $g.L^{-1}$ dry weight with a volumetric pigment concentration, based on absorbance at 500 nm, of 24 OD units. This related to a yield of pigment on biomass of 2.86 OD units.g_x^{-1}.

Maximum biomass productivity observed between approximately 30 and 120 hours of cultivation was determined to be 0.089 ± 0.007 $g_x.L^{-1}.h^{-1}$, with maximum pigment productivity of 0.72 ± 0.18 OD units.h^{-1} observed over the period of approximately 78 to 102 hours. Pigment production was estimated to start between 54 and 69 hours of cultivation. Using the Luedeking-Piret model of product formation, pigment production was shown to not be growth-associated. It is however, biomass associated and can be formed when the culture is actively growing. The non-growth associated specific pigment production rate, β, defining the base case cultivation was estimated at 0.23 OD units.$g_x^{-1}.h^{-1}$.

The pigmentation generated by *P. purpurogenum* DSM 62866 was shown to be the result of a mixture of multiple polar pigments. Extraction of pigment products was achieved using ethyl acetate, with intense red colouration still, however, observed in the aqueous medium. The pigment products in the organic and aqueous phases were processed further, with isolated products submitted for mass spectrometry analysis. A major red product present in the ethyl acetate extract was suggested to be an

alanine derivative of the *Monascus* pigment rubropunctamine based on absorbance maxima and mass spectrometry analysis. The complex nature of the medium could support the formation of a number of related pigment derivatives with properties, such as solubility, dependent on incorporation of various side chains.

Investigation of shaking speed during flask cultivation and agitation speed during benchtop bioreactor cultivation revealed a relationship between pigment production by *P. purpurogenum* and the rate of oxygen transfer into the cultivation medium. Residual oxygen concentration was demonstrated to not be a major factor affecting pigment production. A direct relationship was observed between pigmentation and k_La defining the system over the k_La range of 20 to 25 h^{-1}, corresponding to a maximum oxygen transfer rate of approximately 150 to 188 $mg.L^{-1}.h^{-1}$. During bioreactor cultivation, red pigmentation increased from 0 OD units to approximately 25 OD units as agitation speed was increased. Potential antioxidant properties of the pigment products could explain this trend.

The impact of medium composition was also investigated over a range of growth scales, namely agar plate, multiwell plate, shake flask and bioreactor cultivation. Changes to medium composition included altering the ratio of malt extract to soya peptone and investigating the impact of replacing malt extract with a marshmallow-based substrate as a simple representation of a confectionery waste stream. Across growth scales, soya peptone was demonstrated to be an important medium component for pigment production. Replacing soya peptone with peptone of animal origin during agar plate cultivation inhibited pigment production by *P. purpurogenum*.

The altered malt extract, soya peptone medium taken forward into bioreactor cultivation was composed of half the amount of malt extract, in comparison to the base case medium, with the concentration of soya peptone unchanged (Half MESP medium). This supported equivalent volumetric pigment concentrations, but approximately half the amount of biomass in comparison to MESP medium. As observed under base case conditions, scale-up from shake flasks to the bioreactor system using the Half MESP medium had little effect on volumetric biomass and pigment concentrations achieved.

The highest specific pigment productivity was, therefore, achieved when cultivating *P. purpurogenum* on a medium composed of 15 $g.L^{-1}$ malt extract and 3 $g.L^{-1}$ soya peptone, maintained at a pH of 5 through the application of a citrate buffer, with a cultivation temperature of 30 °C. Yield of pigment on biomass was calculated to be 6.13 OD units.g_x^{-1}, representing a 2.1-fold increase over the base case cultivation. Maximum biomass productivity was shown to be similar to that obtained in MESP medium at 0.077 ± 0.006 $g_x.L^{-1}.h^{-1}$, but over a shorter period of approximately 30 to 54 hours of cultivation. Maximum pigment productivity was, however, observed over approximately the same period as that in the MESP medium and was defined by a value of 0.71 ± 0.11 OD units.h^{-1}. The β value in the Half MESP medium was significantly higher, at 0.98 OD units.$g_x^{-1}.h^{-1}$, showing an increase over the base case value of 4.3-fold.

When replacing the malt extract in the Half MESP medium weight-for-weight with marshmallow confectionery, to simulate a sugar-rich confectionery waste stream, growth and pigment production of

P. purpurogenum was supported. Biomass concentration achieved was similar to that obtained in Half MESP medium, while the volumetric pigment concentration was significantly lower. The result was a yield of pigment on biomass of 3.01 OD units.g_x^{-1}, which is similar to that obtained using the base case medium. The ability of this organism to grow and produce pigments when cultivated on this alternative substrate demonstrates an opportunity for improved resource efficiency through utilisation of waste resources for conversion to a product, thereby improving economic feasibility of the process. Potential exists for improving product yields through further optimisation or supplementation studies.

When considering the combined results of the MESP, Half MESP and marshmallow-based medium cultivations, it was observed that sugar concentration in the medium was a determining factor for maximum volumetric biomass concentration achieved, but not for pigment productivity. Residual sugar concentration was also demonstrated to not be a trigger for the onset of pigmentation. Pigment production was seen to coincide with sporulation of the culture, indicating that some endogenous or external factor, such a medium composition, could be involved in the simultaneous onset of these two cellular processes. Medium components supplied were, however, shown to affect maximum volumetric pigment concentration. This could be attributed to the presence of a growth factor, or equivalent component, in the malt extract or soya peptone.

Given the high pigment productivity achieved in this study when using a malt extract and soya peptone based medium, *P. purpurogenum* DSM 62866 is a promising candidate for the production of natural colourant alternatives. Further work should focus on downstream processing and formulation as well as investigating pigment properties which are important considerations for commercialisation, such as stability and ease of application.

The full data set for this study has been published on the open-access institutional data repository of the University of Cape Town, ZivaHub. DOI: 10.25375/uct.21371169

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Abbreviations

CDW	Cell dry weight
DCM	Dichloromethane
ESI	Electrospray ionisation
FAB	Fast atom bombardment
FT-IR	Fourier-transform infrared (spectroscopy)
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IR	Infrared
LC	Liquid chromatography
MESP	Malt extract (30 $g.L^{-1}$), soya peptone (3 $g.L^{-1}$) medium used for agar plate (15 $g.L^{-1}$ agar added) and liquid cultivation experiments, as recommended by the DSMZ culture collection
MS	Mass spectrometry
NMR	Nuclear magnetic resonance (spectroscopy)
OTR	Oxygen transfer rate
OTR_{max}	Maximum oxygen transfer rate
PCU	Primary control unit (BioFlo 110 fermentor)
PDE	Permitted daily exposure
ppm	Parts per million
RI	Refractive index
RP	Reversed phase (chromatography)
rpm	Revolutions per minute
TLC	Thin layer chromatography
UV	Ultraviolet (light or radiation)
vvm	Volume (air) per volume (liquid) per minute

Nomenclature

a	Interfacial area per unit volume (m^{-1})
atm	Atmosphere
C	Oxygen concentration in the liquid
C*	Oxygen concentration in the liquid in equilibrium with the gas phase/ oxygen solubility ($mg.L^{-1}$)
dO_2	Dissolved oxygen (%)
g_s	Grams of substrate
g_x	Grams of biomass
k_Ga	Volumetric mass transfer coefficient for oxygen transfer from the atmosphere into flask headspace (h^{-1})
k_La	Volumetric mass transfer coefficient for oxygen transfer into liquid medium (h^{-1})
P	Product (pigment) concentration ($g.L^{-1}$)
R_f	Retention factor
S	Substrate concentration ($g.L^{-1}$)
UV-vis	UV-visible light
$v.v^{-1}$	Volume per volume
X	Biomass concentration ($g.L^{-1}$)
$Y_{P/S}$	Yield of pigment on substrate (OD units.g_s^{-1})
$Y_{P/X}$	Yield of pigment on biomass (OD units.g_x^{-1})
$Y_{X/S}$	Yield of biomass on substrate ($g_x.g_s^{-1}$)

1 Introduction

1.1 Background

Colour is an important sensory feature in our everyday lives and plays a significant role in the consumer perception of products such as food, cosmetics, textiles, pharmaceuticals and nutraceuticals (Downham and Collins, 2000; Kirti et al., 2014). The colour of food, for example, affects perception of whether an item is suitable and safe to consume, the expected taste of the product and perceived ripeness or freshness (Dufossé, 2006; Mortensen, 2006; Malik et al., 2012). The application of colourants in order to influence consumer opinion is not a novel practice. Ancient reports detail textile dyeing (Bechtold and Mussak, 2009), and the addition of colourants to food products has long since served the purpose of altering or enhancing the appearance, replacing colour lost during processing or improving product uniformity (Downham and Collins, 2000; Mortensen, 2006; Burrows, 2009).

Historically, colouring of products relied solely on naturally available sources of pigments such as plant and insect extracts and mineral or inorganic pigments. Typical examples of organic sources included flower petals, such as those of cornflower and marigold; spices such as paprika and saffron; and the lac and cochineal insects (Bechtold and Mussak, 2009; Burrows, 2009). These natural colourants have associated limitations such as low yield of colourant from the source, the need for high doses to achieve desired colour, lack of brightness and limited stability upon application (Rodriguez-Amaya, 2016). These colourants were, therefore, replaced by chemical dyes which showed better colour fastness, were required in lower doses and had the additional benefit of cheaper and easier processing (Downham and Collins, 2000; Joshi et al., 2003; Burrows, 2009).

Current regulation regarding the application of chemical colourants is, however, concerned with safety of the consumer and the environment. Some synthetic colourants are no longer approved for use as a result of toxic effects of precursors or the pigments themselves, or environmental concerns surrounding the disposal of process waste streams (Downham and Collins, 2000; Numan et al., 2018). The result is that only a limited number of artificial colourants remain available for application in food and cosmetic products (Arnold et al., 2012). This, together with growing consumer demand for natural products which are perceived to be the healthier alternative, has shifted interest back towards natural colourant sources (Dufossé, 2006; Sigurdson et al., 2017). The utilisation trend of natural and synthetic colourants has been represented in Figure 2.1.

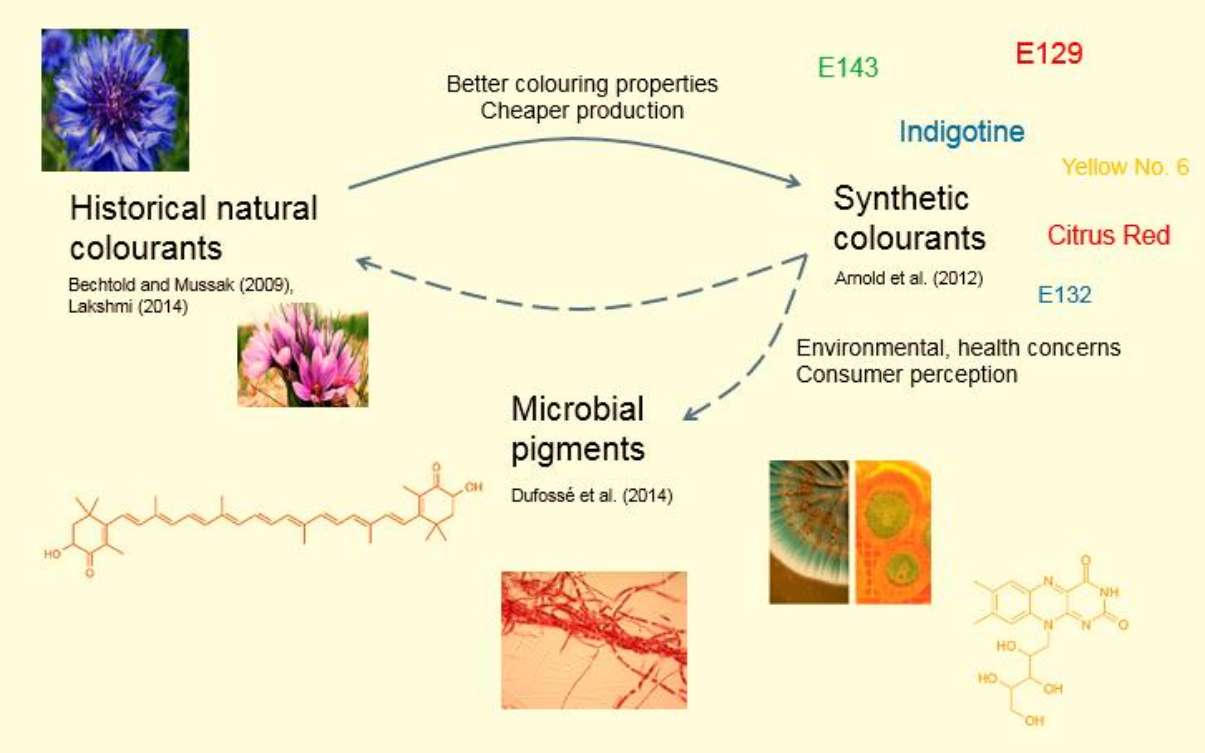


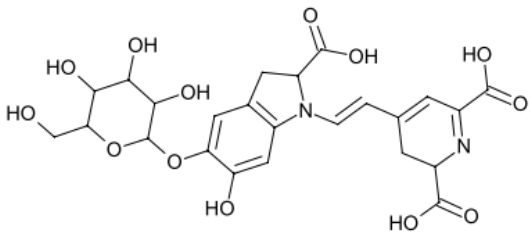
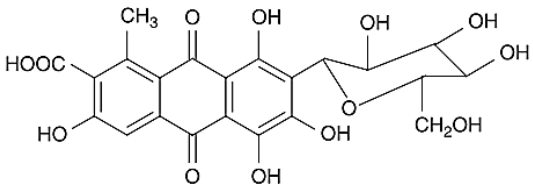
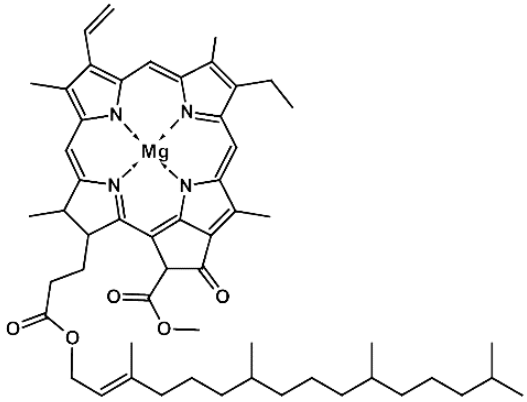
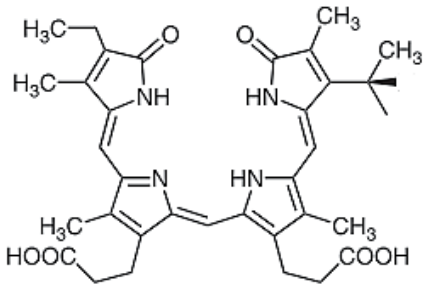
Figure 1.1 Application trends of natural and synthetic colourants.

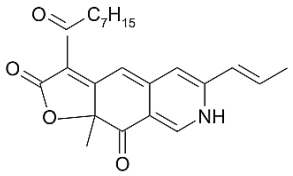
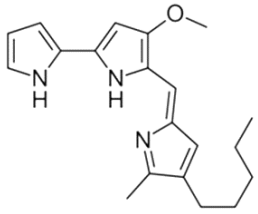
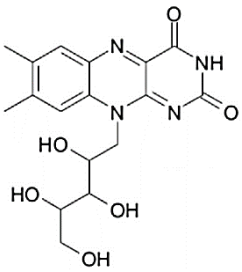
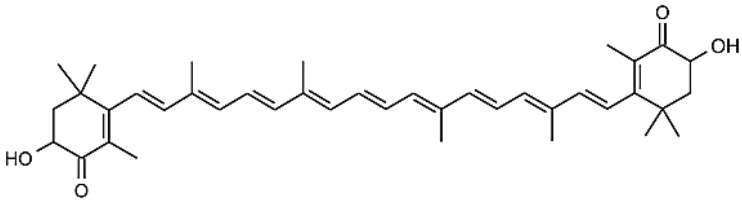
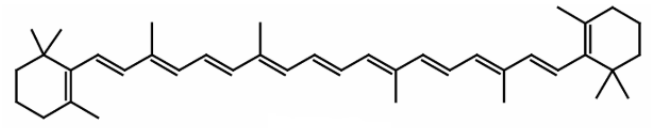
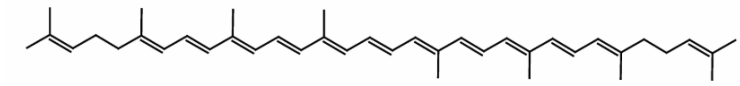
The shift towards natural colourants is demonstrated by the size and predicted growth of the natural colourants market, with food colourants being a good example. In 2022 the global food colourants market was estimated to be valued at $ 2.6 billion, with natural colourants accounting for 67 % of sales by value. This market share is expected to increase, with the natural food colourants sector predicted to grow at 8.1 % annually, compared to total market growth of 7.3 %, for the forecast period of 2022-2032 (Future Market Insights, 2022). This holds promise for continued research into natural colourant alternatives. The sources of these pigment products are extremely diverse, ranging from plants and insects to various microorganisms such as bacteria, yeast and filamentous fungi. Examples of these natural pigments are shown in Table 1.1, with the colour, chemical structure and source of the compound provided.

This table highlights the varied nature of available colourants, with the chemical structure indicating properties and application potential. It is also interesting to note that some pigments occur across a range of species and can, therefore, be produced by or extracted from a variety of sources. This is particularly evident in the carotenoid group of pigments and may be attributed to their conserved light-harvesting and photo-protective roles (Mortensen, 2006; Dawson, 2007).

Microbial sources of natural colourants are gaining interest as a result of ease of production and down-stream processing (Malik et al., 2012). These organisms exhibit fast growth and consistent, controllable pigment production through fermentation processes, which are independent of weather conditions and seasonal raw materials. This addresses some of the limitations associated with other natural colourant sources (Joshi et al., 2003; Mortensen, 2006; Mohanasrinivasan et al., 2013).

Table 1.1 Natural colourants and their sources.

Colouring compound	Colour	Source	
Betanin 	Red-purple	Plants	Beetroot (Mortensen, 2006; Sigurdson et al., 2017)
Carminic acid 	Red	Insects	Cochineal insect (Downham and Collins, 2000)
Chlorophyll 	Green	Cyano-bacteria	Dawson (2007)
		Marine and freshwater algae	
		Plants	Spinach (Eastaugh et al., 2004)
Phycocyanin 	Blue	Cyano-bacteria	*Arthrospira platensis*, *Arthrospira maxima* (Mortensen, 2006)

Monascorubramine 	Red	Filamentous fungi	*Monascus* sp. (de Carvalho et al., 2005; Dufossé et al., 2005)
Prodigiosin 	Red	Bacteria	*Serratia marcescens* (Venil et al., 2014)
Riboflavin 	Yellow	Bacteria	*Bacillus subtilis* (Stahmann et al., 2000; Venil et al., 2014)
		Yeast	*Candida famata* (Stahmann et al., 2000)
		Filamentous fungi	*Ashbya gossypii* (Stahmann et al., 2000)
Carotenoids			
Astaxanthin 	Red	Algae	*Haematococcus pluvialis* (Dufossé et al., 2005)
		Bacteria	*Agrobacterium aurantiacum* (Malik et al., 2012)
		Yeast	*Xanthophyllomyces dendrorhous*/ *Phaffia rhodozyma* (Reynders et al., 1997; Dufossé, 2006)
β-carotene 	Yellow-orange	Algae	*Dunaliella* sp. (Dufossé et al., 2005)
		Filamentous fungi	*Blakeslea trispora* (Schmidt et al., 2005; Choudhari and Singhal, 2008)
		Plants	Carrot, palm oil (Berman et al., 2015; Sigurdson et al., 2017)
Lycopene 	Red	Filamentous fungi	*B. trispora* (Mantzouridou and Tsimidou, 2008)
		Plants	Watermelon, tomato (Sigurdson et al., 2017)

1.2 Scope of the project

This study aims to investigate pigment production by the filamentous fungus *Penicillium purpurogenum* DSM 62866, selected on the basis of pigment type and colour produced by this fungal species, although pigment production in this strain has not previously been reported. The study investigates the growth and pigment production response to changes in cultivation conditions and medium composition, the potential for using a commercial or industrial waste stream as a nutrient source, scale-up of cultivation into a benchtop bioreactor system and characterisation of the pigmentation produced. This work addresses the current gap which exists in curiosity- and discovery-based studies, which dominate the microbial pigment production space, by investigating the factors affecting process yields and productivity thereby aiding the move towards a robust process for pigment production. This study provides insight into whether potential exists for commercial production and application of natural red colourant alternatives using *P. purpurogenum* DSM 62866.

1.3 Structure of the thesis

A review of literature relevant to the project has been presented in Chapter 2. This includes an introduction to current sources of natural pigments, including plants, insects and microorganisms, with a specific focus on filamentous fungi. Current commercial pigments obtained from fungal sources are also discussed, with *P. purpurogenum* presented as a potential source of novel commercial pigments. Commercial potential of microbial pigments has been discussed in the context of improving yields and reducing process costs through the modification of cultivation conditions and medium composition. An introduction to pigment analysis and characterisation is also provided. Chapter 3 presents the methods applied during the course of the study. This includes medium composition, cultivation techniques, pigment characterisation and statistical analysis. The experimental approach taken is outlined before presenting the results.

Results of this study have been split into three chapters. Chapter 4 details the investigation of the impact of cultivation conditions on pigment production. Conditions investigated were temperature, pH, shaking speed during flask cultivation and stirring speed during bioreactor cultivation. The base case cultivation in the New Brunswick Scientific BioFlo 110 7 L fermentor is presented in this chapter. Characterisation of the observed pigmentation is presented in Chapter 5. This includes preliminary characterisation through solvent extraction and thin layer chromatography, and further characterisation of both the ethyl acetate extract and aqueous phase after solvent extraction of a full batch cultivation performed in the benchtop bioreactor system. Chapter 6 details the investigation of the impact of medium composition on growth and pigment production. This includes modifications to the malt extract, soya peptone medium recommended by the culture collection from which the organism was obtained, scale-up of a selected medium composition into the bioreactor system and investigation of the impact of replacing the main carbon source with a confectionery-based substrate. Chapter 7 presents the conclusions drawn from the study as well as recommendations for further work which could add value to this research.

2 Literature Review

The review of literature relevant to the production of alternative red colourants using *Penicillium purpurogenum* DSM 62866 first considers current use of natural colourants and the range of potential sources. A main focus is pigment production by filamentous fungi, with discussion around the drivers for pigment production, current commercial examples and requirements for commercially successful processes. Factors considered are the effect of various cultivation conditions and medium composition, including the potential for producing fungal pigments as part of a waste biorefinery, where a selected waste stream is utilised as a substrate for cultivation of the organism of interest. Finally, general methods of monitoring pigment production and isolating pigment products are discussed, with specific examples provided for characterisation of red, water-soluble, fungal pigments.

2.1 Natural colourants

Growing interest in natural colourant alternatives applied in food, cosmetics, nutraceuticals and other related products comes as a result of a market shift, largely driven by consumers. This stems from a preference for products which are perceived to be natural and healthy, negative environmental and health implications of synthetic colourants and government legislation limiting the application of certain synthetic pigments (Downham and Collins, 2000; Joshi et al., 2003; Rodriguez-Amaya, 2016). This is further strengthened by the reported health benefits associated with some natural colourants used in food and cosmetic applications, such as the antioxidant and provitamin A activity of β-carotene, where this pigment can be converted to vitamin A in the intestines (Downham and Collins, 2000; Dufossé et al., 2005), and the antitumour effects of *Monascus* pigments (Akihisa et al., 2005)

A large number of natural colourants currently in use are obtained from plants (Mortensen, 2006), but these sources are affected by limitations such as natural variation, low yield from the source material and seasonal availability (Gunasekaran and Poorniammal, 2008; Li and Vederas, 2009; Mapari et al., 2009b). Microbial pigment production represents an interesting alternative. It is possible to produce pigments of similar colour and chemical structure, as shown in Table 1.1, with production methods addressing some of the limitations associated with other natural pigment sources.

2.1.1 Plant and insect sources

Colouring compounds from plant and insect sources, traditionally used to impart colour to consumer products, have received renewed interest as a result of the trend toward natural colourant alternatives. The natural colourant market is currently dominated by compounds obtained from plant sources, with the definition of a ‘colouring food’, a food source used as a colouring compound, fitting perfectly the trend for ‘clean label’ ingredients. Plant sources are usually coloured by four main pigment types: tetrapyrrols such as chlorophyll, tetraterpenoids such as carotenoids, flavonoids such as anthocyanins, and betalains (Mortensen, 2006; Rodriguez-Amaya, 2016). These compounds cover a wide colour range and are obtained from a diverse range of sources. Examples have been provided in Table 2.1.

Table 2.1 Colourants obtained from plant sources, classified by colour.

Colour	Colouring compound	Plant source	Notes	Reference
Red	Annatto/ bixin	Annatto/ achiote tree (*Bixa orellana*)	Extracted from seeds of the achiote tree	Mortensen (2006)
	Betanin	Beetroot (*Beta vulgaris*)	Colour is pH stable but shows low heat stability	Mortensen (2006); Sigurdson et al. (2017)
	Lycopene	Watermelon, guava, tomato	Bright red, oil-soluble pigment	Sigurdson et al. (2017)
	Paprika extract	Capsicum (*Capsicum annuum*)	Extract is comprised of a range of carotenoid pigments	Mínguez-Mosquera and Hornero-Méndez (1993); Mortensen (2006)
Yellow - Orange	β-carotene	Carrots (*Daucus carota*), palm oil, alfalfa	Oil-soluble, provitamin A activity	Berman et al. (2015); Sigurdson et al. (2017)
	Crocin/ Crocetin	Saffron (*Crocus sativus*)	Saffron refers to the stigma of the *C. sativus* flower. The pigment is usually not extracted, with the whole stigma rather added to food	Eastaugh et al. (2004); Mortensen (2006); Lakshmi (2014)
	Curcumin	Turmeric (*Curcuma longa*)	Insoluble in water and light-sensitive	Mortensen (2006)
	Lutein	Aztec marigold (*Tagetes erecta*)	Extracted from petals of the marigold flower	Mortensen (2006); Lakshmi (2014)
Green	Chlorophyll	Spinach, grass	Forms part of the photosynthetic apparatus of plants	Eastaugh et al. (2004)
Blue - Purple	Anthocyanin	Grapes, red cabbage, sweet potato, elderberry	Colour is pH dependent, ranging from red in acidic conditions, colourless at around pH 5, and blue-green in neutral to alkaline conditions	Mortensen (2006); Sigurdson et al. (2017)
	Cornflower blue	Cornflower (*Centaurea cyanus*)	Extracted from petals of the cornflower. Limited stability	Eastaugh et al. (2004); Bechtold and Mussak (2009); Lakshmi (2014)
	Indigo	Indigo (*Indigofera tinctoria*)	Extracted from the leaves of the indigo plant. Light-stable	Bechtold and Mussak (2009); Lakshmi (2014)

Plants are a valuable resource in the search for natural colourants as they are an abundant source of pigments, with the flowers, fruits, vegetables and even the leaves serving as potential sources of colouring compounds. This is attributed to the various roles of pigments in plants, including photosynthesis, protection from UV damage and attraction of pollinators (Sigurdson et al., 2017).

Colourants from insect sources traditionally used for textile and food colouring included the anthraquinone pigments laccaic, kermesic, and carminic acids obtained from the lac, kermes, and cochineal insects, respectively (Mortensen, 2006; Bechtold and Mussak, 2009). Cochineal extract and carmine are still widely applied in a variety of food types including beverages, meat and dairy products as a result of their desirable red colour and pigment stability. The low yield of colourant from this insect source, however, is a limiting factor, with large numbers of insects required to yield significant colourant material (Burrows, 2009).

2.1.2 Microbial sources

The majority of natural colourants currently in use are obtained from plant and insect sources, but the diverse range of pigments able to be produced by microorganisms is gaining interest. Although production of natural pigments using microbial sources addresses some of the challenges associated with plant and insect colourants, further limitations are, however, common to most natural colourants irrespective of the source. This includes the generally higher cost of production, larger required doses as a result of low colour intensity and limited stability to factors such as temperature, pH and oxygen (Malik et al., 2012). Microbial pigment producers are, however, extremely diverse and hold potential for production of colourants which could overcome these restrictions. Furthermore, process optimisation holds potential for improvement of yields and lowering of production costs. Microbial pigment sources including bacteria, algae, yeast and filamentous fungi are introduced below.

2.1.2.1 Bacteria and cyanobacteria

Bacteria are prokaryotic organisms, with some species exhibiting pigment production encompassing a wide range of colours. Some bacterial pigments are also reported to confer a number of therapeutic benefits, including anticancer, antibacterial and antioxidant effects (Numan et al., 2018). Advantages associated with these production systems include ease of culturing and rapid growth cycles, which result in quick fermentation processes and shorter production times (Venil et al., 2013; Numan et al., 2018). Currently yields are not sufficient to meet industrial demand, but the ease of strain improvement through genetic engineering holds promise for future production using these organisms (Venil et al., 2014).

The cyanobacteria are autotrophic prokaryotes containing the cellular machinery required for photosynthesis. The components of these photosystems are coloured in order to harness the light energy required for the process. The main pigment, chlorophyll (green), is arranged along with phycobiliprotein accessory pigments such as phycocyanin (blue) and phycoerythrin (red) in phycobilisomes. Carotenoids are other light-harvesting pigments produced by these organisms (Saini

et al., 2018). Carotenoids confer advantages to the producing organism as a result of photoprotective properties upon exposure to ultraviolet (UV) radiation (Mapari et al., 2005; Dawson, 2007).

Spirulina (*Arthrospira platensis, Arthrospira maxima*) is a well-known source of the natural blue pigment phycocyanin. Spirulina extracts are approved as dietary supplements, and as such approval was obtained for the pigment (Mortensen, 2006; Malik et al., 2012). Chr. Hansen, a company which produces natural ingredients reports production of a *Spirulina* extract which functions as a food colourant (Chr. Hansen, n.d.). The application of *Spirulina* extract as a colourant is predicted to grow rapidly over the coming years ("Natural food colours market…", 2015) as other natural blue colourants typically lack stability required for diverse applications.

2.1.2.2 Algae

Algae are autotrophic eukaryotes, also able to produce the components required for growth via photosynthesis. The pigments produced by this group of organisms once again include chlorophyll, phycobiliproteins and carotenoids (Dufossé et al., 2005), with examples of the latter including β-carotene, lycopene, astaxanthin, lutein and zeaxanthin (Saini et al., 2018). These coloured components are of interest to the natural food colourants market both as a result of their colour as well as their associated health benefits, as discussed below.

Members of the genus *Dunaliella* are reportedly the best algal carotenoid pigment producers. Commercial production of β-carotene has been achieved using a species of *Dunaliella*, where the biomass is either used directly as a food additive, or the pigment is extracted and applied in an oil-based form (Dufossé et al., 2005). This pigment product typically contains 5 to 6 % other carotenoid pigments (Mortensen, 2006). β-carotene imparts an orange colour while also providing antioxidant and provitamin A activity (Downham and Collins, 2000; Saini et al., 2018).

Another algal carotenoid pigment approved as a dietary supplement is astaxanthin produced by *Haematococcus pluvialis*. This red pigment is applied in aquaculture to improve pigmentation of fish and crustaceans for human consumption, and is also reported to have antioxidant properties (Dufossé et al., 2005; Berman et al., 2015). Although these organisms produce pigments with desirable colour and potential health benefits, the major limitation affecting commercial production of pigments and other products using algae is low culture productivity (Hejazi and Wijffels, 2004).

2.1.2.3 Yeast

Yeasts are unicellular eukaryotic fungi (Karanjgaokar and Tarfe, 2017), the most well-studied and commercially relevant of which is *Saccharomyces cerevisiae*, used in industrial processes such as brewing and baking (Ostergaard et al., 2000). Other yeast species have commercial potential as carotenoid pigment producers, for example *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) and members of the genus *Rhodotorula* (Dufossé, 2006). Production of the yellow, water-soluble pigment riboflavin has also been reported in the yeast *Candida famata* (Stahmann et al., 2000).

X. dendrorhous is considered, along with the green alga *Haematococcus pluvialis*, to be a significant microbial producer of astaxanthin. This red xanthophyll pigment is widely used in aquaculture to enhance the colour of salmon and shellfish. Experimentation has focused on enhancing production of this pigment through strain selection, mutagenesis, altering fermentation conditions and adjusting the substrate supplied during cultivation, as reviewed by Rodríguez-Sáiz et al. (2010).

2.1.2.4 Filamentous fungi

Filamentous fungi are multicellular eukaryotic organisms, reported to produce a diverse range of pigments which are classified into two main groups, namely the carotenoids and polyketides (Mapari et al., 2008; Mapari et al., 2010). These pigments exhibit varied properties and application potential.

The carotenoid group of pigments exhibit a poly-isoprenoid structure, are oil-soluble and vary in colour between yellow, orange and red (Schmidt et al., 2005; Berman et al., 2015). They are generally sensitive to heat, light and oxidation, but exhibit stability towards pH changes (Joshi et al., 2003; Mapari et al., 2005). The carotenoids can be further divided into two groups, the carotenes and the xanthophylls. The carotenes are composed of only carbon and hydrogen, and include compounds such as α- and β-carotene, phytoene and lycopene. The xanthophylls contain carbon, hydrogen and oxygen, with examples being lutein and zeaxanthin (Mortensen, 2006).

The market for carotenoids is large and continues to grow, with these pigments widely applied as food and feed colourants (Kirti et al., 2014; Berman et al., 2015). They are desirable as a result of their colour as well as their antioxidant properties and other health benefits (Downham and Collins, 2000). The sensitivity of these pigments can, however, limit their potential applications as a result of the loss of colour that could result from exposure to heat and light during processing, or once formulated into the final product. The oil-soluble nature of these pigments can also have implications for application, with these colourants generally applied as solutions or suspensions in vegetable oils (Kirti et al., 2014). Methods of processing oil-soluble pigments for application in aqueous media have been investigated, with emulsions and spray-dried powders reported as successful approaches (Downham and Collins, 2000; Mortensen, 2006).

The polyketide compounds are diverse and include products such as toxins, pharmaceuticals and pigments. These compounds differ in biological function and structure, but share a common biosynthetic pathway for formation of the carbon backbone (Chooi and Tang, 2012), as demonstrated in Figure 2.1.

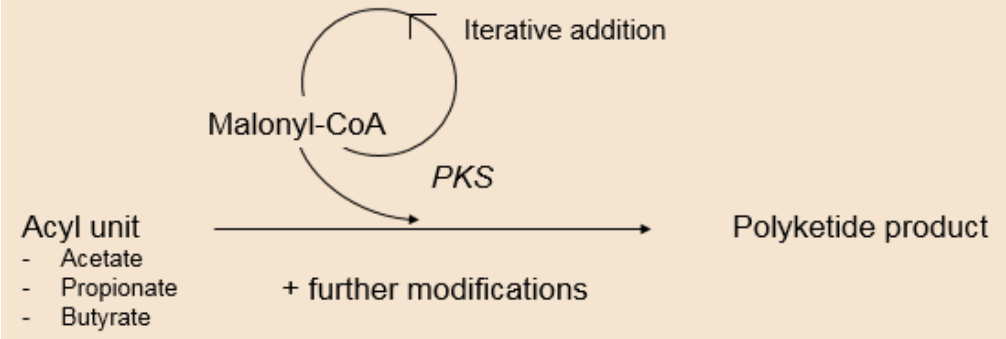


Figure 2.1 Biosynthetic pathway for the production of the carbon backbone used to generate a wide variety of polyketide compounds (adapted from Chooi and Tang (2012)).

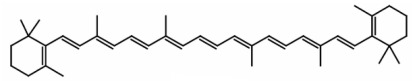
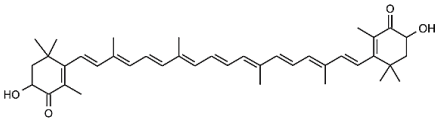
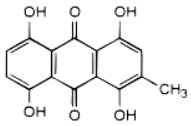
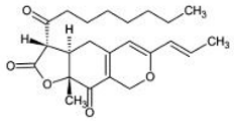
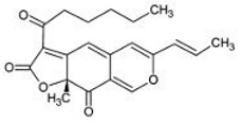
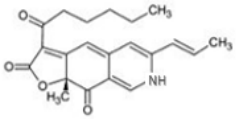
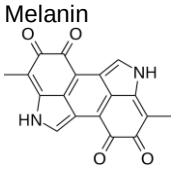
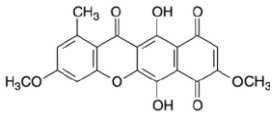
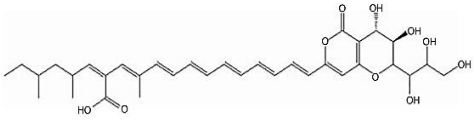
Their synthesis, via the polyketide pathway, involves the decarboxylative condensation of an acyl unit with a malonyl unit. This process is repeated, with successive malonyl units used for extension of the molecule. This process requires the action of polyketide synthases (PKS), multi-enzyme units containing multiple catalytic domains in a single polypeptide (Mapari et al., 2010; Chooi and Tang, 2012). The minimum enzyme components required in a PKS are β-ketoacyl synthase which catalyses the condensation reaction, acyl carrier protein (ACP), and malonyl-CoA:ACP transacylase (Chooi and Tang, 2012). The majority of fungal PKSs are iterative type I PKSs (IPKSs), which contain single copies of multiple functional domains. These enzyme units use these functional domains iteratively to achieve chain extension (Shimizu et al., 2005; Chooi and Tang, 2012).

Polyketide pigments are generally tetraketide to octaketide, i.e. containing 4-8 C_2 units, and are divided into a number of classes, including anthraquinones, hydroxyanthraquinones, oxopolyenes, naphthoquinones and azaphilones (Mapari et al., 2010). Advantages associated with this group of pigments stem from their diversity. This relates to a greater colour range than observed in the carotenoid pigments; variable stability towards heat, light, pH and oxidation; and water-solubility of certain pigments (Jung et al., 2005; Mapari et al., 2005; Mapari et al., 2009; Mapari et al., 2010; Silveira et al., 2013).

The potential application of this group of pigments is, therefore, greater than that of the carotenoids, with improved stability to heat and light, and water-solubility of certain pigments improving the probability of identifying a colouring compound suited to a specific application. Water-solubility, in particular, improves application potential in aqueous-based food products.

Both the carotenoid and polyketide pigments have their place in the natural colourant market, with fungal carotenoids such as β-carotene and lycopene, for example, already produced and widely applied commercially (see Section 2.2.2.1). Polyketide pigments, with their diverse properties, have the potential to broaden the application of natural pigments of fungal origin. The main classes of fungal carotenoid and polyketide pigments have been shown in Table 2.2, with a known production organism and characteristic features of an example pigment provided.

Table 2.2 Main classes of fungal pigments, with typical examples.

Class of compound	Typical example		
	Pigment	**Colour and Characteristic features**	**Production organisms**
Carotenoids			
Carotenes	β-carotene 	Yellow-orange, oil-soluble	*Blakeslea trispora* (Schmidt et al., 2005; Choudhari and Singhal, 2008)
Xanthophylls	Astaxanthin 	Red, antioxidant, applied as feed additive in aquaculture	*Xanthophyllomyces dendrorhous*/ *Phaffia rhodozyma* (Rodríguez-Sáiz et al., 2010)
Polyketides			
Anthraquinones	Cynodontin 	Bronze, can be converted to blue biodye	*Curvularia lunata* (Hobson et al., 1997; Durán et al., 2002)
Azaphilones	Ankaflavin 	Yellow	*Monascus* sp. (Mapari et al., 2008a)
	Rubropunctatin 	Orange	
	Rubropunctamine 	Purple-red, water-soluble	
Melanins	Melanin 	Black-brown	*Aspergillus* sp. (Brakhage, 2013; Kirti et al., 2014)
Naphthaquinones	Bikaverin 	Red, water-soluble, production affected by nitrogen availability and pH	*Fusarium* sp. (Limón et al., 2010)
Oxopolyenes	Orevactaene 	Yellow, water-soluble	*Epicoccum nigrum* (Mapari et al., 2006, 2010)

2.2 Commercialisation of pigments produced by filamentous fungi

Filamentous fungi are of particular interest as microbial sources of natural colourants as a result of the diverse range of pigments produced by this group of organisms, both in terms of colour and chemical structure, and the limited degree to which the pigments from this group of organisms have been harnessed for commercial use thus far (Mapari et al., 2005; Mapari et al., 2010). The filamentous fungi were, therefore, investigated further, considering the role of pigments, drivers for pigment production and current commercial examples.

2.2.1 Why do filamentous fungi produce pigments?

The reason for production of pigments by fungal organisms is poorly understood, with the biological function of the resulting pigments commonly unknown (Schroeckh et al., 2009; Mapari et al., 2010). Various reports suggest that pigments are produced in response to metabolic or environmental stresses or signals, such as light, salt concentration, nutrient or oxygen limitation, or the presence of competing organisms. The carotenoid pigments, for example, confer photoprotective effects to the producing organism upon exposure to ultraviolet radiation (Mapari et al., 2005; Dawson, 2007), which stems from their biological role of light absorbance in plants and other photosynthetic organisms. Upregulation of carotenoid production has been reported in fungal species in response to increased light exposure (Haggblom and Unestam, 1979).

As a result of the diversity of polyketide products, identifying a trigger for this group of pigments may be more challenging. A majority of these pigment products are produced as secondary metabolites and are, therefore, not involved in or required for normal growth of the organism (Yin and Keller, 2011; Boruta, 2018). Secondary metabolites are only produced when it is beneficial for the organism and may have a role in preservation of the species under stress conditions. Production could be in response to endogenous or environmental signals such as temperature, pH, oxidative stress or oxygen limitation (Shwab and Keller, 2008; Brakhage, 2013).

Some pigments produced by filamentous fungi act as toxins, antifungals, or antibiotics (Weissman, 2004; Chooi and Tang, 2012) and the presence of, or interaction with, competing organisms could also cause upregulation of these products (Schroeckh et al., 2009). Production of some polyketide compounds has also been shown in response to changes in light, and may be linked to their light absorbent properties (Miyake et al., 2005).

Identification of the trigger for pigment production of a species of interest for commercialisation is expected to represent an important parameter for process optimisation. This could allow improved control of process timing and lead to an increase in overall yields and productivities.

2.2.2 Current commercial colourants from fungal sources

A number of factors affect the commercial potential of natural pigments produced by filamentous fungi. These include growth characteristics of the organism, pigment yield, ease of extraction and purification, economic viability of the process, and approval for use based on safety of the organism and the pigment product (Dufossé et al., 2005; Kirti et al., 2014). A small number of fungal pigments have satisfied the conditions for commercialisation and are being produced and applied in products for both human and animal consumption. Three examples are outlined below.

2.2.2.1 Carotenes produced by *Blakeslea trispora*

β-carotene is an oil-soluble carotenoid pigment varying in colour from yellow to orange (Downham and Collins, 2000; Kirti et al., 2014). Commercial production of this pigment is achieved using chemical synthesis; extraction from plant sources such as carrots, palm oil and alfalfa; microbial fermentation and photoautotrophic algal cultivation (Berman et al., 2015). The filamentous fungus *B. trispora* was the first commercial microbial source, with the β-carotene produced by this organism exhibiting purity equivalent to chemically synthesised forms (Dufossé et al., 2005). Besides the colouring properties imparted by this compound, β-carotene also confers health benefits, including antioxidant and provitamin A activity, as discussed previously (Downham and Collins, 2000; Roukas and Mantzouridou, 2001; Dufossé et al., 2005).

B. trispora is able to produce large amounts of β-carotene in submerged culture, a feature not observed in other closely related carotenoid producers (Schmidt et al., 2005). It has, however, been noted that sexual interaction of opposite mating types is important for improving production by this organism, with mixtures of the two mating types ((+) and (-)) exhibiting higher pigment yields. The reason for this is the production of a hormone-like substance containing trisporic acid when opposite mating types interact (Adrio and Demain, 2003; Joshi et al., 2003). Trisporic acid is a degradation product of the pigment β-carotene (Schmidt et al., 2005).

Carotenoids are synthesised via the mevalonate pathway in fungi. The first step in the pathway is catalysed by the enzyme phytoene synthase and involves the condensation of two geranylgeranyl pyrophosphate molecules to form phytoene. The enzyme phytoene dehydrogenase then catalyses four successive dehydration reactions, which introduce double bonds into the molecule, leading to the formation of lycopene. The introduction of ring structures at either end of the lycopene molecule by the enzyme lycopene cyclase results in the formation of β-carotene (Rodríguez-Sáiz et al., 2004; Schmidt et al., 2005). This pathway is shown in Figure 2.2.

The genes responsible for carotene biosynthesis in *B. trispora* were described by Rodríguez-Sáiz et al. (2004). The *carB* gene was found to encode phytoene dehydrogenase activity and the *carRA* gene, both lycopene cyclase and phytoene synthase activity, with the resulting protein exhibiting two separate domains. Comparison of the transcription in mixed and single mating type cultures revealed that *carRA* and *carB* transcription was increased when both mating types were present. A maximum fold increase of 148 and 128 in *carB* and *carRA*, respectively, was observed after 48 hours. The maximum increase

observed in single mating type cultures in the same period was 10-fold (Schmidt et al., 2005). The activity of these genes leads to the formation of high levels of β-carotene in this organism.

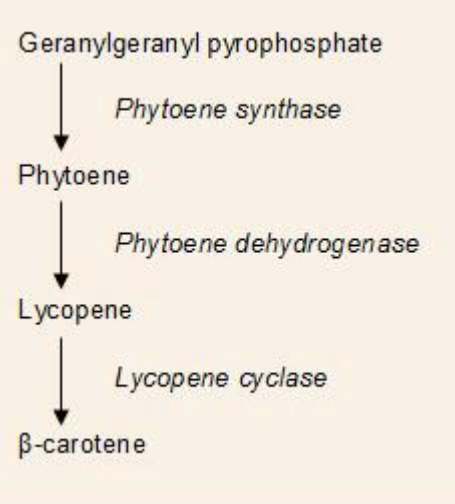


Figure 2.2 Pathway leading to the formation of β-carotene (adapted from Wang et al. (2014))

The red carotene, lycopene, formed one step prior to β-carotene is also of commercial interest and is commercially produced by this fungus (Rodríguez-Sáiz et al., 2004). The use of lycopene cyclase inhibitors in the cultivation medium results in high lycopene productivity. These compounds can, however, only be introduced at low concentrations and must be removed from the final product to allow use in, for example, the food industry. One such lycopene cyclase inhibitor, 2-isopropylimidazole, was found to increase the percentage lycopene in the total carotenoids from 42 to 96 % when added in the range of 50 to 300 $mg.L^{-1}$. The benefit of this lycopene cyclase inhibitor is its solubility in water, allowing easy separation from the oil-soluble pigments produced (Wang et al., 2014).

2.2.2.2 Riboflavin production by *Ashbya gossypii*

Riboflavin, or vitamin B_2, is a yellow, water-soluble pigment currently applied as an animal feed additive and food colourant in products such as breakfast cereals, fruit drinks and yoghurt (Stahmann et al., 2000; Malik et al., 2012). Riboflavin occurs naturally in many food products including milk, eggs, broccoli and almonds. Fortification of diary and cereal-based products is permitted as a result of its role as a B vitamin in cellular respiration, growth and enzyme function (“Riboflavin,” 2008).

Production of this additive using biotechnological processes has grown to compete with chemical synthesis as a result of reduced cost, use of renewable resources for pigment production, as well as reduction in process waste and energy requirements (Stahmann et al., 2000; Dufossé, 2006; Malik et al., 2012). One of these processes involves the filamentous fungus *Ashbya gossypii*, which is a natural overproducer of riboflavin. Production of the colourant is not growth-associated and can be enhanced through supplementation with required pigment precursors, such as glycine (Stahmann et al., 2000).

2.2.2.3 *Monascus* pigments

The so-called *Monascus* pigments are azaphilone-type polyketide pigments produced by fungi belonging to the genus *Monascus*. The pigmented products of these organisms are the oldest example of fungal colourants applied in food (Dufossé et al., 2005; Torres et al., 2016) which continue to be widely used either directly as food products or as food additives in Asia (Ogihara et al., 2000b; Wang et al., 2005; Feng et al., 2012). Traditional production involves cultivation of a selected *Monascus* species on steamed rice. Following growth and pigment production, the fermented angkak, red koji or red mould rice is either dried, ground and applied directly as a food colourant, or the pigments may be extracted using various solvents (Dufossé, 2006; Carvalho et al., 2007). The American chemical manufacturing company FMC Corporation currently produces *Monascus* pigments using this solid state fermentation approach (FMC, 2016). Application of *Monascus* pigments includes use as a food colourant in dairy and meat products (Dufossé et al., 2005).

Investigation of these colouring products has focused on their structure, synthesis and safety. The six main *Monascus* pigments are ankaflavin and monascin which are yellow in colour, monascorubrin and rubropunctatin which are orange, and monascorubramine and rubropunctamine which are red (Hajjaj et al., 1997; Dufossé et al., 2005; Mapari et al., 2010). The structure of these pigments is shown in Figure 2.3. There are, however, other known pigments produced by *Monascus* species, including the yellow azaphilone pigments monascusone A and monascusone B (Jongrungruangchok et al., 2004).

The orange pigments monascorubrin and rubropunctatin exhibit high affinity for amino groups as a result of their structure. The reaction of these pigments with amino groups results in the formation of the water-soluble red pigments monascorubramine and rubropunctamine, respectively (Mapari et al., 2005, 2010). Red pigments are of particular interest given their widespread application in food and cosmetic products, as well as the reduction in synthetic red colourants approved for use in these industries (Carvalho et al., 2007; Bechtold and Mussak, 2009; Burrows, 2009).

The co-production of the mycotoxin citrinin has, however, limited the application of the *Monascus* pigments as natural food colourants (Mapari et al., 2005). Potential contamination with this mycotoxin, which exhibits nephrotoxic and hepatotoxic effects, has prevented approval of *Monascus* pigments as food colourants in both the European Union and the United States (Jia et al., 2010; Dufossé et al., 2014). Contamination of pigment solutions with citrinin has potential implications beyond the food industry, with citrinin in a 70 % ethanol solution exhibiting the ability to permeate human skin. The low molecular weight and lipid-solubility of mycotoxins aids their permeation through the skin (Boonen et al., 2012), potentially limiting the use of *Monascus* pigments in, for example, cosmetic products.

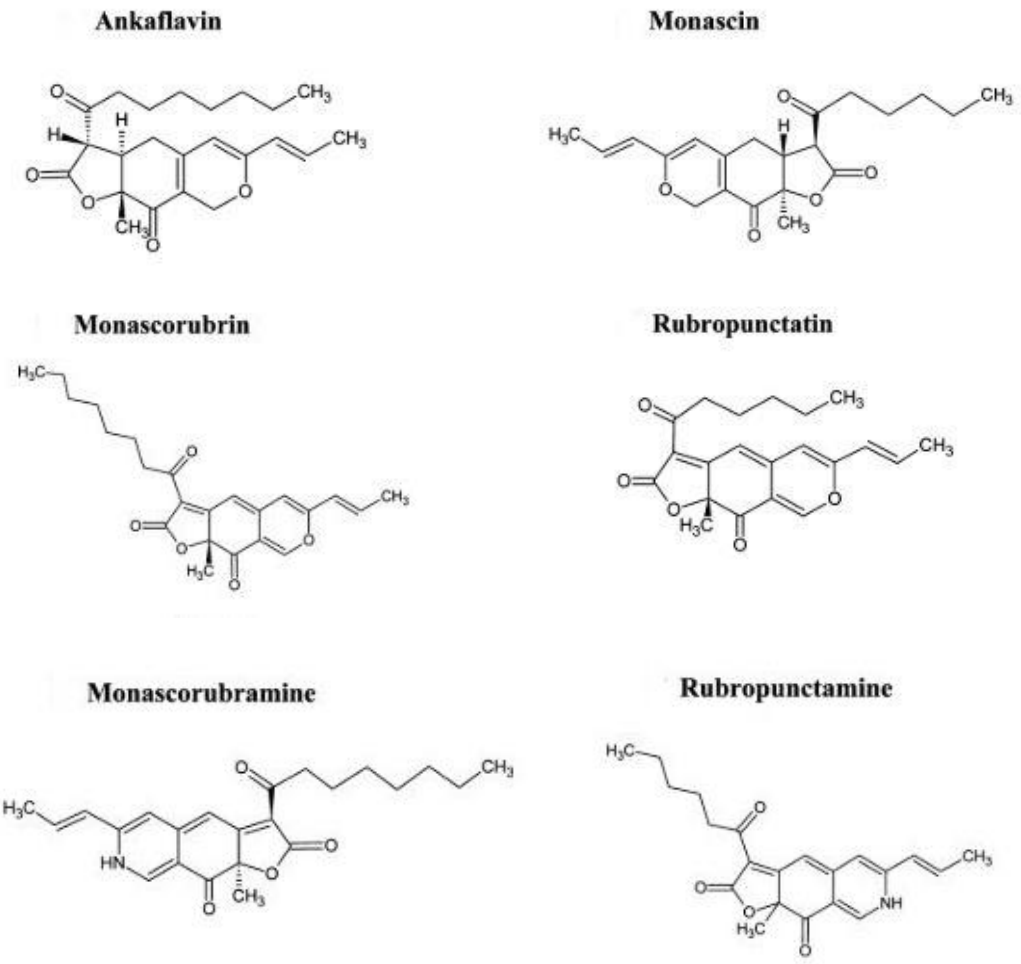


Figure 2.3 Six main azaphilone polyketide *Monascus* pigments (adapted from Mapari et al. (2008a))

Citrinin, like many other mycotoxins, is a polyketide compound and is, therefore, produced via the same pathway as the pigments of interest (Dufossé et al., 2014). A study investigating the prevalence of citrinin production in *Monascus* detected this mycotoxin in all 23 fungal cultures tested. It was, however, noted that citrinin production varied depending on strain as well as culture conditions (Wang et al., 2005). This is consistent with later reports noting the variability of citrinin levels with strain, medium composition and culture conditions (Mapari et al., 2010).

Hajjaj et al. (1999b) investigated pigment and citrinin production in *Monascus ruber* in a benchtop bioreactor system. It was noted that citrinin production started after pigment production and appeared to continue after glucose had been depleted. A branched pathway for the production of red *Monascus* pigments and citrinin was proposed, as shown in Figure 2.4. It has been suggested that modification of cultivation conditions could potentially alter the relative production of the pigment and mycotoxin (Hajjaj et al., 1999a).

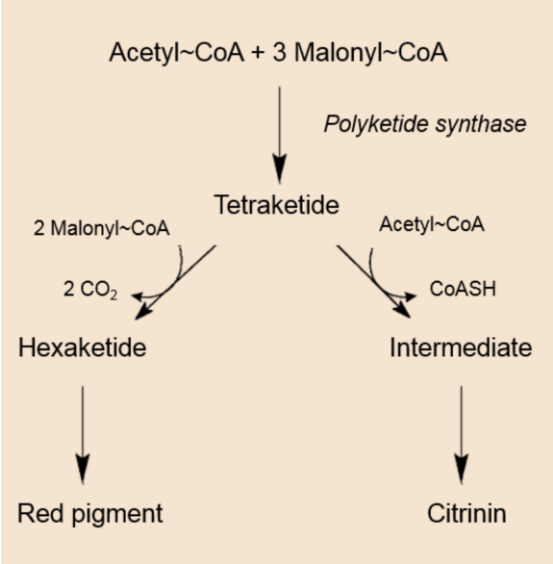


Figure 2.4 Branched pathway suggested for the production of the red *Monascus* pigments and the mycotoxin citrinin (adapted from Hajjaj et al. (1999b)).

A study conducted using *Monascus purpureus* was able to identify the polyketide synthase gene responsible for citrinin formation, *pksCT* (Shimizu et al., 2005). A polymerase chain reaction (PCR) using degenerate primers targeted towards PKS genes yielded a single product. Transcription of this product was higher in citrinin producing strains, and also showed a correlation with citrinin formation. In order to confirm the role of this gene in citrinin formation, the gene was disrupted. This caused inhibition of citrinin formation but had no impact on pigment production. This supports the idea of a branched pathway for pigment and citrinin formation. A later study which investigated the impact of disrupting the *pksCT* gene found similar results. The resulting mutant was morphologically similar to the parent strain, exhibited no significant differences in pigment levels and produced no citrinin (Jia et al., 2010).

Another potential means of addressing citrinin contamination may be linked to the solubility differences of pigments of interest and this mycotoxin. The study by Liu et al. (2005) detected citrinin in all tested *Monascus* fermentation products, but not in any aqueous extracts of these products. This indicates low water-solubility of this compound, which could be taken into account during downstream processing of the pigment product.

Further opportunity, however, lies in the discovery and investigation of other potential fungal pigment sources. Organisms of particular interest would be those producing pigments with similar structure and colouring properties, without the co-production of mycotoxins.

2.2.3 *Penicillium purpurogenum* as a potential source of commercially valuable colourants

Research into the pigment products of *Penicillium* species has been driven by the production of, amongst others, *Monascus* pigment homologues. Some species exhibit pigmentation without the co-production of mycotoxins (Mapari et al., 2008a, 2009b), thus improving the application potential of these

pigment products. One such species of interest is *P. purpurogenum*, which has been reported to produce desirable red pigmentation (Méndez et al., 2011; Santos-Ebinuma et al., 2013; Patil et al., 2015) with characterisation confirming the polyketide nature of these products in some cases.

A *Penicillium* isolate, later identified as *P. purpurogenum* (Arai et al., 2012), was reported to produce a deep red, water-soluble, azaphilone pigment known as PP-V. This pigment compound was shown to be a homologue of the *Monascus* pigment monascorubramine through high resolution fast atom bombardment-mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR) spectroscopy (Ogihara et al., 2000b). A comparison of the structure of monascorubramine and the PP-V homologue is provided in Figure 2.5.

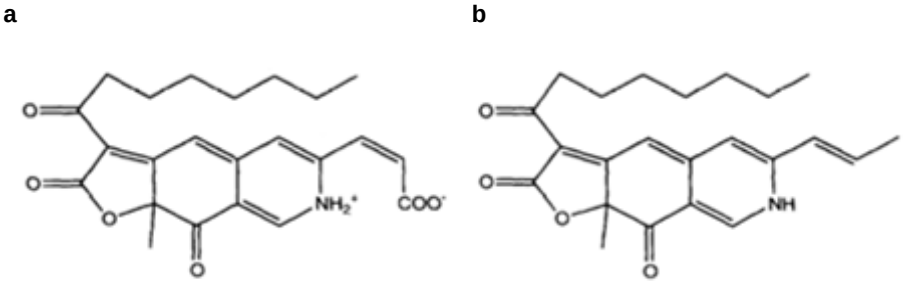


Figure 2.5 Comparison of the PP-V (a) and monascorubramine (b) structures (Ogihara et al., 2000b).

The similarity of these two compounds suggests a shared polyketide pigment synthesis pathway between this *Penicillium* species and members of the *Monascus* genus. This was confirmed by ^{13}C-labelling studies (Ogihara et al., 2000a) which demonstrated production along the pathway represented in Figure 2.6.

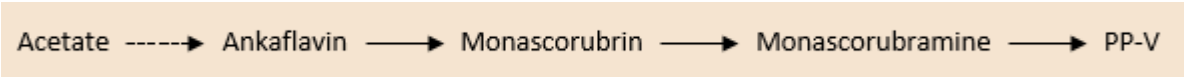


Figure 2.6 Pathway for production of PP-V in *P. purpurogenum* IAM 15392, demonstrated through ^{13}C-labelling studies (adapted from Ogihara et al. (2000a), Ogihara et al. (2001))

A second monascorubramine homologue was identified in the same *Penicillium* isolate. This pigment, PP-R, is also red in colour but is maintained within the mycelia rather than being excreted like the PP-V pigment (Ogihara et al., 2001). The structure of PP-R is shown in Figure 2.7. This highlights the ability of this organism to produce derivatives of the same pigment compound, through the addition of different functional groups or side chains. These changes to the molecules can alter pigment properties such as solubility and absorbance in the UV-visible light range. Similar abilities to produce pigment derivatives have been reported in *Monascus* species (Jung et al., 2003; Yuliana et al., 2017).

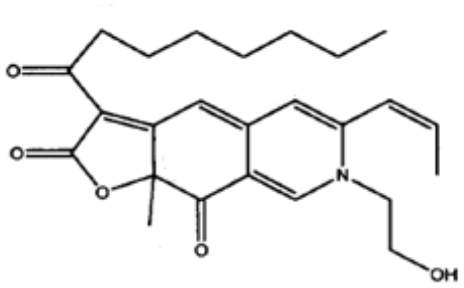


Figure 2.7 Structure of the *Penicillium* pigment PP-R (Ogihara et al., 2001).

It has also been reported that fungal pigment production can be modulated by altering medium composition or culture conditions (Mapari et al., 2010; Méndez et al., 2011). Literature regarding pigment production by *P. purpurogenum* indicates that highest pigment yields can be expected at a temperature of 30 °C (Ogihara et al., 2000b; Gunasekaran and Poorniammal, 2008; Arai et al., 2013) and a pH value of 5 (Ogihara et al., 2000b; Méndez et al., 2011). Ogihara et al. (2000) investigated the effect of applying various buffers as a means of maintaining the pH at a value of 5. Citrate buffer was shown to result in improved pigment concentrations, when compared to acetic acid, succinic acid and phosphate buffers. These parameters which support the highest pigment yields are not necessarily associated with highest biomass concentrations.

P. purpurogenum has been shown to grow and produce pigmentation on a variety of nutrient sources, ranging from defined media to complex mixtures, including a range of industrial waste streams (Ogihara et al., 2000b; Padmapriya et al., 2014; Morales-Oyervides et al., 2015a; Santos-Ebinuma et al., 2015; Kantifedaki et al., 2018). Cultivation kinetics are, however, rarely reported.

The potential impact of physicochemical conditions and medium composition is discussed in Section 2.3, with culture kinetics required to evaluate process performance and product potential as described in Section 2.3.3.

2.3 Factors affecting fungal pigment production

A number of parameters affect biomass and product yields during cultivation of microorganisms as a result of their impact on growth and metabolism. These parameters include environmental conditions, such as temperature and oxygen availability, and the composition of available nutrient sources. These factors can be investigated using a range of cultivation methods, such as agar plate cultivation, solid state fermentation and submerged liquid cultivation over a range of growth scales.

The effect of temperature, pH and oxygen availability on growth and pigment production is described in Section 2.3.1. This includes consideration of optimal, or even obligatory, conditions for growth, and whether these remain unchanged when trying to achieve maximum product yields. The impact of medium composition is discussed in Section 2.3.2, with the potential for using waste sources as a substrate for cultivation discussed while also introducing the concept of a waste biorefinery. Finally, the

way these factors interplay to affect the production process and commercial potential of the pigment product is discussed in Section 2.3.3.

2.3.1 Physicochemical conditions

Process parameters such as temperature, pH, agitation speed and aeration rate can be modified to suit a specific production system and, therefore, represent a potential means of optimising process performance. The production of fungal secondary metabolites, in particular, often requires a specific set of cultivation conditions which induce their gene clusters for biosynthesis. This is linked to these products not being directly involved in growth of the organism, while still contributing to survival in a given environment, or upon exposure to a specific condition (Brakhage, 2013; Boruta, 2018).

Cultivation-based strategies, such as these, are aimed at improving production through altering cultivation conditions to simulate the environmental conditions which usually trigger production (Boruta, 2018). Investigation of the impact of these conditions, also considering their combined effect, could lead to improved product yields and ultimately a reduction in process costs.

2.3.1.1 Temperature and pH

Temperature and pH are basic parameters which need to be controlled, or at least monitored, during any microbial process. This is as a result of their impact on organism metabolism as well as practical considerations for cultivation processes (Rosso et al., 1995). All microorganisms are defined by an optimal value or range for these parameters which is best suited to their cultivation.

Microbes can be broadly classified as thermophiles (high temperature), mesophiles (moderate temperature) or psychrophiles (low temperature) depending on their optimal temperature for growth. This feature defines the environment from which these organisms are likely to be isolated, but can also be utilised during controlled cultivation. Mesophilic conditions are generally preferred for industrial processes, as this reduces the cost associated with temperature control through heating (electrical, heat exchangers and steam) and cooling (Moset et al., 2015). Thermophilic organisms are, however, of interest as a result of the inherent thermostability of their products, such as pigments and enzymes (Chen and Berns, 1980; Benassi et al., 2014).

Microorganisms can also be classified based on their pH requirements, with neutrophiles, acidophiles and alkaliphiles growing optimally around a neutral pH of 7.0, below pH 5 and above pH 8, respectively. Fungi are reported to prefer slightly acidic pH values of around 5-6 (Parker et al., 2016). The control of medium pH during cultivation can be achieved in a number of ways, depending on degree of control required and the tolerance of the organism to pH shifts. This includes pH adjustment to a desired value only at the start of cultivation or the maintenance of a selected pH value during cultivation. Culture pH can be maintained through the application of a selected buffer or the addition of suitable acids or bases in response to observed pH shifts. This requires continuous monitoring of culture pH and can be performed manually or through online measurement and automatic pH adjustment.

Optimal temperature and pH conditions for growth may not, however, result in highest product yields. A stress response to non-optimal growth conditions could result in upregulation of pigment production, as described in Section 2.2.1, where nutrients which would be utilised for growth could be diverted to product formation.

2.3.1.2 Oxygen availability during submerged liquid cultivation

Oxygen requirements also define microorganisms as aerobes, which require oxygen, and anaerobes, which do not. Microbes with a strict requirement for or intolerance to oxygen are called obligate aerobes or anaerobes, respectively. Other groups include facultative anaerobes which grow optimally in the presence of oxygen but can also grow without it; aerotolerant anaerobes which are able to grow in the presence of oxygen, but do not require it; and microaerophiles which require oxygen for growth, but at concentrations lower than that naturally occurring in the atmosphere (Parker et al., 2016).

Oxygen availability has a significant impact on the cultivation of aerobic organisms as a result of its effect on organism metabolism. During cellular respiration oxygen acts as the terminal electron acceptor (Parker et al., 2016). Oxygen provided during cultivation can, therefore, support the growth of the organism as defined by nutrient availability, or oxygen can be the growth-limiting parameter. Various cultivation methods require different considerations for oxygen supply, with modifications to the system having the potential to improve medium oxygen concentration.

A review discussing the advantages and disadvantages associated with shake flask cultivation was presented by Büchs (2001), where the possible metabolic responses to oxygen limitation were listed as:

- Slowing of the entire microbial metabolism;
- Switch to partially anaerobic metabolism, with the production of compounds such as acids and alcohols, where the corresponding pH changes may be inhibitory to the organism;
- Alteration of product metabolism, with growth of the organism not necessarily affected;
- Complete alteration of the microbial metabolism, with different secondary metabolites produced under different conditions of oxygen availability; and
- Culture death due to the accumulation of toxic compounds, as toxin removal or detoxification processes are limited as a result of impaired microbial metabolism.

It is, therefore, important to consider the conditions which play a role in altering the oxygen availability within the culture. Oxygen availability in shake flask cultures, for example, is affected by various factors including shaking platform speed, flask size and filling volume. This is as a result of the impact of these factors on the k_La (volumetric mass transfer coefficient) which defines the system and, therefore, the oxygen transfer rate (OTR) into the medium. A number of models have been developed in an attempt to define the relationship between these system parameters and the oxygen availability within shake flask cultures. Maier and Büchs (2001) defined a proportionality for maximum oxygen transfer rate (OTR_{max}) based on experimental results obtained using open shake flasks, shown in Equation 1.

$$OTR_{max} \sim N^{0.84} V_L^{-0.84} d_0^{0.27} d^{-1.25}$$ Equation 1

where N is shaking platform speed (rpm)
V_L is liquid volume (L)
d_0 is shaking diameter (cm), and
d is flask diameter (m)

Based on this relationship, an increase in OTR is expected when shaking speed is increased or fill volume is decreased. The same is true of k_La, when considering the model developed by Nikakhtari and Hill (2005) for prediction of k_La in shake flask cultures. This was based on experimental data gathered used flasks of various sizes, with different liquid fill volumes and shaking speeds. The resulting equation for calculation of k_La is:

$$k_La = 0.0182 \times (A \times T)$$ Equation 2

where A is stationary liquid surface area, and
T is liquid turbulence factor, calculated using the equations:

$$A = 142 \times (V_F - V_L)^{2/3}$$ Equation 3

$$T = \frac{V_F^{0.463}}{V_L} \times \frac{N}{60}$$ Equation 4

where V_F is flask volume (L)
V_L is liquid volume (L), and
N is shaking platform speed (rpm)

Another consideration for oxygen availability in shake flask cultures is the impact of flask neck diameter and type of closure used. These parameters represent a further barrier to oxygen transfer in terms of replenishment of oxygen in the headspace of the flask. Whereas k_La defines the movement of oxygen between the flask headspace and the culture medium, another mass transfer coefficient, k_Ga, defines the movement of oxygen between the atmosphere and the headspace within the flask (Nikakhtari and Hill, 2006).

Nikakhtari and Hill (2006) investigated the effect of various closures on the k_Ga value defining the system, and reported a decrease in this oxygen transfer coefficient from 55.6 h^{-1} to 1.67 h^{-1} when comparing 500 mL open flasks and those stoppered using a foam plug (Identi-Plugs; Jaece Industries, Inc.), both with the same fill volume and shaking speed. This provides an indication of the limitation that closures impose on oxygen diffusion into shake flasks. The implications of this include reduced air

saturation within the flask, which affects oxygen solubility in the liquid phase and hence oxygen transfer rate (OTR), as demonstrated in Equation 5.

$$OTR = k_La(C^* - C) \quad \text{Equation 5}$$

where k_La is volumetric mass transfer coefficient for oxygen transfer into liquid medium (h^{-1})
where a is the interfacial area per unit volume (m^{-1})
C is oxygen concentration in the liquid ($mg.L^{-1}$)
C* is oxygen concentration in the liquid in equilibrium with the gas phase/ oxygen solubility ($mg.L^{-1}$)
and (C* - C) is the difference between the maximum possible and actual oxygen concentration/ concentration-difference driving force for mass transfer (Doran, 1995)

At 100 % air saturation, solubility of oxygen in water at 30 °C is 7.539 $mg.L^{-1}$ (European Inland Fisheries Advisory Commission, 1986). This value is also supported by the correlation for oxygen solubility in water reported by Doran (1995), shown in Equation 6. If the air saturation decreases to, for example 60 %, oxygen solubility drops to approximately 4.5 $mg.L^{-1}$. This then results in lower oxygen transfer into the liquid phase as a result of reduced concentration driving force. The k_La value, however, remains unchanged. This was represented by Nikakhtari and Hill (2006) as a concurrent decrease in oxygen concentration in the gas phase (flask headspace) and the liquid phase during shake flask cultivation.

$$C^* = 14.161 - 0.3943T + 0.007714T^2 - 0.0000646T^3 \quad \text{Equation 6}$$

where T is temperature (°C)

This shows that oxygen availability in shake flasks can be modified in a variety of ways to suit the needs of the culture. This is important, given that shake flask cultures are often used to investigate growth and productivity of an organism in response to changing conditions before scaling up cultivation. Small scale cultivations are useful for evaluating optimum conditions for growth and product formation, including physicochemical parameters and medium composition. Benchtop bioreactor systems allow the effect of these conditions to be confirmed on a larger scale, while also more closely mimicking conditions in larger cultivations conducted on industrial scale.

Bioreactor systems do, however, offer advantages over shake flasks in terms of investigation of oxygen and mass transfer requirements. The dissolved oxygen concentration in the medium is more easily monitored as well as adjusted. Parameters which affect oxygen availability in a bioreactor system include agitation speed, aeration rate and oxygen concentration in the inlet gas stream (Pereira et al.,

2008). The impact of oxygen on the production of both carotenoid and polyketide fungal pigments has been investigated using bioreactor systems of various scales.

The effect of aeration rate and agitation speed on production of β-carotene by *B. trispora* has been investigated using benchtop bioreactor systems. The study by Mantzouridou et al. (2002) found that highest pigment concentration was achieved at a k_La value of approximately 200 h^{-1}, with values in the range of 36 – 1188 h^{-1} considered. It has been shown that moderate aeration rates (1.5 vvm) and lower agitation speeds (60 – 150 rpm) are best suited to production of β-carotene (Mantzouridou et al., 2002; Goksungur et al., 2004). This can be attributed to the balance required between adequate mixing and mass transfer, while avoiding shear stress. Goksungur et al. (2004) noted that, at the higher aeration rates and agitation speeds considered, a reduction in the occurrence of sexual structures, known as zygospores, was observed. The results of Mantzouridou et al. (2002) also demonstrated a relationship between zygospore area (as a percentage of total mycelium area) and pigment production. A reduction in zygospore formation reduces sexual interaction between the opposite mating types and, therefore, reduces pigment yields. López-Nieto et al. (2004) also considered the effect of dissolved oxygen on lycopene production by *B. trispora* when using the lycopene cylase inhibitor imidazole. Using a semi-industrial scale fermentation of 800 L, it was shown that lycopene production increased with aeration rate and agitation speed (up to 300 rpm), as well as with increased dissolved oxygen concentration as a result of enriching the air inlet with oxygen.

Production of polyketide pigments by members of the *Monascus* genus has been conducted in benchtop bioreactor systems. The impact of changing aeration rate, agitation speed and dissolved oxygen concentration was investigated. In general, a positive relationship between pigmentation and increases in these parameters was demonstrated (Hajjaj et al., 1999a; Pereira et al., 2008). Synthesis of the co-produced mycotoxin, citrinin, was also positively affected, such that the ratio of pigment to toxin declined with increasing oxygen availability. The result of this is that optimum oxygen conditions for pigment production cannot be maintained unless a method of eliminating toxin co-production is introduced.

Ogihara et al. (2000) cultivated *P. purpurogenum* in a jar fermentor using 5 L working volume to produce *Monascus* pigment homologues, without citrinin co-production. The effects of aeration rate and agitation speed were not considered, but dissolved oxygen was maintained at 2.5 ppm.

2.3.2 Medium composition

Medium composition plays a determining role in microbial pigment production, and has been reported to be of greater importance than cultivation conditions in some cases (Santos-Ebinuma et al., 2014). Altering the type of nutrient source supplied or the concentration of medium components can affect biomass and product yields, with certain components potentially determining whether or not pigmentation is observed at all.

2.3.2.1 Carbon and nitrogen sources

The type of carbon or nitrogen source supplied during fungal cultivation can modulate secondary metabolism of the organism and thus alter pigment productivity. Glucose, for example, has been reported to be a good carbon source for growth, but it may interfere with product formation, given that secondary metabolite production is generally improved during sub-optimal growth (Brakhage, 2013). A suggested means of addressing this is the use of a mixture of carbon sources. This could potentially allow growth followed by product formation (Adrio and Demain, 2003).

Ogihara et al. (2000) reported that significant PP-V pigment production was only observed when soluble starch was supplied to *Penicillium* sp. AZ as the sole carbon source. Other carbon sources such as glucose, sucrose, galactose and fructose allowed biomass formation, but limited pigmentation.

Growth and pigment production of other fungal organisms is, however, supported on glucose, with sugar concentration applied shown to have an impact on productivity. A study by Hajjaj et al. (1997) was able to show that pigment production by *M. ruber* was higher when the glucose concentration in the medium was high (20 $g.L^{-1}$ as opposed to 5 $g.L^{-1}$).

A study which investigated the effect of sugar concentration in *B. trispora* found that when sugar content was increased from 30 $g.L^{-1}$ to 50 $g.L^{-1}$, the number of sexual structures (zygospores) increased and a corresponding increase in β-carotene was observed. This is consistent with sexual interaction of *B. trispora* improving carotene productivity (Adrio and Demain, 2003; Joshi et al., 2003). A further increase in sugar supply from 50 $g.L^{-1}$ to 70 $g.L^{-1}$, however, caused a decrease in both sexual structures and β-carotene production (Goksungur et al., 2004). A similar result was noted by Choudhari and Singhal (2008), where an increase in glucose concentration from 50 $g.L^{-1}$ to 60 $g.L^{-1}$ caused an increase in β-carotene yields, while any further increases in glucose concentration had a negative effect on pigment production.

The nitrogen source supplied during fungal cultivation can also have an impact on pigment production, with a variety of inorganic and organic sources available for use. Inorganic sources are beneficial when the culture medium composition needs to be clearly defined, for example, when monitoring the impact of specific nitrogen compounds or when it is required to make up a specified mix of nitrogen-containing components. Organic nitrogen sources are typically richer, with their nitrogen components not always specified. They usually also contain a mix of carbon sources and other growth factors.

When considering the impact of nitrogen source on pigment production in *B. trispora*, yeast extract was found to yield the highest biomass and pigment concentrations. The pigment yield when using this nitrogen source was approximately double that observed when using soya peptone, the nitrogen source which resulted in the second highest β-carotene yield. It was also reported that the use of inorganic nitrogen sources such as ammonium nitrate and ammonium chloride resulted in very low pigment yields (Choudhari and Singhal, 2008).

Inorganic nitrogen sources such as ammonium nitrate and ammonium sulphate do not support the growth of *P. purpurogenum*, with growth and pigment production instead requiring sources such as malt extract or yeast extract (Ogihara et al., 2000b). It has, however, been reported that nitrogen sources which are overly favourable to growth may reduce secondary metabolite yields (Adrio and Demain, 2003).

A study investigating the impact on *Monascus* pigments when providing different amino acids in the culture medium found that red pigment shade varied depending on the amino acid which had been provided (Jung et al., 2003). The fungi were cultivated in shake flasks using glucose as the carbon source with 3 $g.L^{-1}$ ammonium nitrate and 7 $g.L^{-1}$ of one of twenty investigated amino acids as the nitrogen source. This was compared to 10 $g.L^{-1}$ ammonium nitrate as a nitrogen source control. The crude extracts were analysed using the CIELAB colorimetric system, explained by Mapari et al. (2006). The results indicated that red pigments obtained when amino acids were supplied differed in shade to those of the control. It was also noted that depending on the amino acid used, the hydrophobicity/ hydrophilicity of the red pigments was altered. This result indicates that the amino acid was incorporated into the structure of the pigment (Jung et al., 2003). This was, however, not the case for the yellow and orange pigments, which is consistent with the reaction between orange *Monascus* pigments and amino groups yielding red pigments (Mapari et al., 2005, 2010).

Another study investigating the effect of providing different amino acids as precursors for pigment production in *Monascus* revealed that amino acid derivatives were more stable when exposed to light than pigments obtained when ammonium nitrate was supplied as the only nitrogen source (Jung et al., 2005). This indicates that the nitrogen source may have an impact on stability and, therefore, potential applications of the pigment.

2.3.2.2 Potential for using waste substrates

Natural pigments have, in general, a greater cost of production than chemically synthesised alternatives, limiting their commercialisation (Feofilova et al., 2006; Kirti et al., 2014; Rodriguez-Amaya, 2016). While improvement of product yields and productivity represents a means of addressing this issue, additional cost-reduction approaches are also required. Significant costs associated with production of microbial pigments include energy requirements, medium components and down-stream processing. The identification of cheaper or alternative nutrient sources, such as agro-industrial waste products, which support pigment production may, therefore, significantly reduce process costs and improve potential for competitive production of natural colourants (Meinicke et al., 2012; Venil et al., 2013). The potential application of the resulting pigments must, however, be considered when selecting the source of the nutrient stream.

The ability of various filamentous fungi to produce pigments of interest when grown on waste substrates has been demonstrated. These include food waste and industrial by-products. Cultivation methods investigated include solid state fermentation (SSF), semi-solid state fermentation (semi-SSF) and submerged liquid cultivation.

During solid state fermentation the organism is cultivated on the surface of the selected substrate, with the initial moisture content adjusted to suit the requirements of the fungus (Pandey, 2003). SSF is the traditional production method for *Monascus* pigments (Dufossé et al., 2005). Submerged liquid cultivation involves dissolving all substrates in a liquid medium (water) and cultivating the organism in this suspension. This cultivation method offers improved process control, with suitable mixing of the culture improving access to nutrients and hence reducing cultivation times (Silveira et al., 2013). Semi-SSF offers advantages of both systems, with substrate particles of selected sizes suspended in a suitable medium providing a support on which the organism can grow, while the system also allows improved mixing (Kantifedaki et al., 2018).

Examples of food wastes shown to be suitable substrates for cultivation and pigment production of fungi by SSF are dried corn cob waste for the production of yellow and red polyketide *Monascus* pigments (Velmurugan et al., 2011), orange processing waste for yellow, orange and red pigments produced by *M. purpureus* and *P. purpurogenum* (Kantifedaki et al., 2018) and cassava processing waste for the production of red pigments using *P. purpurogenum* (Padmapriya et al., 2014).

By-products of the sugar processing industry have also been used as nutrient sources for the cultivation of filamentous fungi. Molasses is the dark, viscous syrup which remains after sugar extraction. It still contains a high concentration of sugars, along with nitrogen sources and other trace elements. Molasses is cheap and widely available and has been shown to be a suitable substrate for the production of β-carotene by *Blakeslea trispora* (Goksungur et al., 2004).

Sugarcane bagasse, however, is the cellulose-rich waste remaining after crushing and extracting the juice from sugarcane. It has been successfully applied as a carbon source for cultivation of *M. purpureus* to produce red polyketide pigments. Nitrogen supplementation was required, with an inorganic nitrogen source (NH_4Cl) not supporting pigment production, while use of organic sources such as peptone, soy protein and cheese whey resulted in varying pigment productivities (Silveira et al., 2013). Maltose syrup residue has also been shown to support growth and pigment production of *Monascus ruber* during solid cultivation on a solid medium (de Oliveira et al., 2019)

Glycerol, which is a by-product of biodiesel production, has also been investigated as a potential carbon source for the cultivation of the fungus *M. ruber*. Although a market exists for glycerol, the production of red pigments by *M. ruber* represents a means of valorising this by-product (Meinicke et al., 2012).

The use of these alternative low-cost substrates for microbial cultivation represents a means of valorising these waste streams, while also reducing process costs and the environmental burden that otherwise would have been caused by these waste products (Mata-Gómez et al., 2014; Tuli et al., 2015; Harrison et al., 2016). Processes such as these hold potential for application as part of a waste biorefinery, where waste is recognised as a substrate and the fungal pigments represent a potential high-value product.

Waste biorefinery concept

A biorefinery has been defined as the sustainable processing of biomass into a range of valuable products and energy (International Energy Agency Bioenergy, 2018). Energy outputs include heat, power and fuels, while potential products include end-products, which can either be sold or used on site, and intermediate products, including platform chemicals, which require further processing elsewhere. The shift towards closed-loop thinking is demonstrated by the concept of a waste biorefinery, which is differentiated by the utilisation of waste streams as feedstocks for the production of energy or value-added products (Harrison et al., 2016; Venkata Mohan et al., 2016a).

Biorefineries offer many options in terms of potential input and output streams, ranging in complexity from single feedstock-single product or single feedstock-multiple product systems to fully integrated multiple feedstock-multiple product systems. Process integration improves biorefinery efficiency and potentially expands the product range, while further supporting the move toward a sustainable circular economy, where effluents from one process can be used as a feedstock for another (Dahiya et al., 2018).

A waste biorefinery has additional benefits of addressing waste disposal issues (Nizami et al., 2017), and improving biorefinery competitiveness and acceptance (Venkata Mohan et al., 2016b). A waste biorefinery needs to meet two criteria: generation of a feasible product and treatment of waste. This can be achieved through multiple-unit operations, where production can be maximised in one unit, while waste remediation can be optimised in another (Harrison et al., 2016). Waste remediation and valorisation achieved through these processes has environmental benefits while also addressing economic and social aspects, thereby satisfying the three requirements of sustainability. The waste utilised essentially represents a renewable feedstock for the process.

Biorefineries, including waste biorefineries, can make use of a range of technologies to effect the conversion required. The conversion processes currently applied can be classified into four main groups, namely biochemical, thermo-chemical, chemical and physical technologies. Biochemical methods use biological organisms or systems, either directly or as catalysts, to convert the feedstock into energy and bio-based products. Thermo-chemical, chemical and physical processes make use of high temperatures, chemical or mechanical means for conversion of the feedstock, respectively. Chemical and physical methods may be used to process mixed feedstocks non-specifically, whereas in biochemical processes mixed feedstocks may be selected to provide the balance of nutrients essential for efficient performance, while still processing a major waste or biomass component.

Selection of the applied technology is dependent on the type of feedstock (moisture content, biodegradability, propensity to form emissions or toxins, among other features), desired products, associated costs and biorefinery location (Nizami et al., 2017). Cultivation of filamentous fungi on selected waste products to produce high-value pigments would be an example of a biochemical conversion.

2.3.3 Evaluating commercial potential of fungal pigments

Commercialisation of natural pigments is dependent on both the properties of the colouring compounds which make them desirable as marketable products, and the required production process (Charkoudian et al., 2010). Pigment properties of commercial importance include colour and ease of application. Stability of the pigments towards heat, light, oxidation, pH and other potential conditions which may result in degradation and loss of colour must also be evaluated. Considerations regarding the production process include the ability to achieve an appropriate yield of the pigment product, at suitable productivity, while also remaining economically competitive and demonstrating a robust process option (Morales-Oyervides et al., 2015b; Torres et al., 2016). This can be achieved by maximising production and reducing associated costs.

Means of improving productivity include appropriate organism selection (Dufossé, 2006), optimisation of process conditions and use of suitable medium components for both biomass and pigment production (Choudhari and Singhal, 2008; Mapari et al., 2009b; Celestino et al., 2014). Further improvement could be achieved by understanding and manipulating the trigger for pigment production or extending the production period, using for example fed-batch or continuous cultivation systems to maintain the desired production conditions.

Using the basis of the Luedeking-Piret model for product formation (Luedeking and Piret, 1959), it is observed that in order to maximise productivity both biomass production and specific pigment production need to be optimised on the available substrate, under a given set of process conditions. The biomass yield needs to be high enough that the specific pigment production rate achieved results in highest possible pigment production. Both of these factors are likely dependent on substrate availability and utilisation.

The Luedeking-Piret model, as it relates to pigment production, is described by Equation 7:

$$\frac{dCp}{dt} = \alpha . \frac{dCx}{dt} + \beta . Cx \qquad \text{Equation 7}$$

where $\frac{dCp}{dt}$ is pigment productivity

Cx is biomass concentration, and

α and β are growth and non-growth associated specific rates of pigment production, respectively

Potential for maximising pigment production exists in manipulation of both cultivation conditions and medium composition, as described in Section 2.3.1 and Section 2.3.2, respectively.

2.4 Pigment analysis and characterisation

Methods for evaluating pigment products are required in order to monitor production during cultivation as well as to isolate and identify the compounds produced. Procedures required include extracting the colouring compounds from the fungal culture, isolating the pigments from other compounds which may have been co-extracted, determining the amount of pigment produced and, finally, identifying the compounds responsible for observed pigmentation. These methods, additionally, allow comparison of pigmentation achieved in response to changes in cultivation conditions or medium composition.

2.4.1 Monitoring pigment production

The simplest method of monitoring and quantifying pigmentation in a solution is through absorbance measurement using a spectrophotometer. The pigment content can then be expressed as optical density (OD) units per mL of solution (Jia et al., 2010).

The OD reading is generally reported at the wavelength of maximum absorbance, which is related to the colour of the pigment. Pigments absorb light maximally at a specific wavelength which is dependent on their colour, with the remaining light reflected. This is, therefore, perceived as the complementary colour of the wavelength absorbed (Charkoudian et al., 2010). Maximum absorbance at approximately 500 nm corresponds to red pigmentation, while yellow pigments absorb maximally in the range of 400 – 420 nm (Carvalho et al., 2007), as demonstrated in Figure 2.8.

Evaluation of pigment colour, beyond wavelength of maximum absorbance, is often performed using the CIELAB colorimetric system (Jung et al., 2003; Mapari et al., 2006, 2008b). The basis of this system is that light reflected from a surface of any colour can be matched using a mixture of red, blue and green light (Mapari et al., 2006). The output is given as a pigment shade, based on a hue angle of 0, 90, 180 or 270 ° which corresponds to red, yellow, green or blue, respectively, and pigment lightness given by a value between 0 (black) and 100 (white). Complete wavelength scans over the UV-visible light range can also provide an indication of the colour of a solution by indicating the proportion of compounds of different colours. This method of monitoring pigmentation can also be used to evaluate how changing conditions affect the production of pigments of various colours. An example is the proportion of yellow and red pigments produced by *M. ruber* when cultivated under different pH conditions (de Oliveira et al., 2019)

Absorbance can be used to monitor the production of extracellular pigments by determining the relevant OD measurement of the culture filtrate, without any further processing. The UV-visible absorbance maxima can also be used to validate pigment identification through comparison to spectra for known compounds provided in literature (Mapari et al., 2005).

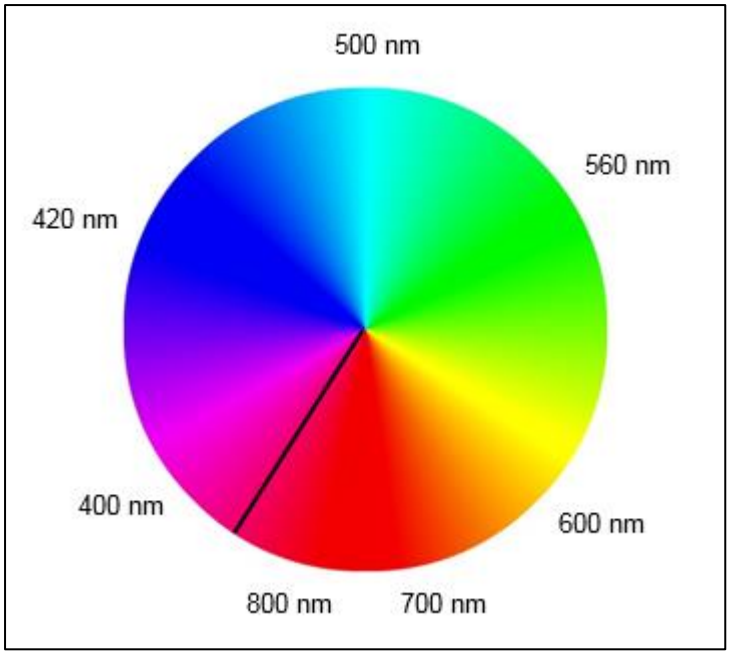


Figure 2.8 Colour wheel representing the complementary nature of maximum absorbance and perceived colour. Adapted from Charkoudian et al. (2010).

It is widely accepted to report maximum pigment yield as the absorbance value at the wavelength of maximum absorbance of the target colour for application (Morales-Oyervides et al., 2015b), however, in order to relate this OD value to a pigment concentration, it is possible to create a calibration curve using a pigment standard. This has been previously performed and reported in studies investigating production of the yellow-orange pigment β-carotene. The OD_{450} measurements of prepared solutions containing the pigment were plotted against the corresponding concentrations. This allowed the β-carotene concentration of tested samples to be expressed in $mg.L^{-1}$ through measurement of absorbance alone (Choudhari and Singhal, 2008; Papaioannou et al., 2008).

An issue specific to this system is that the absorbance of β-carotene overlaps with that of another carotenoid pigment product, lycopene. Soroka et al. (2012), however, described a spectrophotometric method for the simultaneous determination of β-carotene and lycopene concentrations produced by *B. trispora* cultures. This was achieved by mixing pure solutions of β-carotene and lycopene in selected proportions to yield known concentrations of the two compounds. Evaluation of the spectral scans of these resulting mixtures revealed that the extinction coefficients, specifically of lycopene, were not constant and instead depended on the ratio of measured absorbance peaks at 470 and 502 nm. It was reported that through the use of these new extinction coefficients it is possible to evaluate the concentration of these two pigments in an unknown pigment mixture. Results of this spectrophotometric method were shown to be comparable to those obtained by HPLC. This does, however, have implications for other pigment mixtures, especially those containing multiple pigments of similar colour hues.

HPLC and other chromatography techniques are the most accurate in terms of pigment quantification when working with a known pigment. These methods are, however, laborious and expensive, with

method development often required for effective separation and quantification of a pigment of interest. The lack of standards for some pigment molecules can also hinder quantification using these analytical methods (Joshi et al., 2003).

When performing exploratory work or producing pigment compounds or mixtures of unknown composition, the use of these techniques is not practical as comparison to a pigment standard is required to effectively quantify pigment concentration. Unknown pigments would, therefore, first need to be isolated and identified.

2.4.2 Isolation and characterisation of pigment products

In order to further analyse pigment products, it is necessary to separate these compounds of interest from their production medium. This involves the use of a suitable extraction method and subsequent isolation techniques. Selection of appropriate process routes is dependent on the location of the products, the properties of the pigments and the cultivation method applied.

2.4.2.1 Fungal pigment extraction and recovery

Fungal pigments can either be retained within the biomass or be secreted into the surrounding medium, therefore, being referred to as intracellular or extracellular, respectively. For submerged liquid cultivation, initial processing involves separating the fungal biomass from the spent medium. Suitable processes for this purpose include centrifugation or filtration. The biomass or the cell-free medium, depending whether the pigment is intra- or extracellular, is then reserved for further processing. Following solid state fermentation the entire substrate, and attached biomass, is generally processed to yield desired pigment products. The fermentation product can either be dried and used directly as a colourant, or the pigments can be extracted using suitable solvents (Dufossé, 2006; Carvalho et al., 2007), for example, a polar, water miscible solvent, such as ethanol (Johns and Stuart, 1991; de Carvalho et al., 2005; Babitha et al., 2008). A study by Carvalho et al. (2007) compared the extraction of *Monascus* pigments from solid substrates using various polar and non-polar solvents. The results showed that the more polar solvents were better suited to extraction of these molecules, with the best extraction achieved using methanol, dimethyl sulfoxide, and ethanol. The latter two exhibited 94.9 % and 91.6 % extraction, respectively, of that observed when using methanol. Ethanol was, however, recommended for further experimentation as this solvent is better suited to large scale applications as a result of its non-toxic nature and lower cost.

The acceptable residual levels of solvents applied during synthesis or extraction of fungal pigments is governed by the guidelines set out for dietary and other related products such as pharmaceuticals. The United States Pharmacopeia, for example, classifies solvents into three classes based on toxicity and environmental impact. Class 1 solvents, such as benzene, are highly toxic and should be avoided. Class 2 solvent application should be limited, with permitted daily exposure (PDE) values specifically defined. Examples include chloroform, with a concentration limit of 60 ppm, and methanol, at 3000 ppm. Class 3 solvents are considered less toxic and, therefore, lower risk. A concentration limit of 5000 ppm

of each solvent is acceptable. Example solvents from this class include acetone, 1-butanol, dimethyl sulfoxide, ethanol and ethyl acetate (United States Pharmacopeial Convention, 2019)

Pigment extraction methods, in addition to product location, are also dependent on the pigment properties, such as solubility and polarity, with fungal pigments broadly categorised as oil- or water-soluble. Oil-soluble pigments are hydrophobic and generally intracellular in nature, with the carotenoid pigments being significant examples. The intracellular nature of these pigments means the biomass needs to be processed to release the pigment products. Comparison of biomass treatments applied to *B. trispora* prior to pigment extraction found yields from wet, filtered and washed biomass to be the highest. Freeze drying, grinding after freezing, and drying the biomass at 50 °C were found to cause pigment degradation and lower pigment yields (Papaioannou et al., 2008).

Recovery of oil-soluble pigments generally requires the use of organic solvents, which have associated concentration limits for application and, therefore, need to be removed (Mortensen, 2006). The carotene pigments of *B. trispora*, for example, have been shown to be effectively extracted using solvents such as acetone, ethyl ether, 2-propanol, ethanol and ethyl acetate (Roukas and Mantzouridou, 2001; Papaioannou et al., 2008). When considering pigment yields and extraction time, Papaioannou et al. (2008) found the most effective solvents to be acetone, 2-propanol and ethanol, with methanol, pentane and hexane being less effective. Some solvents also preferentially extracted certain pigments. Acetone exhibited favourable lycopene recovery and methanol, ethanol and ethyl ether showed increase β-carotene extraction. Solvent concentration can also have an impact on pigment extraction. An ethanol concentration of 100 %, rather than 60 or 40 %, has been shown to result in highest pigment recovery (Roukas and Mantzouridou, 2001). This can be attributed to the solubility properties of the pigments.

Water-soluble pigments can be retained in the biomass or secreted, with suitable extraction methods varying depending on location. Intracellular pigments can be extracted directly from the fungal biomass using suitable solvents, for example lower alcohols (Mortensen, 2006), or liberation of the products by cell disruption may be required (Velmurugan et al., 2010).

Water-soluble pigments secreted into the cultivation broth during fermentation, or liberated into an aqueous medium after fermentation, need to be isolated from the other soluble components. Suitable methods include precipitation, such as acetone or ethanol precipitation (Lin et al., 1992), or liquid-liquid extraction. Liquid-liquid extraction can be achieved through the application of water immiscible solvents or aqueous two-phase extraction, using for example polymer-polymer or polymer-salt systems, where two aqueous solutions form separate phases and the product of interest preferentially partitions into one of these phases (Iqbal et al., 2016).

2.4.2.2 Pigment isolation techniques

Once a suitable extraction procedure has been applied, the extract must be processed such that a single product of interest can be isolated for subsequent characterisation and identification. A variety of methods, of varying complexity and application potential, are available for use. Separation of fungal pigments in solution has been achieved using a variety of chromatography-based methods (Hajjaj et al., 1997; Ogihara et al., 2000b; Jung et al., 2003; Papaioannou et al., 2008; Wang et al., 2014). These include thin-layer chromatography (TLC), column chromatography and high pressure/ performance liquid chromatography (HPLC).

The key factors associated with separation by TLC are the affinity of the sample components for the stationary phase (the TLC plate), and their solubility in the mobile phase, with improved separation of sample components achieved through altering the mobile phase composition. According to Coker and Ayoola (2008) the ideal mobile phase for separation of an unknown solution can be identified by altering the polarity of the mobile phase. This can be achieved through the use of increasingly polar solvents, or altering the ratio of a non-polar and polar solvent mixture. This works on the basis that eluting power of solvents increases with increasing polarity. Solvent eluting power increases in the order: alkanes < toluene < halogenated hydrocarbons < diethyl ether < ethyl acetate < acetone < alcohols < acetic acid.

This holds true for all normal-phase chromatography techniques utilising a hydrophilic stationary phase. Reversed phase chromatography is also possible, where a hydrophobic stationary phase has higher affinity for hydrophobic or less polar molecules, and the more polar molecules are, therefore, eluted first.

These principles can also be applied to other chromatography methods such as HPLC and column chromatography, which allows separation of larger sample volumes. HPLC methods have the added benefit of selection from multiple possible detectors, such as UV-visible light, refractive index, fluorescence and photodiode array (Feng et al., 2012), suited to a wide variety of potential compounds.

2.4.2.3 Pigment compound characterisation

The production and isolation of novel or unknown pigments must be followed by characterisation and identification techniques. Chromatography methods can be coupled to mass spectrometry in order to evaluate the molecular weight of the molecule (Hajjaj et al., 1997). Mass spectra along with UV-vis absorbance maxima can be used to inform pigment structure and identity by comparison to known values or previous spectral analyses (Mapari et al., 2005).

A number of techniques are also available in order to elucidate the structure of an unknown pigment. These include Fourier-transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction (Lian et al., 2015). The elemental composition of an isolated pigment can also be evaluated.

2.4.3 Characterisation of red, water-soluble fungal pigments

Water-soluble, extracellular pigments are of particular interest for commercial production given the wider application potential of water-soluble pigments over those which are oil-soluble, and the easier and cheaper downstream processing associated with extracellular products (Velmurugan et al., 2010).

The search for natural red pigments is attributed to changes in regulation regarding the use of synthetic pigments. The toxic effects of the pigments or their precursors, and their potential environmental impact are considered (Downham and Collins, 2000; Numan et al., 2018), with red pigments particularly affected by the reduction in synthetic pigments available for use in food and cosmetic applications (Carvalho et al., 2007; Bechtold and Mussak, 2009). Red pigments which are no longer approved for use in food and/ or cosmetic products in the United States include amaranth (Federal Food, Drug, and Cosmetic Act Red No. 2) and erythrosine (FD & C Red No. 3). These have largely been replaced by Allura Red AC (FD & C Red No. 40) which is an azo dye (Burrows, 2009; Arnold et al., 2012; Batada and Jacobson, 2016).

The *Monascus* pigments, and their derivatives, also reported to be produced by members of the *Penicillium* genus, are examples of red, water-soluble pigments which could be used in place of these synthetic pigments. Characterisation techniques previously applied to these and other red, water-soluble pigments are detailed in Table 2.3.

These methods, although diverse, have all been effective in isolating and characterising red, water-soluble pigments from various fungal sources. These techniques have also been crucial for further study of these pigment molecules. This includes investigation of their solubility (Bhardwaj et al., 2007), stability (Lin et al., 1992; Hajjaj et al., 1997; Jung et al., 2011), antioxidant potential (Padmapriya and Murugesan, 2016; Jin et al., 2018), and antimicrobial and other medicinal properties (Choi and Park, 2018; Jin et al., 2018).

Table 2.3 Isolation and characterisation methods applied to red, water-soluble pigments produced during fungal cultivation.

Pigment and/or production organism	Extraction, isolation methods	Characterisation, identification techniques	Reference
Monascus ruber N-glucosyl-rubropunctamine, N-glucosyl-monascorubramine	Lyophilised culture filtrate re-dissolved in water, extracted with water-saturated *n*-butanol	NMR spectroscopy, mass spectrometry	Hajjaj et al. (1997)
	Column chromatography [chloroform/ methanol at 90:10, followed by 50:50]; TLC [chloroform/ methanol/ water (65:25:4)]; HPLC		
Monascus sp.	Equal volume 95 % ethanol added to culture broth, 30 °C, 180 rpm, filtered	LC-MS, NMR spectroscopy	Jung et al. (2003, 2011)
	TLC [chloroform/ methanol/ water (90:25:4)], major spots excised, dissolved in ethanol; HPLC		
Monascus sp.	Filtrate extracted with ethyl ether, precipitate removed; 5x volume 100 % ethanol added to aqueous phase, precipitate removed; precipitates combined and dissolved in methanol	Infrared spectroscopy, NMR spectroscopy	Lin et al. (1992)
	HPLC		
Monascus red pigments	Sequential extraction of pigment powder; hexane, ethyl acetate (yellow and orange pigments) and methanol (red pigments)	FT-IR spectroscopy, ESI-MS, NMR spectroscopy, X-ray diffraction	Lian et al. (2015)
	Methanol extract dried, separated by two dimensional TLC [ethanol/ petroleum ether (7:3), and methanol/ dichloromethane (1:1)]		
Paecilomyces sinclairii	Culture filtrate extracted with acidified ethyl acetate. Extract evaporated under vacuum, dissolved in water, filtered. Water extract dried, dissolved in absolute ethanol, filtered. Ethanol extract crystallised by adding chloroform/ n-heptane (1:2) and then dried	ESI-MS	Cho et al. (2002); Choi and Park (2018)
	Medium pressure flash chromatography over silica gel using an ethyl acetate/ hexane/ water/ acetonitrile step gradient		

Penicillium marneffei	Agar containing diffused pigment extracted with water at 50 °C. Extract filtered and lyophilised. Reversed phase HPLC, HPLC-MS-MS	UV-vis absorption and fluorescence, Atomic Absorption spectroscopy, FT-IR spectroscopy, NMR spectroscopy, CHNS elemental analysis, LC-MS	Bhardwaj et al. (2007)
Penicillium sp. PP-V	Culture filtrate extracted with ethyl acetate, evaporated, dissolved in acetone TLC [*n*-butanol/ acetic acid/ water (12:3:5)] for pigment detection; repeated column chromatography of acetone solution (acetone as developing solvent) for pigment purification	IR spectroscopy, FAB-MS, NMR spectroscopy	Ogihara et al. (2000b); Arai et al. (2012)
Penicillium purpurogenum	Aqueous two-phase extraction using polyethylene glycol (PEG) and sodium polyacrylate, where the pigment preferentially partitions into the PEG phase	/	Santos-Ebinuma et al. (2015)
Penicillium purpurogenum	5 x volume 90 % methanol added to culture broth TLC [*n*-butanol/ acetic acid/ water (12:30:50)]	GC-MS of separated bands, FT-IR spectrometry	Padmapriya and Murugesan (2016)
Penicillium purpurogenum	Culture filtrate extracted with ethyl acetate, concentrated by rotary evaporation Column chromatography [ethyl acetate/ methanol (1:0, 50:1, 30:1)] followed by TLC. Target band excised and dissolved in methanol	LC-MS, FT-IR spectrometry, NMR spectrometry	Jin et al. (2018)

2.5 Study background, aims and objectives

2.5.1 Literature summary

Natural pigments are receiving renewed interest as a result of the limited number of synthetic colourants still approved for use in food and other applications (Burrows, 2009; Arnold et al., 2012), and the associated health benefits of these natural products (Downham and Collins, 2000). Microbial sources of colourants, specifically, are being investigated as a result of the diverse range of products that this group represents, as well as the potential for continued and controlled production, generally unattainable with plant and insect sources (Joshi et al., 2003; Mortensen, 2006).

Filamentous fungi are well-known producers of secondary metabolites of potential interest. The pigments produced by this group of organisms can be broadly categorised into two groups, the carotenoids and polyketides, which have variable solubility in oil and water (Mapari et al., 2008b, 2010). While the carotenoid, and other oil-soluble, pigments are suited to certain applications, the use of water-soluble pigments in a range of food and related products is generally simpler given their aqueous nature (Velmurugan et al., 2010). Red and yellow pigments, in particular, are widely applied in these types of products, with limited colouring options available (Batada and Jacobson, 2016).

The production of red, water-soluble pigments has been reported in the fungal species *Monascus*. These products are applied as food colourants in Asia, but the co-production of mycotoxins has limited widespread approval (Jia et al., 2010; Dufossé et al., 2014). *Penicillium* sp. have been demonstrated as alternative producers of these pigments and their derivatives, reportedly without co-production of toxic metabolites (Ogihara et al., 2000b; Mapari et al., 2008a; Kojima et al., 2016). Literature provides limited information regarding production aspects of these pigments, including conditions favouring their production, biokinetics for process design and the impact of scale-up.

This study aims to confirm pigment production by *P. purpurogenum* DSM 62866, a strain not previously reported to produce pigmentation, investigate growth and pigment production response to changes in process parameters and characterise the pigmentation produced. Cultivation parameters such as temperature, pH, oxygen supply and medium composition are investigated to identify conditions which improve, or are responsible for, pigment production by this organism. Cultivation is scaled up and the biokinetic parameters are evaluated to analyse process and product potential. Finally, the potential for improving resource efficiency and, therefore, process feasibility is investigated by applying a potential waste stream as a substrate for growth. Confectionery waste serves as a case study, with marshmallow confectionery applied as a consistent feed for proof of concept.

2.5.2 Objectives and key questions

To address the aims set out above, this study has been structured around a set of objectives and associated key questions. The experimental approach outlined in Section 3.7 details the work undertaken to address each facet of the study.

1. Confirm red pigment production by *P. purpurogenum* DSM 62866 and investigate the effect of physicochemical parameters on growth and pigment production by this organism.
    - Is pigmentation produced on the complex medium recommended for growth of this organism?
    - Are the pigment products secreted into the surrounding medium?
    - Do physicochemical conditions impact growth and pigment production of this organism?
    - What temperature and pH conditions are best suited to pigment production (within a range of considered values)?
2. Determine whether the small-scale concept can be replicated in a benchtop scale stirred tank reactor. This system allows enhanced control of cultivation parameters, including modification of oxygen transfer rate by altering aeration rate or agitation speed.
    - Are growth and pigment production trends consistent when scaling up cultivation?
    - Does oxygen availability play a role in pigment production?
3. Characterise the pigment products, also considering the properties that influence application potential.
    - Is culture pigmentation the result of a single pigment or a collection of pigments, and what is the identity of this/these molecule(s)?
    - What extraction and isolation methods are suitable for the pigment products?
4. Investigate the effect of medium composition on growth and pigment production.
    - What key nutrients are required for pigment production?
    - What impact does altering the carbon to nitrogen ratio in the cultivation medium have on growth and pigment production?
    - Does the onset of pigment production coincide with depletion of sugars?
    - Can pigment production be replicated when substituting cultivation medium components with confectionery products, applied as an example of agro-industrial waste?
5. Evaluate the kinetics which define the cultivation of *P. purpurogenum* DSM 62866 under a given set of conditions. These kinetics inform process decisions and influence product potential.
    - What is the effect on biomass or pigment productivity of altering cultivation parameters or medium composition?
    - Is the pigment product growth or non-growth associated?
    - Can the yield of extracellular pigments per gram of biomass be increased such that the specific product formation rate, β, and the biomass concentration achieved are both maximised for product formation, as demonstrated in the second term of the Luedeking-Piret equation describing production of secondary metabolites ($dC_P/dt = \alpha.dC_X/dt + \beta.C_X$)?

3 Materials and Methods

This chapter describes the methodology for cultivation of *Penicillium purpurogenum* DSM 62866, with a specific focus on monitoring and improving pigment production by this organism. Section 3.1 describes the maintenance conditions for the organism, and Section 3.2 provides the composition of all medium types used in agar plate and liquid cultivation experiments. In Section 0 the various cultivation methods employed during agar plate, multiwell plate, shake flask, and bioreactor cultivation are described. Section 3.4 details the evaluation of culture kinetics and the calculation of various yields during liquid cultivation. Section 3.5 describes the pigment characterisation methods used. In Section 3.6 the statistical analyses applied to cultivation data are introduced, and finally, in Section 3.7 the experimental approach taken in this study is described.

3.1 Microorganism and maintenance conditions

The filamentous fungus selected for investigation in this study was *Penicillium purpurogenum* DSM 62866, obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen). This organism is classified under risk group 1, based on the German Technical Rules for Biological Agents. Organisms in this risk group represent no risk of infectious disease. Toxin production and allergenic effects are also considered and noted (DSMZ, 2014; Bundesantalt für Arbeitsschutz und Arbeitsmedizin, 2016). This organism was selected for investigation based on literature reports of significant red pigment production by members of this species, although pigment production by this strain was not previously reported.

In 2011 the species *P. purpurogenum* was renamed *Talaromyces purpurogenus* (Stoll) Samson, Yilmaz, Frisvad & Seifert (Mycobank MB 560667), based on phylogenetic analyses (Samson et al., 2011). As this name change has not yet been adopted by the culture collection, the strain used has been referred to as *P. purpurogenum* DSM 62866 throughout this study.

The organism was maintained on malt extract, soya peptone (MESP) agar plates composed of 30 $g.L^{-1}$ malt extract (Biolab, Merck), 3 $g.L^{-1}$ soya peptone (peptone from soybean, enzymatic digest, Sigma-Aldrich), and 15 $g.L^{-1}$ bacteriological agar (Biolab, Merck), and was subcultured monthly. Glycerol stocks (10% $v.v^{-1}$ glycerol) of harvested fungal spores were also prepared and stored at -60 °C.

3.2 Cultivation media

Throughout this study *P. purpurogenum* DSM 62866 was cultivated using a variety of solid and liquid medium types to evaluate the impact of different nutrient sources, and their concentration, on growth and pigment production. Solid media composition is detailed in Table 3.1, while the composition of various liquid media used is provided in Table 3.2.

Table 3.1 Composition of agar plate media used for the cultivation of *P. purpurogenum* in this study.

Agar plate medium	**Composition (g.L^{-1})**								**Notes**
	Agar	Glucose	Malt extract	Marsh. / Choc.	Oat flakes (boiled)	Peptone	Yeast extract	Soya peptone	
MESP	15	-	30	-	-	-	-	3	Recommended for cultivation of *P. purpurogenum* DSM 62866 (DSMZ, 2014)
meSP	15	-	10	-	-	-	-	3	
MEsp	15	-	30	-	-	-	-	1	
MESPG	15	10	30	-	-	-	-	3	
MESPY	15	-	30	-	-	-	10	3	
MESPGY	15	10	30	-	-	-	10	3	
MEP	15	-	30	-	-	3	-	-	
MEYE	15	4	10	-	-	-	4	-	
MEA	15	20	20	-	-	1	-	-	Wang et al. (2005)
Oat flake agar	15	-	-	-	30	-	-	-	
Confectionery	15	-	-	15	-	-	-	-	Marshmallow or chocolate confectionery, dissolved and solidified using agar
Potato dextrose agar	Prepared as per manufacturer's instructions								

The majority of experiments involved cultivation of *P. purpurogenum* on the malt extract, soya peptone (MESP) medium recommended by the DSMZ culture collection from which it was obtained (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], 2007). This included both agar plate and liquid cultivation studies, where the bacteriological agar component was omitted. The fungus was also cultivated on agar plates with the MESP medium composition modified by either adjusting the concentration of the malt extract or soya peptone, replacing soya peptone with peptone of animal origin (peptic digestion of animal tissue), or by including glucose and/ or yeast extract as additional medium components. Agar plates of other compositions typically used for the cultivation of fungi were also investigated.

Some liquid cultivation studies required the application of a buffer to the cultivation medium in order to maintain the pH at a selected value, as noted in Table 3.2. Following on the work of Ogihara et al. (2000), the buffers considered in this study were acetic acid (glacial, 100%) – sodium acetate (further referred to as acetate buffer), citric acid monohydrate – trisodium citrate dihydrate (citrate buffer), and sodium dihydrogen phosphate – disodium hydrogen phosphate (phosphate buffer), all applied at 50 mM concentration.

Table 3.2 Composition of liquid media used for the cultivation of *P. purpurogenum* in this study.

Liquid medium	**Composition ($g.L^{-1}$)**								**Notes**
	NH_4NO_3	Glucose/ Maltose	Malt extract	Marsh-mallow	Sodium nitrate	Soluble starch	Soya peptone	Yeast extract	
MESP	-	-	30	-	-	-	3	-	Recommended by DSMZ culture collection (DSMZ, 2007), w/o agar. pH controlled through buffer application, as stated.
SYN	3	-	-	-	-	20	-	2	Soluble starch filter sterilized and added to other, autoclaved, components. pH 5, 50 mM citrate buffer.
ME:SP ratio	-	-	Variable	-	-	-	Variable	-	Impact of altering ratio of ME:SP investigated. See Table 6.1, Table 6.2 for full composition list. pH 5, 50 mM citrate buffer.
Half MESP	-	-	15	-	-	-	3	-	pH 5, 50 mM citrate buffer.
Half MESP + Glucose/ Maltose	-	15	15	-	-	-	3	-	
Marsh.	-	-	-	15	-	-	-	-	Evaluation of confectionery stream as substrate. With or without (w/o) 50 mM citrate buffer, pH 5 See Table 6.8 for full composition list.
Marsh. + SP	-	-	-	15	-	-	Variable (as stated)	-	
Marsh. + $NaNO_3$	-	-	-	15	Variable (as stated)	-	-	-	

All medium and buffer components, with the exception of soya peptone (Sigma-Aldrich), were obtained from Merck (Appendix A).

3.3 Cultivation method: Inoculation procedure, monitoring and evaluation

Agar plate cultivation and various forms of liquid cultivation were used in this study to evaluate culture conditions best suited to the growth and pigment production of this fungal strain. The various cultivation methods required different inoculation procedures, culture monitoring and evaluation, as described below.

3.3.1 Agar plate cultivation

Initial evaluation of the effect of cultivation temperature and medium composition on growth and pigment production was performed using agar plate cultivation. Spot-inoculation was used in this case, with a sterile pointed instrument being touched onto the surface of a 7-day old MESP *P. purpurogenum* plate culture and then onto a freshly prepared agar plate of required composition. The effect of cultivation temperature was evaluated using MESP agar plates, while the range of agar plates used to evaluate the impact of altered medium composition are listed in Table 3.1. Visual monitoring was used for evaluation of growth and pigment production on agar plates, with pictures taken to allow later comparison of the various incubation conditions. The diameter of the resulting colony after the required number of incubation days was recorded as a measure of growth in some cases.

3.3.2 Multiwell plate liquid cultivation

Liquid cultivation in multiwell plates was used as an in initial screening method for sets of experiments requiring evaluation of large numbers of conditions. These included selection of pH and buffer conditions during liquid cultivation, investigating the impact of altering the ratio of malt extract and soya peptone in the cultivation medium, and evaluating supplementation of the confectionery substrate.

3.3.2.1 Culture conditions

Multiwell plate cultivations were performed in 12-well multiwell plates (Greiner Bio-one) using a 2 mL medium volume in 3 mL wells. Triplicate wells were used for each medium composition or condition evaluated and the plates were sealed with Parafilm M ® (Bemis) to minimise evaporation. In order to account for evaporation, the weight of the multiwell plate after inoculation was noted and monitored daily. Weight loss due to evaporation was corrected by adding sterile deionised water at discrete intervals, with the total volume divided equally between the wells.

3.3.2.2 Inoculation procedure

Multiwell plate cultivations were inoculated using either a 10 % ($v.v^{-1}$) growing culture inoculum or directly using a harvested spore solution to yield an initial spore concentration of $1x10^5$ $spores.mL^{-1}$. The growing culture inoculum was prepared as a shake flask culture using 100 mL MESP medium in a 500 mL Erlenmeyer flask. The medium was inoculated with a spore solution to yield a starting spore concentration of $1x10^5$ $spores.mL^{-1}$, and was incubated on an orbital shaking platform (MRC Ltd.) in a 30 °C temperature-controlled room, with shaking at 125 rpm. After 3 days the culture was used to inoculate the wells by adding 200 µL of culture to 1.8 mL of selected medium.

Preparation of the spore solution used to inoculate the shake flask growing culture inoculum involved harvesting *P. purpurogenum* spores from 7-day old MESP agar plates into sterile deionised water (dH_2O) or culture medium, determining the spore concentration by spore counting using a Neubauer Improved counting chamber (Marienfeld), and calculating the volume required for inoculation, based on an initial spore concentration of $1x10^5$ spores.mL^{-1}. This is the same procedure followed for direct spore inoculation. The details regarding spore counting and calculations are provided in Appendix B.1.

3.3.2.3 Culture monitoring and evaluation

pH and growth

Following inoculation, the plates were incubated at 30 °C, with shaking. The pH of the wells was monitored daily using a pH meter (Cyberscan 2500 pH meter), with care taken to maintain the monoseptic nature of the cultivation. This allowed pH trends to be tracked and compared, while in the case of the pH – buffer experiments this also provided an indication of the ability of each buffer to maintain the pH at the desired value. The buffering capacity of each buffer at each pH condition was assigned a numerical rating between 1 and 4, as explained in Table 3.3. Growth achieved under each condition was evaluated by determining the average cell dry weight (CDW) of triplicate wells. The entire culture volume in each well was vacuum-filtered onto a pre-dried and weighed 0.45 µm cellulose nitrate membrane filter (Sartorius Stedim Biotech) before being dried to constant weight at 80 °C (EcoTherm LABOTEC oven). The change in weight of the filter, due to biomass, was then used to calculate the biomass concentration achieved. The detailed procedure for CDW determination is provided in Appendix B.2. Given the sacrificial nature of the sampling procedure these were end-point values, unless replicate multiwell plates were inoculated at the start of the cultivation for continued monitoring.

Table 3.3 Rating system for buffering capacity in multiwell plate liquid cultures.

Buffering capacity rating	pH control observed	Explanation
1	Little to none	Shifts of >1 pH unit from desired value
2	Average	Shifts of approximately 1 pH unit
3	Good	Shifts of approximately 0.5 pH units
4	Excellent	No shift from desired value

Pigmentation

Pigment production in multiwell plate liquid cultures was evaluated visually, with the pigmentation observed given a numerical rating between 1 and 4. A description of the rating system used is provided in Table 3.4.

Table 3.4 Rating system used to describe pigment production in multiwell plate liquid cultures.

Pigmentation rating	Description	Colour example
1	No pigmentation observed	
2	Pale pink to pink	
3	Red	
4	Deep red	

3.3.3 Shake flask cultivation

Shake flask cultivation was used in a number of experiments including those investigating the effect of temperature, pH and the application of a buffer, shaking platform speed, and altering medium composition.

3.3.3.1 Culture conditions

Shake flask cultivations were performed in Erlenmeyer flasks, in general making use of a liquid volume equal to one fifth of the flask volume. This included cultivations of 50 mL medium volume in 250 mL flasks, and 100 mL medium in 500 mL flasks. The flasks were stoppered using self-made cotton wool bungs. Following inoculation, flasks were incubated at 30 °C, unless otherwise specified, and were placed on an orbital shaking platform (MRC Ltd.) in a temperature-controlled room or in an orbital shaking incubator (Labcon) for the duration of the cultivation period, which was 5 to 6 days. The shaking speed used was 150 rpm, unless otherwise specified.

3.3.3.2 Inoculation procedure

Shake flask cultures were inoculated directly with a spore solution to yield an initial spore concentration of $1x10^5$ spores.mL^{-1}. The use of direct spore inoculation rather than a growing culture inoculum was based on the reduced time required for the inoculum train, as well as improved standardisation between experiments. Direct spore inoculation is described in Appendix B.1.

3.3.3.3 Culture monitoring and evaluation

Cultivation in shake flasks was monitored through daily sampling, which involved removing a 1 mL sample of the growing culture from each flask and placing it in a 2 mL microcentrifuge tube (SSIbio). All sample tubes were then placed in the microcentrifuge (Centrifuge 5418 R with FA-45-18-11 rotor, Eppendorf) and were spun at 13000 rpm for 10 minutes which, in the majority of cases, was sufficient to cause collection of biomass at the bottom of the tube, but was insufficient to result in complete separation of biomass and the supernatant (i.e. pelleting of the biomass).

Pigmentation

After centrifugation, 200 µL of the supernatant was removed from each microcentrifuge tube for analysis of pigmentation. Pigment production was evaluated indirectly by measuring the absorbance of cell-free supernatant at 500 nm (A_{500}), which relates to red colouration. In order to confirm maximum absorbance at 500 nm, a spectral scan (A_{400} – A_{800}) of the supernatant was performed at each sampling point, with the absorbance at each wavelength measured and recorded. The supernatant samples were placed in the wells of a 96-well multiwell plate (Greiner Bio-one) and absorbance was measured within the UV-visible light range using a spectrophotometer (FLUOstar Omega microplate reader, BMG Labtech). If required, samples were diluted such that the maximum absorbance was below 1 OD unit. Absorbance of all samples was blanked against the cultivation medium used or an appropriate dilution thereof.

pH and growth

The remaining volume in the microcentrifuge tube was then used for further analysis of the cultivation. The pH was measured using a pH meter (Cyberscan 2500 pH meter) and growth was evaluated by determining the CDW of the sample (Appendix B.2).

3.3.4 Bioreactor cultivation

Cultivation of *P. purpurogenum* DSM 62866 was scaled up to 5 L working volume in a 7 L BioFlo 110 Modular Benchtop Fermentor (New Brunswick Scientific) which allowed cultivation temperature, agitation speed (rpm) and aeration rate to be controlled, while also performing online monitoring of pH and percentage dissolved oxygen (dO_2) concentration.

The cultivation temperature was monitored using a temperature probe placed within the thermowell housing which was in contact with the *P. purpurogenum* culture. Temperature was controlled through an internal cooling coil with circulating cooling liquid, and an external heating blanket (New Brunswick Scientific). Agitation was achieved through the use of a single central impeller shaft, with two 6-bladed Rushton-type impellers (60 mm diameter) placed 25 mm and 75 mm, respectively, from the bottom of the impeller shaft, driven by an agitator motor (Model C32-E-450X, Magmotor Corporation). Pre-determined values for temperature and agitation speed were set, monitored, and controlled through the BioFlo 110 Primary Control Unit (PCU). Aeration rate was controlled by a rotameter and the air inlet stream was passed through a 0.2 µm membrane filter (Millipore) to prevent contamination of the culture.

Online monitoring of the pH and dO_2 concentration of the system was achieved using the BioFlo 110 PCU. The data was also logged automatically using the New Brunswick Scientific BioCommand software. The culture pH and dO_2 concentration were measured using a pH probe (Mettler Toledo 405-DPAS-SC-K8S pH probe), and dO_2 electrode (Mettler Toledo, InPro 6110) respectively. Agitation speed could also be regulated within a set range using the cascade control of the BioFlo 110 system in order to maintain a minimum required dO_2 concentration.

Pressure build-up in the fermentor was prevented by allowing gas to leave the system. To prevent evaporation, this exhaust gas stream was passed through a condenser cooled using a room temperature water stream. The exhaust gas was also filtered using a self-made cotton wool depth filter and a 0.2 µm membrane filter (Millipore), to prevent spores leaving the system. The depth filter functioned in improving the length of usage of the absolute filter.

Aseptic culture sampling was enabled by the sampling assembly which included a sampler tube submerged in the culture, an external sample bottle, and a syringe used to draw the culture sample into the bottle.

A base case set of conditions for bioreactor cultivation was determined based on the results of agar plate, multiwell plate, and shake flask cultivations and was performed using the malt extract, soya peptone (MESP) medium described previously (Table 3.2), with modifications to the base case involving changes to cultivation parameters or medium composition. Cultivation runs were performed in duplicate for each set of conditions, with each cultivation lasting 6 days.

3.3.4.1 Reactor and medium preparation

Reactor preparation included cleaning and assembly, electrode calibration, autoclaving the system, and setting the cultivation parameters, and is described in detail in Appendix C.1. Five litres of the required cultivation medium was prepared, with 4.5 L placed in the reactor vessel prior to autoclaving and 0.5 L autoclaved separately to be used for spore harvesting and inoculation. Antifoam 204 (Sigma-Aldrich), used at a concentration of 250 $\mu L.L^{-1}$, was also added to the medium prior to autoclaving. The fully assembled reactor system is shown in Figure 3.1, with associated equipment demonstrated in Figure 3.2.

Figure 3.1 New Brunswick Scientific BioFlo 110 modular benchtop fermentor system used for 5 L working volume cultivation of *P. purpurogenum*. The reactor is shown, with agitator motor, pH and dO2 probes and heating jacket visible.

Figure 3.2 Duplicate New Brunswick Scientific BioFlo 110 reactors, with associated PCUs and common data logging and coolant circulation systems.

3.3.4.2 Determination of volumetric mass transfer coefficient (k_La)

The volumetric mass transfer coefficient, or k_La value, as a function of agitation and aeration rate in the New Brunswick Scientific BioFlo 110 benchtop bioreactor was determined by the gassing out method, prior to inoculation of the cultivation medium.

The dO_2 probe was first calibrated, by sparging with nitrogen to obtain a 0 % reference and sparging with air to obtain a 100 % dO_2 concentration set point. Following calibration, the oxygen concentration in the medium was decreased to 0 % once again by sparging with nitrogen. The system was then sparged with air at the required aeration and agitation rates, and the increase in dO_2 concentration with time was recorded. This was performed using online monitoring of the bioreactor system, with values recorded every 15 seconds.

The relationship between oxygen transfer rate (OTR), k_La and the concentration driving force was provided in Equation 5. Integrating this to give Equation 8 it is seen that a plot of $\ln(1 - \frac{C}{C^*})$ as a function of time should give a straight line with slope of $-k_La$. This was performed in duplicate for each agitation speed considered.

$$\ln(1 - \frac{C}{C^*}) = -k_La.t \qquad \text{Equation 8}$$

where C is oxygen concentration in the liquid ($mg.L^{-1}$ / %)
C* is oxygen saturation concentration in the liquid ($mg.L^{-1}$ / %)
and t is time

The maximum OTR can then be calculated using the k_La value and the saturation dissolved oxygen concentration, as demonstrated below.

$$OTR_{max} = k_La.(C^* - C) \qquad \text{Equation 9}$$

where (C* - C) is concentration-difference driving force, where
C* is equal to 7.539 $mg.L^{-1}$ (or 0.236 $mmol.L^{-1}$),
under 1 atm air pressure at 30 °C,
assuming 100 % oxygen saturation (EIFAC, 1986),
and C tends towards 0

3.3.4.3 Inoculation procedure

Inoculation of the system followed reactor sterilisation and stabilisation of the cultivation parameters at the desired values. Direct spore inoculation was used once again to achieve an initial spore concentration of $1x10^5$ $spores.mL^{-1}$, however, some adjustments were made to the inoculation procedure. The spores were harvested into the cultivation medium and were counted to determine the

volume of spore solution required for reactor inoculation. Before inoculation additional cultivation medium was added to the calculated volume of spore solution to bring the inoculum volume to 500 mL, which was the volume required to increase the total cultivation volume to 5 L. This inoculation procedure is outlined in Appendix C.2.

3.3.4.4 Culture monitoring and evaluation

In addition to the online monitoring and logging of temperature, culture pH and dO_2 concentration described in Section 3.3.4, evaluation of pH, growth, pigmentation and utilisation of medium components was also performed through manual sampling of the system. Samples were taken from the system twice daily, with one sample taken every 24 hours from the time of inoculation and the second sample taken 6.5 hours later. The culture was withdrawn from the reactor using the sampling assembly, with three separate samples taken at each sample point, and the total volume removed ranging between 12 and 40 mL.

pH and growth

The pH of the withdrawn samples was measured (Cyberscan 2500 pH meter) and compared to the online pH value, and a 1 mL volume from each of 3 separate samples was used for CDW determination (Appendix B.2). The culture was also viewed under magnification in order to determine whether any obvious morphology changes were linked to observed pH shifts or the onset of pigment production. A 20 µL sample of appropriately diluted culture was placed on a microscope slide in order to prepare a wet mount. The sample was then viewed at magnifications ranging from 100 to 1000 times using an Olympus BX40 microscope, with images of the fungal biomass captured using the attached camera (Olympus U-TV0.5XC adapter, ColorView soft imaging system camera), and analysed using the analySIS® FIVE Digital Imaging Solutions software. The remaining volume was then filtered through a 0.45 µm cellulose nitrate filter (Sartorius Stedim Biotech) under vacuum, and the resulting filtrate was used for further analysis.

Pigmentation

Absorbance of the culture filtrate at 500 nm (A_{500}) was used as a measure of pigmentation and was monitored as described for the shake flask cultivation supernatant in Section 3.3.3.3, with a spectral scan performed over the UV-visible light range of 400 – 800 nm. This analysis of pigmentation was performed in triplicate at each sampling point.

Utilisation of medium components

Additional monitoring of bioreactor cultivations was performed as a result of the increased sample volume available for analysis, particularly the utilisation of supplied medium components by *P. purpurogenum* as the cultivation progressed. At each sampling point, samples of the culture filtrate were aliquoted into 2 mL microcentrifuge tubes (SSIbio) and were immediately placed at minus 20 °C, frozen for later analysis. Once the bioreactor cultivation run was completed, the entire set of samples

was analysed simultaneously for residual sugar concentration using either the sulphuric acid-UV method or High Pressure Liquid Chromatography (HPLC).

Carbohydrate quantification by the sulphuric acid-UV method

As a result of the absorbance in the visible light range of the pigmentation produced by *P. purpurogenum*, the use of carbohydrate quantification assays which rely on colour changes and measurement of resulting absorbance in the range of 400 to 600 nm was excluded. A method relying on UV absorbance of hydrolysed sugar samples was, therefore, selected for investigation (Albalasmeh et al., 2013). The method measures the absorbance in the UV range of furfural derivatives produced when carbohydrate compounds are hydrolysed using concentrated sulphuric acid. Absorbance of the hydrolysed samples was measured at 316 nm using a UV spectrophotometer (Thermo Scientific Genesys 10S UV-vis Spectrophotometer) with the samples placed in a quartz cuvette (High precision cell, Quartz SUPRACIL®, Hellma Analytics) and blanked against dH_2O.

The concentration of sugars present in the cultivation samples was calculated using a calibration curve generated by applying the assay to sugar samples over a range of known concentration. As the main sugar present in the MESP medium used during bioreactor cultivation was maltose (Neogen Corporation, 2009; Merck, 2016), this sugar was used to generate the calibration curve, and the residual sugar concentration in the cultivation samples was expressed in terms of maltose-equivalent units ($g.L^{-1}$).

As a result of interference caused by protein present in samples of the culture filtrate, the assay was preceded with a protein precipitation step. This protein precipitation method was originally described as a means of deproteinising blood before evaluating blood sugar concentrations (Somogyi, 1945), but has since been applied to extracts of malted barley prior to performing carbohydrate determination (Holmes, 1991), supporting its use in this study. The precipitation makes use of barium hydroxide and zinc sulphate which react to form barium sulphate, which precipitates, and zinc hydroxide, which precipitates the proteins in the sample (Poon, 2006). The protein precipitation and sulphuric acid-UV assays are described in detail in Appendix B.3.1 and Appendix B.3.2, respectively.

Quantification of individual sugars using High-Performance Liquid Chromatography (HPLC)

Sucrose, glucose and fructose were quantified by HPLC during bioreactor cultivations using the marshmallow-based medium. Sugars were quantified using a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm, 9 µm) coupled to an RI detector. Elution was performed using 5 mM H_2SO_4 as the mobile phase, at a flow rate of 0.6 $mL.min^{-1}$ for 15 minutes at 65 °C.

Calibration curves for the sugars of interest were constructed over a concentration range of 0 – 1 $g.L^{-1}$, with samples containing unknown concentrations diluted appropriately. Data was analysed using Chromeleon Chromatography Data System software (Thermo Scientific).

3.4 Kinetics and yield calculations during liquid cultivation

In order to compare conditions within, as well as between, cultivations the kinetics and yields of biomass and pigment production were determined as a function of culture conditions. Evaluation was dependent on the variables measured in each cultivation system.

Biomass and pigment formation kinetics were evaluated in shake flask and bioreactor cultivations, with the growth rate (dX/dt), specific growth rate (μ or lnX/t), rate of pigment formation (dP/dt) and specific pigment formation (dP/dX) evaluated by least squares regression. Least squares regression analysis was performed using the Microsoft Excel statistical array function LINEST. This function provides outputs in the form of an array, as demonstrated in Table 3.5.

Table 3.5 Output of the Microsoft Excel statistical array function LINEST

Slope (m)	Intercept (b)
Standard error associated with the slope (SE_m)	Standard error associated with the intercept (SE_b)
r^2	Standard error associated with the y estimate (SE_{est})
F statistic	Degrees of freedom (d_f)
Regression sum of squares (SS_{reg})	Residual sum of squares (SS_{res})

Slope calculated by least squares regression is obtained using Equation 10:

$$m = \frac{n\sum(xy) - \sum x \sum y}{n\sum x^2 - (\sum x)^2} \qquad \text{Equation 10}$$

The total, residual and regression sum of squares values are obtained as follows:

$$SS_{total} = \sum (y_i - \bar{y})^2 \qquad \text{Equation 11}$$

$$SS_{res} = \sum (y_i - y_{est})^2 \qquad \text{Equation 12}$$

$$SS_{reg} = SS_{total} - SS_{res} \qquad \text{Equation 13}$$

where y_i is the individual y values in the data set

$\bar{y}$ is the mean of the y values in the data set

y_{est} is the estimated y value at each point based on regression analysis

The standard error associated with the estimate and the slope are then calculated as shown below.

$$SE_{est} = \sqrt{\frac{SS_{res}}{n-2}} \qquad \text{Equation 14}$$

$$SE_m = \frac{SE_{est}}{\sqrt{\sum (x_i - \bar{x})^2}} \qquad \text{Equation 15}$$

where n is the number of data points
x_i is the individual x values in the data set
$\bar{x}$ is the mean of the x values in the data set

These growth and pigment formation rates, evaluated in selected shake flask and bioreactor cultivations, are defined by the slope value for a specified selection of x and y values in the total data set. Replicate cultivations under specified culture conditions are evaluated separately, with the average rate reported. Standard deviation reported along with these values is calculated as described in Section 3.6.

The monitoring of residual sugar concentration throughout bioreactor cultivation also allowed the calculation of various yields, as outlined below:

$$Y_{X/S} = \frac{\text{Biomass produced (g)}}{\text{Substrate consumed (g)}} \qquad \text{Equation 16}$$

$$Y_{P/X} = \frac{\text{Product (pigment) produced (OD units)}}{\text{Biomass produced (g)}} \qquad \text{Equation 17}$$

with $Y_{X/S}$ calculated over a defined period, and
$Y_{P/X}$ calculated for a specified sample point (generally at maximum biomass and pigment concentrations)

The yield of biomass on substrate was calculated over rolling 24 hour periods, beginning at each sampling point, with 2 samples taken daily (e.g. sample 1 – 3, 2 – 4, 3 – 5, etc.). The average yield over a specified period was then calculated. Yields in replicate cultivations were evaluated separately, before calculating the average $Y_{X/S}$ value and associated standard deviation (Section 3.6).

3.5 Pigment characterisation

Monitoring of the pigmentation produced by *P. purpurogenum* was based on the absorbance spectrum of the cell-free culture broth. Biomass was separated from the spent medium and products through either centrifugation or filtration (0.45 µm cellulose nitrate filter, Sartorius Stedim Biotech). Absorbance at 500 nm was used as a measure of the red pigment production during submerged liquid cultivation,

as this wavelength represents the peak of absorbance of a red solution (Section 2.4.1). The absorbance spectrum, and the position of the various absorbance peaks within it, exhibited by the culture sample can, however, also be used as a tool for identification of the pigment compounds based on previous reports in literature; hence absorbance scans were collected in the UV-visible light range between 400 and 800 nm.

In order to analyse the pigmentation produced, beyond the absorbance spectrum, the pigment compounds were separated from the culture broth using solvent extraction. Selected solvent extracts were then subjected to thin layer chromatography (TLC) in order to provide an indication of whether the observed pigmentation results from a single pigment compound or a pigment mixture. These techniques formed part of the preliminary pigment characterisation and are detailed in Section 3.5.1 and Section 3.5.2. Large-scale extraction, following bioreactor cultivation, allowed further characterisation of the pigment products, with both the solvent extract and aqueous phase remaining after extraction subjected to analysis. Section 3.5.3 describes the techniques applied, which included rotary evaporation and various chromatography and spectroscopy techniques.

3.5.1 Solvent extraction

Liquid-liquid extraction using water immiscible solvents was selected as the means of separating the pigment compounds from the culture filtrate. Ethyl acetate had previously been reported as an effective solvent for the extraction of *Monascus* pigment homologues produced by *P. purpurogenum* in liquid culture (Ogihara et al., 2000b; Arai et al., 2012). Extraction using this solvent was compared to that observed using other water-immiscible solvents including 1-butanol, chloroform, dichloromethane, diethyl ether, petroleum ether, and toluene. The solvent miscibility table consulted for selection of water-immiscible solvents across a range of chemical groups is provided in Appendix D.

The extraction procedure involved adding 5 mL culture filtrate and 2.5 mL selected solvent to a test tube. This improved ease of identification of the separate phases. The test tubes were vortexed for 30 seconds to ensure the contents were well-mixed and were then allowed to stand at room temperature until 2 clear phases were visible. Extraction was evaluated through visual comparison of the resulting colour of the organic phase, and was given a rating between 1 and 4. These ratings are explained in Table 3.6. Pictures of the resulting extraction mixtures in test tubes were also taken to allow later comparison of the extraction achieved.

Table 3.6 Rating system used to describe extraction using water-immiscible solvents.

Extraction rating	Description
1	No colour visible in organic phase
2	Organic phase pale red and lighter than aqueous phase
3	Organic phase red, colour equivalent or slightly darker than aqueous phase
4	Organic phase darker in colour than aqueous phase

All solvents, with the exception of dichloromethane (Sigma-Aldrich) were obtained from Merck.

3.5.2 Thin Layer Chromatography (TLC)

The two solvents which resulted in extraction of the most colour from the culture filtrate (evaluated visually) were used for further investigation of the colouring compounds produced by *P. purpurogenum*. Following extraction, the organic phase was subjected to TLC analysis in an attempt to separate different pigment components in the solution, where more than one colouring compound was present. TLC analysis was performed by loading the extract sample onto a TLC plate (Macherey-Nagel GmbH & Co. KG, ALUGRAM® pre-coated TLC sheets, 0.20 mm silica gel 60), allowing the sample to dry completely through evaporation of the solvent, placing the plate in a TLC chamber containing the selected solvent mobile phase, and allowing the solvent to move up the plate, thereby separating out the components in the extract. This process is described in detail in Appendix B.4.

The first TLC experiment performed was based on the work of Ogihara et al. (2000), where the pigment components in an ethyl acetate extract of *P. purpurogenum* culture filtrate were separated using a mobile phase consisting of *n*-butanol/ acetic acid/ water in the ratio 12:3:5. Further experimentation involved changes to the mobile phase, in an attempt to improve separation of the components of the extract. As it has been reported that the ideal solvent for separation can be identified by manipulating the polarity of the mobile phase (Coker and Ayoola, 2008), the types of mobile phase applied to the system included various ratios of chloroform/ methanol as a result of their differing polarity, and a number of other relatively polar solvents, including ethyl acetate and 1-butanol.

Once effective separation of the components was achieved, the retention factor (or R_f value) for the various components was calculated based on the ratio of the distance travelled by the component and the distance travelled by the solvent. This R_f value can be compared to values reported in literature as an indication of whether the same products have been obtained in separate studies.

3.5.3 Large scale extraction and characterisation

In order to obtain sufficient pigment sample for the characterisation techniques detailed below, *P. purpurogenum* DSM 62866 was cultivated under base case conditions in the New Brunswick Scientific BioFlo 110 fermentor, as described in Section 3.3.4. The colouring compounds were then extracted from the resulting cell-free culture broth using ethyl acetate (Section 3.5.3.1), before further characterising the resulting extract and the compounds remaining in the aqueous phase (Sections 3.5.3.2 and 3.5.3.3)

3.5.3.1 Ethyl acetate extraction

The biomass produced during cultivation in the BioFlo 110 bioreactor was separated from the spent medium and products through centrifugation (Avanti J-E Centrifuge with JA-10 rotor, Beckman Coulter). The broth was divided into 300 mL volumes and centrifuged at 10000 rpm for 10 minutes before pouring off the supernatant and discarding the cell pellet.

Approximately 4.4 L of cell-free culture broth was then extracted using ethyl acetate. One litre of supernatant was combined with 750 mL of ethyl acetate in a 5 L glass separating funnel. The liquids were well-mixed before being left to separate. The aqueous phase was then tapped off the bottom of the funnel and set aside for re-extraction. The organic layer was also set aside for further processing. The extraction of the aqueous phase was then repeated twice more using the aqueous phase from each previous extraction, with 650 mL and then 600 mL of ethyl acetate added. The overall result was extraction of the supernatant with twice the volume of ethyl acetate, as demonstrated in Figure 3.3. The entire procedure was repeated multiple times to extract the full liquid volume from the bioreactor cultivation, with the supernatant to solvent ratios maintained for bigger or smaller volumes.

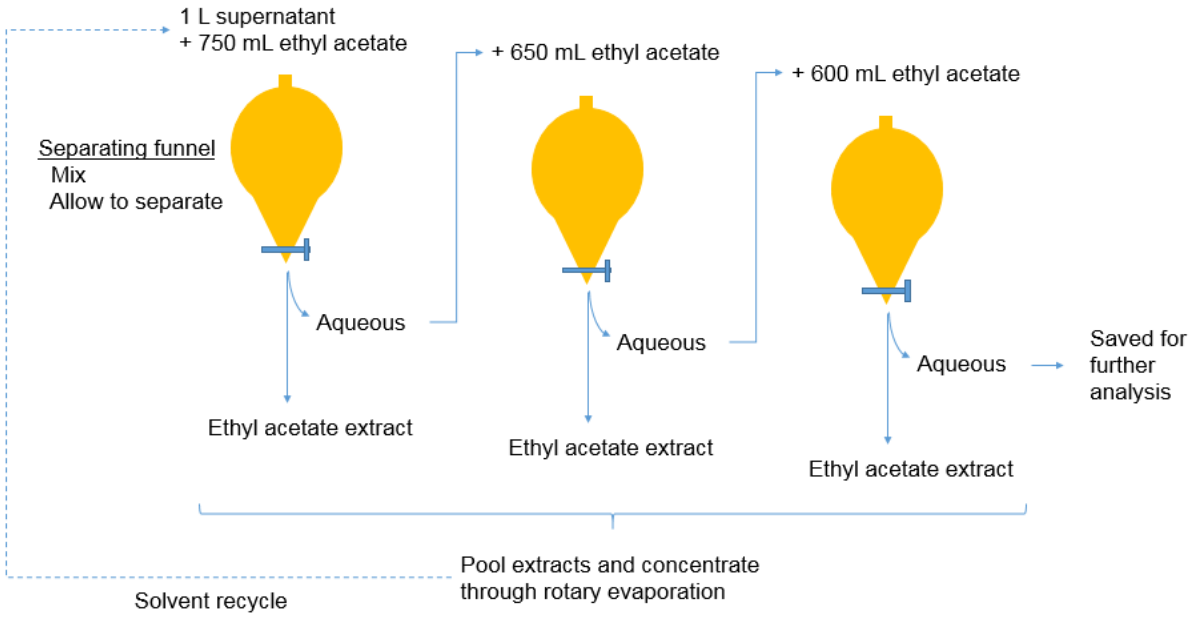


Figure 3.3 Ethyl acetate extraction procedure

Rotary evaporation of the organic extract was performed continually during the extraction procedure to allow reuse of the solvent. This was performed using the Rotavapor R-210 (Buchi, Switzerland) at a temperature of 40 °C. The equipment used during extraction and rotary evaporation is shown in Figure 3.4. Once all the ethyl acetate extracts had been pooled, magnesium sulphate was added to the concentrated liquid to remove any water, before filtering and then concentrating it further into a thick syrup-like liquid. This extraction product was used for further analysis, as detailed in Section 3.5.3.2.

The aqueous phase remaining after ethyl acetate extraction was concentrated using the same equipment (Figure 3.4b), under a higher temperature of 50 °C. Further analysis of the compounds which remain in the aqueous phase is described in Section 3.5.3.3.

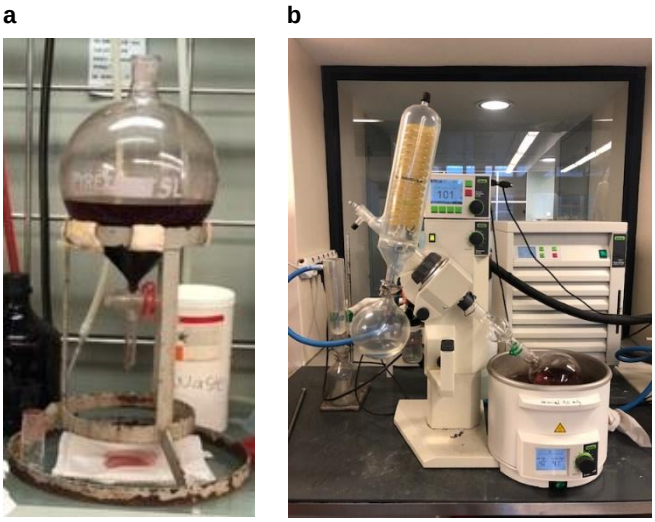


Figure 3.4 Equipment used for extraction (**a**) and rotary evaporation (**b**)

3.5.3.2 Further analysis – organic phase

The concentrated extract was separated into its various components using chromatography techniques. TLC (detailed in Section 3.5.2 and Appendix B.4) was used for visualisation of the components in the extract, and column chromatography was used to separate colouring compounds of interest into individual fractions. Column chromatography was performed using an automated high-performance flash purification system, the Biotage Isolera One (Figure 3.5). The stationary phase in the column was flash silica (Sigma-Aldrich), with 2 mobile phase components fed at a specified ratio throughout the run. Fractions were collected and re-run on TLC to evaluate whether components of interest had been effectively separated from other cell products, before concentrating the selected fractions through rotary evaporation.

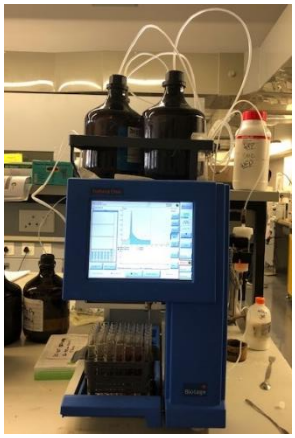


Figure 3.5 Biotage Isolera One system used for column chromatography

These concentrated fractions then contained only red compounds of interest, which were then submitted for liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy. ^{1}H-NMR analysis was performed using a Bruker Avance III 400 spectrometer, with samples dissolved in deuterated chloroform or deuterated water, depending on solubility.

3.5.3.3 Further analysis – aqueous phase

The concentrated aqueous phase was also evaluated using TLC (Appendix B.4), leading to the observation that reversed phase chromatography is likely required to separate the components of this solution. Column chromatography was performed as described in Section 3.5.3.2, with the stationary phase instead being C_{18} reversed phase silica (Sigma-Aldrich). Collected fractions were pooled and concentrated under vacuum before being submitted for high resolution mass spectrometry (Central Analytical Facility, Stellenbosch University).

3.6 Statistical analysis

All statistical analysis was performed using Microsoft Excel. Standard deviation across replicate samples was calculated using Equation 18 for standard deviation of a sample of a population. Where results have been represented graphically, the error bars represent the standard deviation of the measurements.

$$\text{Sample standard deviation} = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i-\bar{x})^2} \qquad \text{Equation 18}$$

where n is the number of data points
x_i is each individual data point, and
$\bar{x}$ is the mean of the data

When required to determine whether the means of 2 sample groups differ significantly, the Microsoft Excel Analysis ToolPak add-in was used to perform an F-Test and t-Test with the data. The F-Test (two sample for variances) is performed in order to determine whether the variances of the 2 groups differ significantly, with the null hypothesis being that the variances of the 2 groups are equal. After performing the F-Test, if the F value for the data is greater than the $F_{critical}$ value, the null hypothesis is rejected. This is followed by the t-Test, two sample assuming equal/ unequal variances, depending on the result of the F-Test. The t-Test is performed to determine whether the means of the 2 groups differ significantly, with the null hypothesis being that the means of the 2 groups are equal. If the t_{stat} value for the data is greater than the $t_{critical}$ value (one- or two- tail depending on the requirements of the experiment) or less than $-t_{critical}$, the null hypothesis is rejected, meaning that the means of the 2 groups differ significantly. If, however, the t_{stat} value is within the range of $-t_{critical}$ to $t_{critical}$, the null hypothesis is supported, and the means of the 2 groups do not differ significantly. Statistical significance was determined using a confidence interval of 95 % in all cases.

3.7 Experimental approach

The research approach has been presented in five sections, aligning with objectives of the study. These include investigating the effect of cultivation conditions, scaling up cultivation into a benchtop bioreactor system and evaluating cultivation kinetics, characterising the pigment(s) produced by *P. purpurogenum* and determining the effect of altering medium composition, including the impact of utilising a potential waste stream, namely confectionery, as the main carbon source for cultivation of the organism.

3.7.1 Proof of concept of red pigment production by *P. purpurogenum* DSM 62866 on a complex nutrient source and identification of cultivation conditions which favour pigment production

The medium recommended by the DSMZ culture collection for the cultivation of *P. purpurogenum* DSM 62866 is composed of 30 $g.L^{-1}$ malt extract and 3 $g.L^{-1}$ soya peptone, with the medium pH adjusted to 5.6 prior to autoclaving (DSMZ, 2007). Initial studies, using both agar plate and submerged liquid cultivation, were conducted to demonstrate whether the organism is able to produce pigmentation of interest when grown on this medium.

The impact of selected cultivation conditions were evaluated, determining their effect on both pigmentation and biomass production owing to the combined effect of these on productivity. Parameters considered were cultivation temperature, pH and the application of buffers, and shaking speed during incubation of flask cultures. The impact of cultivation temperature was evaluated using agar plate cultivation, with the plates incubated at room temperature, 30 °C and 37 °C for a period of 14 days. Culture pH during submerged liquid cultivation in multiwell plates and shake flasks was either adjusted to pH 5.6 prior to autoclaving (with no further pH control) or maintained at pH values of 5, 6 or 7, using one of the following buffer systems: acetic acid – sodium acetate, citric acid monohydrate – trisodium citrate dihydrate, or sodium dihydrogen phosphate – disodium hydrogen phosphate buffer, each applied at 50 mM concentration. The final parameter investigated is shaking speed during incubation of flask cultures, to determine the effect of mass transfer and mixing on both pigmentation and growth.

3.7.2 Scale-up of cultivation to benchtop bioreactor scale and investigation of the impact of oxygen availability

The conditions selected to result in improved pigment production by *P. purpurogenum* DSM 62866 were then applied during 5 L batch cultivation in the New Brunswick Scientific BioFlo 110 modular benchtop fermentor. Comparison to shake flask cultivation allowed the impact of cultivation scale on growth and pigment production to be evaluated. Further, the results of media screening experiments (Section 3.7.5) were validated at the bioreactor scale under conditions of enhanced reproducibility.

The improved control of cultivation parameters in the bioreactor system also allowed further investigation of the impact of oxygen availability on growth and pigment production. The initial approach was to not adjust agitation rate during the cultivation, thereby allowing the residual oxygen concentration in the reactor to reach 0 % (based on a calibration of nitrogen and air). The second approach was to

conduct a series of reactor cultivations at discrete agitation rates and to evaluate the growth and pigment production in response to changing the rate of oxygen transfer into the medium. This allowed the effect of residual oxygen concentration and oxygen transfer rate to be compared.

3.7.3 Evaluation of cultivation kinetics

The larger volume available for sampling in the BioFlo 110 reactor system allowed more frequent monitoring of growth and pigment production and evaluation of residual sugar concentration throughout the cultivation period, thereby allowing kinetic analysis. This enabled determination of various biomass and pigment yields and productivities as well as investigation of whether pigment production is growth associated. Process kinetics were used to compare cultivations performed under varying conditions or using different medium compositions.

3.7.4 Characterisation of the pigmentation produced

In order to characterise the pigments produced by *P. purpurogenum* DSM 62866, these compounds need to be isolated from the culture broth. Various solvents were investigated to evaluate their potential for extracting red pigments from cell-free filtrate or supernatant. Ethyl acetate was selected for large-scale extraction of pigments from a batch cultivation performed in the BioFlo 110 reactor. Chromatography methods were then applied to further isolate and purify the red pigment products. Various methods were used to characterise the components which contribute to the observed pigmentation. These included spectroscopy and mass spectrometry techniques. This was performed with the aim of characterising the partially purified pigment product.

3.7.5 Investigation of the effect of medium composition on growth and pigment production

Further investigation of altering cultivation parameters in order to improve pigment production focused on the impact of medium composition on biomass concentration and specific pigment production rate. As mentioned previously, the recommended medium for cultivation of *P. purpurogenum* is composed of 30 $g.L^{-1}$ malt extract and 3 $g.L^{-1}$ soya peptone.

3.7.5.1 Evaluation of the impact of type of medium components supplied, and their concentration

The composition of the recommended medium was altered by changing the concentration of either component, thereby affecting both the medium concentration and the resultant C:N ratio, with the ratio of malt extract to soya peptone altered in most cases. The effect of adding supplementary medium components was also investigated.

This was performed initially using agar plate and small-scale submerged liquid cultivation. Based on these results, the most promising medium composition was selected for bioreactor cultivation. Biomass and pigment production using this medium were compared to cultivation using the original medium, to validate its benefit.

3.7.5.2 Replication of pigment production in a complex medium formulated using a potential waste stream

The potential for production of pigments by *P. purpurogenum* DSM 62866 on a cheaper or alternative nutrient source was evaluated as a means of improving resource productivity. Confectionery industry waste was selected as a case study with confectionery products used as a consistent feed for proof of concept. Supplementation of the confectionery-based medium with selected nitrogen sources was investigated using small-scale submerged liquid cultivation and biomass and pigment production was validated at bioreactor scale using the New Brunswick Scientific BioFlo 110 fermentor.

Shake flask and bioreactor cultivations using the confectionery-based medium were compared to confirm trends observed during scale-up with the malt extract-based medium. Bioreactor cultivations using the confectionery-based medium and the conventional complex media based on malt extract and soya peptone were also compared, with volumetric biomass and pigment concentrations, rates of production, and yields assessed under each condition.

4 Results and Discussion: Physicochemical parameters

Effect of physicochemical conditions on growth and pigment production of *P. purpurogenum* DSM 62866

Penicillium purpurogenum DSM 62866 was obtained from the DSMZ culture collection, with recommended cultivation conditions of 24 °C and initial adjustment of the medium to pH 5.6 (DSMZ, 2014). This chapter considers the effect of these, and other, parameters on cultivation of the organism.

In Section 4.1 the growth and pigment production response of the organism to changes in temperature and pH were considered, using agar plate, multiwell plate and shake flask experiments. Section 4.2 considers a further system parameter, namely the impact of oxygen availability, through modification of shaking speed during flask cultivations.

Based on observed results, selected parameters were applied to a base case bioreactor cultivation in the New Brunswick Scientific BioFlo 110 system, as reported in Section 4.3. Finally, in Section 4.4 the effect of oxygen availability was investigated further by varying the gas-liquid oxygen transfer rate through altering agitation speed applied during cultivation in the bioreactor system. These results were compared to those obtained under base case conditions.

4.1 Optimisation of temperature and pH conditions for *P. purpurogenum* pigment production

Investigation of the temperature and pH conditions best suited to pigment production by *P. purpurogenum* DSM 62866 was undertaken. This was based on literature which reported changes in growth and pigment production trends of *P. purpurogenum* strains, and other microorganisms, in response to altering these cultivation conditions (Ogihara et al., 2000b; Dufossé et al., 2005; Méndez et al., 2011; Mukherjee and Singh, 2011; Santos-Ebinuma et al., 2014). The impact of cultivation temperature was evaluated using agar plate cultivation and is discussed in Section 4.1.1. Culture pH and the application of buffers was investigated using small-scale liquid cultivation in multiwell plates, with the results provided in Section 4.1.2. In Section 4.1.3 results are presented of shake flask experiments which served as validation of the temperature and pH conditions proposed to be best suited to pigment production.

4.1.1 Cultivation temperature

To investigate the impact of cultivation temperature, *P. purpurogenum* was spot-inoculated onto MESP agar plates and incubated at room temperature (approximately 24 °C), 30 °C, and 37 °C for 14 days. Relative growth and pigment production at these temperatures was evaluated visually, with pictures of the plates taken daily to allow later comparison. Pictures of the growth observed after 7 and 14 days of incubation are provided in Figure 4.1 along with the recorded colony diameter, clearly showing that growth and pigment production by *P. purpurogenum* on MESP agar plates is dependent on cultivation temperature.

A cultivation temperature of 30 °C was shown to cause better growth and pigment production by this organism than incubation at either of the other temperatures considered (room temperature or 37 °C). Growth was observed sooner and also progressed faster when incubated at 30 °C, as shown by the greater colony diameter at this temperature after either 7 or 14 days. Pigment production was also improved when incubated at 30 °C, with red pigmentation observed within the fungal mycelium as well as diffused into the surrounding agar after 14 days. After the same period of incubation at room temperature, red pigmentation was observed within the fungal biomass but not diffused into the agar. Fungal colonies resulting from 37 °C incubation exhibited only a pale brown colour, possibly suggesting a different suite of pigments.

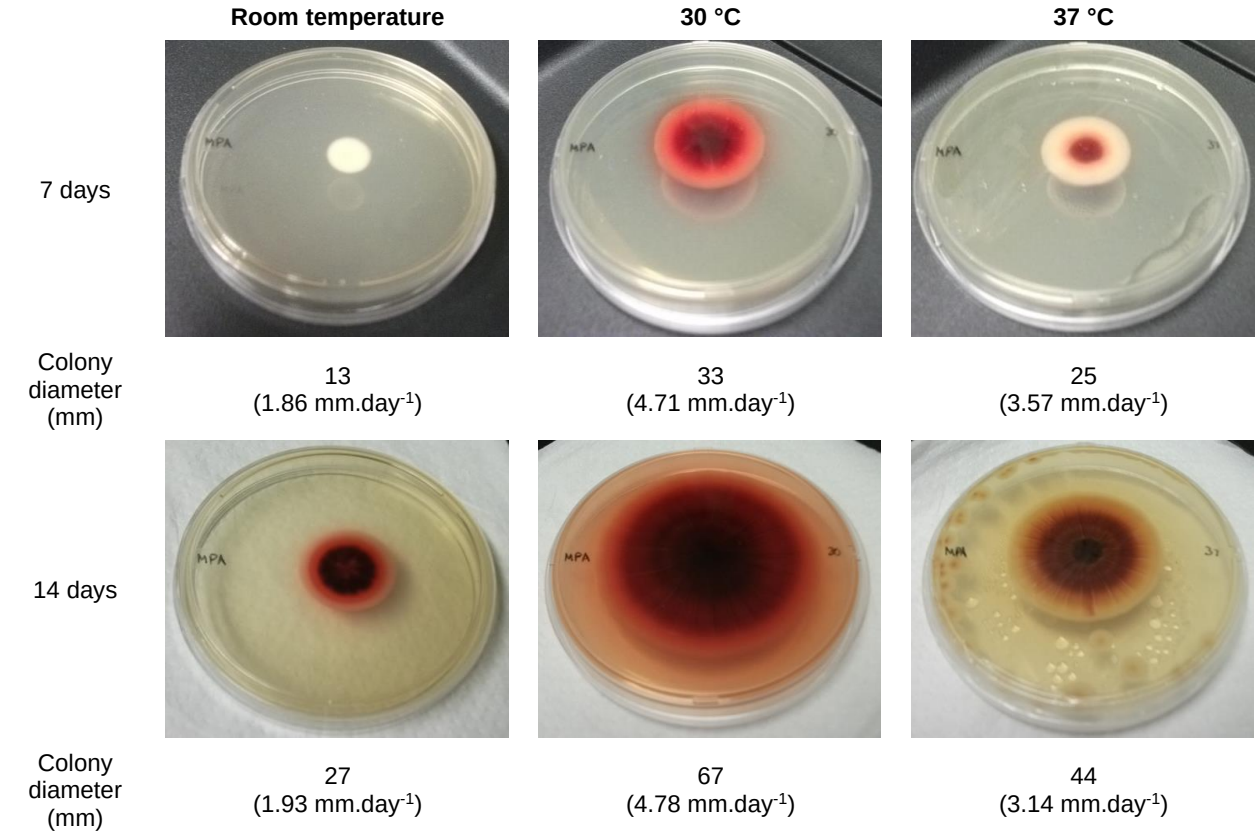


Figure 4.1 Growth and pigment production exhibited by *P. purpurogenum* DSM 62866 on MESP agar plates incubated at room temperature, 30 °C, or 37 °C, after 7 and 14 days. Colony diameter (mm) achieved on the pictured plate is provided as a measure of growth.

Although the recommended incubation temperature for this fungal strain was 24 °C (DSMZ, 2014), the improved growth and pigment production observed at 30 °C compares well with literature, as demonstrated in Table 4.1. Red pigment production by *P. purpurogenum* in liquid culture was investigated at temperatures of 24 °C and 34 °C by Méndez et al. (2011), with better production obtained at 24 °C. Production at 30 °C was not investigated, however, the results align well with those reported in this study, with incubation at room temperature supporting better pigment production than observed at 37 °C. Other studies investigating pigment production by *Penicillium* species compared pigmentation observed during incubation at a greater range of temperatures (Gunasekaran and Poorniammal, 2008; Padmapriya and Murugesan, 2014). Highest levels of red pigment production were reported at 30 °C, followed by 25 °C and then 35 °C, which is consistent with the results of this study.

Table 4.1 Investigation of the effect of temperature on red pigment production by *Penicillium* species. Temperatures are listed in order from most to least pigmentation achieved.

Organism	Cultivation conditions	Temperature (°C)	Reference
Penicillium purpurogenum DSM 62866	Spot-inoculated on MESP agar plate, initial pH 5.6, 14 days	30 > room temperature > 37	This study
Penicillium purpurogenum GH2	40 mL Czapek-Dox modified broth in 250 mL flask, initial pH 5, 200 rpm, 10 days	24 > 34	Méndez et al. (2011)
Penicillium purpurogenum	100 mL yeast phosphate soluble starch medium in 250 mL flask, 7 days	30 > 25 > 35 > 40 > 45	Padmapriya and Murugesan (2014)
Penicillium sp.	25 mL potato dextrose broth in 250 mL flask, 200 rpm, 7 days	30 > 25 > 35 > 15 > 10	Gunasekaran and Poorniammal (2008)

The study by Ogihara et al. (2000), using a *P. purpurogenum* strain to produce *Monascus* pigment homologues, also reported highest growth and pigment production in cultivations performed at 30 °C.

4.1.2 Culture pH and the application of buffers

The impact of pH on growth and pigment production of *P. purpurogenum* was investigated in liquid cultivation experiments using an incubation temperature of 30 °C, identified as being best suited to growth and pigment production of this organism. In the work by Ogihara et al. (2000), where production of a violet pigment by *P. purpurogenum* was investigated, it was reported that stable production required the application of a buffer to maintain the pH at a selected value. In order to investigate whether the same was true of the fungal strain used in this study, initial liquid cultivation experiments were performed in shake flasks using 100 mL volumes of MESP medium, without applying a buffer to the system. These flasks were incubated at 30 °C on an orbital shaking platform for 6 days.

These experiments revealed pH shifts which occurred over the cultivation period, while also providing an indication of the growth that can be achieved using the MESP medium. During the 24 hours following inoculation the culture pH increased from an initial value of approximately 5 to a value greater than 7, and over the next 3 days decreased slowly back to approximately pH 5. Beyond day 4 of the cultivation an increase in culture pH was observed once again. The trend of the pH rising and falling over the first

4 days of the cultivation was followed, inconsistently, with the onset of rapid pigment production. This is shown in Figure 4.2.

Duplicate flasks showed consistent pH and growth trends, both achieving dry weight values of greater than 10 $g.L^{-1}$ by day 5 of the cultivation. Pigment production, however, varied significantly indicating that this strain may require pH control through the application of a buffer for stable production to be achieved, as reported by Ogihara et al. (2000). In order to investigate this, *P. purpurogenum* DSM 62866 was cultivated in MESP medium, buffered to pH values of 5, 6, or 7, by applying a 50 mM citrate, acetate, or phosphate buffer (details regarding buffer composition are provided in Section 3.2).

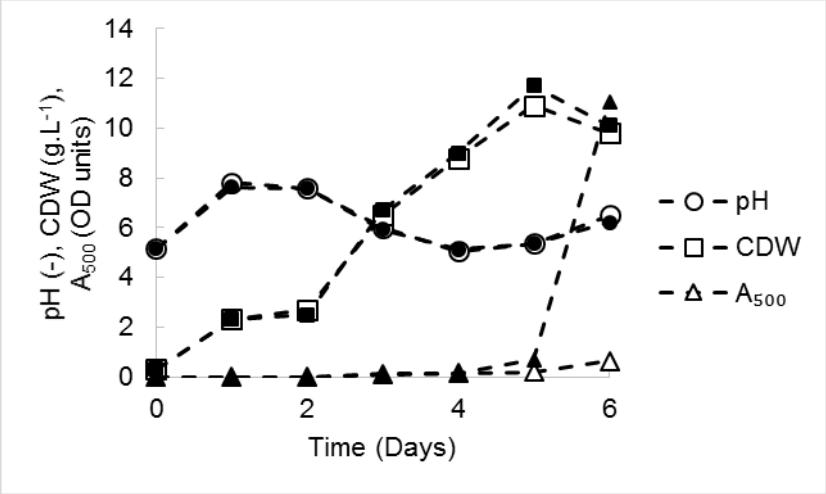
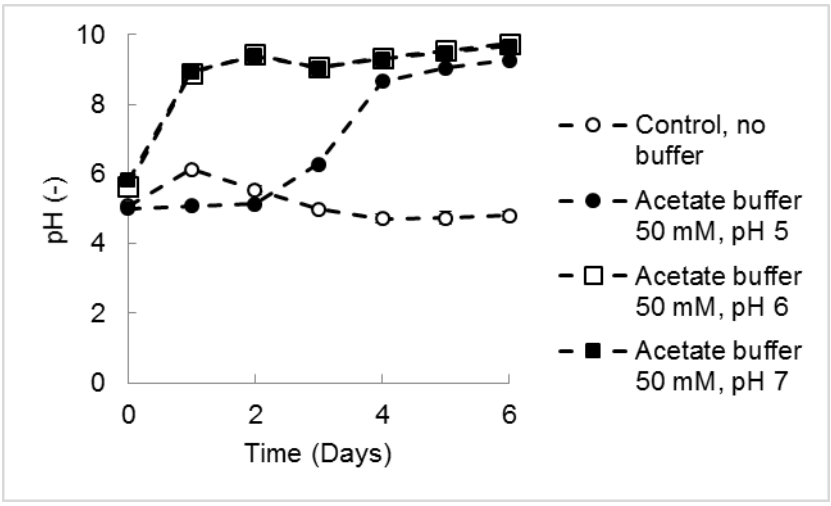


Figure 4.2 pH, growth and pigment production by *P. purpurogenum* in MESP medium in two shake flasks inoculated and incubated in the same way (open symbols – Flask A, closed symbols – Flask B). Error bars are not shown as each set of data represents a single flask.

As a result of the number of buffer/ pH combinations to be tested (10 conditions, including a non-buffered MESP control) the decision was made to use small-scale cultivation to investigate the impact of buffers and pH on growth and pigment production. *P. purpurogenum* was, therefore, cultivated in 2 mL volumes in 12-well multiwell plates, with duplicate sets of triplicate wells used for each buffer/ pH condition. The cultivations were inoculated using a 10 % ($v.v^{-1}$) growing culture inoculum, as described in Section 3.3.2.2. The pH of the wells was monitored daily, and growth was evaluated on day 5 of the cultivation by sacrificing one set of triplicate wells for CDW determination. The second set of wells was used for continued pH monitoring on day 6.

The pH trends during the multiwell plate liquid cultivation are provided in Figure 4.3, with Figure 4.3a showing the trend observed with application of an acetate buffer at pH values of 5, 6, or 7, Figure 4.3b the trend for citrate buffer application, and Figure 4.3c the trend for phosphate buffer application. The pH was also measured in MESP control wells without the application of a buffer, and showed a starting pH value of around 5 which increased to approximately 6 over the first 24 hours, and proceeded to decline back to a value approximately equal to the starting value and remained at this level for the remainder of the cultivation period. These pH shifts are slightly different to those observed previously in shake flasks (Figure 4.2), but still show the trend of rising and falling as growth proceeds.

a



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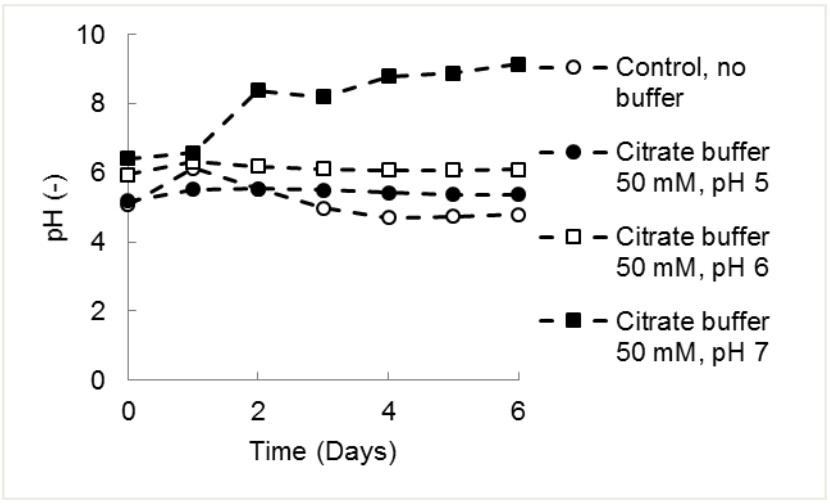


c

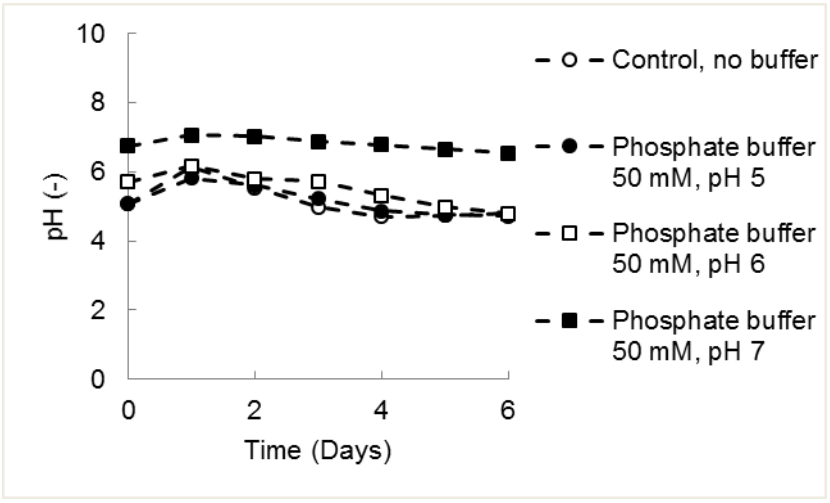


Figure 4.3 pH trend observed during cultivation of *P. purpurogenum* in MESP medium with the application of acetate buffer (a), citrate buffer (b), and phosphate buffer (c), to maintain the pH at a value of 5, 6, or 7 when incubated at 30 °C for 6 days. Results plotted are the average pH values measured across triplicate wells. Error bars represent the standard deviation of the data.

The pH trend in the control wells was compared to that observed with application of each of the buffers. Acetate buffer was shown to have insufficient capacity to maintain the pH of the *P. purpurogenum* culture at any of the selected values. A pH value of 5 was maintained for the first 2 days of the cultivation, but then increased rapidly, reaching a value of greater than 8.5 by day 4, and remaining above this level for the rest of the cultivation period. When this buffer was applied to maintain the pH at values of 6 and 7, the trends observed were the same (data overlaps in Figure 4.3a), with pH values of between 8.9 and 9.8 recorded for the period of day 1 to day 6 of the cultivation. The phosphate buffer also showed limited ability to maintain the pH at a selected value, with a pH value of 7 adequately maintained during the cultivation, but cultures buffered to a value of 5 or 6 behaving much like the MESP control without any buffer applied.

It was only through citrate buffer application that a pH value of 5 or 6 could be adequately maintained, with a pH range of 5.2 – 5.5, and 6 – 6.3, respectively. As expected, citrate buffer was not able to maintain the pH at a value of 7, with the pH increasing to, and remaining above, a value of 8 from day 2 of the cultivation onwards. The buffering capacity of the various buffer/ pH combinations investigated was given a numerical rating between 1 and 4, with 1 being little to no buffering capacity, 2 relating to poor to average pH control, 3 being good buffering capacity, and 4 indicating that no shift from the desired pH value is observed (Table 3.3). The ratings for the different conditions are provided in Table 4.2.

Growth measurement by CDW determination on day 5 of the cultivation revealed that the conditions which exhibited poor buffering capacity also had a detrimental impact on growth, with all acetate and phosphate buffer cultures, and those buffered to a pH of 7 using citrate buffer, exhibiting reduced average CDW values across triplicate wells in comparison to the non-buffered MESP control. The use of citrate buffer to maintain the pH at 5 or 6, however, caused an increase in observed growth. The average CDW measured in triplicate wells for each condition is plotted in Figure 4.4, while the numerical CDW values are provided in Table 4.2.

Of the buffer/ pH conditions investigated, it was also only through the application of the citrate buffer, to maintain the pH at a value of 5 or 6, that pigment production was observed, with a pH of 5 resulting in pigmentation comparable to the control, while pH 6 resulted in lighter colouration. In order to summarise the pigment production trends across all of the conditions considered, the pigmentation observed in the triplicate wells was given a numerical rating between 1 and 4, ranging from 1 being no visible pigment production and 4 being dark red colouration, as described in Table 3.4. The colour of the bar in Figure 4.4 represents the pigmentation achieved, while the ratings assigned to all conditions have been provided in Table 4.2, along with the other data summarising the cultivation.

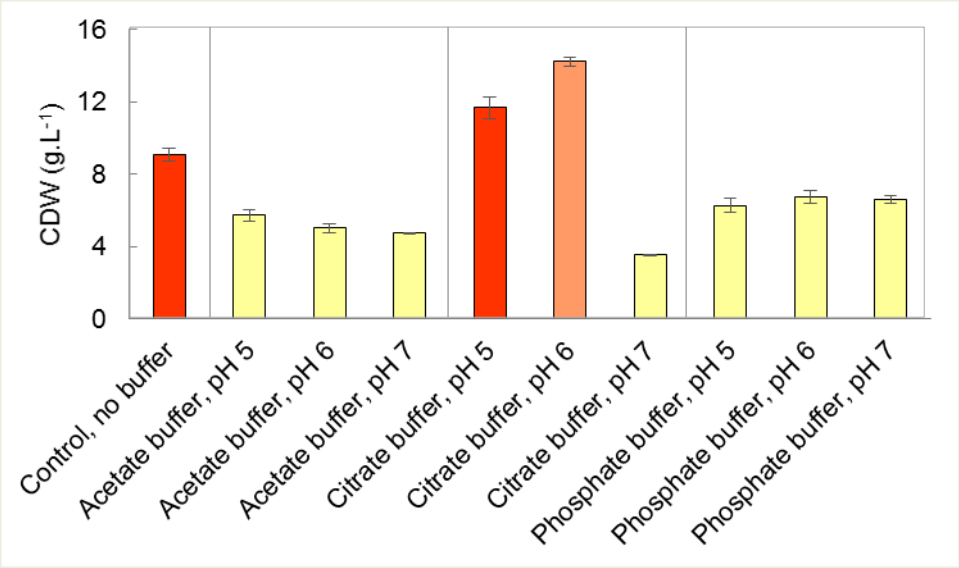


Figure 4.4 Average cell dry weight achieved during multiwell plate liquid cultivation of *P. purpurogenum* in MESP medium when evaluating the impact of applying 50 mM acetate, citrate, or phosphate buffers to maintain the pH at a value of 5, 6, or 7. Error bars represent the standard deviation across triplicate wells. The colour of the bar represents the pigmentation achieved under each buffer/ pH condition.

The varying growth and pigment production responses of *P. purpurogenum* when applying different buffers at the same pH, as summarised in Table 4.2, suggests that rather than a mere pH effect, the actual buffer components may play a role in improving pigment production and reducing variation. Best growth and pigment production were obtained when pH was maintained in the range of 4.7 – 6.3, as observed in the control and selected citrate buffer cultures. Conversely a decrease in growth and pigmentation was observed with the phosphate buffer, even though pH was maintained in this favourable range in some cultures. The benefit associated with citrate buffer application may suggest a link to the pigment production pathway of *P. purpurogenum*, with components of the buffer either functioning as pigment pre-cursors or having some other stimulatory effect on production metabolism.

The impact of pH on growth and pigment production of *Penicillium* species has been investigated previously by either adjusting the starting pH value without applying pH control during the cultivation, or through the application of buffers to maintain selected pH values.

In general, studies which considered only adjusting the initial pH value of the medium reported results which were not consistent with those observed in this study. Gunasekaran and Poorniammal (2008) reported that best growth (5.42 $g.L^{-1}$) of a *Penicillium* species was achieved when adjusting the starting pH to 8. This was followed by growth at pH 9 (4.75 $g.L^{-1}$) and then pH 10 (3.47 $g.L^{-1}$). An initial pH of 9 was also reported to be most favourable for pigment production. Another study investigating the growth of *P. purpurogenum* reported highest growth when the starting pH of a xylose-based medium was adjusted to 7 (approximately 6 $g.L^{-1}$). Highest pigment production was, however, reported at pH 5, showing some agreement with the results of this study (Méndez et al., 2011). The reason for the observed differences may be that initial pH adjustment has little effect on the pH trends during

cultivation. The results of Gunasekaran and Poorniammal (2008) showed that irrespective of starting pH, the final pH of the *Penicillium* culture was in the range of 4.2 to 4.9.

Table 4.2 Numerical summary of the *P. purpurogenum* liquid cultivation performed in multiwell plates to evaluate the impact of applying an acetate, citrate, or phosphate buffer to maintain the pH at 5, 6, or 7. Shaded data indicates runs in which the pH remained within the range of 4.7 to 6.3.

Buffer	**pH**	**pH control rating (1-4)** [a]	**pH range during cultivation**	**Growth (CDW, $g.L^{-1}$)**	**Pigmentation rating (1-4)** [b]
Control, no buffer	/	/	± 4.7 – 6.1	9.10 ± 0.35	3
Acetate	5	1	± 5.0 – 9.3	5.73 ± 0.30	1
	6	1	± 5.6 – 9.8	5.02 ± 0.25	1
	7	1	± 5.8 – 9.7	4.73 ± 0.06	1
Citrate	5	3	± 5.2 – 5.5	11.68 ± 0.60	3
	6	3	± 6.0 – 6.3	14.23 ± 0.24	2
	7	1	± 6.4 – 9.2	3.53 ± 0.03	1
Phosphate	5	2	± 4.7 – 5.8	6.28 ± 0.38	1
	6	2	± 4.8 – 6.2	6.77 ± 0.35	1
	7	3	± 6.5 – 7.1	6.63 ± 0.21	1

a pH control rating system explained in Table 3.3

b Pigmentation rating system explained in Table 3.4

The effect of applying various buffers, including those used in this study, to the liquid cultivation of *P. purpurogenum* in a soluble starch-based medium was investigated previously by Ogihara et al. (2000). A culture pH of 5 was reported to be best suited to pigment production, as seen in this study, and the effect on growth and pigment production when applying buffers at this value was, therefore, evaluated. The starch-based medium was able to support growth, irrespective of the buffer used, but amount of growth varied. Growth, evaluated as wet mycelial weight, was shown to increase with the change from phosphate buffer (20 $g.L^{-1}$), to citrate buffer (35 $g.L^{-1}$), to acetate buffer (40 $g.L^{-1}$). The improvement observed when replacing phosphate buffer with citrate buffer is consistent with the results of this study, however, acetate buffer had a detrimental effect on the growth of *P. purpurogenum* DSM 62866 in this study. The application of citrate buffer resulted in best pigment production, which is consistent with the results of this study, even though a different cultivation medium was used.

These results suggested that the application of citrate buffer to maintain the pH of MESP medium at a value of 5 would enable pigment production equivalent to that observed with the same medium when no buffer is applied, while reducing variability of production as reported by Ogihara et al. (2000). The potential for more reproducible pigment production through application of the buffer was, therefore, investigated further.

4.1.3 Validation of proposed temperature and pH conditions using shake flask cultivation

Of the temperature and culture pH conditions considered, those which result in improved pigment production, with or without a simultaneous improvement in growth of the filamentous fungus *P. purpurogenum*, were determined to be a cultivation temperature of 30 °C, identified through varying the incubation temperature of agar plates, and a culture pH of 5 maintained through the application of a 50 mM citric acid monohydrate – trisodium citrate dihydrate buffer, determined using small-scale liquid cultivation of the organism in multiwell plates.

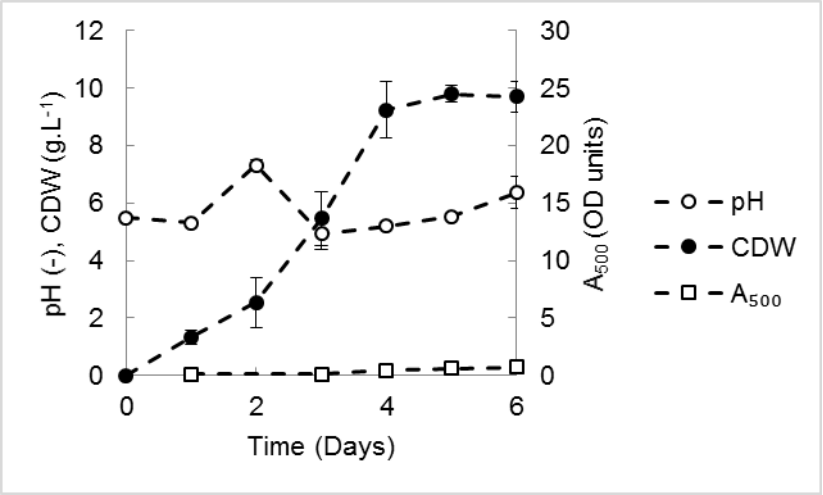
These cultivation parameters were then applied to shake flask cultures of *P. purpurogenum* DSM 62866 to validate that the citrate buffer application at pH 5 results in equivalent or improved pigment production in comparison to MESP medium without buffer application. A potential benefit of buffer application is also reported to be reduced variability in terms of pigment production (Ogihara et al., 2000b), which was evaluated by directly comparing the shake flask cultivations using MESP medium with and without buffer application.

The experiment made use of 100 mL medium in 500 mL flasks, inoculated directly with $1x10^5$ spores.mL $^{-1}$, and incubated at 30 °C, with shaking at 150 rpm, for 6 days. The pH trends, growth, and pigment production recorded during the cultivation are provided in Figure 4.5, with Figure 4.5a summarising the cultivation without the application of the buffer, and Figure 4.5b summarising the cultivation with the application of 50 mM citrate buffer.

The trends observed during MESP cultivation without the application of the buffer were compared to results observed in previous shake flask experiments (Figure 4.2). The expected pH shift from the starting value of around 5 to approximately 7.5, before declining again, was observed in both cases. This change did, however, occur more rapidly, taking place between day 1 and day 3 of the cultivation rather than over the first 4 days, as shown in Figure 4.2. Another difference observed was slightly reduced growth, as well as that, rather than inconsistent pigment production, pigmentation was not produced to a significant degree in any of the replicate flasks.

When comparing the cultures with and without the application of the buffer a number of differences are clear. The large pH shifts observed in non-buffered MESP cultures are effectively prevented in flasks containing the 50 mM citrate buffer, with the pH remaining in the range of 5 to 5.5 over the 6-day cultivation period. This compares well with the observed pH range when applying the citrate buffer at pH 5 during cultivation in multiwell plates (Table 4.2). Growth, measured as cell dry weight, showed an overall decrease following application of the buffer, with the average CDW across triplicate flasks reaching a maximum value of 9.80 ± 0.30 g.L $^{-1}$ by day 5 of the cultivation in non-buffered cultures, while cultures with the application of the buffer reached a maximum CDW of 8.57 ± 0.15 g.L $^{-1}$ by day 6. This is in contrast to the results observed during multiwell plate cultivation, where application of the citrate buffer at pH 5 caused an increase in growth, in comparison to the non-buffered control medium, from 9.10 ± 0.35 g.L $^{-1}$ to 11.68 ± 0.60 g.L $^{-1}$ after 5 days.

a



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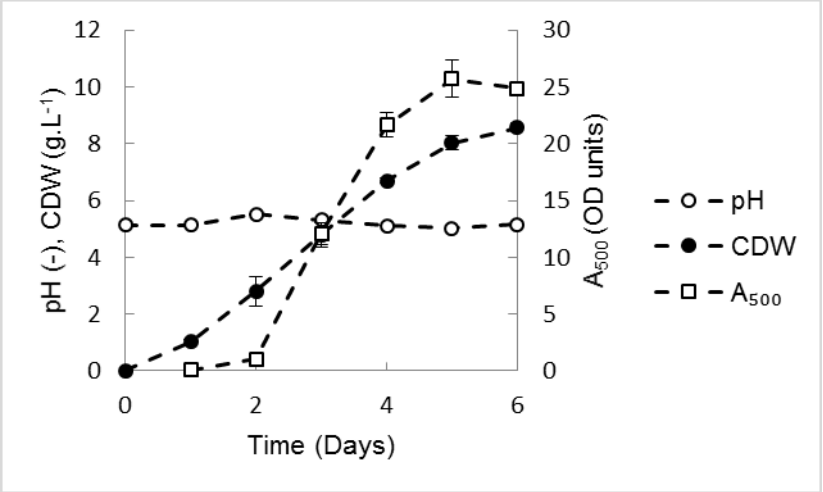


Figure 4.5 pH trends, growth and pigment production observed during shake flask cultivation of *P. purpurogenum* at 30 °C in MESP medium, without (a) and with (b) application of 50 mM citrate buffer to maintain the pH at a value of 5. The average result across triplicate flasks is plotted, with error bars representing the standard deviation of the data.

Growth with and without the application of the buffer also showed slightly different trends over the cultivation period. Similar growth rates were achieved in both sets of flasks at the beginning of the cultivation, but differences were observed beyond the first 24 hours. Flasks with buffer application exhibited increased biomass productivity beyond day 1, with growth increasing linearly between approximately day 1 and day 4 of the cultivation. An increase in biomass productivity was only observed beyond day 2 in flasks without buffer application, with growth increasing linearly between day 2 and day 4. The greater biomass productivity in the non-buffered flasks, compared to the buffered flasks, over this period ultimately resulted in a higher maximum CDW. The maximum biomass concentrations and productivities achieved in both sets of flasks are provided in Table 4.3.

Table 4.3 Maximum CDW and biomass productivity of *P. purpurogenum* in MESP medium, with and without the application of 50 mM citrate buffer to maintain the pH at a value of 5. Flasks were incubated at 30 °C, with shaking at 150 rpm.

MESP, no buffer				**MESP, pH 5, 50 mM citrate buffer**			
Maximum CDW [a]	Average biomass productivity [a]			Maximum CDW [a]	Average biomass productivity [a]		
$g.L^{-1}$	Time (days)	$g.L^{-1}.day^{-1}$	$g.L^{-1}.h^{-1}$	$g.L^{-1}$	Time (days)	$g.L^{-1}.day^{-1}$	$g.L^{-1}.h^{-1}$
9.80 ± 0.30	0 – 2	1.27 ± 0.44	0.053 ± 0.018	8.57 ± 0.15	0 – 1	1.03 ± 0.06	0.043 ± 0.002
	2 – 4	3.35 ± 0.39	0.140 ± 0.016		1 – 4	1.90 ± 0.05	0.079 ± 0.002

a Results presented as average ± standard deviation across triplicate flasks

Results of multiwell plate cultivations also indicated that pigmentation equivalent to that observed in non-buffered cultures could be expected when applying the citrate buffer at pH 5, with the potential benefit of reduced variability between replicate cultivations. Flasks without buffer application in this experiment, however, consistently showed little to no pigment production over the 6-day cultivation, with an A_{500} value of < 1 OD unit observed across triplicate flasks. This was significantly improved in cultures with the application of the buffer where a deep red colour, defined by an average A_{500} value of approximately 25 OD units, was achieved by day 5 of the cultivation. The full wavelength scan of the red pigment solution over the range of 400 to 600 nm is provided in Figure 4.6. This demonstrates maximal absorbance at 500 nm, corresponding to red pigmentation, with a second peak in absorbance at approximately 420 nm.

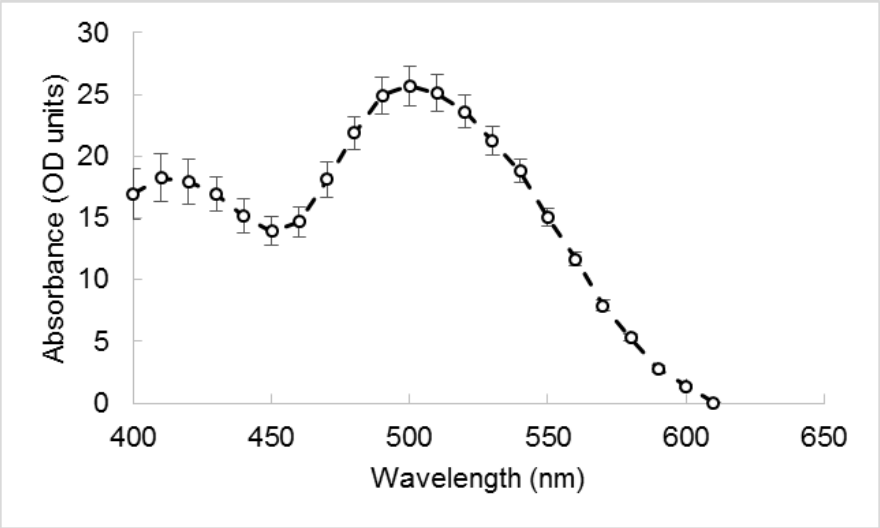


Figure 4.6 Wavelength scan over 400 to 600 nm for the red pigment solution produced during cultivation of *P. purpurogenum* DSM 62866 on malt extract-based medium. Error bars represent standard deviation across triplicate flasks.

The volumetric pigment concentration achieved with buffer application compares well with reported production of the *Monascus* pigment homologue PP-V by *P. purpurogenum* (Ogihara et al., 2000b), where it was observed that, through the application of citrate buffer, stable maximum pigment production in the culture filtrate was defined by an A_{500} value of 19.3 OD units.

Limited variation in pigmentation across triplicate flasks was also noted in this experiment, which is consistent with reports of more stable pigment production following the application of a buffer. Another feature of the cultivations was that improved pigment production was not necessarily associated with increased growth of the culture. This has been reported previously for this species (Ogihara et al., 2000b; Méndez et al., 2011), and is consistent with trends observed during production of secondary metabolites, rather than growth-associated products.

It is important to remember that biomass concentration achieved is still an important factor in the production of secondary products. Cultivation parameters are altered with the aim of maximising specific product formation rates and volumetric production is then increased by increasing biomass formation, as demonstrated by the Luedeking-Piret model (Equation 7). The increase in pigmentation achieved in this experiment without simultaneous increase in biomass does, however, indicate that specific pigment formation rate has increased in response to the changes made.

The deep red colouration achieved when applying the 50 mM citrate buffer at a pH of 5, along with the limited variability in pigment production observed between replicate flasks, supports the use of this buffer in further liquid cultivation experiments.

4.2 Considering the impact of oxygen availability

Availability of oxygen in liquid microbial cultures is known to have an impact on the growth and product metabolism of aerobic organisms (Büchs, 2001). Therefore, it is possible that oxygen transfer into the cultivation medium or the resulting oxygen concentration may affect pigment production by *P. purpurogenum*. This was investigated by monitoring the growth and pigment production response of the organism to changes in shaking platform speed during cultivation in shake flasks using MESP medium. The temperature and pH conditions previously identified as being best suited to pigment production were maintained in these experiments and only shaking platform speed was varied.

4.2.1 Effect of shaking platform speed on shake flask cultures

Oxygen availability in shake flasks is affected by a number of factors, including flask size, medium volume, shaking speed during incubation and type of closure used, as these parameters have an impact on the mass transfer coefficients which define the system (k_La and k_Ga) and, therefore, the oxygen transfer rate into the medium. Adjusting any of these cultivation parameters will, therefore, result in a change in oxygen availability within the culture. In order to investigate the impact of shaking speed on pigment production of *P. purpurogenum*, all other parameters, including flask size, filling volume, type of closure, and shaking platform used were kept constant throughout the experiment.

Cultivations were performed using 100 mL MESP medium in 500 mL flasks. The medium was buffered to a pH value of 5 using a 50 mM citrate buffer, and flasks were incubated at 30 °C for 5 days, following inoculation with a spore solution to yield a starting concentration of $1x10^5$ spores.mL^{-1}. *P. purpurogenum* was cultivated using shaking speeds of 120, 130, 140 and 150 rpm, with the 150 rpm data previously reported in Figure 4.5b. The pH, growth and pigment production trends observed at all speeds considered are compared in Figure 4.7.

Shaking speed was shown to have little effect on pH during MESP cultivation. The trends observed remained relatively unchanged, with the characteristic peak occurring around day 2 of the cultivation, irrespective of the shaking speed during incubation. This peak is likely due to the release of ammonia as proteins are broken down by the organism (Nanou et al., 2007).

The impact on growth of *P. purpurogenum* was also limited, with a slight trend of decreasing growth with increasing shaking speed. Growth at 120 rpm reached a maximum of approximately 9.5 $g.L^{-1}$ CDW by day 5 of the cultivation, while growth at 150 rpm reached a concentration of around 8 $g.L^{-1}$ CDW in the same period. The results of the MESP medium cultivations did, however, reveal a trend of increasing pigmentation with increasing shaking speed. The A_{500} values recorded were approximately 1, 10, 14, and 25 OD units for shaking speeds of 120, 130, 140, and 150 rpm, respectively.

In order to evaluate whether this effect is likely to be a result of improved oxygen availability within the culture in response to increased shaking speed, the k_La prediction model defined by Nikakhtari and Hill (2005), as discussed in Section 2.3.1.2, was investigated. The model was validated by comparing experimental results for maximum oxygen transfer rate (OTR_{max}) reported by Maier and Büchs (2001) to predicted k_La values based on the system parameters. The k_La values corresponding to shaking speeds of 100, 150, 200, and 250 rpm were calculated using the model, and are presented in Table 4.4. The corresponding OTR_{max} values were calculated using Equation 9.

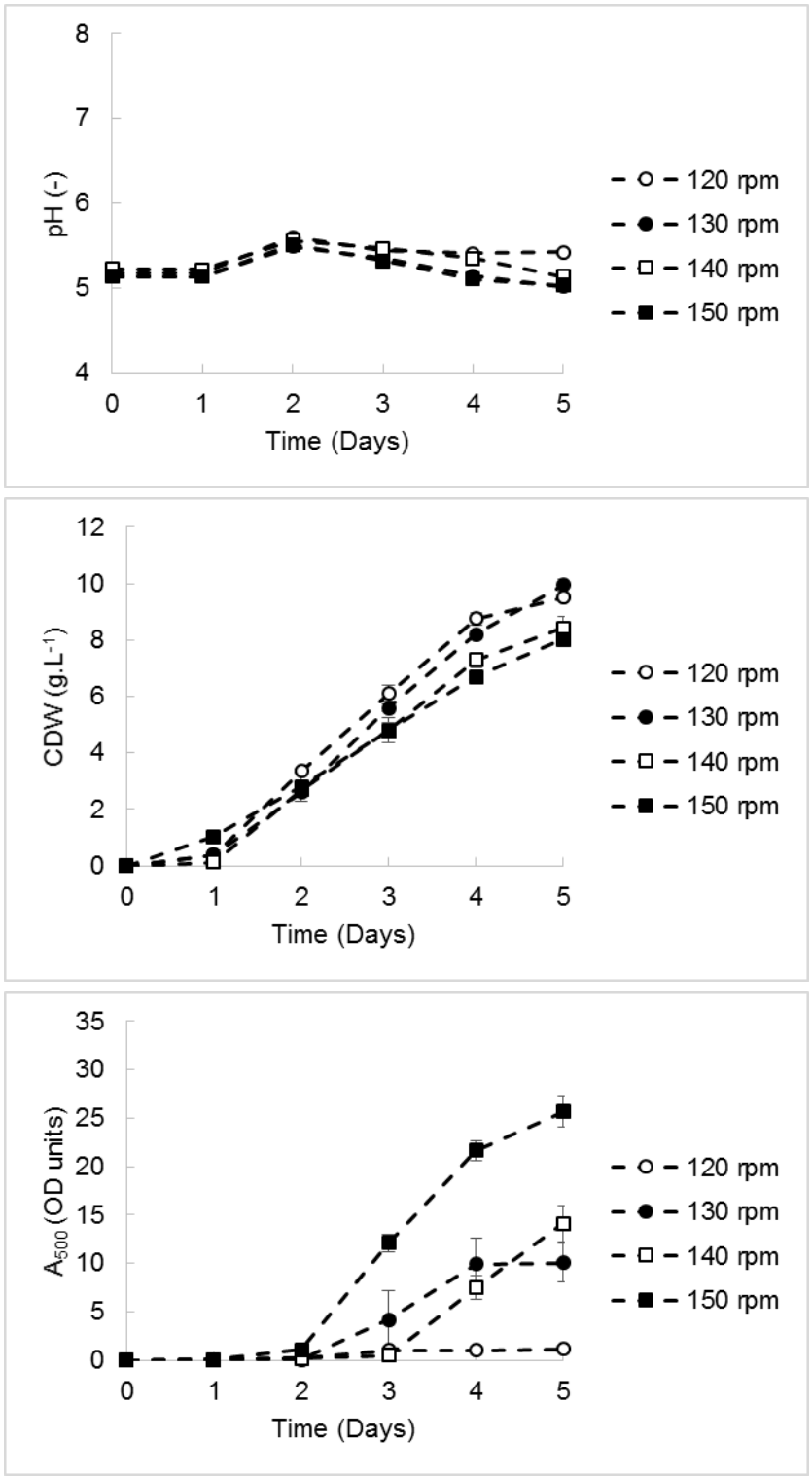


Figure 4.7 Comparison of the pH (a), growth (b), and pigment production (c) trends observed in response to altered shaking speed during liquid cultivation of *P. purpurogenum* at 30 °C for 5 days in MESP medium, buffered to a pH of 5 using 50 mM citrate buffer. Error bars represent the standard deviation associated with replicate flasks.

Table 4.4 Predicted k_La values, based on the model developed by Nikakhtari and Hill (2005), and corresponding OTR values in a 250 mL flask, with 26 mL fill volume, at various shaking speeds, for comparison to the results reported by Maier and Büchs (2001).

V_F (L)	V_L (L)	N (rpm)	Stationary liquid surface area (cm^2)	Liquid phase turbulence factor	kLa (h^{-1})	OTR_{max} ($mmol.L^{-1}.h^{-1}$)
			$A = 142 \times (V_F - V_L)^{2/3}$	$T = \frac{V_F^{0.463}}{V_L} \times \frac{N}{60}$	$k_La = 0.0182 \times (A \times T)$	
0.25	0.026	100	52.37	33.74	32.16	7.58
		150		50.61	48.24	11.36
		200		67.48	64.32	15.15
		250		84.34	80.40	18.94

The experimental results for OTR_{max}, measured in a 250 mL flask, with a 26 mL fill volume, with varied shaking speed (Maier and Büchs, 2001) are shown in Figure 4.8. Good agreement was observed between the calculated OTR values in Table 4.4 and the experimental results presented in Figure 4.8, providing support for the model developed by Nikakhtari and Hill (2005). The model was thus applied using the cultivation conditions of the current experiment in order to evaluate k_La under the varying conditions of shaking speed. The results are presented in Table 4.5.

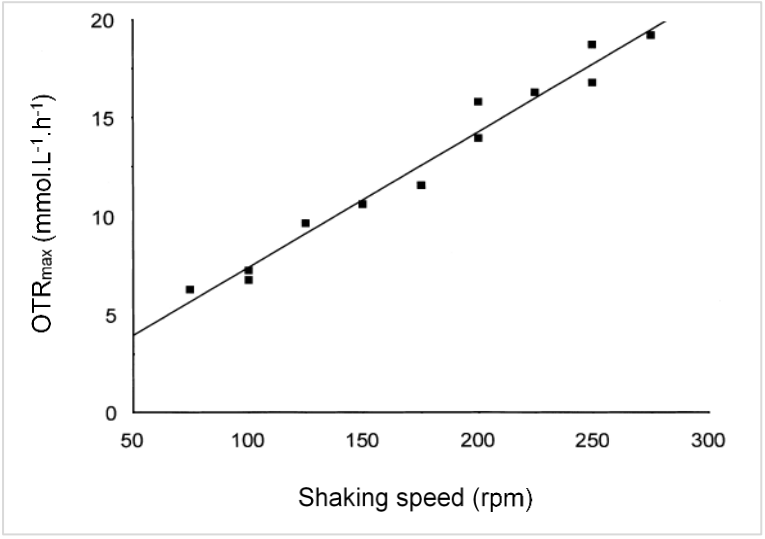


Figure 4.8 Relationship between shaking speed and OTR_{max}, in a 250 mL flask, with a 26 mL fill volume (Maier and Büchs, 2001).

Although this model does not take into account all of the factors which affect oxygen availability within the culture (Nikakhtari and Hill, 2005), it does provide an indication of the expected oxygen transfer in the shake flask cultures given the parameters of these experiments. The results indicate that a k_La value of 20.36 h^{-1} is expected with a shaking speed of 120 rpm, while an increase in shaking speed to 150 rpm raises this k_La value to 25.45 h^{-1}. This represents an increase in k_La of approximately 25 %, which would correspond to an increase in OTR of 25 %, assuming that the oxygen saturation and solubility conditions within the flasks remain the same, irrespective of changes to shaking speed.

Table 4.5 Predicted k_La values for a 100 mL culture in a 500 mL flask, with varying shaking speed, based on the model developed by Nikakhtari and Hill (2005).

V_F (L)	V_L (L)	N (rpm)	Stationary liquid surface area (cm^2) (A) [a]	Liquid phase turbulence factor (T) [a]	kLa (h^{-1}) [a]
0.5	0.1	120	77.09	14.51	20.36
		130		15.72	22.05
		140		16.93	23.75
		150		18.14	25.45

a Formulae for calculation of A, T and k_La provided in Table 4.4

The significant change in k_La value with altered shaking speed, based on this model, supports the theory that the varied pigmentation observed with changes in shaking speed (Figure 4.7c) may be due to the resulting difference in oxygen availability within the liquid culture. This is further supported by the observed relationship between calculated k_La value and A_{500} value defining the pigmentation under different shaking speed conditions, as shown in Figure 4.9. Growth observed under each condition has also been plotted against the predicted k_La value.

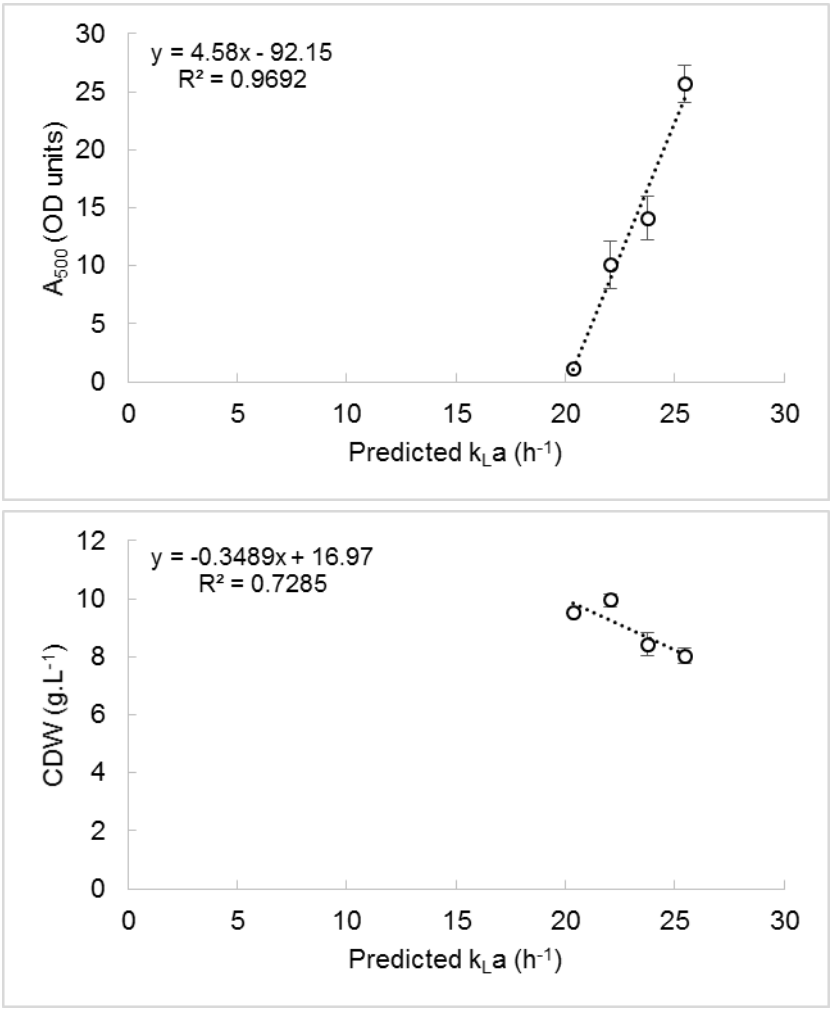


Figure 4.9 Relationship between predicted k_La value and maximum pigment production (**a**) or biomass concentration (**b**) in MESP medium, pH 5, 50 mM citrate buffer, with varying shaking speed after 5 days. Error bars represent the standard deviation associated with replicate flasks.

Considering this, it can be postulated that as shaking speed is lowered, oxygen serves as the limiting factor for pigment production by the *P. purpurogenum* culture. Potential metabolic responses to oxygen limitation described by Büchs (2001) are discussed in Table 4.6, considering the relevance of each to the observed growth and pigmentation of *P. purpurogenum* in this experiment.

Table 4.6 Metabolic responses of microbial cultures to oxygen limitation (Büchs, 2001), and the relevance of these to *P. purpurogenum* DSM 62866 cultivation.

Response	Relevance
Entire microbial metabolism slows down.	Reduced shaking speed has limited impact on pH trends and growth of *P. purpurogenum.*
Metabolism becomes partially anaerobic, with the production of compounds such as acids and alcohols, where the corresponding pH changes may be inhibitory to the organism.	Overall pH trends remain relatively unchanged with altered shaking speed. Cultures are, however, buffered so these products may be formed without having a major impact on pH. Therefore, likely not a pH effect but may be an altered metabolism effect.
Product metabolism changes, with growth of the organism not necessarily affected.	Of the parameters tested, only pigment production by *P. purpurogenum* has been shown to be significantly affected by changes in shaking speed.

Potential oxygen limitation as a result of reduced shaking speed is, therefore, hypothesised to affect product metabolism. In fact, a slight trend of increased growth with lower shaking speed was observed, as pigment production declined (Figure 4.7, Figure 4.9). This suggests that substrate is diverted from growth to pigment production when conditions are suited to product formation, or when product formation is favourable for the organism based on conditions experienced by the culture.

These results provide further evidence that pigment production is not growth-associated, given that an increase in pigment production is not accompanied by a corresponding increase in growth, but rather a slight decline. The growth-associated versus biomass-associated nature of pigment production is investigated during bioreactor cultivation of this organism (Section 4.3.4).

4.2.2 Oxygen availability considerations for bioreactor cultivation

The implications of oxygen availability on pigment production, as demonstrated by altering the shaking platform speed during incubation of MESP flask cultures (Figure 4.7), also need to be considered in bioreactor systems. During bioreactor cultivation, enhanced control of oxygen availability is possible, with a number of parameters affecting the dissolved oxygen concentration in the system. These parameters, which can be altered to suit the oxygen requirements of the organism include agitation speed, aeration rate, pressure in the reactor vessel and oxygen concentration in the inlet gas stream (Doran, 1995; Pereira et al., 2008). It is also important to note that, contrary to shake flask cultivation where oxygen is obtained via surface aeration alone, the bioreactor system makes use of direct air sparging from the bottom of the culture. The air passes through the culture, with the bubbles dispersed and broken up due to the agitation of the system, enhancing oxygen transfer into the medium.

Oxygen transfer rate in a bioreactor is also affected by other properties of the microbial cultivation, including temperature (as a result of its effect on gas solubility), the concentration of solutes in the

growth medium, the addition of chemicals such as antifoam, and the presence of microbial cells which can have an influence on the properties of the liquid (Doran, 1995).

The impact of these parameters on oxygen availability can be evaluated by considering the equations which define oxygen transfer rate (Equation 5) and the volumetric mass transfer coefficient (Equation 19), as demonstrated in Table 4.7.

$$OTR = k_La(C^* - C) \qquad \text{Equation 5}$$

$$k_La = K(\frac{P}{V})^{\alpha}.v_s^{\beta} \qquad \text{Equation 19}$$

where a is interfacial area per unit volume (m^{-1})
K is a constant
α, β are exponents
v_s is superficial gas velocity (volumetric gas flow rate/ cross sectional area) ($m.s^{-1}$)
P is power input (W)
V is fluid volume (m^3), and
$(\frac{P}{V}) = N^{\alpha 1}D^{\alpha 2}$

where N is stirrer speed (s^{-1}), and
D is stirrer diameter (m)

(Van't Riet, 1979)

Table 4.7 Factors influencing oxygen transfer rate (Van't Riet, 1979; Doran, 1995)

Term		**Parameters influencing this term**	**Effect on OTR of increasing this parameter**
k_La	a	Bubble size	Decrease
	$(\frac{P}{V})$	Agitation speed	Increase
	v_s	Aeration rate	Increase
$(C^* - C)$		Temperature	Decrease
		Solute concentration	Decrease
		Total gas pressure	Increase
		Oxygen partial pressure	Increase

As described in Section 3.3.4, monitoring of dissolved oxygen concentration is performed during cultivation in the New Brunswick Scientific BioFlo 110 benchtop fermentor, providing an indication of when cultivation parameters should be adjusted in order to prevent oxygen limitation. Previous cultivation of *P. purpurogenum* for pigment production in a 5 L reactor was performed under a dissolved

oxygen concentration of 2.5 ppm (Ogihara et al., 2000b), which is equivalent to 2.5 $mg.L^{-1}$. Given that the solubility of oxygen in water at 30 °C, under air pressure of 1 atm, is 7.539 $mg.L^{-1}$ (EIFAC, 1986), and assuming this holds for the cultivation medium, a concentration of 2.5 $mg.L^{-1}$ relates to a dO_2 concentration of approximately 33 % of saturation at atmospheric pressure, as shown below. A dissolved oxygen concentration of 35 % of saturation was, therefore, selected as the minimum level during cultivation in the New Brunswick Scientific BioFlo 110 fermentor, and was maintained through modification of agitation speed. This minimum concentration value serves as a starting point for the investigation of the impact of oxygen availability on pigment production in the base case cultivation (Section 4.3). The comparison of residual oxygen concentration and oxygen transfer rate in this system is detailed in Section 4.4.

$$\text{Percentage } dO_2 \text{ concentration} = \frac{2.5\ mg.L^{-1}}{7.539\ mg.L^{-1}} \times 100$$

$$= 33.16\ \%$$

A previous study in this bioreactor system determined the k_La value for water at 30 °C, at agitation speeds in the range of 200 to 1200 rpm, and aeration rates in the range of 0.5 to 1.5 vvm (Williams, 2005). It was shown that agitation speed had a greater impact on k_La than aeration rate. Agitation speed was thus selected for further investigation, with an aeration rate of 1 vvm designated as an arbitrary set point for the current study.

Although the relationship between k_La and agitation speed is recognised not to be linear (Van't Riet, 1979), it can be approximated as such for the data presented by Williams (2005) across a selected range of data, 200 to 600 rpm at 1 vvm, as shown in Figure 4.10.

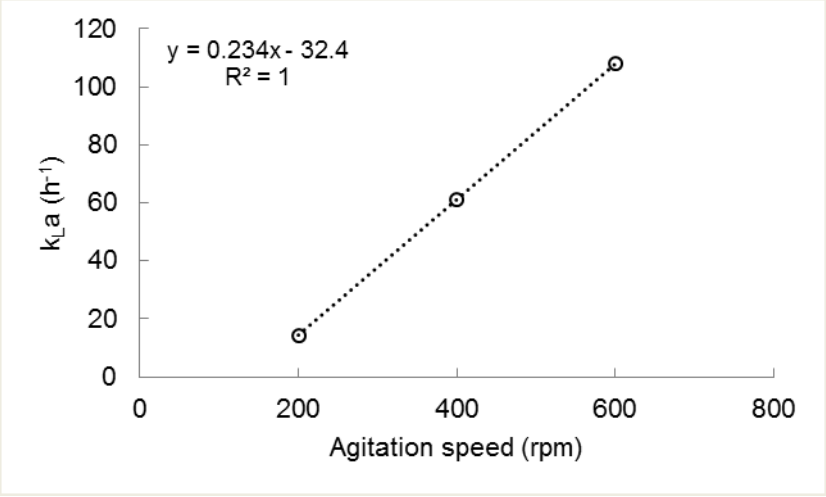


Figure 4.10 Best-fit line for k_La in water at 30 °C in the New Brunswick Scientific BioFlo 110 bioreactor at an aeration rate of 1 vvm, and an agitation speed of between 200 and 600 rpm (Williams, 2005).

In order to allow selection of a suitable agitation rate for the base case bioreactor cultivation of *P. purpurogenum* DSM 62866, the data presented by Williams (2005) and the k_La values calculated for the shake flask cultures (Section 4.2.1) were both considered.

The volumetric pigment concentration was found to increase with increasing shaking speed during incubation of shake flasks, with A_{500} values of > 20 OD units only observed in the MESP medium when a shaking speed of 150 rpm was used. As a result, an equivalent or greater k_La value than 25.45 h^{-1} (Table 4.5), achieved during this shake flask cultivation, should be applied during bioreactor cultivation.

An agitation speed of 300 rpm was selected as the starting point for bioreactor cultivation based on the relationship between k_La and agitation speed depicted in Figure 4.10. Using the equation of the best-fit line, k_La (h^{-1}) = (0.234 x agitation speed in rpm) – 32.4, the k_La corresponding to this agitation speed is greater than that required for pigment production, based on the results of shake flask experiments. This, along with the other conditions selected for application during bioreactor cultivation, is summarised in Section 4.3.

4.3 Base case BioFlo 110 reactor cultivation of *P. purpurogenum* DSM 62866

Following the investigation of the effect of various cultivation conditions on growth and pigment production of *P. purpurogenum* DSM 62866 using agar plates and small-scale liquid cultivation, the conditions identified as being best suited to pigment production by this organism were applied to 5 L fermentations in the New Brunswick Scientific BioFlo 110 benchtop bioreactor. The aim of performing these cultivations was to determine whether pigment production would be maintained or enhanced at this cultivation volume, given the improved control of system parameters such as temperature and dissolved oxygen concentration. Details regarding cultivation in the bioreactor system, including reactor preparation, inoculation procedure, and culture monitoring, are provided in Section 3.3.4.

The conditions applied during bioreactor cultivation, based on the results of previous agar plate and liquid cultivation experiments, are outlined in Section 4.3.1. Sections 4.3.2 to 4.3.5 describe the base case cultivation in the bioreactor, including the method used to monitor carbohydrate utilisation, the overall trends observed and a comparison of the results to those obtained during shake flask cultivation.

4.3.1 Selection of base case BioFlo 110 reactor cultivation conditions

Cultivation conditions best suited to pigment production by *P. purpurogenum* DSM 62866 were investigated using agar plate, multiwell plate, and shake flask cultivation. The parameters applied during base case bioreactor cultivation are summarised in Table 4.8, along with justification for their selection and details regarding how they are maintained or adjusted during cultivation. The medium selected for base case cultivation in the bioreactor was the MESP medium recommended for cultivation of *P. purpurogenum*. This medium was used during the cultivation temperature and culture pH optimisation studies described in Section 4.1, and shaking speed investigation detailed in Section 4.2.1.

Table 4.8 System parameters for 5 L cultivation of *P. purpurogenum* in the New Brunswick Scientific BioFlo 110 benchtop fermentor.

Parameter	Set value	Basis for selection	Notes
Temperature	30 °C	Agar plate experiments described in Section 4.1.1	Controlled using an external heating blanket and an internal cooling coil.
pH	5	Multiwell plate and shake flask experiments described in Section 4.1.2 and Section 4.1.3, respectively	Controlled through the use of a 50 mM citrate buffer. No supplementary acid or base addition during the cultivation.
Aeration	1 vvm	Arbitrary starting value, based on the results of the study by Williams (2005), as reported in Section 4.2.2.	Reduced to 0.5 vvm towards the end of the cultivation to control foaming.
Agitation speed	300 rpm	Calculated k_La value at this agitation speed is greater than that calculated for shake flasks at 150 rpm (see Section 4.2.2)	Increased to 400 rpm during the course of the cultivation to maintain dO_2 concentration at, or above, 35 %.

As seen in Table 4.8, temperature and pH were not actively varied during the bioreactor cultivation, while parameters affecting oxygen availability were regulated to maintain favourable conditions for growth and pigment production. Aeration was reduced once the culture had entered stationary phase in order to reduce the incidence of foaming in the vessel, while agitation rate was varied between 300 rpm and 400 rpm using cascade control of the BioFlo 110 system in order to maintain the dO_2 concentration at or above 35 % (approximately 2.6 $mg.L^{-1}$), as discussed in Section 4.2.2.

System parameters were set and allowed to stabilise before inoculating the medium with a *P. purpurogenum* spore solution to yield a starting concentration of $1x10^5$ $spores.mL^{-1}$ (described in Appendix C.2). Duplicate cultivations were performed, with manual sampling for monitoring of culture pH, growth, pigmentation, and utilisation of medium components performed twice daily for 6 days, as described in Section 3.3.4.4.

4.3.2 Establishing a method for monitoring carbohydrate utilisation

Given the larger liquid volume available for culture sampling in the bioreactor in comparison to shake flask cultivation, it was possible to monitor the utilisation of medium components as growth and pigment production proceeded. This additional data allowed calculation of biomass and pigment yields and provided further means of comparing cultivations performed in the bioreactor under varying conditions.

Considering the MESP medium, which is composed of 30 $g.L^{-1}$ malt extract, and 3 $g.L^{-1}$ soya peptone, it is apparent that the majority of the utilisable components are carbohydrates. Malt extract, which is the main component of this medium, contains approximately 60 % reducing sugars (Neogen Corporation, 2009), mainly in the form of maltose (Merck, 2016). Based on this, monitoring of the carbohydrate concentration was selected as a means of tracking medium utilisation during *P. purpurogenum* cultivation. As a result of the absorbance of the pigment product in the visible light range (particularly in the region of 400 to 600 nm), however, spectrophotometric assays for carbohydrate determination which require detection within this range had to be considered with caution, especially given the expected concurrent decrease in carbohydrate concentration with increasing pigment concentration.

One such colorimetric assay, the phenol-sulphuric acid method for carbohydrate determination, described by DuBois et al. (1956) was adapted such that determination of the carbohydrate concentration relied solely on the UV absorbance of sulphuric acid-treated samples (Albalasmeh et al., 2013).

The original phenol-sulphuric acid method (DuBois et al., 1956) involves the hydrolysis of sugars, in the presence of phenol, using concentrated sulphuric acid. This results in a colour change to yellow-orange, with the intensity of the colour dependent on the sugar concentration in the sample as well as the phenol concentration. This reaction is shown in Figure 4.11, with glucose provided as an example. A constant phenol concentration in excess of requirement is selected and applied to both the standard sugar solutions used to generate the calibration curve as well as the unknown samples. The colour change is detected by measuring the absorbance of the samples at 490 nm using a spectrophotometer. This prevents the use of this method in the pigment study, as the wavelength is affected by absorbance of the red pigment.

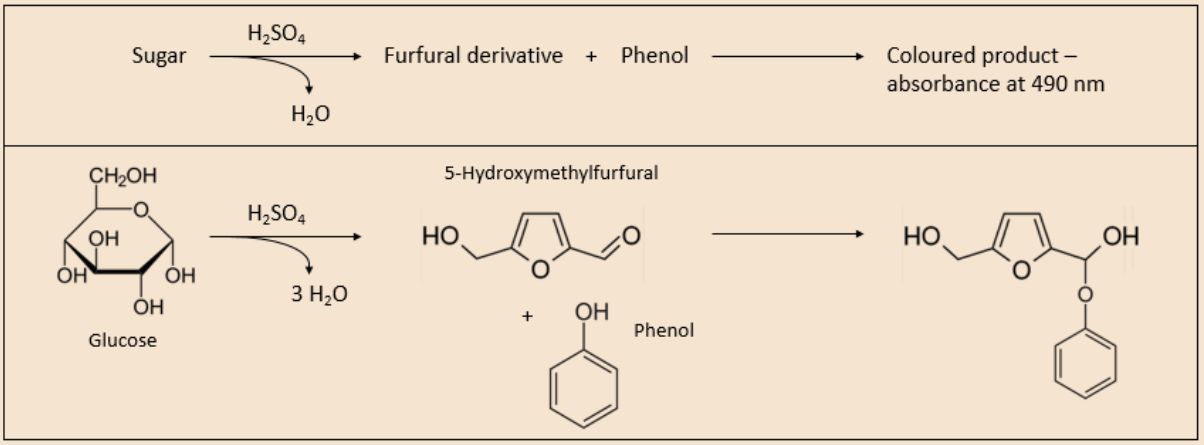


Figure 4.11 Reaction forming the basis of the phenol-sulphuric acid method for carbohydrate determination. Glucose is provided as an example (adapted from Panagiotopoulos and Sempéré (2005)).

The modified version of this assay presented by Albalasmeh et al. (2013) overcomes this drawback by bypassing the colouration step. Carbohydrates in the sample are once again hydrolysed using concentrated sulphuric acid, but the furfural derivatives resulting from this hydrolysis step are then quantified directly based on their UV absorbance. The wavelength of maximum absorbance is evaluated through the use of a spectral scan as it can shift based on the concentration of the sulphuric acid used.

The calibration curve for quantification of sugars in unknown samples was generated using maltose as the standard sugar, given that this is the predominant sugar in the cultivation medium. Initial evaluation of MESP medium samples revealed an overestimation of the sugar concentration, given an expected starting value of 60 – 70 % ($w.w^{-1}$) of the total medium components added. This overestimation could be attributed to interference of protein in the samples, given their inherent UV absorbance (Albalasmeh et al., 2013), and the sulphuric acid-UV method was, therefore, preceded by a protein precipitation step (Section 3.3.4.4, Appendix B.3.1).

The blank, standard and unknown samples were all subjected to protein precipitation prior to sulphuric acid hydrolysis, and sugar concentrations in the MESP medium were then found to align with expected values. The maltose calibration curve generated using this full method is shown in Figure 4.12.

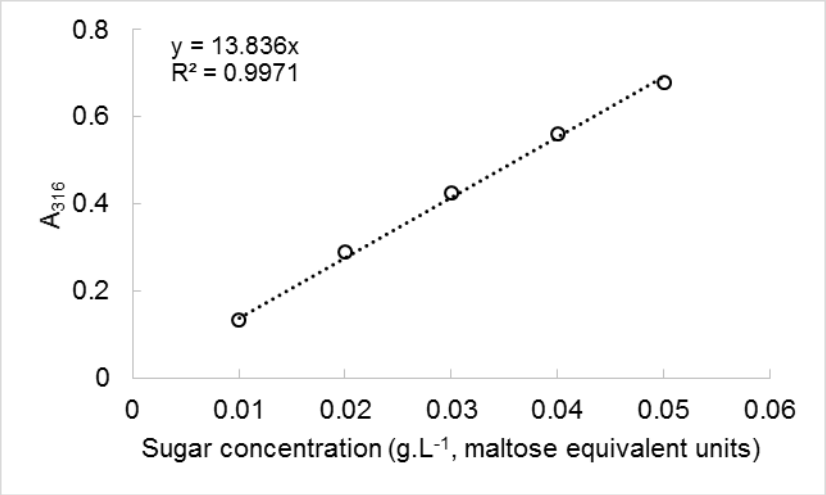


Figure 4.12 Calibration curve for sulphuric acid-UV assay used to quantify total carbohydrate content in BioFlo 110 bioreactor cultivation samples, following the protein precipitation step. Calibration curve was constructed using maltose with a range of 0.01 to 0.05 g.L^{-1}, and concentrations in unknown samples are expressed as maltose equivalent units. Error bars represent the standard deviation associated with triplicate measurements.

The method was also validated by spiking MESP medium samples with known amounts of maltose. Prepared MESP medium was diluted 1:1 with either sterile deionised water, 20 g.L^{-1} maltose stock solution or sterile deionised water and 20 g.L^{-1} maltose stock solution in ratios of 3:1 or 1:1. The resulting sugar concentrations determined in the assayed solutions are summarised in Table 4.9, with the difference in actual spiked amount and measured value provided.

Table 4.9 Validation of sulphuric acid-UV assay by spiking MESP medium with known concentrations of maltose. Results represent the average value across triplicate measurements.

Sample	**Composition**			**Measured sugar conc. (g.L^{-1})**	**Difference from X/ Measured spiked value (g.L^{-1})**	**Difference between actual and measured spiked values**	
	MESP	20 g.L^{-1} maltose	dH_2O			g.L^{-1}	% of amount spiked
X [a]	1	0	1	11.84 ± 0.57	/	/	/
X + 2.5 g.L^{-1}	4	1	3	14.15 ± 0.43	2.31	0.19	7.44
X + 5 g.L^{-1}	2	1	1	16.90 ± 0.61	5.06	0.06	1.27
X + 10 g.L^{-1}	1	1	0	21.28 ± 0.91	9.45	0.55	5.52

a X represents the sugar concentration in MESP medium diluted to half its original concentration

The results show a deviation of less than 10 % between spiked and calculated values in all assayed samples. This supported the use of this assay to quantify residual sugar concentration in samples taken during bioreactor cultivation of *P. purpurogenum* DSM 62866.

4.3.3 Overview of the base case reactor cultivation

The cultivation of *P. purpurogenum* DSM 62866 in the BioFlo 110 bioreactor proceeded for 6 days, during which time pH, growth, pigment production and sugar utilisation were monitored. The observed trends have been plotted in Figure 4.13.

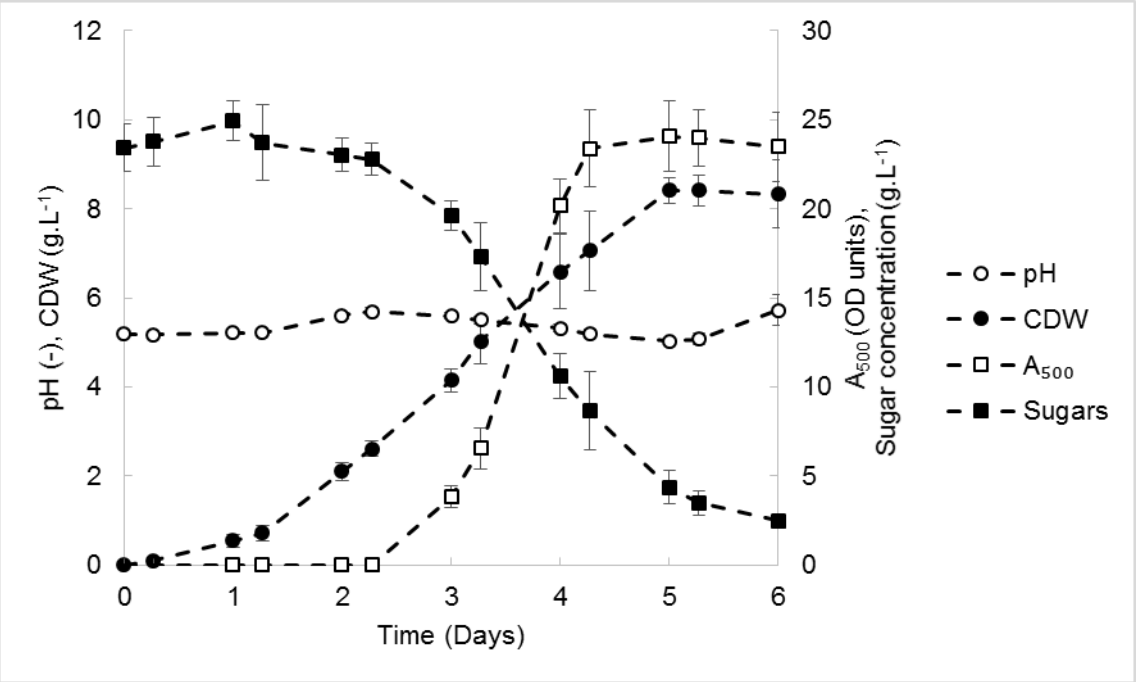


Figure 4.13 Cultivation summary of *P. purpurogenum* DSM 62866 grown in MESP medium, pH 5 (50 mM citrate buffer), at 30 °C, with agitation of 300 to 400 rpm, and aeration of 0.5 to 1 vvm, in the BioFlo 110 modular benchtop fermentor for 6 days. Results show the average pH, growth, pigment production, and sugar concentration for triplicate measurements performed during duplicate cultivations, with error bars representing the standard deviation of the data.

The pH of the culture grown in the bioreactor displayed the expected trends, with the characteristic peak during cultivation of *P. purpurogenum* (Figure 4.5b, Figure 4.7a) observed around day 2. The pH was maintained within the range of approximately 5.0 to 5.7 over the first 5 days of the cultivation, with indications of culture pH beginning to rise beyond this level only observed by day 6, once stationary phase was reached. Growth of the organism was visible by the 24-hour sampling point, beyond which biomass began to accumulate more rapidly. The culture grew as freely dispersed hyphae, exhibiting an overall homogenous appearance. By the 48 hour time point higher biomass concentration and associated increased oxygen demand of the culture meant that the agitation speed had to be increased above 300 rpm to maintain the dO_2 concentration of the system above 35 % (approximately 2.6 $mg.L^{-1}$). This was achieved using cascade control, which regulates agitation rate based on measured dO_2 concentration, with a maximum allowed agitation speed of 400 rpm. At around 100 hours, the aeration rate of the culture was reduced from 1 vvm to 0.75 vvm to control foaming, and by 125 hours it was decreased further to 0.5 vvm, while the agitation speed was decreased back down to 300 rpm. Given that the oxygen demand of the culture had declined by this stage, dissolved oxygen was maintained at

or above the pre-determined level of 35 % even with reductions in agitation speed and aeration rate. Maximum CDW of approximately 8.4 $g.L^{-1}$ was achieved by day 5 of the cultivation.

Red pigmentation was first observed at the 72-hour sampling point, exhibiting an A_{500} value of approximately 3.8 OD units. Extrapolating backwards using the period of approximately linear increase in pigmentation between 78.5 and 102.5 hours of cultivation, the latest point at which pigment production could have started was calculated to be approximately 69 hours. This is shown in Figure 4.14, and indicates that coloured products began to be formed between 54.5 and 69 hours of cultivation.

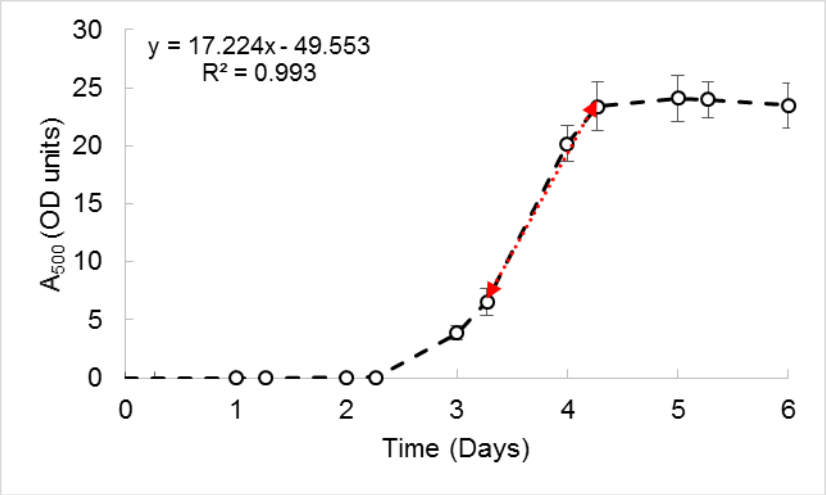


Figure 4.14 Period of linear increase in pigmentation during base case BioFlo 110 bioreactor cultivation of *P. purpurogenum* denoted by the arrows, with the corresponding trendline equation provided.

Pigmentation then increased rapidly, reaching approximately 20 OD units within the following 24 hours. Beyond day 4 of the cultivation pigment production began to slow, reaching a maximum A_{500} value of approximately 24 OD units by the 120-hour sampling point. Numerical data summarising the cultivation, obtained at sequential 24-hour sampling points, is provided in Table 4.10.

Table 4.10 Cultivation data recorded every 24 hours during growth of *P. purpurogenum* DSM 62866 on MESP medium, pH 5, 50 mM citrate buffer at 30 °C in the New Brunswick Scientific BioFlo 110 fermentor.

Day	**pH**	**Growth**		**Pigmentation**			**Sugar conc.**
		CDW, $g.L^{-1}$	$g.L^{-1}.h^{-1}$	OD units	$OD.h^{-1}$	$OD.g_x^{-1}.h^{-1}$	Maltose-equivalent units, $g.L^{-1}$
1	5.22 ± 0.01	0.55 ± 0.15	0.02 ± 0.01	ND	-	-	24.94 ± 1.15
2	5.61 ± 0.07	2.10 ± 0.19	0.06 ± 0.00	ND	-	-	23.03 ± 0.93
3	5.60 ± 0.08	4.15 ± 0.26	0.08 ± 0.00	3.84 ± 0.62	0.16 ± 0.03	0.05 ± 0.01	19.62 ± 0.83
4	5.32 ± 0.08	6.58 ± 0.84	0.10 ± 0.04	20.18 ± 1.53	0.68 ± 0.12	0.13 ± 0.01	10.60 ± 1.25
5	5.03 ± 0.06	8.42 ± 0.29	0.08 ± 0.04	24.07 ± 1.97	0.16 ± 0.02	0.02 ± 0.00	4.36 ± 0.93
6	5.73 ± 0.34	8.33 ± 0.76	-	23.48 ± 1.95	-	-	2.50 ± 0.23

ND Not detected

The morphology of the culture was also monitored visually at the sequential 24-hour sampling points to determine whether any obvious changes were linked to observed pH or growth trends, or the onset of pigment production. Representative images of the fungal morphology observed as the cultivation progressed are provided in Figure 4.15.

The only notable morphological change occurred around day 3 of the cultivation, where the production of conidiospores was observed for the first time. Conidiospores are observed at the tips of specialized hyphae known as conidiophores, where successive spores are produced by specialized cells known as phialides (Campbell et al., 2013). The reason for the onset of sporulation is unclear. Sporulation can be linked to endogenous signals or external factors such as light, pH or nitrogen limitation (Roncal and Ugalde, 2003; Bayram and Braus, 2012). Interestingly, the onset of sporulation does coincide with the onset of pigment production.

The link between sporulation and secondary metabolism has been reported previously, with the environmental conditions required for these cellular processes often exhibiting similarities (Calvo et al., 2002; Bayram and Braus, 2012). Calvo et al. (2002) reported that secondary metabolites associated with sporulation can either activate sporulation, be required to be incorporated into sporulation structures, or merely be secreted at the approximate time of sporulation.

Previous reports suggest a link between sporulation and carbohydrate limitation (Jičínská, 1968; Roncal and Ugalde, 2003), however, it is observed in Figure 4.13 that on day 3 of the cultivation when pigmentation and sporulation are first observed, the residual sugar concentration in the medium is high. A relationship between nitrogen metabolism and secondary metabolism has been suggested during cultivation of *P. purpurogenum* for pigment production (Kojima et al., 2016) and could be investigated further as a potential trigger for the onset of pigment production.

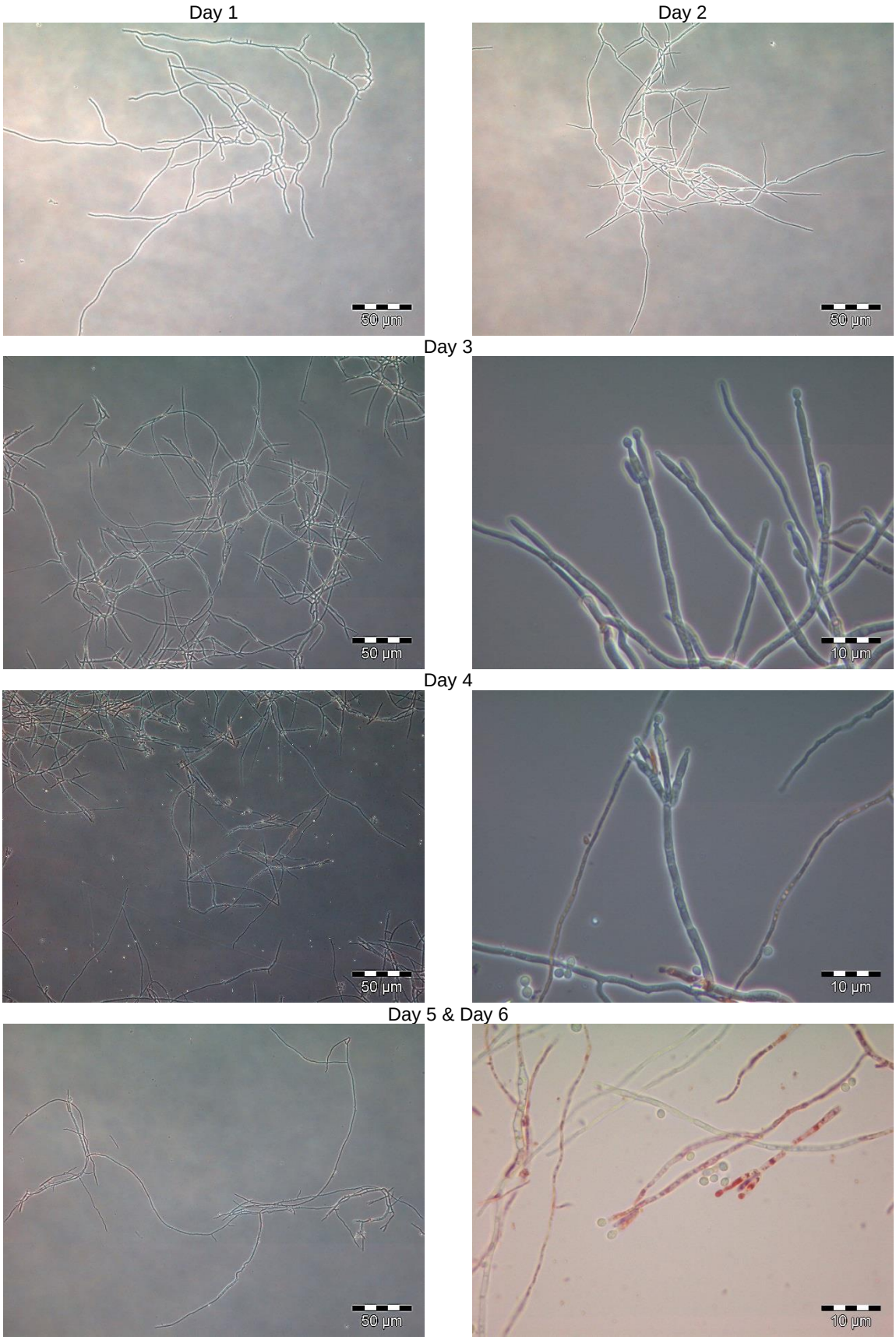


Figure 4.15 Morphology of *P. purpurogenum* DSM 62866 during cultivation in the BioFlo 110 fermentor using MESP medium, pH 5, 50 mM citrate buffer, at 30 °C.

4.3.4 Growth kinetics and yields

To further analyse the relationship between growth, pigment production and the utilisation of sugar substrate, the overall trends for these cultivation parameters were investigated and compared. This allowed calculation of the maximum growth rate of the organism, as well as various yields which define the cultivation, as described in Section 3.4.

Observation of the growth trend of *P. purpurogenum* in the bioreactor system revealed 3 approximate periods defined by different biomass productivities. The first growth period, between 0 and 30 hours of cultivation, was defined by a linear increase in biomass concentration of approximately 0.024 $g.L^{-1}.h^{-1}$. Although this growth rate is low, the linear trend indicates that germination of the fungal spores occurs rapidly in this culture medium. The second period also exhibited a linear increase in CDW, up to the 120 hour sampling point, but at a significantly higher rate of approximately 0.089 $g.L^{-1}.h^{-1}$. These biomass productivities are provided in Table 4.11. The third period, beyond 120 hours of cultivation was defined by no further increase in biomass concentration, indicating entry into the stationary phase of growth.

The linear increase in biomass concentration which defined the major growth periods of the cultivation can be explained by the filamentous nature of *P. purpurogenum*, where growth is achieved predominantly via elongation of the fungal hyphae rather than by cell division. Spore germination initially results in exponential increase in hyphal length, but this becomes limited by incorporation rates at the tip or transport of nutrients to the tip from increasingly distant regions. Fungal hyphae then extend at a linear rate, with exponential growth possible through hyphal branching. New hyphal branches do, however, continue to extend at the linear rate (Gow and Gadd, 1995). The shift from apical (tip extension) to lateral growth (branching) is generally the result of accumulation of vesicles at the hyphal tip, or for improved nutrient acquisition (Harris, 2008). The increase in biomass productivity observed at approximately 30 hours of cultivation indicates that branching may have occurred, but that continued, exponential branching was not required to cope with the accumulation of cell products. The result was continued linear growth, but at a higher growth rate.

A maximum specific growth rate of $0.099 \pm 0.010\ h^{-1}$ was achieved during the *P. purpurogenum* cultivation on MESP medium in the BioFlo 110 bioreactor, as determined by regression analysis. This maximum rate was recorded over the first 24 hours of the cultivation, which correlates well with the observation that growth is largely achieved through elongation of the fungal hyphae. At the start of the cultivation the fungal spores germinate and develop into hyphae, resulting in the greatest specific change in biomass concentration (gram of biomass formed per gram of biomass present per unit time). Beyond this point, rate of growth is dependent on the rate of elongation of the fungal hyphae, and is a function of nutrient availability and the number of hyphal tips (Gow and Gadd, 1995).

This maximum specific growth rate was, however, evaluated based on only 2 time points. If this is extended to include the third sampling point at approximately 30 hours, this rate declines to 0.087 ± 0.008 h^{-1}, indicating that the culture, given its filamentous nature, has entered a period of linear growth.

Table 4.11 Biomass productivity during different growth phases and maximum specific growth rate of *P. purpurogenum* DSM 62866 when cultivated in the BioFlo 110 bioreactor using MESP medium, pH 5, 50 mM citrate buffer, at 30 °C.

Time (hours)	Average biomass productivity [a]		Specific growth rate [a]	
	$g.L^{-1}.h^{-1}$	R^2 [b]	h^{-1}	R^2 [b]
0-30	0.024 ± 0.007	0.995	-	-
6-24	-	-	0.099 ± 0.010	-
6-30	-	-	0.087 ± 0.008	0.980
30-120	0.089 ± 0.007	0.997	Decreases with time	-

a Results presented as average ± standard deviation across duplicate cultivations
b Determined through linear regression analysis

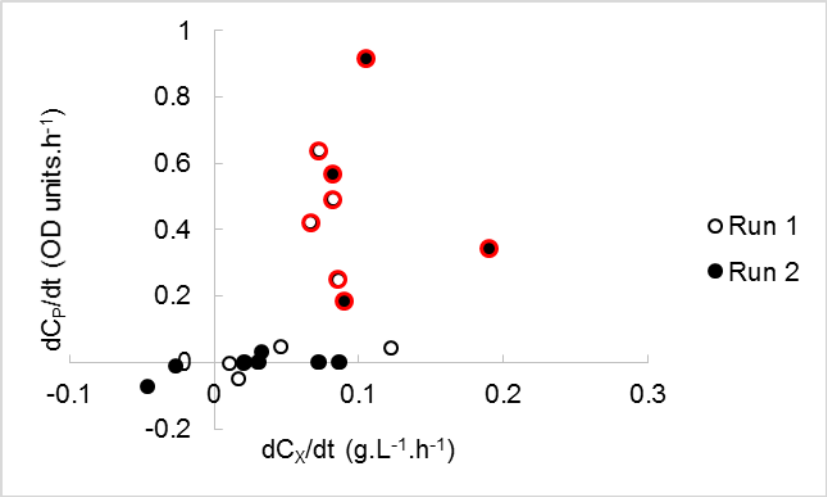
Linear trends were also observed for pigment production and sugar utilisation. Pigmentation was estimated to start between 54.5 and 69 hours of cultivation (Figure 4.14), with a linear increase observed between approximately 72 and 100 hours. Maximum pigment productivity during this period was recorded between 78 and 100 hours and was defined by a rate of 0.72 ± 0.18 OD units.h^{-1}, determined through linear regression analysis. It was noted that pigment production began after the initiation of growth and continued during the linear growth phase. Pigmentation also stopped increasing before the maximum biomass concentration was reached.

Pigment production was evaluated by plotting the change in volumetric pigment concentration with time against either the change in biomass concentration with time, or the volumetric biomass concentration to determine the growth (α) and non-growth (β) associated specific rates of pigment production, based on the Luedeking-Piret model of product formation (Luedeking and Piret, 1959) shown in Equation 7.

$$\frac{dCp}{dt} = \alpha.\frac{dCx}{dt} + \beta.Cx \qquad \text{Equation 7}$$

The change in volumetric pigment concentration against change in biomass concentration showed no discernible trend, with the data from the duplicate runs plotted separately in Figure 4.16a. The data points indicated in red represent the period of linear increase in volumetric pigment concentration. A correlation was, however, observed between change in pigment concentration with time and volumetric biomass concentration, as shown in Figure 4.16b. The trendline equation (y = 0.2305x – 0.7397) relates to the time period of 72 to 96 hours, with the slope of this line defining the non-growth associated specific rate of pigment production. The results indicate that pigment production by *P. purpurogenum* DSM 62866 is non-growth associated, and dominantly biomass associated, with a β value of 0.23 OD units.$g_x^{-1}.h^{-1}$ (SE_m = 0.002).

a



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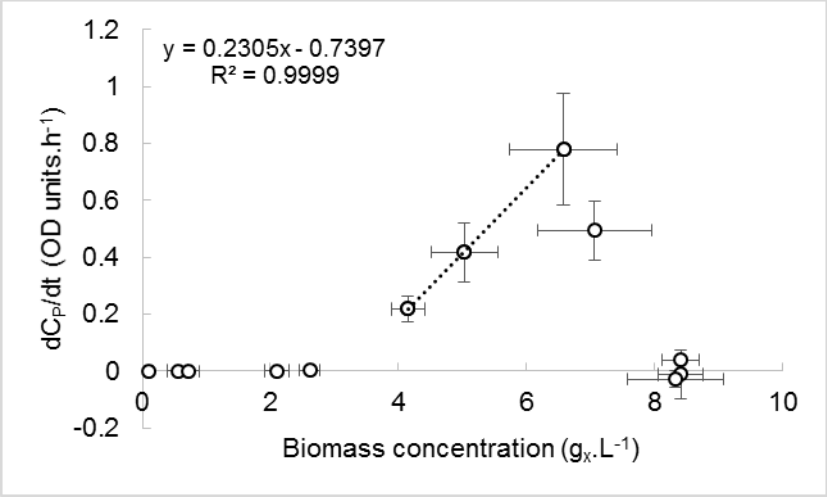


Figure 4.16 Change in volumetric pigment concentration (dC_P/dt) as a function of change in biomass concentration (dC_X/dt, a) or volumetric biomass concentration (b) to evaluate the growth and non-growth associated specific rates of pigment formation during base case BioFlo 110 reactor cultivation of *P. purpurogenum* DSM 62866. Error bars indicate the standard deviation across duplicate cultivations, unless plotted separately.

The majority of the sugars in the medium were consumed over the period of approximately 48 to 120 hours of cultivation, corresponding to maximum biomass and pigment productivity. While biomass concentration continued to increase to day 5 of the cultivation, pigment production appeared to cease by day 4 when residual sugar concentration was still high. The volumetric pigment concentration remained approximately stable until the end of the cultivation.

Based on the assumption that minimal sugars are consumed for the generation of red pigment products, the biomass formed and sugars consumed over the period of 48 to 120 hours was used to calculate the yield of pigment on biomass ($Y_{X/S}$). This value was calculated using Equation 16, as described in Section 3.4. The yield of pigment on biomass ($Y_{P/X}$) was calculated based on maximum CDW and A_{500} values, achieved after 120 hours of cultivation, using Equation 17. The yields in this system were calculated to be 0.37 ± 0.01 $g_x.g_s^{-1}$ ($Y_{X/S}$) and 2.86 ± 0.29 OD units.g_x^{-1} ($Y_{P/X}$).

These yields and productivities (Table 4.11) define the cultivation of *P. purpurogenum* DSM 62866 on this medium under the given cultivation conditions. These values can be used to compare cultivation performance when changing operating parameters or medium composition, thereby helping to inform process and product potential.

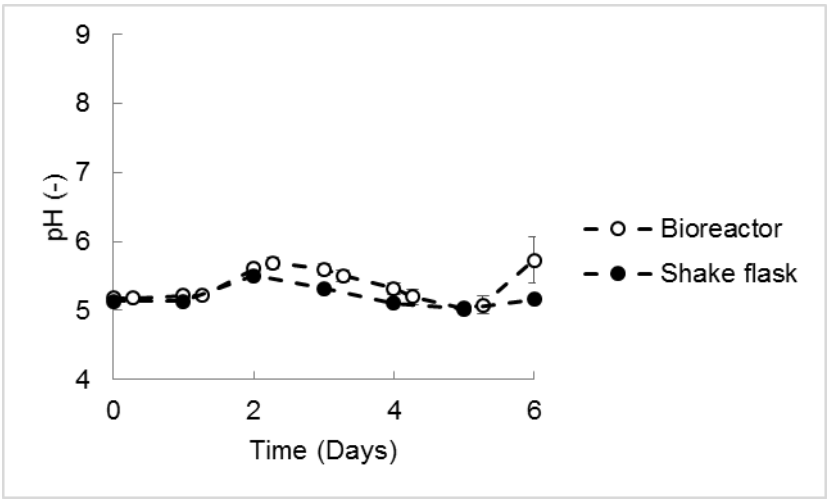
4.3.5 Comparison of shake flask and bioreactor cultivation performance using MESP medium

The base case cultivation in the bioreactor was compared to the results obtained in shake flasks using the MESP medium, maintained at a pH of 5 through the application of 50 mM citrate buffer, incubated at 30 °C with shaking at 150 rpm. These represent the same conditions of temperature and pH used in the bioreactor cultivation, and the shaking condition which resulted in highest volumetric pigment concentrations, of approximately 25 OD units. This comparison was performed in order to determine whether pigment production could be maintained or improved when scaling up cultivation, as well as to investigate the impact of this cultivation system on growth of the organism. Comparison of the pH, growth and pigment production trends in the 100 mL shake flask cultivation and the 5 L bioreactor cultivation is shown in Figure 4.17.

The culture pH in the shake flask and bioreactor cultivations exhibited the same trend, rising from a starting pH of approximately 5 to a peak of between 5.5 (shake flask) and 5.7 (bioreactor) around day 2 of the cultivation. The pH then declined again to a value close to the initial pH value by day 5, before starting to increase once again. The differences between the cultivations were a slightly broader pH range in the bioreactor cultivation, with higher pH values observed in this system between day 2 and day 5 of the cultivation, as well as a more rapid increase in pH observed between day 5 and day 6, when compared to shake flask cultures.

Similar growth trends were observed in the shake flask and bioreactor cultivations, exhibiting volumetric growth rates of 0.079 ± 0.002 $g.L^{-1}.h^{-1}$ (Table 4.3) and 0.089 ± 0.007 $g.L^{-1}.h^{-1}$ (Table 4.11), and achieving maximum CDW values of 8.57 ± 0.15 $g.L^{-1}$ and 8.42 ± 0.29 $g.L^{-1}$, respectively. This is consistent with the findings of Nikakhtari and Hill (2005), who stated that given the high k_La values which can be achieved in shake flasks under conditions of low fill volume and high shaking speed, the surface aeration achieved may be sufficient to allow aerobic growth kinetics similar to those observed in scaled-up bioreactors, where aeration and mixing are more easily controlled.

a



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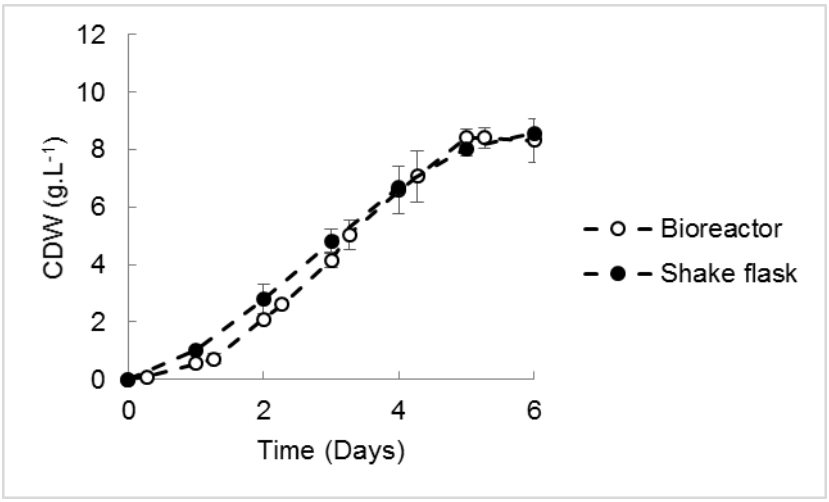

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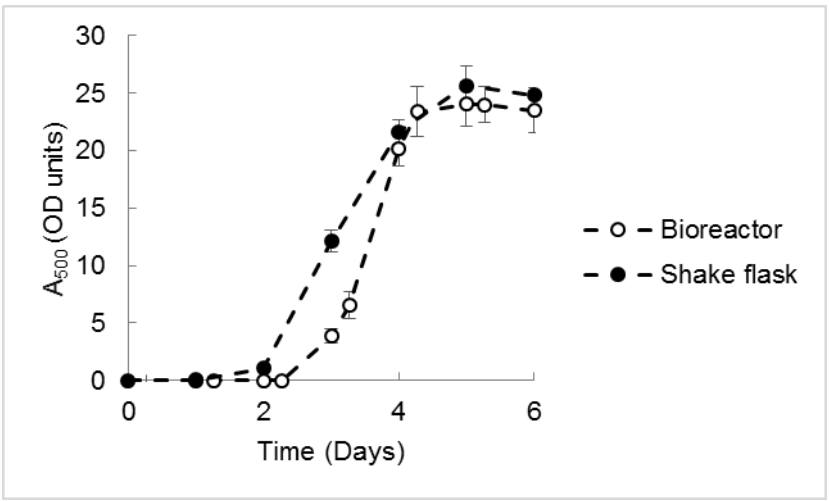


Figure 4.17 Comparison of the pH (a), growth (b), and pigment production (c) trends observed during cultivation in MESP medium, pH 5, 50 mM citrate buffer, at 30 °C in the BioFlo 110 fermentor and shake flasks (150 rpm). Bioreactor data represents the average of triplicate measurements performed during duplicate cultivations, while shake flask data represents the average measurement across triplicate flasks. Error bars represent the standard deviation of the data.

Pigment production trends were similar in the two cultivation systems, but varied in terms of time of onset, with pigmentation first visualised on day 2 of the shake flask cultivation, but only by day 3 of the bioreactor cultivation. The rate of pigment production also differed between the cultivation systems, with a production rate of 0.43 OD units.h^{-1} between 48 and 96 hours in shake flasks, and 0.72 OD units.h^{-1} between 78.5 and 102.5 hours during bioreactor cultivation. The specific pigment production over these same periods was found to be 5.28 OD units.$(g_x.L^{-1})^{-1}$ in shake flasks and 8.40 OD units.$(g_x.L^{-1})^{-1}$ in the bioreactor as determined by linear regression using the Microsoft Excel LINEST function. These cultivation parameters are summarised in Table 4.12, with the plot of A_{500} against biomass provided in Figure 4.18 as a visual representation of this production trend.

Table 4.12 Average pigment productivity and specific pigment production achieved during shake flask and BioFlo 110 bioreactor cultivation of *P. purpurogenum* DSM 62866 in MESP medium, pH 5 (50 mM citrate buffer) at 30 °C.

	Maximum average pigment productivity		**Specific pigment productivity**	
	OD units.h^{-1}	R^2 **a**	OD units.$(g_x.L^{-1})^{-1}$	R^2 **a**
Bioreactor **b**	0.72 ± 0.18	0.993	8.40 ± 2.07	0.997
Shake flask **c**	0.43 ± 0.03	0.998	5.28 ± 0.32	0.999

a Determined through linear regression analysis
b Results presented as average ± standard deviation across duplicate cultivations
c Results presented as average ± standard deviation across triplicate flasks

Although pigment production started later in the bioreactor, productivity was higher, resulting in volumetric pigment concentrations comparable to those in shake flasks being achieved. The shake flask and bioreactor cultivations achieved maximum A_{500} values of 25.7 OD units and 24.1 OD units, respectively, after 5 days. A t-Test, two sample assuming unequal variance, revealed that no significant difference exists between the maximum pigmentation achieved in each system (data shown in Table G.1). This shows that, during scale-up of the cultivation, pigment production was able to be maintained, but bioreactor cultivation conferred no additional benefit in terms of volumetric pigment concentration achieved. The higher volumetric and specific rates of pigment production in the bioreactor system do represent areas of potential interest, if the production period could be extended thus increasing volumetric pigment yields.

Considering that pigment production was shown to increase with increasing shaking speed during incubation of shake flask cultures, up to a maximum A_{500} value of approximately 25 OD units at a shaking speed of 150 rpm (Section 4.2.1), volumetric pigment concentrations of approximately 25 OD units or greater were expected in the bioreactor. This was based on the ability to maintain the dO_2 concentration at or above 35 % through adjustment of the agitation rate in the system, while also achieving equivalent or greater k_La values than those in shake flask cultures (Section 4.2.2). Equivalent levels of pigmentation were achieved when the cultivation was scaled-up into the bioreactor system, indicating that oxygen availability in the system was suited to pigment production by the organism. This could not, however, be attributed to either a minimum required residual oxygen concentration or oxygen transfer rate. Control of parameters influencing k_La in the BioFlo 110 bioreactor allowed these aspects to be investigated further.

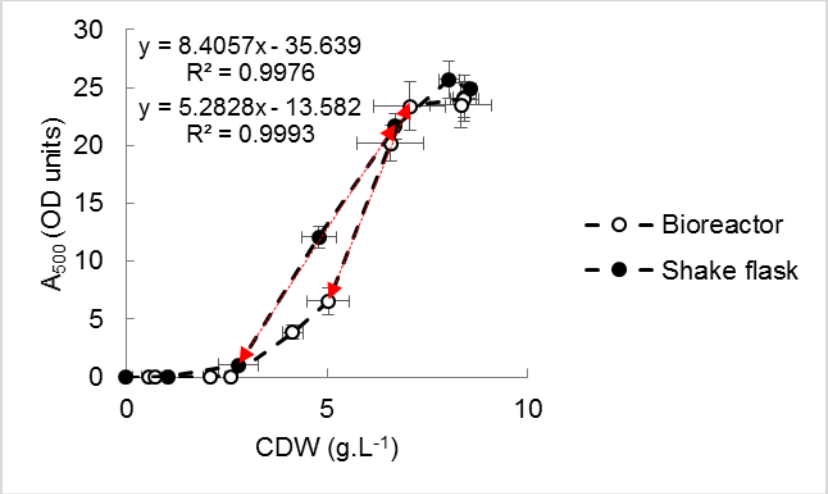


Figure 4.18 Pigment production (A_{500}) plotted against growth (CDW) achieved by *P. purpurogenum* DSM 62866 in BioFlo 110 bioreactor and shake flask cultures, using MESP medium, pH 5, 50 mM citrate buffer, at 30 °C. Bioreactor results are average values of triplicate measurements performed in duplicate cultivations, and shake flasks results are average values across triplicate flasks, with error bars representing the standard deviation of the data. The trendline equations correspond to the period of maximum specific pigment production as denoted by the arrows.

4.4 Investigation of oxygen availability on pigment production using the BioFlo 110 bioreactor system

The New Brunswick Scientific BioFlo 110 bioreactor system was used to further assess the impact of oxygen availability on growth and pigment production, verify the trends of increasing pigmentation with increasing oxygen mass transfer coefficient and oxygen transfer rate as observed during shake flask cultivation (Section 4.2.1), and determine whether there is an optimum oxygen transfer rate beyond which no further improvement in pigment production is observed.

It is important to remember that a number of other factors are affected by altering shaking platform speed or agitation speed. Overall mass transfer in the culture is altered, affecting transfer of nutrients to the biomass and removal of waste products. The focus is however, on oxygen transfer and oxygen availability as this is hypothesised to have a dominant effect on growth and pigment production, while also having easily measurable outcomes.

A set of experiments was performed using the same pH, temperature and aeration parameters as the base case bioreactor cultivation, listed in Table 4.8. Impeller speed was varied, with a range of set points selected and applied. Duplicate 6-day cultivations were performed for each agitation speed using the MESP medium. Direct spore inoculation and monitoring of pH, growth and pigmentation proceeded as described for the base case cultivation (Section 4.3.1).

Monitoring of the dO_2 concentration during BioFlo 110 cultivation allowed residual oxygen concentration in the medium and rate of oxygen transfer into the medium to be evaluated independently with regard to the effect of these factors on growth and pigmentation of *P. purpurogenum*. Experiments presented

in Section 4.4.1 explored whether pigmentation is a function of residual oxygen concentration, while in Section 4.4.2 the effect of oxygen transfer rate on pigmentation is investigated.

4.4.1 Pigmentation as a function of residual oxygen concentration

Base case cultivation of *P. purpurogenum* in the BioFlo 110 bioreactor was performed using an initial agitation speed of 300 rpm. This was selected based on evaluation of the oxygen mass transfer coefficient, or k_La, associated with this agitation speed in the bioreactor system and the k_La required for pigment production based on experimental results of shake flask cultivations, as described in Section 4.2.1. Although the k_La value at this speed was calculated to be greater than that required for pigmentation, it was insufficient to maintain the dO_2 concentration in the culture above the 35 % minimum set point. To investigate whether residual oxygen concentration is an important factor for pigment production by *P. purpurogenum*, the agitation speed of the system was set and maintained at 300 rpm throughout the cultivation. The cultivation at this set agitation speed in the BioFlo 110 bioreactor proceeded for 6 days, with pH, growth and pigment production monitored twice daily. The cultivation trends are provided in Figure 4.19.

The pH of the culture was maintained within the range of 5.1 to 5.9, with the highest pH value recorded around day 3, when pigmentation began to be formed. Growth exhibited an almost linear increase in CDW recorded between day 2 and day 5. This was described by a volumetric growth rate or biomass productivity of 0.089 ± 0.031 $g.L^{-1}.h^{-1}$. Beyond 120 hours, no further increase in biomass was observed, with the maximum biomass concentration achieved being approximately 8.1 $g.L^{-1}$. Pigmentation was observed by day 3 of the cultivation, reaching a maximum of 24 OD units by day 5.

By the 48-hour time point, oxygen demand of the culture had reduced the dO_2 concentration to 0%, meaning that the oxygen utilization rate of the culture was equal to or greater than the rate of oxygen transfer into the liquid medium. During base case cultivation in the system, this was prevented by allowing the agitation speed to increase to a maximum of 400 rpm, thereby maintaining the dO_2 concentration above 35 %. These two cultivations were, therefore, compared in order to observe whether residual oxygen concentration was an important parameter for pigment production by *P. purpurogenum*. The pH, growth and pigment production trends for the base case and 300 rpm cultivations are plotted in Figure 4.20.

The pH during the cultivation of *P. purpurogenum* in MESP medium at 300 rpm continued to increase beyond day 2, reaching a maximum of approximately 5.9 by day 3. The pH trends at 300 rpm, therefore, differed from those under base case conditions, but did not appear to impact either growth or pigment production of the organism. Maximum biomass concentrations achieved were 8.4 ± 0.3 $g.L^{-1}$ and 8.1 ± 1.6 $g.L^{-1}$ by day 5 of the cultivation under base case agitation conditions or 300 rpm, respectively. Maximum biomass productivities of 0.089 ± 0.007 $g.L^{-1}.h^{-1}$ (base case) and 0.089 ± 0.031 (300 rpm) were recorded as shown in Table 4.13.

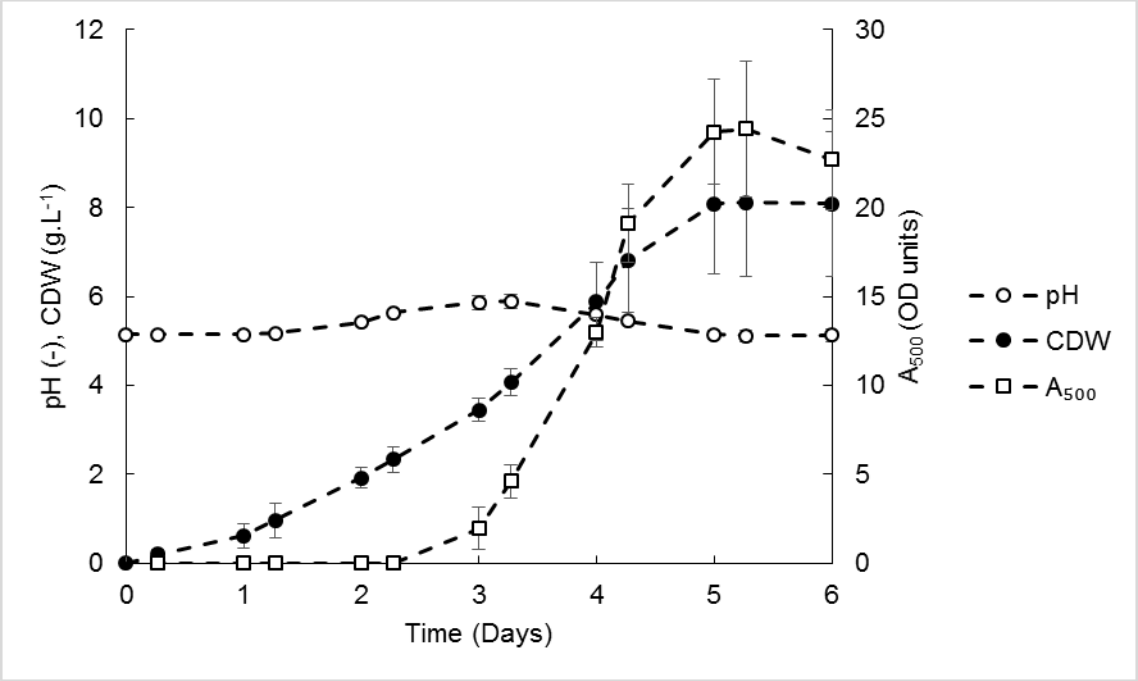
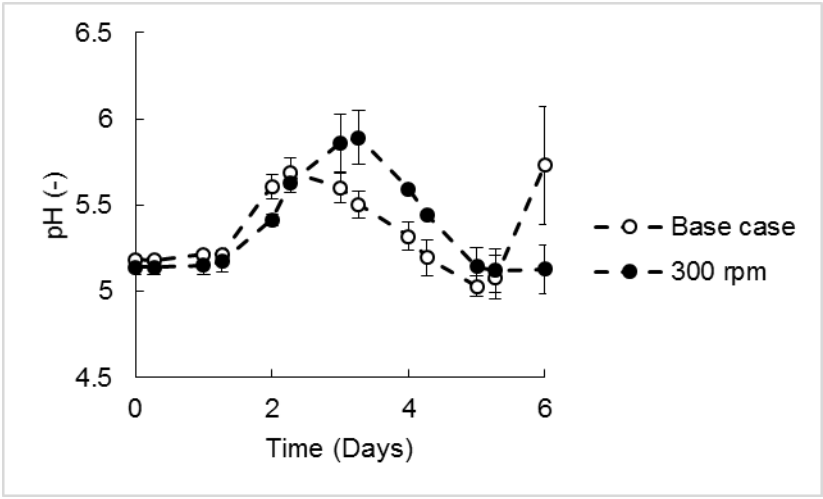


Figure 4.19 Cultivation summary for *P. purpurogenum* DSM 62866 grown in MESP medium, pH 5 (50 mM citrate buffer), at 30 °C, with agitation maintained at 300 rpm, and aeration of 0.5 to 1 vvm, in the BioFlo 110 modular benchtop fermentor for 6 days. Results show the average pH, growth and pigment production trends based on triplicate measurements performed during duplicate cultivations. Error bars represent the standard deviation of the data.

As observed in Figure 4.20c, the rate of increase in pigmentation was lower in the 300 rpm cultivation, but an equivalent maximum volumetric pigment concentration, defined by 24 OD units, was achieved in both the base case and 300 rpm cultivations. This maximum was observed by day 5 in the 300 rpm cultivation rather than day 4 under base case conditions.

These results indicate that residual oxygen concentration in the cultivation broth does not have a notable effect on growth or pigmentation of *P. purpurogenum* where ongoing gas-liquid mass transfer is maintained. Whether dO_2 concentration was maintained above 35 % or allowed to decrease to 0 %, very similar growth and pigment production trends were observed. Therefore, the conclusion was drawn that pigmentation is likely not affected by oxygen limitation, or dependent on a certain residual oxygen concentration in the medium. The positive correlation observed between pigmentation and shaking speed during shake flask cultivation (Section 4.2.1) does, however, indicate that oxygen availability plays a determining role in pigment production by *P. purpurogenum* and should, therefore, be further explored in terms of its rate of supply.

a



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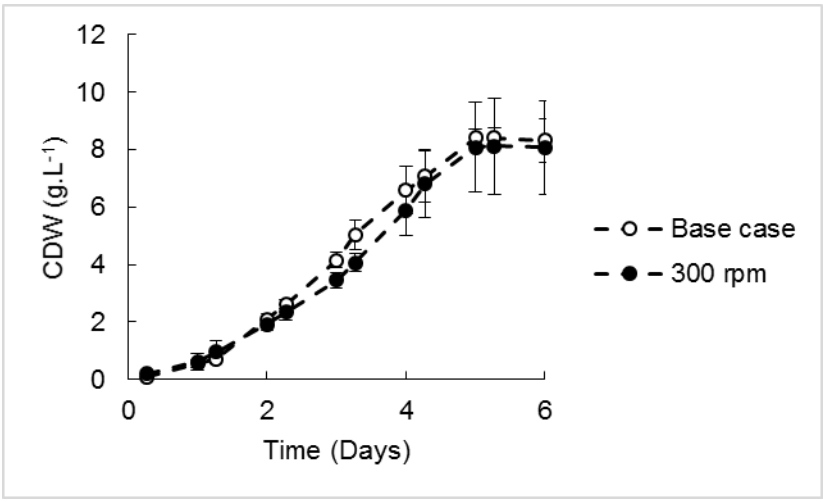

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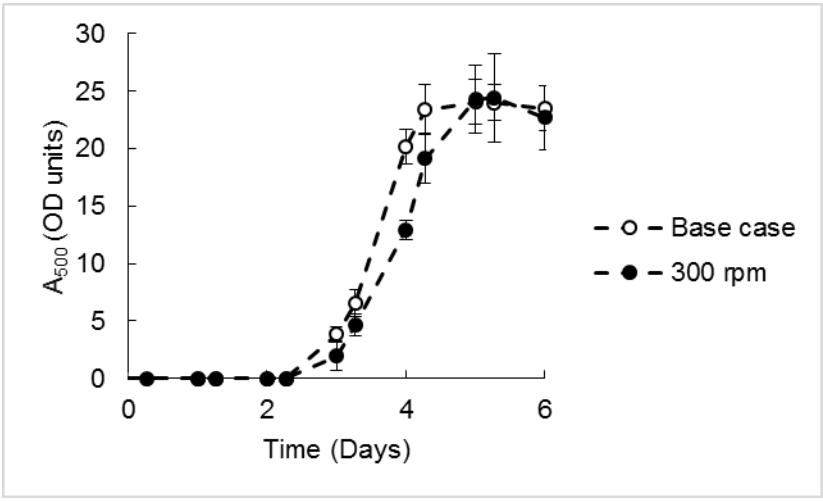


Figure 4.20 Comparison of the pH (a), growth (b), and pigment production (c) trends observed during cultivation in MESP medium, pH 5, 50 mM citrate buffer, at 30 °C in the BioFlo 110 fermentor with agitation either ranging between 300 and 400 rpm (base case), or maintained at 300 rpm throughout. Data presented is the average of triplicate measurements performed during duplicate cultivations, with error bars representing the standard deviation of the data.

Table 4.13 Maximum biomass productivity of *P. purpurogenum* DSM 62866 during cultivation in the BioFlo 110 bioreactor using MESP medium, pH 5, 50 mM citrate buffer, at 30 °C under base case conditions or with agitation maintained at 300 rpm throughout.

	Average biomass productivity [a]		
	Time period (h)	$g.L^{-1}.h^{-1}$	R^2 [b]
Base case	30.5 – 120	0.089 ± 0.007	0.997
300 rpm	48 – 120	0.089 ± 0.031	0.988

a Results presented as average ± standard deviation across duplicate cultivations
b Determined through linear regression analysis

4.4.2 Pigmentation as a function of oxygen transfer

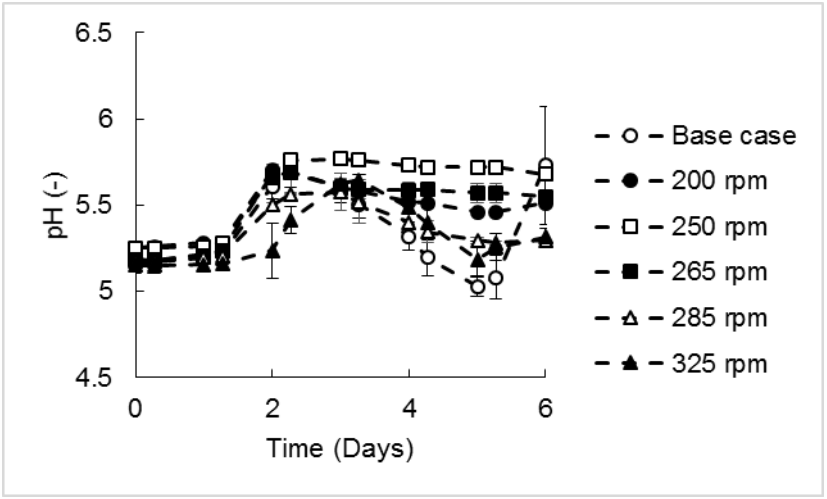
The impact of rate of oxygen supply on pigmentation was investigated further by varying the gas-liquid mass transfer coefficient and thus affecting rate of oxygen transfer into the culture broth. This was achieved by altering agitation rate in the BioFlo 110 reactor system across the range of 200 to 325 rpm, while maintaining aeration at a set point of 1 vvm.

An agitation speed of 200 rpm was selected as the starting point for this set of experiments, with successive speeds selected based on the observed pigmentation. The full set of agitation speeds investigated was 200, 250, 265, 285 and 325 rpm, all of which were compared to the base case BioFlo 110 cultivation. The 300 rpm set point cultivation was not included in these analyses given its similarity to the base case cultivation (Section 4.4.1). Cultivation parameters other than agitation speed were consistent with those applied during the base case cultivation (Table 4.8), including the use of the MESP medium. All cultivations, except those at 200 rpm and 250 rpm, were completed in duplicate.

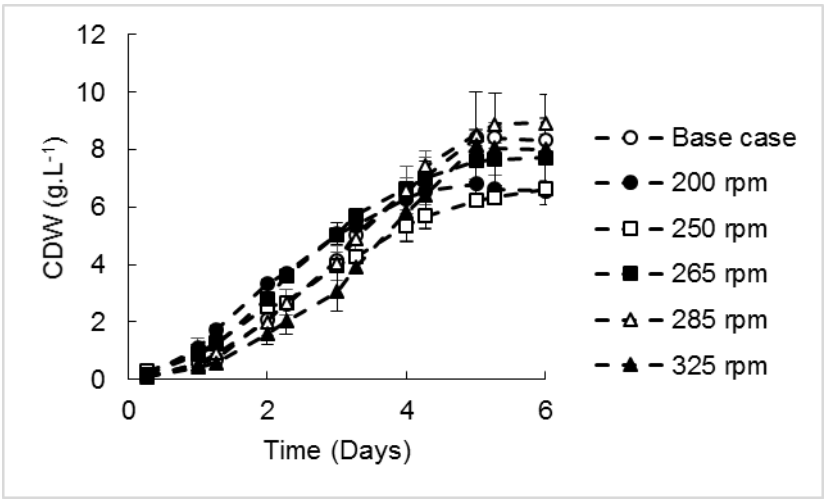
It was expected that pigmentation would decrease with decreasing agitation speed, with the base case cultivation considered to represent 300 rpm, the agitation rate at the start of the cultivation. Agitation at 325 rpm was included to determine whether raising the agitation speed above that applied as the starting value in the base case cultivation would result in improved culture pigmentation. The pH, growth and pigment production observed during cultivation of *P. purpurogenum* over this range of agitation speeds is plotted in Figure 4.21.

The conditions showed similar pH trends over the first 2 days of the cultivation, with the maximum pH exhibited by this time point under most conditions, with the exception of 325 rpm. Maximum pH value achieved in all cases was in the range of 5.5 to 5.8. Beyond day 2, cultivations with agitation speeds in the range of 200 rpm to 265 rpm showed only a slight downward trend to the end of the cultivation period. The other speeds considered, however, resulted in a more notable decrease, exhibiting pH values in the range of 5 to 5.3 units by day 5.

a



b



c

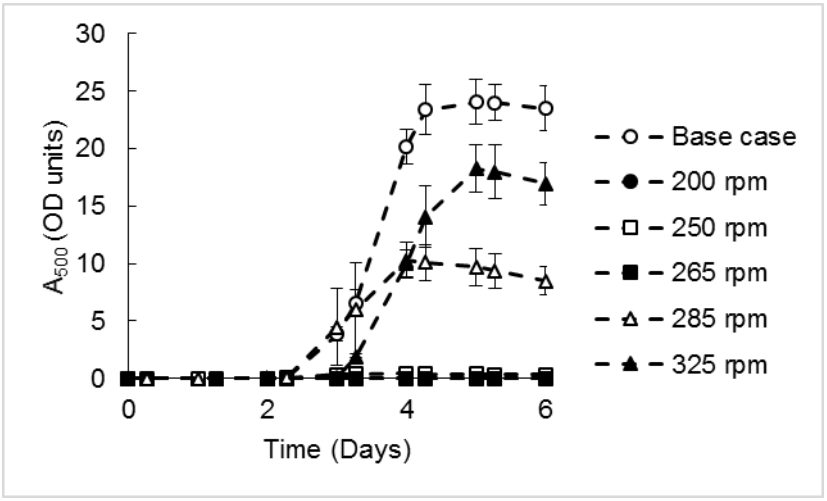


Figure 4.21 pH (a), growth (b), and pigment production (c) trends observed during cultivation in MESP medium, pH 5, 50 mM citrate buffer, at 30 °C in the BioFlo 110 fermentor while applying a range of agitation speeds. Data is reported as the average of triplicate measurements performed during duplicate cultivations, except in the case of 200 rpm and 250 rpm where single cultivations were performed. Error bars represent the standard deviation of the data.

The majority of conditions resulted in a maximum biomass concentration in the range of 8 to 9 $g.L^{-1}$. Cultivations performed at 200 rpm and 250 rpm, however, exhibited less growth, with maximum CDW reaching approximately 6 to 7 $g.L^{-1}$. Maximum biomass productivity was achieved during a period of approximately linear increase in CDW, continuing to day 4 or day 5 in all cultivations. This has been summarised, along with maximum volumetric biomass concentration, in Table 4.14. Biomass productivity achieved again demonstrates a difference in growth when agitating at 200 or 250 rpm in comparison to the higher speeds considered. Cultivations performed at or above 265 rpm all exhibited very similar maximum biomass productivities.

Table 4.14 Maximum biomass concentration and biomass productivity achieved when cultivating *P. purpurogenum* DSM 62866 in the BioFlo 110 bioreactor system under varying conditions of agitation speed.

Agitation condition	**Maximum biomass concentration**	**Average biomass productivity**		
	$g.L^{-1}$	Time period (h)	$g.L^{-1}.h^{-1}$	$R^{2\,a}$
Base case [b]	8.42 ± 0.29	30 – 120	0.089 ± 0.007	0.997
200 rpm [c]	6.80 ± 0.10	24 – 78	0.079 ± 0.003	0.994
250 rpm [c]	6.63 ± 0.21	30 – 102	0.062 ± 0.004	0.997
265 rpm [b]	7.73 ± 0.79	30 – 78	0.093 ± 0.012	0.998
285 rpm [b]	8.93 ± 0.19	30 – 102	0.091 ± 0.002	0.990
325 rpm [b]	8.15 ± 1.86	48 - 120	0.093 ± 0.044	0.989

a Determined through linear regression analysis
b Results presented as average ± standard deviation across duplicate cultivations
c Results presented as average ± standard deviation across triplicate measurements

Pigmentation trends were, however, not aligned with the observations made regarding growth, as demonstrated in Figure 4.21c. At agitation speeds up to and including 265 rpm no pigmentation was observed, while a volumetric pigment concentration of approximately half that obtained under base case conditions was observed at an agitation rate of 285 rpm. Increasing the agitation rate to 325 rpm did not result in a further increase in pigmentation in comparison to the base case, but rather a slight decline was observed. It is possible that while oxygen transfer at 300 rpm is sufficient for pigment production by *P. purpurogenum* an increase to 325 rpm results in unfavourable shear conditions at a crucial period of biomass formation during the first 48 hours of the cultivation, given the increase to 400 rpm in the base case cultivation beyond this time point.

Based on the results presented in Figure 4.21, day 5 was identified as the time point at which comparisons could most easily be drawn between cultivations. Pigmentation was generally at a maximum and no further increase in biomass concentration was observed beyond this point. The pH, growth and pigmentation data relating to the 120-hour sampling point has, therefore, been summarised in Figure 4.22. A trend of increasing pigmentation with increasing agitation speed is observed between 265 rpm and 300 rpm (base case). A trend of decreasing pH at day 5 is also observed over this range of agitation speeds.

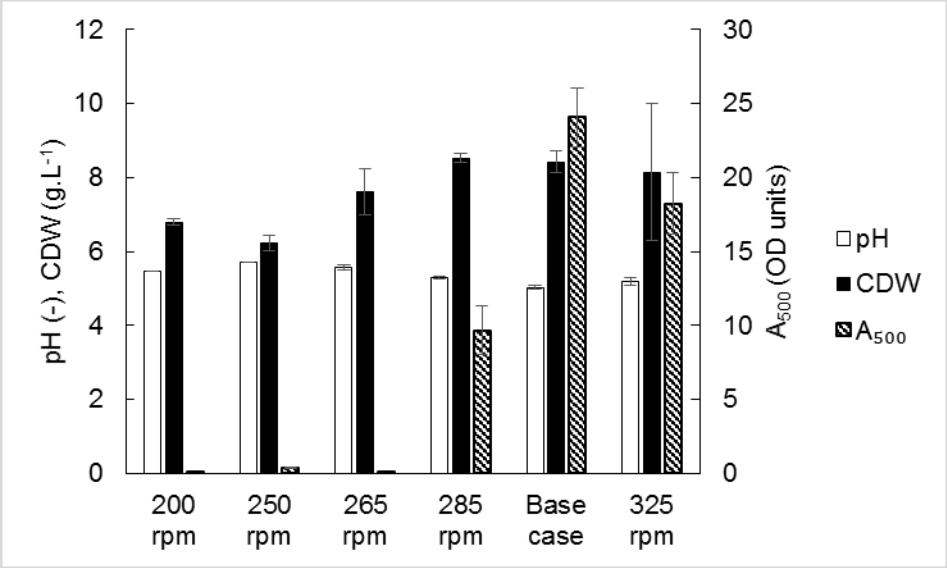


Figure 4.22 Summary of pH, growth and pigmentation at day 5 of *P. purpurogenum* cultivation on MESP medium in the BioFlo 110 bioreactor under different conditions of agitation speed. Error bars represent the standard deviation of triplicate measurements performed during duplicate cultivations (single cultivations for 200 rpm and 250 rpm).

When plotting pigmentation achieved against pH at day 5 for agitation speeds over the range of 265 to 325 rpm, a linear correlation is observed between these two parameters, as shown in Figure 4.23. This could suggest that the pigment product has an influence on culture pH, or that a product such as an organic acid is co-produced.

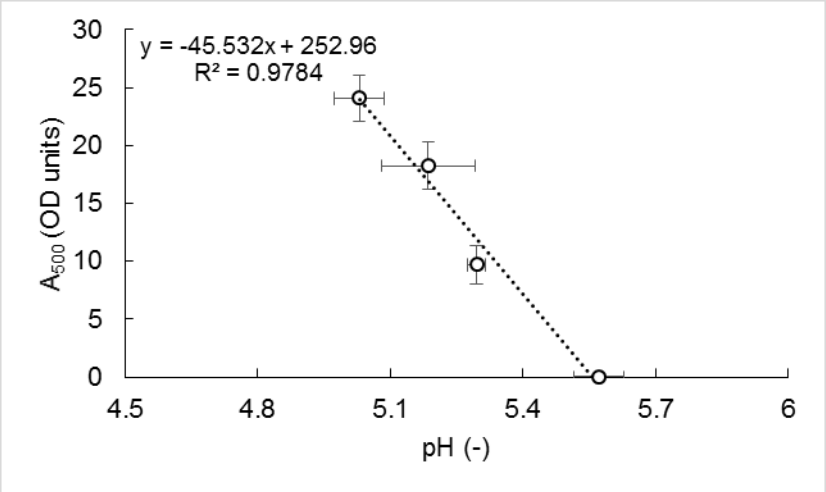


Figure 4.23 Pigmentation plotted against pH value on day 5 for *P. purpurogenum* cultivations performed in the BioFlo 110 bioreactor system using MESP medium, pH 5 at various agitation speeds. Error bars represent the standard deviation associated with triplicate measurements during duplicate cultivations.

In order to relate the pigmentation trends directly to oxygen transfer, impacted by agitation rate, the volumetric mass transfer coefficient in the BioFlo 110 reactor system was evaluated experimentally. The gassing out method as described in Section 3.3.4.2 was used to determine the k_La relating to various stirring speeds over the range of 200 rpm to 500 rpm in MESP medium. Base case cultivation

parameters as defined in Table 4.8 were applied throughout. The resulting relationship between k_La and agitation speed, at a fixed aeration rate of 1 vvm, is plotted in Figure 4.24.

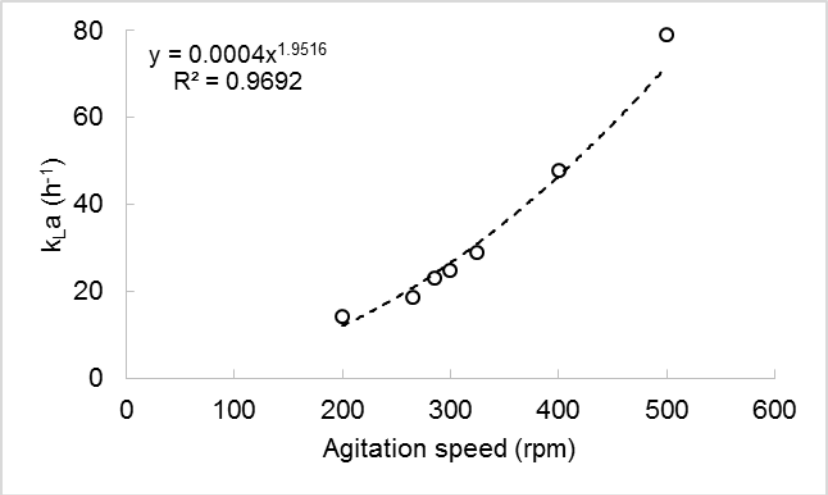


Figure 4.24 Relationship between k_La and agitation speed in the BioFlo 110 bioreactor when using MESP medium at pH 5 maintained using a 50 mM citrate buffer, with aeration supplied at 1 vvm. Error bars show the standard deviation between duplicate k_La measurements.

The mass transfer coefficient was shown to increase with increasing agitation rate, with these parameters related by a power law. This is expected based on the equations and correlations describing k_La, as presented in Section 4.2.2 (Van't Riet, 1979; Doran, 1995). Similar results have been reported previously in stirred tank reactor systems fitted with dual Rushton-type impellers (Karimi et al., 2013). Pigmentation achieved in the bioreactor was plotted as a volumetric concentration against the measured k_La value under varying conditions of agitation. The resulting plot is provided in Figure 4.25.

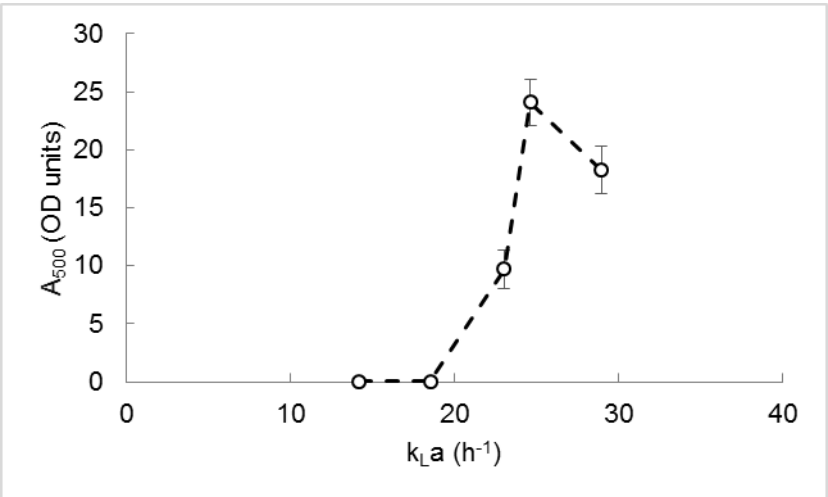


Figure 4.25 Pigmentation observed under varying conditions of agitation speed during cultivation in the BioFlo 110 bioreactor plotted against measured k_La at each speed. Cultivation on MESP medium, pH 5, 50 mM citrate buffer, with aeration maintained at 1 vvm. Error bars for pigmentation represent standard deviation of triplicate measurements of duplicate cultivations, while those for k_La are for duplicate measurements.

A trend of increasing pigmentation with increasing k_La was observed within a subset of the data, relating to stirring speeds of 265 rpm to 300 rpm. The relationship between pigmentation and predicted k_La in shake flask cultivation, as reported in Figure 4.9, was then plotted along with this data to observe any correlation between the two cultivation systems. These plots, including the best-fit line describing the shake flask data, are provided in Figure 4.26. It is observed that pigment production response to changes in k_La in the bioreactor correlates well with shake flask data. This applies within a linear range relating to a k_La of approximately 20 – 25 h^{-1}.

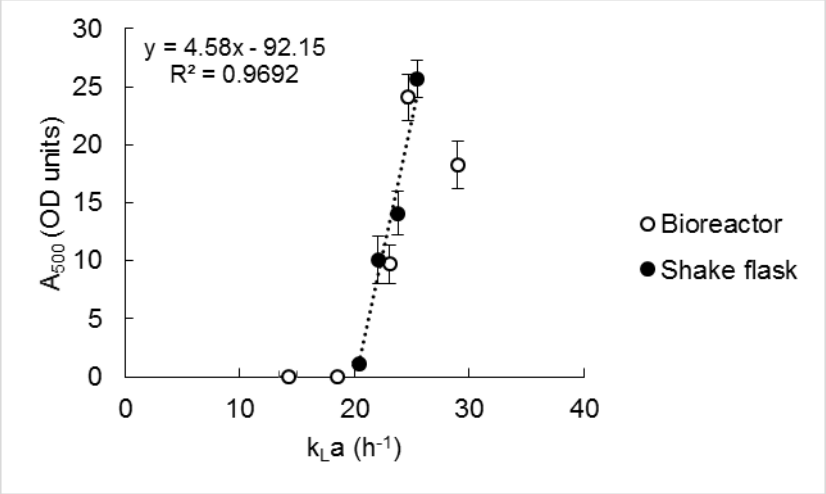


Figure 4.26 Relationship between k_La and pigmentation in the BioFlo 110 bioreactor and shake flask cultivations.

When the two cultivation systems are defined by the same k_La value or a rate of oxygen transfer in a similar range, cells cultivated in these two systems may experience different shear effects. It can, therefore, be hypothesised that oxygen transfer, rather than shear, is responsible for variation in pigmentation, based on the data which shows the same relationship between k_La and pigmentation in both shake flasks and the BioFlo 110 bioreactor.

The similar trends observed in the two cultivation systems suggest that oxygen supply, through varying oxygen transfer rate, is responsible for regulating pigment production by *P. purpurogenum* DSM 62866. Oxygen transfer rate is mediated by k_La, which was in turn mediated by changing shaking platform speed or agitation rate in this study. Based on the results observed during bioreactor cultivation, there appears to be a minimum rate of oxygen transfer below which pigment production does not occur. Combined results in shake flask and bioreactor cultivations indicate that pigment production increases linearly with increasing oxygen supply in the k_La range of approximately 20 to 25 h^{-1}, corresponding to a maximum OTR of approximately 150 to 188 $mg.L^{-1}.h^{-1}$.

At a k_La of approximately 29 h^{-1}, corresponding to 325 rpm in the bioreactor, pigmentation declined. This could be attributed to an optimum OTR, or a response to the shear experienced by the culture at this increased agitation rate. The effect of oxygen transfer rate versus shear on pigment production could be investigated through the use of an oxygen-enriched air stream. This would have the effect of

increasing oxygen transfer into the medium (considering Equation 5, Table 4.7) without influencing shear experienced by the cell culture.

Improved production of polyketide pigments with increased k_La has been reported previously. A study performed with *Monascus ruber* investigated the effect of altering both aeration rate and agitation speed (Hajjaj et al., 1999a). An overall trend of increased red pigmentation with increasing k_La was observed, with the exception of highly aerated conditions (agitation greater than 400 rpm), where accumulation of a by-product had an inhibitory effect on pigment production. A later study revealed that accumulation of organic acids had a negative impact on pigmentation, but that the main inhibitory effect was caused by an unknown product (Hajjaj et al., 2000). A direct relationship between k_La and red pigment production by *P. purpurogenum* is a novel finding.

This increase in pigmentation in response to increasing k_La may have a link to oxidative stress experienced by the cell culture. In filamentous fungi the production of secondary metabolites with antioxidant properties has been reported as a means of counteracting the effect of reactive oxygen species (Aguirre et al., 2006). A range of fungal pigment extracts have been show to exhibit antioxidant properties (Heo et al., 2018), including the polyketide pigment products of *Monascus purpureus* and *P. purpurogenum* (Srianta et al., 2017; Jin et al., 2018; Kantifedaki et al., 2018).

5 Results and Discussion: Characterisation of pigment products

During liquid cultivation in shake flasks and the BioFlo 110 reactor (New Brunswick Scientific) pigment production by *Penicillium purpurogenum* DSM 62866 was evaluated based on absorbance of a cell-free culture filtrate or supernatant at 500 nm. This is the wavelength corresponding to maximum absorbance of any red pigmentation in solution, as described in Section 2.4.1. Further analysis of pigmentation is, however, required in order to identify the compounds responsible and discern defining characteristics of the products which could affect application potential.

Section 5.1 describes preliminary characterisation techniques undertaken to investigate basic properties of the pigments. Solvent extraction was performed for isolation of colouring compounds, while also providing information regarding pigment solubility and polarity. Thin layer chromatography (TLC) was applied as a means of determining whether the observed colour is the result of a single or multiple compound(s). Section 5.2 describes large-scale extraction and characterisation of colouring compounds produced through cultivation in the BioFlo 110 bioreactor system.

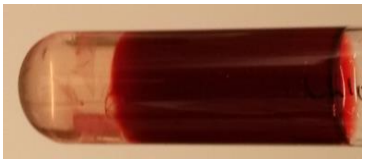
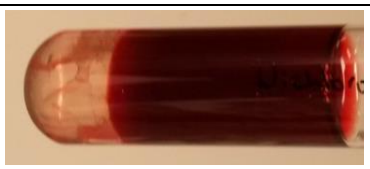
5.1 Preliminary characterisation

Initial characterisation work was concerned with investigating basic properties of the red pigmentation produced by *P. purpurogenum* DSM 62866. Given the extracellular nature of the pigmentation of interest, this first required separation of the biomass from the spent medium and products, achieved through either centrifugation or filtration (0.45 µm cellulose nitrate filter, Sartorius Stedim Biotech). This was followed by extraction of the colouring compounds from the liquid medium, through the use of various solvents, and isolation of red compounds from other components which were also extracted, through TLC.

5.1.1 Solvent extraction

The method selected for separation of the pigment compounds from the culture filtrate was liquid-liquid extraction using the water immiscible solvents 1-butanol, chloroform, dichloromethane, diethyl ether, ethyl acetate, petroleum ether, and toluene, and the procedure outlined in Section 3.5.1. These initial characterisation studies were performed using small liquid volumes, with 5 mL of culture filtrate used per extraction experiment. A summary of the extraction results is provided in Table 5.1, including an extraction rating based on the colour of the organic phase from 1 with no colour in the organic phase to 4 where the organic phase is deeper in colour than the aqueous phase, as described in Table 3.6, and an image of the colour extraction achieved.

Table 5.1 Extraction of cell-free culture filtrate using various water-immiscible solvents.

Solvent	Chemical group	Extraction rating [a]	Extraction result
1-Butanol	Alcohol	3	
Chloroform	Alkyl halide	1	
Dichloromethane	Alkyl halide	1	
Diethyl ether	Ether	2	
Ethyl acetate	Ester	2	
Petroleum ether	Alkane	1	
Toluene	Aromatic	1	

a Extraction rating system explained in Table 3.6

The solvents selected for the extraction study, while all being immiscible in water, represented a range of chemical groups with varying polarity. The requirement for the solvent to be immiscible with water created a limitation in terms of the use of polar solvents, with 1-butanol (the only alcohol tested) being the most polar solvent investigated. The degree of colour extraction achieved with each of the solvents also varied, providing some information regarding pigment characteristics.

Visual evaluation of the pigment extraction revealed that, of the solvents considered, 1-butanol resulted in the best extraction, with the colour in the organic phase being equivalent, if not darker than that remaining in the aqueous phase. This was followed by ethyl acetate, and then diethyl ether which were able to extract some colour from the culture filtrate, but in both cases the resulting organic phase was lighter in colour than the aqueous phase. When applying the remaining solvents (chloroform, dichloromethane, petroleum ether, and toluene) no colour extraction from the aqueous phase was

observed. This trend of decreasing ability to extract the pigment compounds aligns with the decreasing polarity of the solvent molecules. The most polar solvents tested, such as 1-butanol and ethyl acetate, achieved good colour extraction while the least polar solvents, petroleum ether and toluene, were not able to extract any visible colour.

This preferential extraction into the more polar solvents tested indicates that the colouring compounds produced by *P. purpurogenum* during submerged liquid cultivation are likely polar in nature. This relates to both the colouring compounds extracted into the solvent as well as the compounds contributing to the red colour which remain in the aqueous phase during extraction, given the polar nature of water. This is consistent with the reported slightly polar nature of the *Monascus* pigments and their homologues produced by *Penicillium* species (Carvalho et al., 2007), with the red pigments in particular (rubropunctamine, monascorubramine, and their derivatives) being the most polar (Mapari et al., 2008a).

The extraction results observed compare well with existing literature. The extraction of red pigments produced by *Monascus ruber* during submerged liquid cultivation by Hajjaj et al. (1997) involved freeze-drying the culture filtrate, re-dissolving the resulting powder in dH_2O, and then performing liquid-liquid extraction using *n*-butanol. The use of *n*-butanol was reported to improve efficiency of extraction, as observed in this study.

The solvent which resulted in second best extraction in this study, ethyl acetate, has also been used for the extraction of *Monascus* pigment homologues produced by *P. purpurogenum*. This was followed by further processing of the organic phase, ultimately resulting in the isolation of a pure pigment compound (Ogihara et al., 2000b; Ogihara and Oishi, 2002; Arai et al., 2012, 2013). Another study also reported selective fractionation of the pigments produced by *P. purpurogenum* into ethyl acetate rather than hexane or butanol (Celestino et al., 2014), which shows that pigment production is strain-specific, with even slight differences in pigment structure resulting in solubility differences and, therefore, variations in terms of extraction process required and future application potential.

The compounds extracted into the tested solvents were further characterised by thin layer chromatography, as reported below. It is, however, important to note that intense red colouration was still observed in the aqueous phase following extraction. The compounds which contribute to the colour of the aqueous phase are also of potential interest and should not be excluded from further investigation.

5.1.2 TLC of the organic phase pigments

In order to obtain further information about the composition of the pigment extracts, specifically relating to whether the observed colour is the result of a single colouring compound or a pigment mixture, the solvent extracts resulting from the extraction study were subjected to thin layer chromatography (TLC) analysis. The extracts from the two solvents best able to extract the colouring compounds from the *P. purpurogenum* culture filtrate, 1-butanol and ethyl acetate, were used for further experimentation.

As a result of the similarity observed between the results of this study and the work by Ogihara et al. (2000) in terms of optimum temperature, pH, and buffer conditions for production of *Monascus* pigment homologues by *P. purpurogenum*, their TLC protocol reported for separation of the pigments was attempted. Following extraction with ethyl acetate, the pigment mixture was separated by TLC using a mobile phase composed of *n*-butanol/ acetic acid/ water in the ratio 12:3:5. This solvent mixture was able to cause movement of the extract sample along the TLC plate (Macherey-Nagel GmbH & Co. KG, ALUGRAM® pre-coated TLC sheets, 0.20 mm silica gel 60), but streaking rather than a clear banding pattern was observed (Table 5.2).

In order to improve separation of the pigment components, the impact of changing the composition of the mobile phase was investigated. Higher eluting power of the mobile phase is linked to increased polarity, which can be modified by altering the ratio of a selected polar and non-polar solvent in a mixture, or through the use of increasingly polar solvents (Coker and Ayoola, 2008). Both of these methods were attempted in this study.

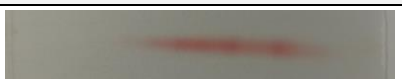
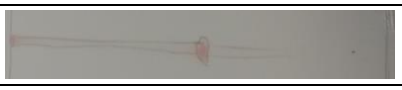
The polar/ non-polar solvent combination used was chloroform/ methanol, based on frequent use in literature for TLC and column chromatography (Hajjaj et al., 1997; Jung et al., 2003, 2011; Kim et al., 2006), with ratios of 2:1, 1:1, and 1:2 (increasing polarity) selected for investigation. With increasing polarity of the mobile phase, increased movement of the sample was observed. With a chloroform/ methanol ratio of 2:1, the sample did not move from the load line, while with a ratio of 1:1 streaking of the sample was observed, with the majority of the sample remaining at the load line. A ratio of 1:2, however, resulted in effective movement of the sample along the plate, but did not cause any separation of components.

The next approach taken was to apply solvents of increasing polarity, with Coker and Ayoola (2008) reporting that polarity increases in the order: alkanes < toluene < halogenated hydrocarbons < ethyl acetate < acetone < alcohols < acetic acid. The results of the chloroform/ methanol TLC runs appeared to indicate that chloroform was not sufficiently polar to cause movement of the extract samples. Based on this, only solvents more polar than the halogenated hydrocarbons were investigated further. These included ethyl acetate, acetone, 1-butanol, ethanol, methanol, and acetic acid. The separation observed when using each of these solvents is shown in Table 5.2.

The least polar solvents investigated (ethyl acetate and acetone) were not able to interact with the sample and the entire sample spot, therefore, remained at the load line. The next group of solvents tested were the alcohols: 1-butanol, ethanol, and methanol, with polarity increasing with decreasing chain length. The result observed with 1-butanol was the same as that achieved when using ethyl acetate and acetone, but the other alcohols yielded more promising results. With the application of 70 % ethanol as the mobile phase, separation of the sample components began to be visible, with 2 separate, but slightly unclear, bands observed after TLC. These bands were more easily observed when separating the components of the 1-butanol extract than the ethyl acetate extract. These results were improved further through the use of methanol, with 2 clear bands observed when separating the

components of the 1-butanol extract of the culture filtrate. The resulting bands appeared to be red and pink in colour, with retention factors (R_f) of 0.58 and 0.63, respectively.

Table 5.2 TLC results obtained using various mobile phase compositions to separate the pigment components of 1-butanol or ethyl acetate extracts of *P. purpurogenum* culture filtrate.

Mobile phase		**Sample extracted using**	**Result**	
Solvent	**Solvent ratio**		**Observed separation**	**R_f value(s)**
1-butanol/ acetic acid/ water	12:3:5	Ethyl acetate		/
Chloroform/ methanol	2:1			/
	1:1			/
	1:2			Band: 0.51
Ethyl acetate	/	1-butanol		/
Acetone	/			/
1-butanol	/			/
Ethanol (70 %)	/	Ethyl acetate		Band 1: 0.78 Band 2: 0.84
		1-butanol		Band 1: 0.75 Band 2: 0.79
Methanol	/	Ethyl acetate		Band 1: 0.57 Band 2: 0.66
		1-butanol		Band 1: 0.58 Band 2: 0.63
Acetic acid	/	Ethyl acetate		/
		1-butanol		/

Increasing the polarity of the mobile phase further by applying acetic acid once again resulted in streaking of the sample across the TLC plate. It is possible that the increased polarity of acetic acid in comparison to methanol results in the sample moving too quickly across the plate, thereby limiting the interaction with the silica stationary phase, and preventing separation of the sample into different components.

The results of the preliminary characterisation studies revealed that the pigmentation produced by *P. purpurogenum* DSM 62866 is likely a combination of several red, polar pigment compounds. Best solvent extraction results were achieved when using the most polar, water-immiscible solvents considered, namely 1-butanol and ethyl acetate, and TLC analysis of these extracts revealed more than one red band, indicating that the observed colour is the result of multiple pigment products.

As stated in Section 5.1.1, red pigmentation was still observed in the aqueous phase following solvent extraction and these compounds, which are not extracted into the organic phase, should not be excluded from further pigment analysis. Both the organic phase and aqueous phase resulting from solvent extraction of a 5 L culture broth were analysed as detailed in Section 5.2.

5.2 Large-scale extraction and characterisation

The subsequent stage of pigment characterisation required a larger amount of sample for analysis and, therefore, a larger cultivation volume. Culture broth resulting from 5 L volume cultivation under base case conditions (Section 4.3.1) in the BioFlo 110 bioreactor system (New Brunswick Scientific) was, therefore, subjected to solvent extraction. The extract was then concentrated before undergoing further analysis including thin layer chromatography (TLC), column chromatography, liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy, as detailed in Section 5.2.1.

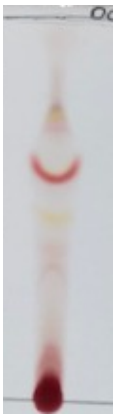
The aqueous phase following ethyl acetate extraction was also further characterised, as described in Section 5.2.2. The liquid volume was concentrated under vacuum and analysed by TLC, reversed phase (RP) chromatography, and high-resolution mass spectrometry (HRMS).

5.2.1 Extraction and organic phase analysis

The liquid volume cultivated in the BioFlo 110 bioreactor was centrifuged to remove the biomass. Red pigment products were then extracted from the cell-free supernatant using ethyl acetate, as described in Section 3.5.3.1. Following rotary evaporation and drying, a small quantity of the extract was subjected to nuclear magnetic resonance (NMR) spectroscopy for analysis of the crude product (Appendix E). This revealed large areas of noise, indicating that further processing and purification was required in order to characterise individual pigment products.

Different TLC methods were considered and attempted, including both 5 % and 10 % methanol in either ethyl acetate or dichloromethane (DCM). These results are provided in Table 5.3. Good separation was observed when using a mobile phase of 5 % methanol in dichloromethane, with multiple coloured products observed. One clear red band was observed as a major product, however, lots of red coloured product remained at the load line.

Table 5.3 Results of TLC analysis performed on concentrated ethyl acetate extract of *P. purpurogenum* culture broth.

Methanol: Ethyl acetate		**Methanol: Dichloromethane**	
1:9	1:19	1:9	1:19
			

The concentrated ethyl acetate extract was then subjected to multiple rounds of column chromatography (Biotage, Sweden) using varying ratios of methanol and DCM in order to separate the red components into individual fractions. Fractions were evaluated using TLC (methanol/ DCM 1:19), to observe which red products they contained. The red products of interest included the isolated red band, as shown in Figure 5.1, and the large amount of product visualised on the TLC load line, which was eluted from the chromatography column by ramping up the proportion of methanol in the mobile phase (10 – 80 %).

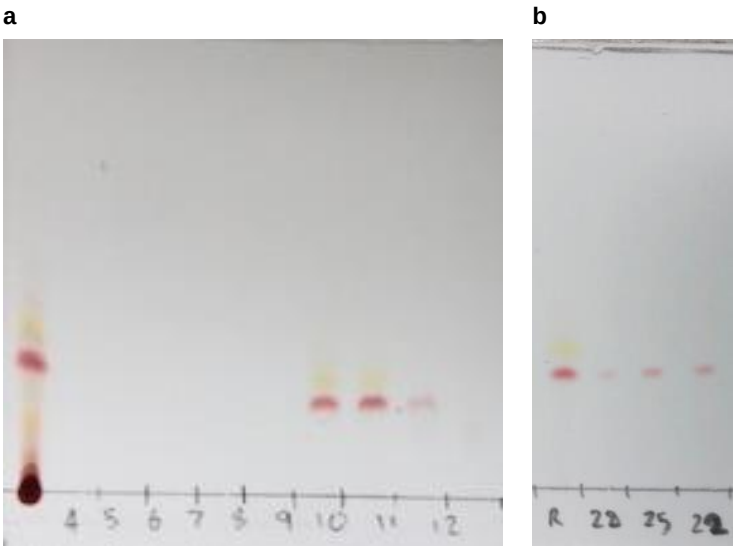


Figure 5.1 Isolation of the major red product into individual fractions through column chromatography using the Biotage Isolera One, as observed through TLC (Macherey-Nagel GmbH & Co. KG, 0.20 mm silica gel 60 plate) using methanol/ DCM (1:19) as the mobile phase.

Once the red products had been isolated into one or more fractions, they were pooled and then concentrated under vacuum (Buchi, Switzerland) for further evaluation. These concentrated products were submitted for and LC-MS analysis.

The mass spectrum for the red band isolated as demonstrated in Figure 5.1 gave a quasimolecular ion at m/z 426 $(M+H)^+$ and is provided in Appendix E.

5.2.2 Aqueous phase analysis

The aqueous phase which remained after extraction with twice the volume ethyl acetate (Section 3.5.3.1) exhibited significant red pigmentation and was, therefore, also subjected to further characterisation. The liquid volume was concentrated under vacuum to yield a thick, syrup-like substance. A small amount of this material was then dissolved in methanol for application in various analysis techniques.

The initial step towards analysis was to compare the properties of the red colourants in the aqueous phase to what was observed in the organic phase. TLC analysis was thus attempted using the solvent system of methanol/ DCM (1:19) which had yielded effective separation of the components of the ethyl acetate extract.

This solvent system did not result in any movement of the products along the TLC plate (Macherey-Nagel GmbH & Co. KG, 0.20 mm silica gel 60), with this result not changing with the application of methanol alone (the polar component of the solvent system) as the mobile phase. This resulted in the observation that reversed phase chromatography is likely to be required in order to separate these coloured components.

Column chromatography was performed using C_{18} reversed phase silica (Sigma-Aldrich), using a mobile phase of acetonitrile and water. This began with 10 % acetonitrile, increasing slowly and incrementally to elute different compounds. A peak in absorbance was observed each time the acetonitrile percentage was increased, either indicating elution of different compounds with slightly different solubilities, or that a higher concentration of the same compound was eluting based on the change in solubility. These peaks were collected as separate fractions, concentrated under vacuum and submitted for HRMS analysis (CAF, Stellenbosch University). The mass spectra for all submitted samples exhibited the same 2 major peaks, with quasimolecular ions at m/z 433 $(M+H)^+$ and 449 $(M+H)^+$. An example is provided in Appendix E.

5.2.3 Summary of techniques and prospective characterisation of pigment class

As reported previously, fungal pigments fall into two main groups: the carotenoids and polyketides (Mapari et al., 2010). The carotenoid pigments are oil-soluble; vary in colour between yellow, orange and red; and are generally sensitive to heat, light and oxidation (Joshi et al., 2003; Mapari et al., 2005; Schmidt et al., 2005; Berman et al., 2015). These features of carotenoids do not all apply to the red pigments synthesised by *P. purpurogenum* DSM 62866 in this study, indicating that these products are not carotenoids and are likely polyketides.

The polyketide group of pigments is extremely diverse, with the compounds divided into a number of groups, including quinones (anthraquinones, hydroxyanthraquinones, naphthoquinones), flavonoids, melanins and azaphilones (Mapari et al., 2010; Dufossé, 2018). The major classes of fungal pigments are summarised in Table 2.2, highlighting characteristic features and listing known producers. As demonstrated in Table 2.2 the polyketide pigments exhibit variable stability and solubility, with water solubility of certain pigments representing an advantage in terms of range of potential application.

Penicillium species have been reported to produce a range of diverse azaphilone polyketide compounds, including a number of pigments (Osmanova et al., 2010). The reported production of *Monascus*-like azaphilone polyketide pigments by *P. purpurogenum* strains, in fact, formed the basis of selection of this organism for this study.

Although the HRMS and NMR results do not allow conclusive identification of the pigments produced by this *P. purpurogenum* strain, it is postulated that the red pigmentation observed could be attributed to the production of azaphilone pigments. Features of the red pigment products in this study which support this hypothesis are agreement in absorbance maxima, effective extraction with the same solvents previously used for these types of pigments and similar solubility trends, as shown in Table 5.4.

Table 5.4 Azaphilone polyketide pigment properties reported along with the features of the red pigmentation produced by *P. purpurogenum* DSM 62866 in this study.

Organism and/ or pigment	**Absorbance maxima (nm)**	**Quasimolecular ion, m/z $(M+H)^+$**	**Soluble in**	**Reference**
P. purpurogenum DSM 62866	420, 500	426	*n*-Butanol, ethyl acetate, methanol, water	This study
		433, 449		
Monascus ruber ATCC 96218, N-glucosylmonascorubramine	422, 500	544	*n*-Butanol, water	Hajjaj et al. (1997)
Monascus ruber ATCC 96218, N-glutarylmonascorubramine	422, 500	512		
P. purpurogenum IBT 11181, *P. purpurogenum* IBT 3645, N-glutarylmonascorubramine	504	512	Water	Mapari et al. (2009b)
P. purpurogenum IBT 11181, N-glutarylrubropunctamine	430, 522	484		
P. purpurogenum GH2	425 – 430, 495 – 500	Not reported	Water	Morales-Oyervides et al. (2015)
P. purpurogenum, 7-(2-hydroxyethyl)-monascorubramine (PP-R)	412, 528	426	Acetone, chloroform, DMSO, ethyl acetate, methanol	Ogihara et al. (2001); Mapari et al. (2006)
P. purpurogenum, monascorubramine derivative (PP-V)	570	412	Acetone, DMSO, ethyl acetate, methanol, water	Ogihara et al. (2000b); Kojima et al. (2016)

Pigments belonging to this group have a reported affinity for amine groups, with the reaction between azaphilones and amine-containing compounds resulting in the formation of red/ purple pyridones with enhanced water solubility (Osmanova et al., 2010). Reactions with different amine-containing compounds results in the formation of pigments with differing side chains and, therefore, different properties. The azaphilone fungal polyketide pigments are highly diverse, with many derivatives reportedly produced by *Monascus* and *Penicillium* species (Mapari et al., 2010; Yuliana et al., 2017).

The quasimolecular ion observed at m/z 426 $(M+H)^+$ for the major red product extracted into ethyl acetate and isolated through column chromatography (Section 5.2.1) matches that obtained for the red monascorubramine derivative PP-R previously isolated from *P. purpurogenum* cultures (Ogihara et al., 2001; Mapari et al., 2006). This pigment derivative is, however, reported to be an intracellular product that is soluble in chloroform and insoluble in water. These trends differ from what was observed for the red pigment products in this study (see Section 5.1.1). Solubility trends for the *P. purpurogenum* DSM 62866 pigments more closely resemble those of the extracellular monascorubramine derivative, PP-V (Ogihara et al., 2000b), but m/z 412 $(M+H)^+$ was not observed in the mass spectra for the organic or aqueous phase products (Appendix E).

The m/z 426 $(M+H)^+$ also corresponds with the mass of the alanine derivative of rubropunctamine, where the amine group is replaced by the amino acid, as shown in Figure 5.2. This pigment derivative has previously been reported to be produced by *Monascus* species (Sato et al., 1997), with supplementation of the culture medium with individual amino acids also resulting in the production of different forms of the original *Monascus* pigment, with specific amino acid groups incorporated (Jung et al., 2003).

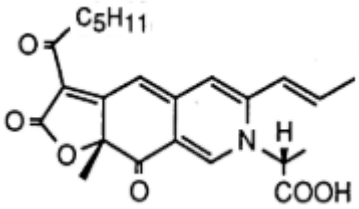


Figure 5.2 Alanine derivative of rubropunctamine ($C_{24}H_{27}NO_6$, 425 $g.mol^{-1}$).

Given the complex nature of the culture medium used in this study, a wide variety of nitrogen compounds, and amino acids, are expected to be available for consumption and could thus be incorporated into pigment products. It is possible that the red pigments extracted into ethyl acetate and those which remain in the aqueous phase following extraction could be derivatives of each other. Incorporation of different amino groups into the same basic pigment structure could alter pigment properties such as solubility, thus affecting extraction and isolation techniques.

6 Results and Discussion: Medium composition

Effect of the type and concentration of medium components supplied during cultivation on growth and pigment production of *P. purpurogenum* DSM 62866

Chapter 4 detailed the investigation of various cultivation conditions and their impact on growth and pigmentation produced by *Penicillium purpurogenum* DSM 62866. In Chapter 5 characterisation of the compounds responsible for the observed pigmentation was discussed. The next major factor responsible for changes in fungal pigment production is the type and concentration of nutrients supplied during cultivation, with reports highlighting the importance of medium composition, sometimes above that of culture conditions, in defining observed pigmentation (Santos-Ebinuma et al., 2014).

This chapter investigates various aspects relating to medium composition, including altering the ratio of medium components and replacing existing components with waste substrate alternatives. Section 6.1 describes the effect of modifying the MESP medium. This includes both agar plate (Section 6.1.1) and submerged liquid cultivation (Section 6.1.2), with a major focus on altering the ratio of the main carbon and nitrogen sources in the cultivation medium (Section 6.1.3). Section 6.2 then describes cultivation in the BioFlo 110 bioreactor system using a different ratio of malt extract to soya peptone than that in the MESP medium, based on the results presented in Section 6.1.3. The results obtained in the bioreactor system were compared to those observed when using the original recommended MESP medium. Finally, Section 6.3 evaluates the impact of replacing the main carbohydrate source in the malt extract-based medium with a confectionery-based substrate to explore the potential of using waste materials from the confectionery industry. Two confectionery streams were considered, with marshmallow selected for investigation in submerged liquid cultivation using both the shake flask and BioFlo 110 bioreactor cultivation systems.

6.1 Modifications to malt extract-based medium

The MESP medium used for cultivation of *P. purpurogenum* in the majority of experiments reported in this study is composed of 30 $g.L^{-1}$ malt extract and 3 $g.L^{-1}$ soya peptone, as recommended by the culture collection from which the organism was obtained (DSMZ, 2014). Following investigation of the effect of temperature and culture pH, as reported in Section 4.1, changes to the cultivation conditions recommended by DSMZ included incubation at 30 °C instead of 24 °C, as well as the use of 50 mM citrate buffer to maintain the culture pH around 5, as a result of their beneficial effect on pigment production.

The potential to further improve pigment yields by altering medium composition was considered. An initial agar plate screening study provided insight into the requirement for malt extract and soya peptone for pigment production by *P. purpurogenum*. This was then confirmed in a liquid cultivation system, before investigating different ratios of these medium components.

6.1.1 Composition of agar plate medium

To evaluate the effect of medium composition on growth and pigment production of *P. purpurogenum*, agar plate cultivation at 30 °C was used with modified versions of the MESP medium and medium compositions typically used for the cultivation of fungi on solid substrates. All agar-based medium compositions used are detailed in Table 3.1. *P. purpurogenum* spores were spot-inoculated onto the surface of these agar plates and growth was monitored visually over 14 days. Images of the growth observed after 14 days are provided in Figure 6.1.

Most medium types considered resulted in growth of *P. purpurogenum* comparable to that achieved with the control MESP medium, based on colony diameter. However, pigment production was shown to be dependent on medium composition. Red pigmentation was only observed on some of the incubated plates, with only the MESP and MEsp (reduced concentration of soya peptone) plates exhibiting diffused pigmentation in the agar surrounding the fungal mycelium. Plates such as meSP (reduced concentration of malt extract), MESPG (supplemented with glucose), and MEA exhibited only slight red colouration within the fungal biomass, while with other media, including MEYE, OA, and PDA, no red pigmentation was observed.

Santos-Ebinuma et al. (2014) investigated the impact of various factors including temperature, pH, shaking speed, and concentration of medium components on *P. purpurogenum* pigment production. The results indicated that medium composition was the main factor responsible for altered pigment production, with varying concentration of sucrose or yeast extract being most closely correlated to changes in pigmentation.

The importance of medium composition was clearly demonstrated in this study as, although agar plate incubation temperature was shown to have an effect on the degree to which pigmentation was produced (Figure 4.1), altering the medium composition in some cases prevented red pigment production entirely (Figure 6.1). Based on the observed results it appears that, of the compositions considered, the MESP medium is best suited to pigment production by this *P. purpurogenum* strain. Modifications to this medium, which included either reducing the concentration of malt extract or soya peptone or supplementing with glucose and yeast extract, appeared to negatively affect growth and/ or pigment production.

Increasing the C:N ratio through the addition of glucose conferred no benefit to the organism. Growth was comparable to that observed on the MESP medium, while pigmentation was reduced and limited to the fungal biomass. A reduction in pigment production when using a cultivation medium containing glucose has previously been reported for this species (Ogihara et al., 2000b). When the medium was supplemented with 10 $g.L^{-1}$ yeast extract a significant decrease in colony diameter and pigmentation was observed. Growth, but not pigment production, was improved through the addition of glucose (MESPGY).

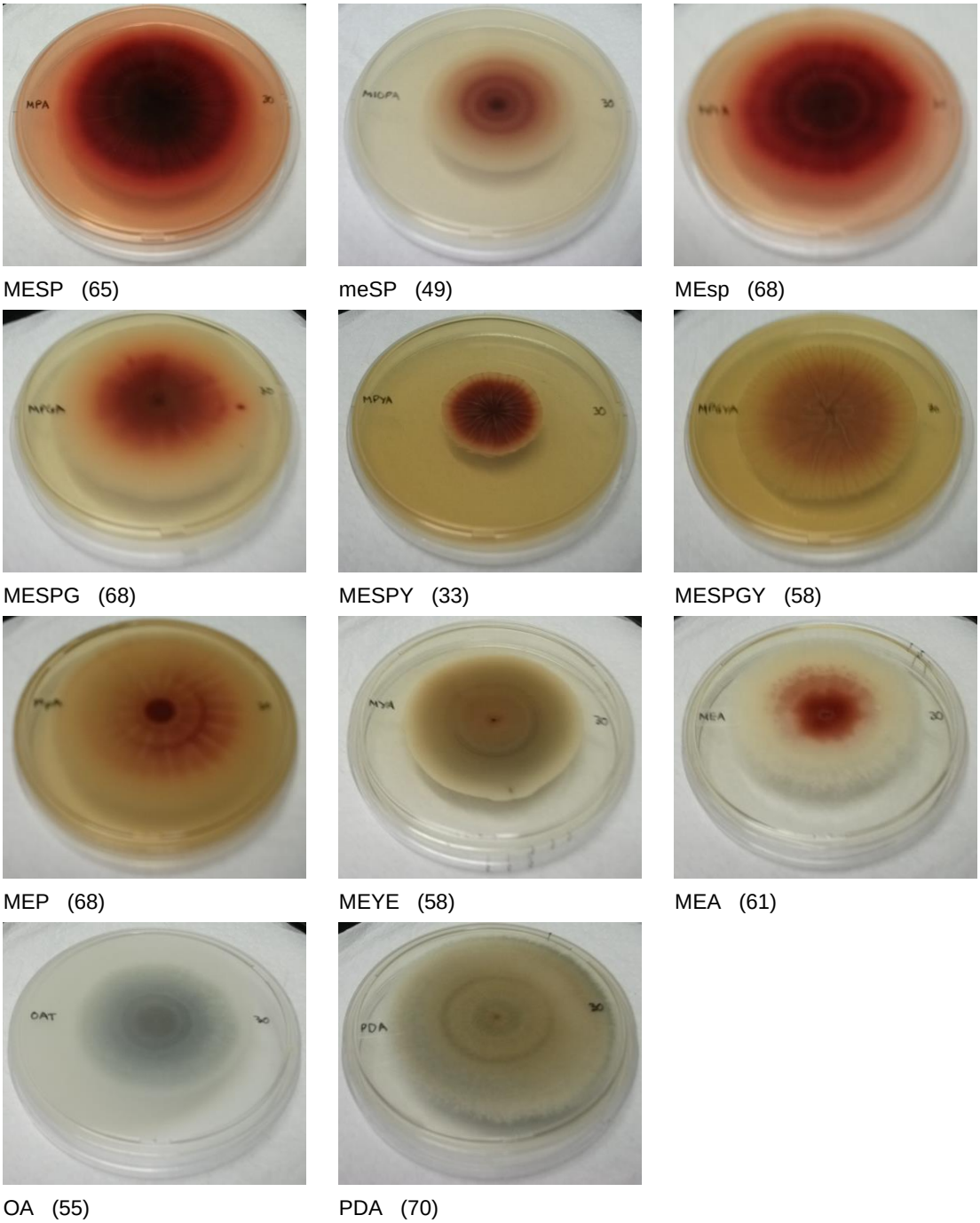


Figure 6.1 Growth of *P. purpurogenum* on agar plates of varying composition (Table 3.1) after incubation at 30 °C for 14 days. Colony diameter (mm) is reported in brackets.

Growth and pigment production were also affected when lowering the concentration of malt extract in the medium (meSP plates). Colony diameter was slightly reduced and only limited pigmentation was observed within the fungal mycelium. This appears to indicate that components within malt extract are required for effective pigment production, but could also show that reduced growth in response to lower carbon supply (lower C:N ratio) had a consequential effect on pigment production. Reducing the soya peptone concentration (MEsp plates), however, had little effect on the growth of *P. purpurogenum* with production of diffused pigmentation retained. While this may support the idea that soya peptone is not

essential for growth or pigment production, replacing soya peptone with peptone of animal origin (MEP plates) results in a drastic decrease in pigmentation, with only slight red colouration observed within the mycelium. This could potentially be linked to the significantly different amino acid profiles which are likely to result from enzymatic digestion of soybean or animal products.

Based on these observations, it appears that both malt extract and soya peptone are required for the effective production of red pigmentation by this strain of *P. purpurogenum*, without it being necessary to supplement the medium with additional sugar or protein sources. This was investigated further through comparison to a pigment production medium used previously for *P. purpurogenum* (Section 6.1.2), as well as through altering the composition of the MESP medium in liquid cultivation experiments (Section 6.1.3).

6.1.2 Comparison of MESP and starch-based production media

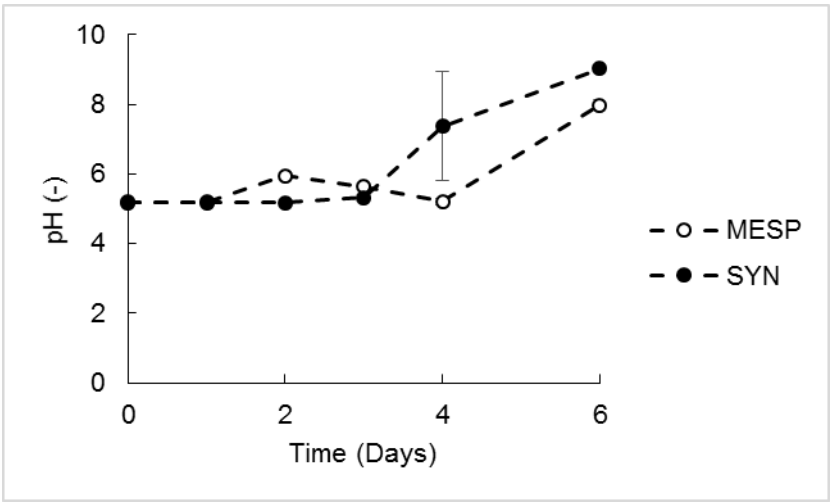
To confirm the requirement for malt extract and soya peptone for pigment production of *P. purpurogenum* DSM 62866, a shake flask cultivation experiment was undertaken to compare growth and pigment production achieved using MESP medium and the SYN starch-based medium, previously used as the pigment production medium for a *P. purpurogenum* strain (Ogihara et al., 2000b; Arai et al., 2013). Both medium types were maintained at a pH of 5 through the application of 50 mM citrate buffer. Medium composition is detailed in Table 3.2.

The cultivations were performed using 50 mL medium in 250 mL flasks, incubated at 30 °C with shaking at 150 rpm for 6 days. The pH, growth, and pigment production trends observed over this period are presented in Figure 6.2.

As expected, the MESP medium cultivation displayed good growth and pigment production, with a maximum CDW of just below 10 $g.L^{-1}$ and maximum A_{500} of approximately 15 OD units recorded over the 6-day period. The starch-based SYN medium, in which the complex nitrogen source is provided as yeast extract with additional nitrogen supplied as NH_4^+, did not support good growth of *P. purpurogenum*. Very little change in biomass concentration was observed over days 1 to 3, followed by an increase to approximately 1 $g.L^{-1}$ on day 4. Growth in the culture showed minimal change beyond day 4. The pH of the SYN medium remained stable over the first 3 days of the cultivation, before rising to a value of approximately 9 by day 6, potentially impacting growth. Pigment production in this culture was also extremely limited, with A_{500} values remaining below 0.1 OD units over the entire cultivation.

The limited growth and lack of pigment production when grown on the starch-based medium indicates strain-specific differences with respect to medium requirements within the *P. purpurogenum* species. The key role of malt extract and soya peptone in effective pigment production by *P. purpurogenum* DSM 62866 was again demonstrated.

a



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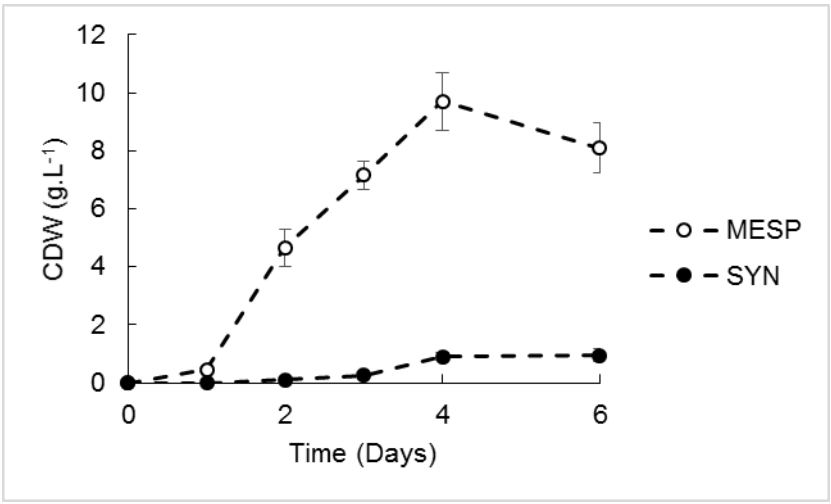

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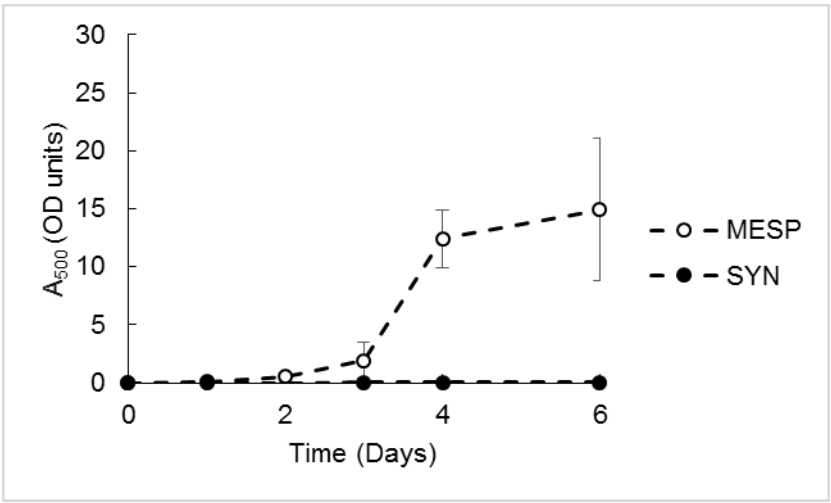


Figure 6.2 Cultivation summary showing pH trends (a), growth (b) and pigment production (c) achieved during shake flask cultivation of *P. purpurogenum* at 30 °C in MESP medium and SYN medium, both with the application of 50 mM citrate buffer at a pH of 5. Results plotted are the average values across duplicate flasks, with error bars representing the standard deviation of the data.

6.1.3 Altering the composition of the MESP medium in liquid culture

Given that both malt extract and soya peptone were shown to be essential components of the pigment production medium for *P. purpurogenum* DSM 62866, the effect of altering the ratio of these two components was considered in Section 6.1.3.1. The impact on growth and pigment production of replacing a portion of the malt extract component, weight-for-weight, with selected sugars was also investigated and is presented in Section 6.1.3.2.

6.1.3.1 Impact of altering the ratio of malt extract to soya peptone

On altering MESP medium composition using agar plate cultivation, it was shown that reducing the soya peptone concentration in the medium had little effect on the observed growth and pigmentation, but that removing soya peptone negatively impacted pigment production (Figure 6.1). Reducing the amount of malt extract applied in the medium was also shown to cause a visible decrease in growth and pigment production by *P. purpurogenum*. It was, however, not evident whether the reduction in malt extract caused a decrease in pigment production or whether the reduced growth on the lower substrate concentration had a resulting effect on pigmentation.

To explore this further, malt extract concentrations of 30 $g.L^{-1}$ and 15 $g.L^{-1}$ were selected as the starting point for the set of experiments investigating the impact of altering the ratio of malt extract to soya peptone, while soya peptone was applied at concentrations in the range of 0.75 $g.L^{-1}$ to 6 $g.L^{-1}$. A total of 8 medium compositions detailed in Table 6.1, with ratios of ME:SP in the range of 2.5 to 40, were considered using a multiwell plate system for submerged liquid cultivation. In all cases the medium was maintained at a pH of 5 through the application of a 50 mM citrate buffer.

Table 6.1 Composition of malt extract, soya peptone-based media applied during multiwell plate cultivation to investigate the impact of altering the ratio of these two components.

Concentration ($g.L^{-1}$)		**Ratio (ME:SP)** [a]
Malt extract	Soya peptone	
30	6	5
	3	10
	1.5	20
	0.75	40
15	6	2.5
	3	5
	1.5	10
	0.75	20

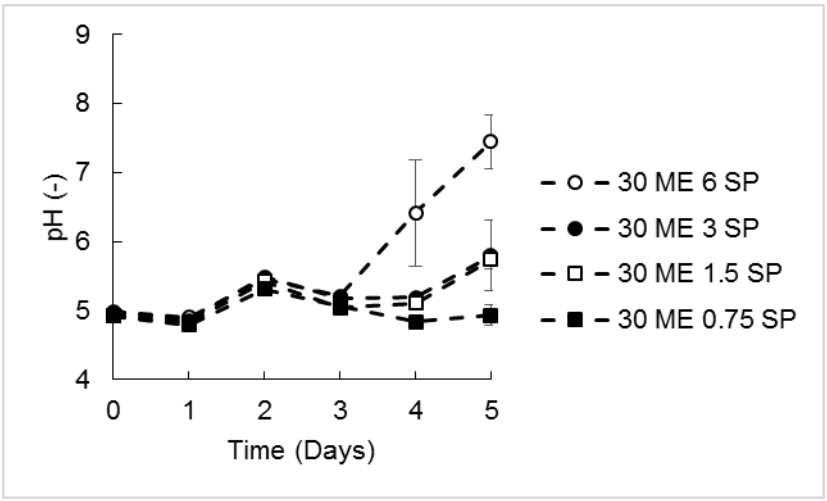
a Ratio calculated by dividing concentration of malt extract by that of soya peptone

The 2 mL medium volume in each well was inoculated at $1x10^5$ spores.mL^{-1} using direct spore inoculation, as described in Appendix B.1, and plates were incubated at 30 °C, with shaking, for 5 days. The culture pH was monitored daily, while CDW and pigmentation were evaluated at the end of the cultivation period. The entire well volume was harvested and centrifuged, with 200 µl of supernatant used to measure absorbance at 500 nm, and the remaining volume vacuum-filtered for CDW determination.

The pH trends over the course of the cultivation are provided in Figure 6.3, with Figure 6.3a and Figure 6.3b showing media containing 30 $g.L^{-1}$ and 15 $g.L^{-1}$ malt extract, respectively. The data shows a trend of increased culture pH at the end of the cultivation with increasing concentration of soya peptone, at both malt extract concentrations.

It is also observed that media containing 15 $g.L^{-1}$ malt extract exhibit lower pH peaks at day 2, but higher overall pH by the end of the cultivation when compared to media containing 30 $g.L^{-1}$ malt extract with the same soya peptone concentration. In fact, final pH value can be correlated to starting ME:SP ratio, with a lower ME:SP ratio resulting in a higher pH by day 5 of the cultivation. Culture pH as a function of soya peptone concentration and ME:SP ratio is shown in Figure 6.4.

a



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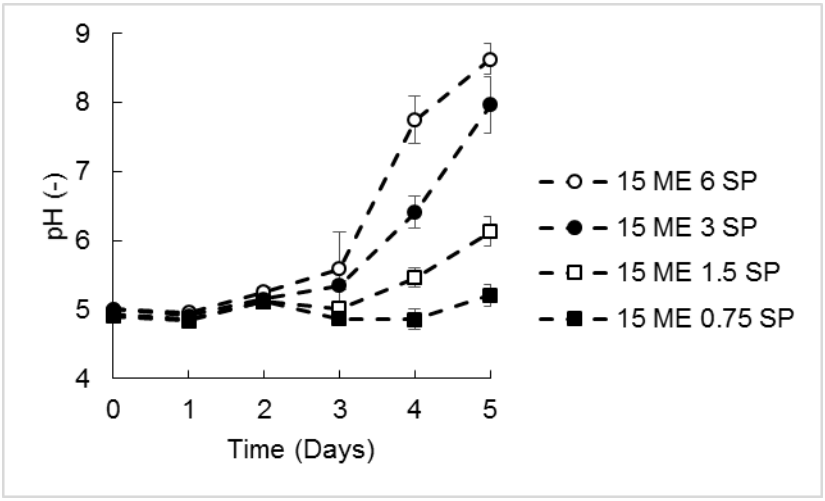
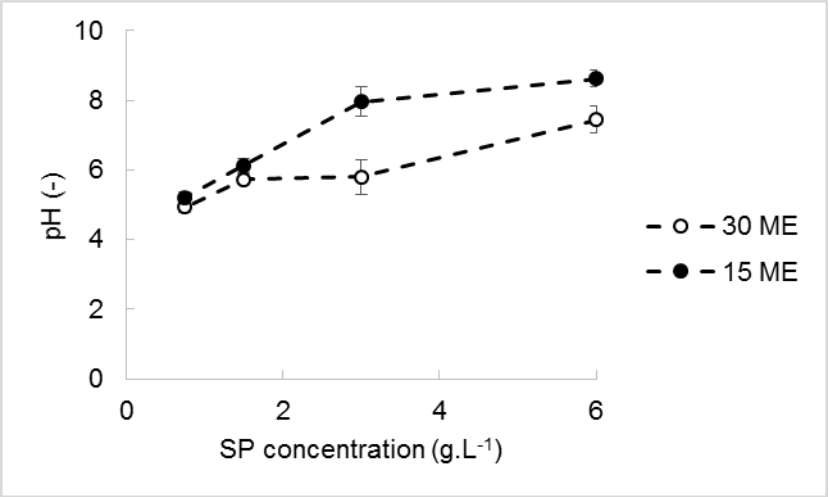


Figure 6.3 pH trends during multiwell plate cultivation at 30 °C with media containing 30 $g.L^{-1}$ (a) or 15 $g.L^{-1}$ (b) malt extract (ME) with various concentrations of soya peptone (SP). Results shown are the average measurements across triplicate wells, with error bars indicating the standard deviation of the data.

a



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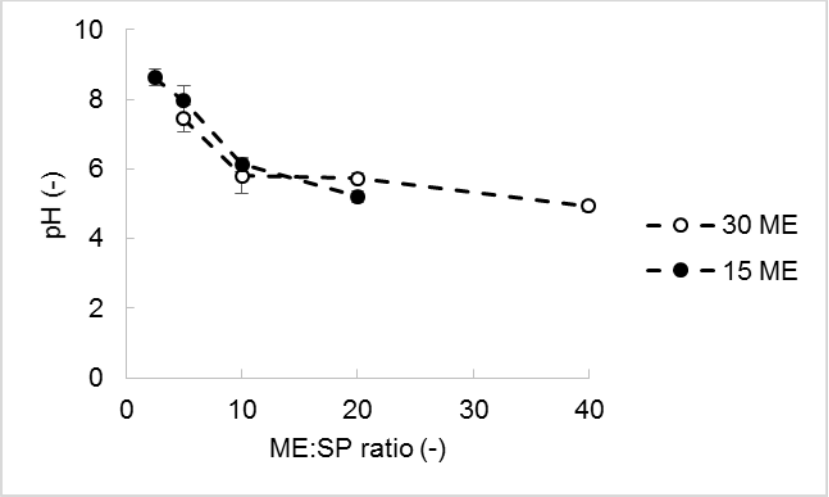


Figure 6.4 Culture pH by day 5 of the cultivation of *P. purpurogenum* as a function of soya peptone concentration (a) or ME:SP ratio (b) in the medium. Error bars represent standard deviation associated with triplicate measurements.

Growth, measured as CDW on day 5 of the cultivation, has been presented in Figure 6.5. Overall, media containing 15 $g.L^{-1}$ malt extract supported less growth in comparison to 30 $g.L^{-1}$ ME media. This is expected given the lower substrate availability for cellular metabolism and is consistent with the results observed during agar plate cultivation with reduced malt extract concentrations (Figure 6.1). An indication of pigmentation achieved under each condition was also provided in Figure 6.5, with the columns coloured according to the pigmentation rating system described in Table 3.4. In contrast to growth, pigment production appears to increase with decreasing substrate concentration. The 15 ME conditions exhibited more pigmentation than 30 ME conditions, and increased pigmentation was also observed with decreasing soya peptone concentration.

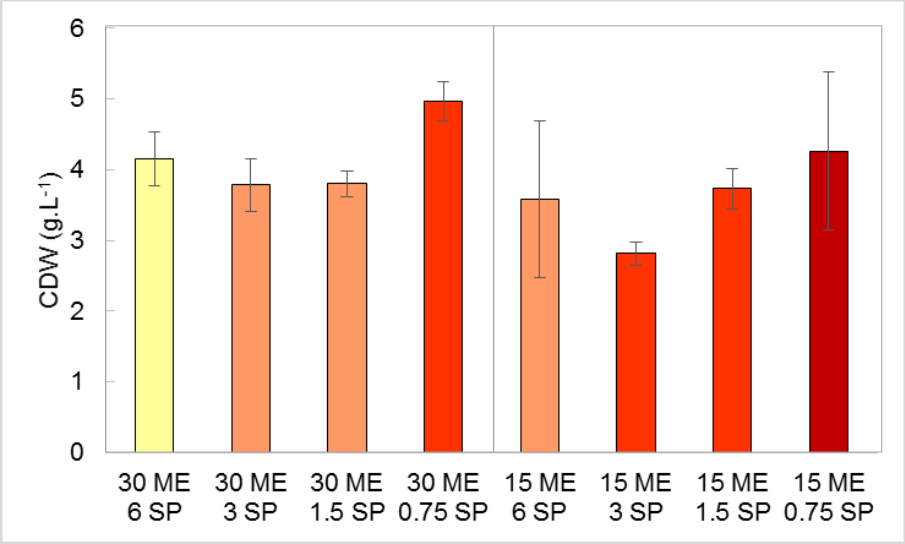


Figure 6.5 Growth, measured as CDW, after 5 days of submerged liquid cultivation in a multiwell plate system using media composed of 30 $g.L^{-1}$ or 15 $g.L^{-1}$ malt extract (ME) and a soya peptone (SP) concentration in the range of 6 – 0.75 $g.L^{-1}$. Results are provided as the average value across triplicate wells, with error bars representing the standard deviation of the data. Colour of each column provides an indication of observed pigmentation.

The measured absorbance value at 500 nm, relating to red colouration, for each medium composition was also plotted against the corresponding ME:SP ratio, as shown in Figure 6.6. This shows a trend of increasing pigmentation with increasing ME:SP ratio with 30 $g.L^{-1}$ and 15 $g.L^{-1}$ malt extract media, as well as increased pigmentation in media containing 15 $g.L^{-1}$ malt extract in comparison to 30 $g.L^{-1}$ malt extract when the ME:SP ratio is the same.

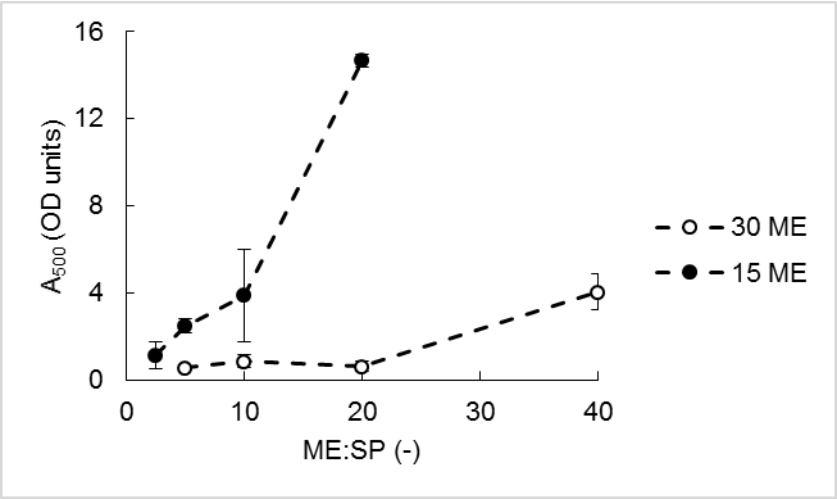


Figure 6.6 Pigmentation achieved during multiwell plate cultivation with media containing 30 $g.L^{-1}$ (30 ME) or 15 $g.L^{-1}$ malt extract (15 ME) plotted against the ME:SP ratio calculated based on the applied soya peptone concentration. Results represent the average of triplicate measurements, with errors bars indicating the standard deviation of the data.

The improvement in pigmentation observed with decreasing soya peptone concentration and an increasing ME:SP ratio when applying a malt extract concentration of 15 $g.L^{-1}$ was investigated further through shake flask cultivation, with the original MESP medium included as a control. A soya peptone

concentration of 6 g.L^{-1} was omitted from further experimentation as a result of the decline in pigmentation observed between 3 g.L^{-1} SP and 6 g.L^{-1} SP in both 15 ME and 30 ME medium conditions. Two additional SP concentrations of 0.5 g.L^{-1} and 0.375 g.L^{-1} were included. The composition of the media used during these shake flask experiments is provided in Table 6.2.

Table 6.2 Composition of malt extract, soya peptone-based media applied during shake flask cultivation to investigate the impact of altering the ratio of these two components.

Concentration (g.L^{-1})		**Ratio (ME:SP)** [a]
Malt extract	Soya peptone	
30	3	10
15	3	5
	1.5	10
	0.75	20
	0.5	30
	0.375	40

a Ratio calculated by dividing concentration of malt extract by that of soya peptone

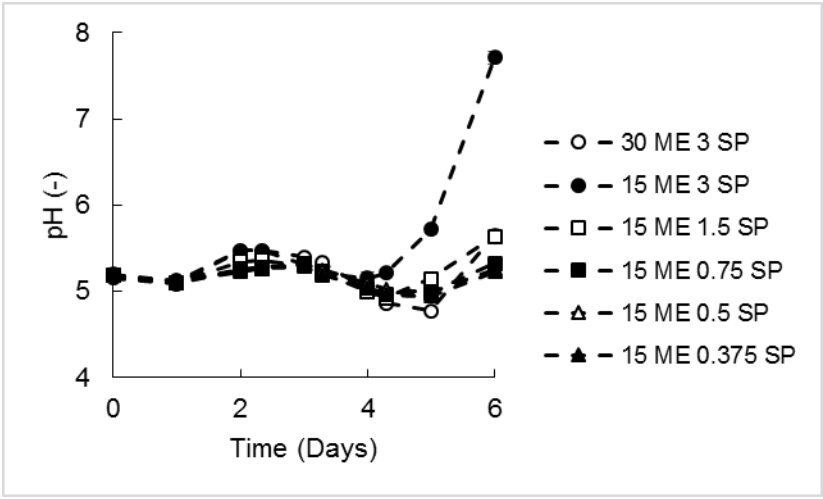
Shake flask experiments were conducted using 100 mL medium volume in 500 mL flasks. Cultures were inoculated at an initial spore concentration of 1x10^{5} spores.mL^{-1}, as described in Section 3.3.3.2, and flasks were incubated at 30 °C, with shaking, for 6 days. The pH, growth and pigment production trends over the course of the cultivation are provided in Figure 6.7.

A peak in the pH profile of between 5.3 and 5.5 units was observed between day 2 and day 3 of the cultivation in all cases. This was followed by a decline in culture pH, before increasing once again. Similar pH trends to those in multiwell plate cultivation were observed, with lower ME:SP ratios being correlated with higher culture pH by the end of the cultivation.

Growth in shake flasks showed some agreement with results observed during multiwell plate cultivation, with media containing 15 g.L^{-1} malt extract supporting less growth than that with 30 g.L^{-1} malt extract, at the same soya peptone concentration. The difference was, however, more pronounced in the shake flask system, with maximum CDW achieved in the 15 g.L^{-1} malt extract, 3 g.L^{-1} soya peptone medium being approximately 60 % of that in the original MESP medium. Overall, higher biomass concentrations were obtained during shake flask cultivation than in multiwell plates, with growth trends showing little agreement between the two systems.

Pigment production during shake flask cultivation also differed from that observed in multiwell plate cultures. The highest volumetric pigment concentration recorded during this experiment was approximately 28 OD units and was obtained when growing *P. purpurogenum* on the MESP control medium. This was followed by the 15 g.L^{-1} ME, 3 g.L^{-1} SP medium and the 15 g.L^{-1} ME, 1.5 g.L^{-1} SP medium, with pigmentation, however, observed earlier in the cultivation. The remaining 3 medium compositions showed very similar pigment production trends, with maximum absorbance values of approximately 18 OD units recorded for each condition.

a



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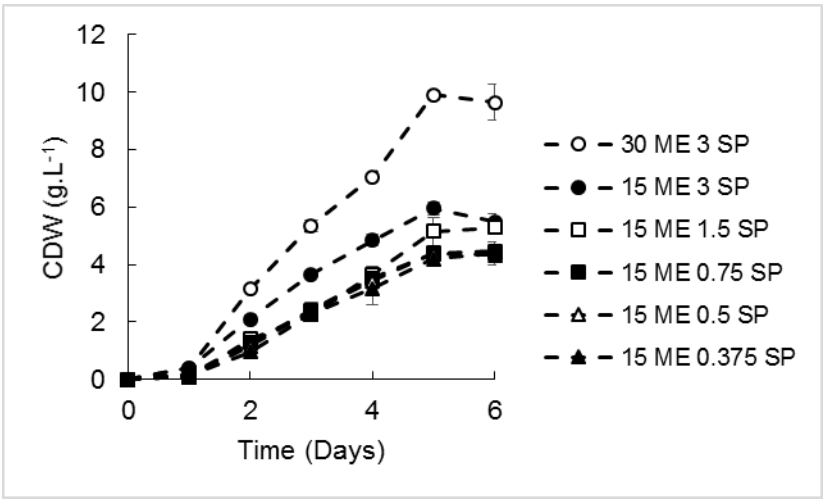

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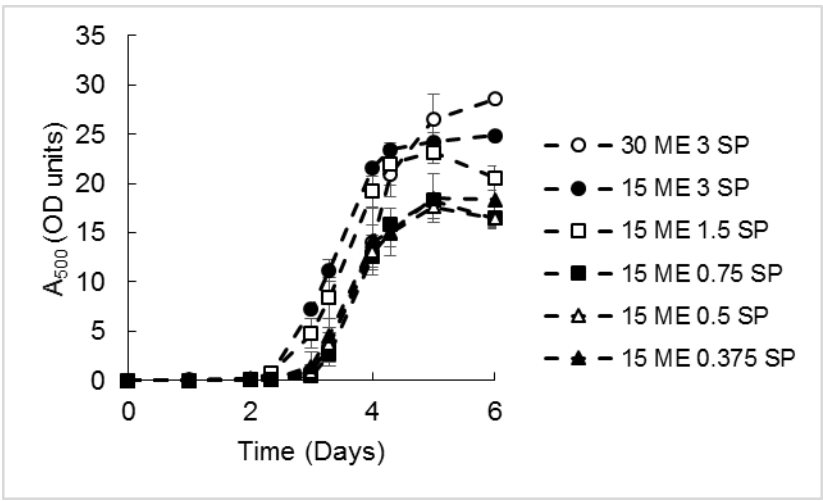

Figure 6.7 Comparison of the pH (a), growth (b), and pigment production (c) trends observed during shake flask cultivation of *P. purpurogenum* in media containing various concentrations of malt extract and soya peptone, buffered to a pH of 5 using 50 mM citrate buffer. Flasks were incubated at 30 °C for 6 days. Results are provided as the average value across triplicate flasks, with error bars representing the standard deviation of the data.

This differs from trends observed during multiwell plate cultivation, with volumetric pigment concentrations being generally higher in shake flask cultures. Volumetric biomass and pigment

concentrations achieved in these two systems are compared in Table 6.3. This demonstrates that only with the 15 g.L^{-1} malt extract, 0.75 g.L^{-1} soya peptone medium (highlighted in Table 6.3) were growth and pigment concentrations within a similar range across the two cultivation systems.

Table 6.3 Volumetric biomass and pigment concentrations achieved during shake flask and multiwell plate cultivation of *P. purpurogenum* DSM 62866 using media of varying concentration of malt extract and soya peptone.

Medium composition [a]	**Biomass concentration (g.L^{-1})** [b]		**Pigment concentration (A_{500}, OD units)** [b]	
	Shake flask	Multiwell plate	Shake flask	Multiwell plate
30 ME 3 SP	9.90 ± 0.14	3.78 ± 0.38	26.49 ± 2.56	0.85 ± 0.31
15 ME 3 SP	5.95 ± 0.21	2.82 ± 0.16	24.20 ± 0.99	2.46 ± 0.31
15 ME 1.5 SP	5.15 ± 0.49	3.73 ± 0.28	23.15 ± 1.07	3.88 ± 2.13
15 ME 0.75SP	4.40 ± 0.17	4.27 ± 1.11	18.26 ± 0.37	14.68 ± 0.29
15 ME 0.5 SP	4.37 ± 0.06	/	17.65 ± 1.28	/
15 ME 0.375 SP	4.20 ± 0.26	/	18.48 ± 2.46	/

a Numbers represent concentration in g.L^{-1} of malt extract (ME) and soya peptone (SP)
b Results presented as average ± standard deviation across triplicate flasks or wells

The postulated dependence of *P. purpurogenum* DSM 62866 pigment production on oxygen availability, as discussed in Section 4.2 and Section 4.4, may be responsible for the variation observed between multiwell plate and shake flask cultivation, with differences in oxygen transfer and bulk liquid mixing reported for these two systems (Hermann et al., 2003; Duetz, 2007). Surface tension has a major effect on oxygen transfer in multiwell plate cultivations, not reported in larger shaking systems. Only above a critical shaking frequency is an increase in OTR_{max} observed with increasing shaking speed (Hermann et al., 2003). The reduced oxygen demand of cultures growing on lower substrate concentrations may provide an explanation for the convergence of results as substrate concentration decreases, as demonstrated in Figure 6.8, in this oxygen-sensitive system.

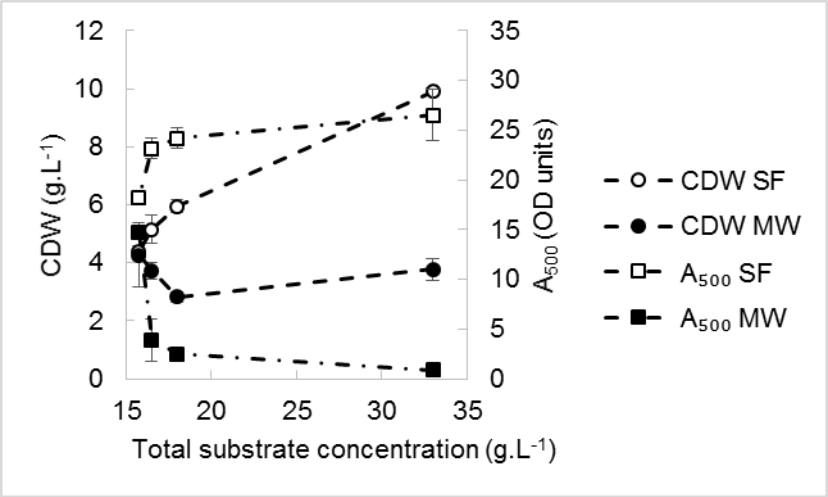

Figure 6.8 Volumetric biomass and pigment concentrations achieved during shake flask (SF) and multiwell plate (MW) cultivation of *P. purpurogenum* DSM 62866 as a function of total substrate concentration in malt extract, soya peptone-based media. Error bars represent standard deviation associated with triplicate flasks or wells.

Given the similarities observed between shake flask and BioFlo 110 reactor cultivation when using MESP medium (Figure 4.17), this shake flask experiment should provide a better indication of the response of the culture to scale-up than the multiwell plate cultivation.

6.1.3.2 Impact of compensating for reduced malt extract concentration with selected sugars

Reducing the malt extract concentration in the MESP medium from 30 $g.L^{-1}$ to 15 $g.L^{-1}$ while maintaining the soya peptone concentration at 3 $g.L^{-1}$ was shown to have a beneficial effect on specific pigment production during shake flask cultivation, as demonstrated in Figure 6.7, where volumetric pigment concentrations remained high while biomass production was significantly reduced. This medium containing 15 $g.L^{-1}$ malt extract and 3 $g.L^{-1}$ soya peptone, maintained at a pH of 5 through the application of 50 mM citrate buffer is referred to as Half MESP medium in all further experiments. The impact of replacing the difference in mass of malt extract between MESP and Half MESP medium, weight-for-weight, with selected sugars was investigated as a potential means of improving pigment production, potentially by increasing biomass yields while maintaining improved specific pigment production.

Maltose was selected for this purpose, as it is the main sugar present in malt extract (Neogen Corporation, 2009; Merck, 2016) and is a dimer of glucose. The impact of using glucose was also investigated in order to confirm its detrimental impact on pigment production observed on agar plates, which may be affected through the reducing sugar groups present. The concentration of soya peptone in the Half MESP, Half MESP + Maltose, and Half MESP + Glucose media was maintained at 3 $g.L^{-1}$. The composition of each medium type is outlined in Table 3.2. Cultivations using these variations of medium composition were performed using 100 mL liquid volumes in 500 mL flasks, incubated at 30 °C, with shaking at 120 rpm, for 5 days. Culture monitoring was performed as described in Section 3.3.3.3, with the data summarising the cultivations provided in Figure 6.9.

The pH of the cultures was maintained at a value of 5 through the application of a 50 mM citrate buffer. This was most effective in the Half MESP + Glucose medium, where hardly any shift in pH was observed throughout the cultivation. The pH trend observed when using the other medium types was the same over the first 3 days, increasing to a maximum of approximately pH 5.5 before declining. Beyond day 3, however, the pH in the Half MESP + Maltose cultures decreased back to the starting value, while that in the Half MESP cultures began to increase, reaching a value of approximately 7.5 by day 5.

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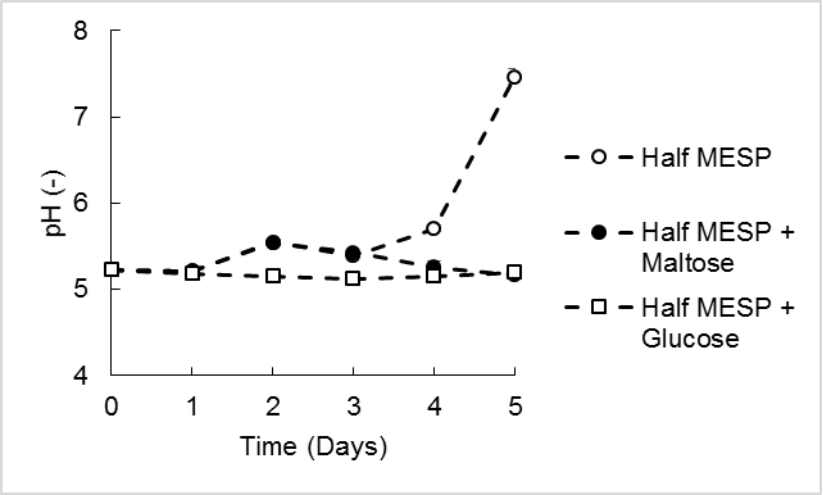

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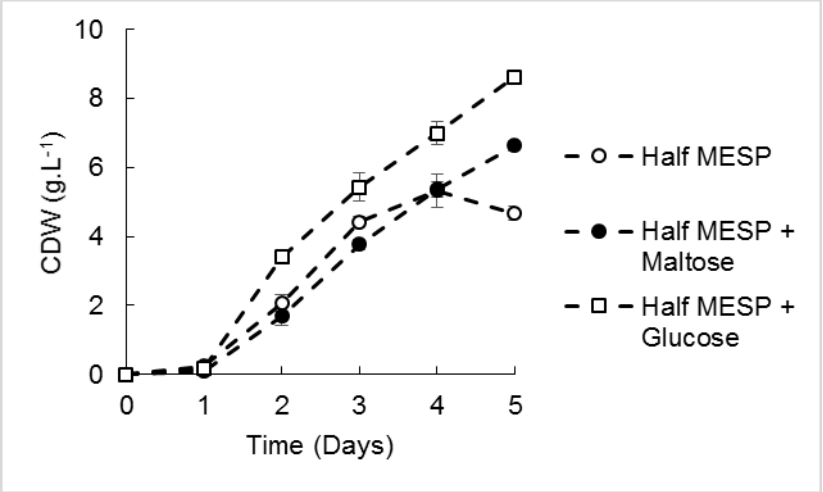

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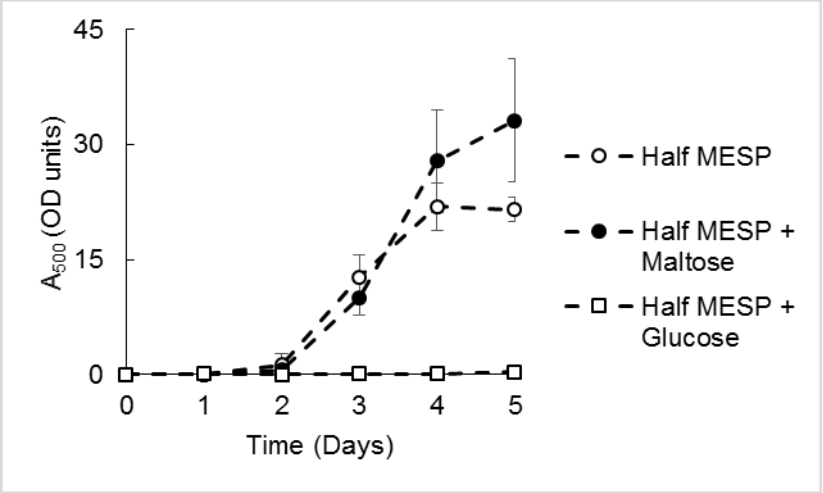

Figure 6.9 Comparison of the pH trends (a), growth (b) and pigment production (c) observed in *P. purpurogenum* shake flask cultures grown using variations of the MESP medium at 30 °C, with shaking at 120 rpm, for 5 days. Average values across triplicate flasks are shown, with error bars representing the standard deviation of the data.

The Half MESP and Half MESP + Maltose cultures exhibited similar growth trends over the first 4 days of the cultivation, despite the additional carbohydrate source supplied in the form of maltose. Beyond day 4, however, the Half MESP + Maltose culture continued to show an increase in biomass concentration which was not observed in the Half MESP culture. The addition of glucose in the Half MESP + Glucose medium had a beneficial effect on growth of *P. purpurogenum*. By day 2 of the cultivation the Half MESP + Glucose cultures exhibited average CDW values which were double that achieved in the Half MESP and Half MESP + Maltose cultures in the same period. The highest overall CDW of approximately 8.6 $g.L^{-1}$ was achieved in Half MESP + Glucose cultures, followed by approximately 6.6 $g.L^{-1}$ in Half MESP + Maltose cultures and approximately 5.4 $g.L^{-1}$ in the Half MESP cultures.

The average absorbance recorded for each of the medium types is shown in Figure 6.9c, with a picture of the pigmentation achieved in representative flasks provided in Figure 6.10. Visually, the pigment production in the Half MESP, and Half MESP + Maltose cultures was very similar, with both medium types resulting in deep red colouration. The absorbance value recorded for these cultures was, however, highest in the Half MESP + Maltose cultures, with an average A_{500} value of approximately 33 OD units, but with large variation observed between replicates. The pigmentation achieved in the Half MESP medium was defined by a maximum A_{500} value just below 22 OD units which aligns well with the volumetric pigment concentrations observed previously using MESP medium (Figure 4.5b).

Despite the increased growth observed when using the Half MESP + Glucose medium, pigment production was absent, with the culture remaining a pale yellow colour throughout the cultivation period. Glucose is a favourable substrate for biomass production during fungal cultivations, while secondary metabolite production is often higher under conditions of sub-optimal growth (Brakhage, 2013), potentially explaining the lack of pigmentation in this growth medium. Reduction in pigment production in response to glucose addition has been reported previously during liquid cultivation of *P. purpurogenum* (Ogihara et al., 2000b).

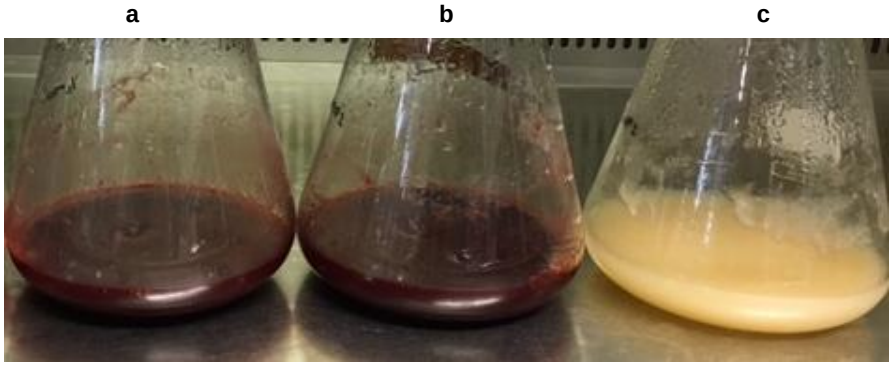

Figure 6.10 Visual comparison of the pigmentation achieved when cultivating *P. purpurogenum* in Half MESP (a), Half MESP + Maltose (b), Half MESP + Glucose (c) media, all buffered to a pH of 5 through the application of 50 mM citrate buffer. Flasks were incubated at 30 °C, with shaking at 120 rpm, for 5 days.

Based on the pigment production observed in this experiment, Half MESP + Glucose was excluded from consideration for further production studies. The Half MESP and Half MESP + Maltose media, however, yielded promising pigment production results.

Cultivation on the Half MESP medium yielded good pigmentation, reproducible production between replicate flasks, and a number of benefits which may be significant when considering future large-scale industrial production. These include reduced medium cost in comparison to the MESP medium and the Half MESP + Maltose medium, and lower overall growth, which would have implications for culture mixing and energy requirements, while also improving the ease with which the culture can be processed to yield the product, given the extracellular nature of the pigment of interest.

Specific pigment production in Half MESP medium was maintained in Half MESP + Maltose medium, with higher biomass and pigment concentrations recorded for the sugar-supplemented medium by day 5 of the cultivation. Specific pigment production of 4.62 ± 0.50 OD units.g_x^{-1} and 4.99 ± 1.20 OD units.g_x^{-1} was recorded for the Half MESP and Half MESP + Maltose media, respectively. These values were shown to not differ significantly using a two-sample t-Test, assuming unequal variances, at a 95 % confidence interval (data is provided in Table G.2). Volumetric and specific pigment production in the Half MESP + Maltose medium showed significant variability between replicates. Means of improving reproducibility in this medium may represent a potential area for further research.

6.2 BioFlo 110 reactor cultivation using modified MESP medium

The modified MESP medium containing 15 g.L^{-1} malt extract and 3 g.L^{-1} soya peptone (referred to as Half MESP medium) was then applied in 5 L working volume cultivation of *P. purpurogenum* in the New Brunswick Scientific BioFlo 110 fermentor. This medium was selected for further investigation based on the results presented in Section 6.1.3, where high volumetric pigment concentrations were maintained even though biomass production decreased in comparison to MESP medium cultivations.

The reactor cultivation was preceded by an investigation of shaking platform speed during flask cultivation (Section 6.2.1) to evaluate whether base case cultivation parameters of agitation and aeration would be suitable for Half MESP cultivation. Following on from this, all bioreactor system parameters were kept constant between this cultivation and the base case experiment (Table 4.8).

Duplicate 6-day cultivations were performed in the BioFlo 110 bioreactor using the modified medium composition, with direct spore inoculation used, once again, to yield an initial concentration of $1x10^5$ spores.mL^{-1}. Cultivation sampling was performed as per the base case cultivation, as described in Section 3.3.4.4, with residual sugar concentration evaluated using the sulphuric acid-UV assay. The growth and pigment production achieved was compared to that obtained in the base case MESP medium cultivation.

6.2.1 Effect of shaking speed when cultivated on Half MESP medium

The effect of shaking speed was investigated using the Half MESP medium in order to determine whether increasing shaking speed during flask cultivation would have the same effect on pigmentation as observed when cultivating *P. purpurogenum* on MESP medium (Section 4.2.1). Cultivations were performed using 100 mL medium in 500 mL flasks, with the medium buffered to a pH of 5 using a 50 mM citrate buffer. The flasks were incubated at 30 °C for 5 days following inoculation with a spore solution to yield a starting concentration of $1x10^5$ spores.mL^{-1}. The pH, growth and pigmentation relating to shaking speeds of 120, 130 and 140 rpm are presented in Figure 6.11.

Altering the shaking speed had little impact on the pH trend during cultivation, as observed with MESP medium (Section 4.2.1), with the characteristic peak around day 2 observed at all shaking speeds considered. By day 5 of the cultivation all cultures exhibited a rapid increase in pH, reaching values of approximately 6.8 to 7.5. Growth trends of *P. purpurogenum* on Half MESP medium also remained similar irrespective of shaking speed, with maximum cell dry weight values ranging between approximately 5.3 and 6.3 $g.L^{-1}$. The cultivation performed at 120 rpm did, however, reach a peak in CDW by day 4 of the cultivation, while cultivations at 130 and 140 rpm exhibited maximum growth by day 5.

Contrary to the results observed in MESP medium, shaking speed was also shown to have little effect on pigment production in Half MESP medium, with maximum A_{500} values recorded for the cultivations all being within the range of approximately 21 OD units (120 rpm) to 26 OD units (130 rpm).

Given the pigment production trends observed in MESP medium with changes in oxygen availability in both shake flasks (Section 4.2.1) and the BioFlo 110 bioreactor (Section 4.4), the lack of variation in pigmentation in Half MESP medium with changing shaking speed suggests that the oxygen requirement of the *P. purpurogenum* culture or the oxygen conditions experienced were altered by changing the composition of the growth medium. This may be linked to the reduced oxygen demand of the culture growing on less available substrate, and is supported by the lower overall growth observed in this medium as well as the lower maximum biomass productivity in Half MESP medium, as shown in Table 6.4. The lower solute concentration in the Half MESP medium would also result in higher oxygen solubility in this growth medium, as shown in Table 4.7.

This discussion focuses on the impact of oxygen transfer and oxygen availability as this is hypothesised to have a dominant effect on growth and pigment production by this organism. Other factors are however, affected when altering shaking speed of the culture including transfer of nutrients to the biomass as well as potential shear effects.

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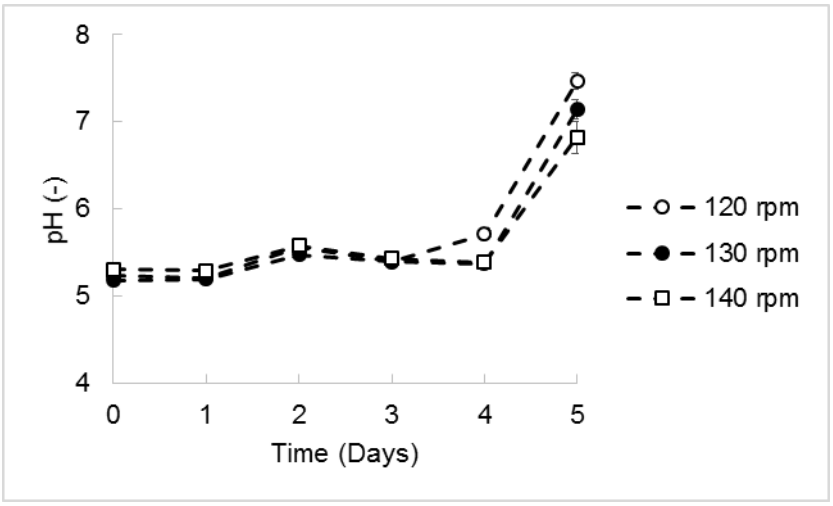

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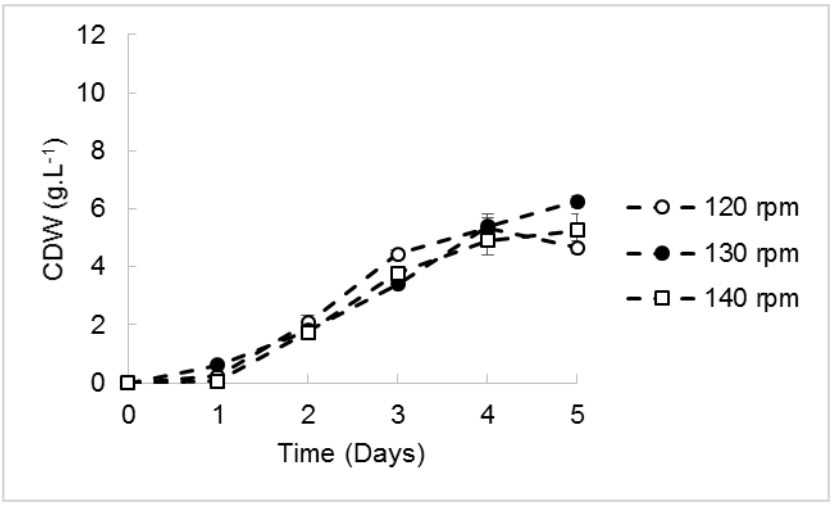

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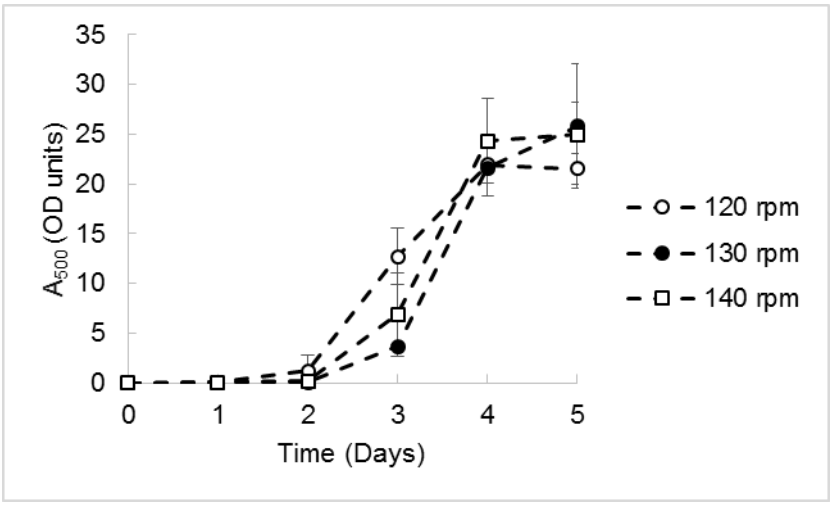

Figure 6.11 pH trends (a), growth (b), and pigment production (c) observed in response to changes in shaking platform speed during liquid cultivation of *P. purpurogenum* at 30 °C in Half MESP medium, buffered to a pH of 5 using 50 mM citrate buffer. Error bars represent the standard deviation across replicate flasks.

Table 6.4 Maximum cell dry weight and maximum biomass productivity of *P. purpurogenum* in MESP or Half MESP medium, pH 5, 50 mM citrate buffer at 30 °C, under varying conditions of shaking speed.

Shaking speed (rpm)	**Maximum CDW [a]**		**Average biomass productivity [a]**			
	$g.L^{-1}$		MESP		Half MESP	
	MESP	Half MESP	Hours	$g.L^{-1}.h^{-1}$	Hours	$g.L^{-1}.h^{-1}$
120	9.50 ± 0.00	5.33 ± 0.49	24 – 72	0.119 ± 0.003	24 – 72	0.088 ± 0.004
130	9.95 ± 0.21	6.25 ± 0.21	48 – 96	0.117 ± 0.003	48 – 96	0.075 ± 0.006
140	8.43 ± 0.38	5.27 ± 0.55	24 – 96	0.098 ± 0.004	24 – 72	0.077 ± 0.006

a Results presented as average ± standard deviation across replicate flasks

Overall, pigment production in the Half MESP medium showed an improvement in comparison to that in the MESP medium at the same shaking speed. However, given that increased shaking speed was shown to result in little to no improvement in pigmentation in Half MESP medium, it can be assumed that the pigment production observed at shaking speeds of 120 rpm to 140 rpm represents the maximum volumetric concentration possible when *P. purpurogenum* DSM 62866 is cultivated on Half MESP medium, under the given conditions of pH and temperature. Considering that the pigment production observed in MESP medium at 150 rpm is comparable to that observed in Half MESP medium at lower shaking speeds, as shown in Figure 6.12, it appears that equivalent volumetric pigment concentrations can be achieved when cultivating *P. purpurogenum* on either medium, when oxygen is not limiting, with the reduced substrate availability affecting growth of the organism rather than pigment production. The pH, growth and pigment production trends at different shaking speeds in the MESP and Half MESP media are compared in Figure 6.12.

To evaluate whether a critical oxygen supply per gram of biomass exists, pigmentation achieved in both media was plotted against specific oxygen transfer rate. The OTR_{max} for each shaking speed was calculated using Equation 9 and the predicted k_La values (Table 4.5) based on the model presented by Nikakhtari and Hill (2005). This data is presented in Figure 6.13.

A trend of increasing pigmentation with increasing specific OTR_{max} was observed in the MESP medium cultivations (trendline described by $y = 2.56x - 37.16$, $R^2 = 0.86$). Higher specific OTR_{max} was experienced in the Half MESP medium cultures, but this did not result in any further trend of increased pigmentation ($y = -0.31x + 33.74$, $R^2 = 0.29$). Above a specific OTR_{max} of 23.88 $mg.g_x^{-1}.h^{-1}$, achieved in the 150 rpm MESP medium cultivation, pigment production by *P. purpurogenum* was maintained in the range of approximately 21 to 26 OD units. The correlation observed between the 150 rpm MESP medium cultivation and the Half MESP medium cultivations suggests that a maximum volumetric pigment concentration exists for the *P. purpurogenum* culture under the given cultivation conditions.

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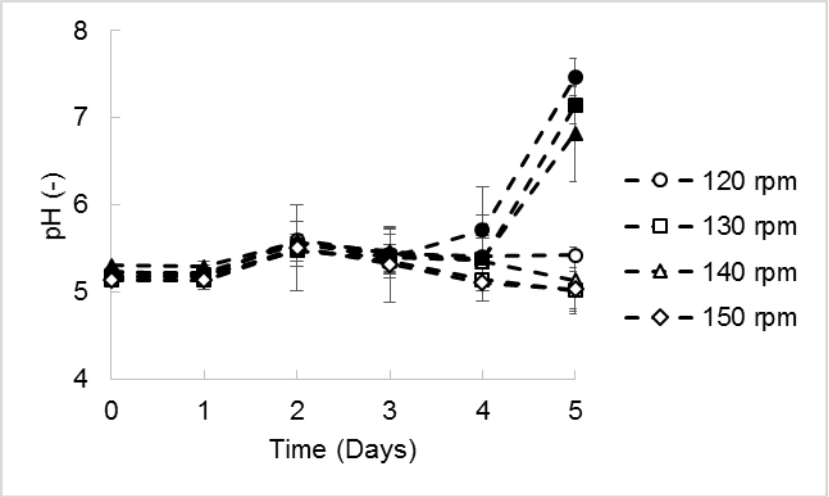

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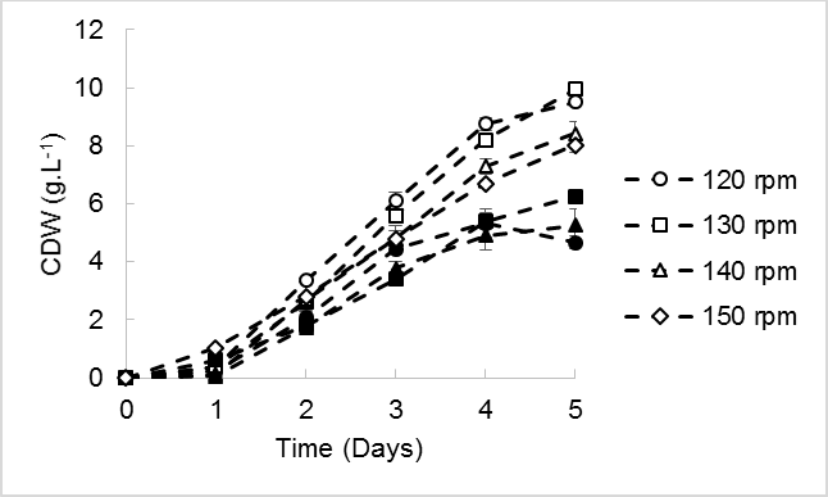

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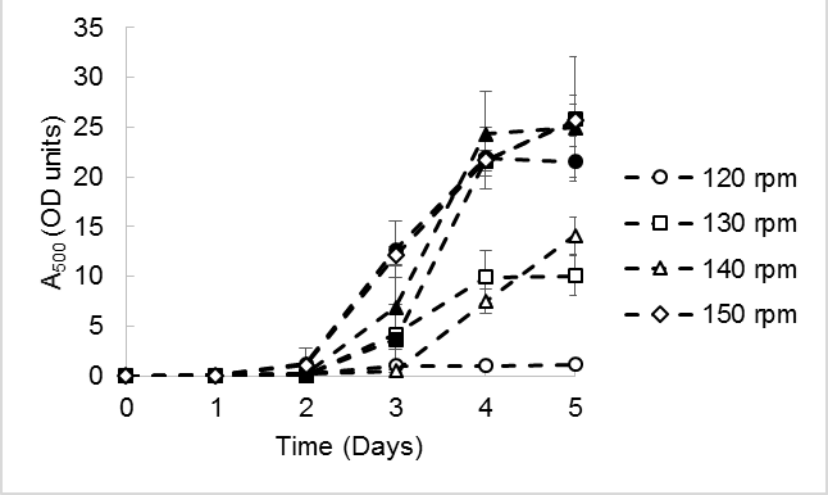


Figure 6.12 pH (a), growth (b) and pigment production (c) response of *P. purpurogenum* to changes in shaking platform speed during cultivation in MESP (open symbols) or Half MESP (closed symbols) medium. Error bars represent the standard deviation associated with replicate flasks.

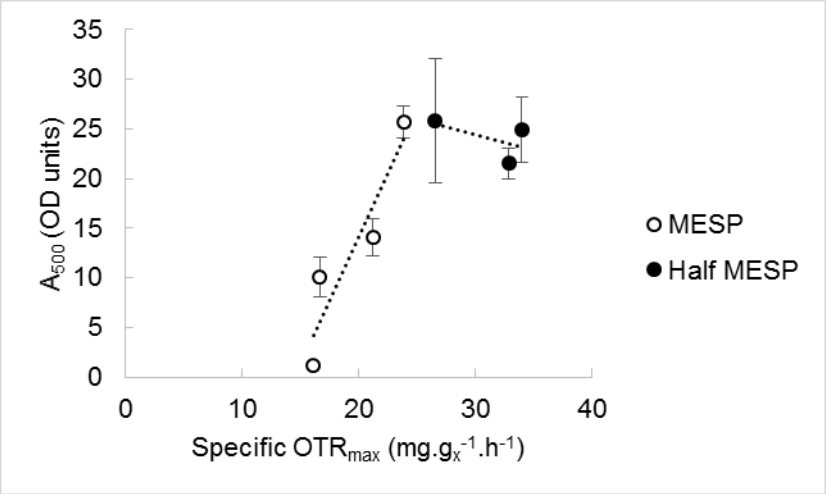


Figure 6.13 Pigment production by *P. purpurogenum* DSM 62866 during shake flask cultivation in MESP and Half MESP media as a function of specific maximum oxygen transfer rate (a) and maximum oxygen transfer rate (b) at different shaking speeds. Error bars indicate the standard deviation associated with replicate flasks.

6.2.2 Overview of the BioFlo 110 reactor cultivation

The pH, growth, pigment production, and sugar utilisation trends during cultivation of *P. purpurogenum* DSM 62866 on Half MESP medium in the New Brunswick Scientific BioFlo 110 fermentor were monitored twice daily for 6 days. This data has been provided in Figure 6.14.

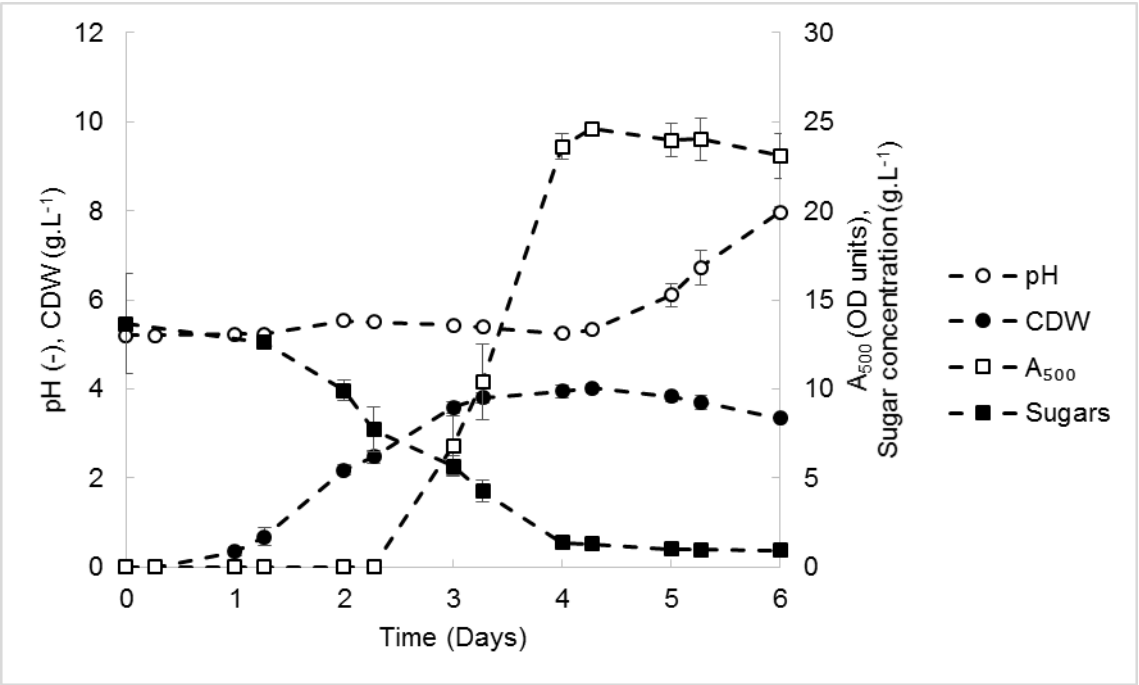


Figure 6.14 Cultivation summary of *P. purpurogenum* DSM 62866 grown in Half MESP medium, pH 5 (50 mM citrate buffer), at 30 °C, with agitation of 300 to 400 rpm, and aeration of 0.5 to 1 vvm, in the BioFlo 110 modular benchtop fermentor for 6 days. Results show the average pH, growth, pigment production, and sugar concentration for triplicate measurements performed during duplicate cultivations, with error bars representing the standard deviation of the data.

The culture pH was maintained within the range of approximately 5.2 to 5.5 over the first 4 days, with the expected peak observed around day 2 of the cultivation. By day 5 of the cultivation the pH had risen to approximately 6.1 and at the final sampling point, on day 6, a pH of approximately 8 was recorded. This is consistent with the shake flask cultures (Figure 6.9a).

Growth of *P. purpurogenum* was observed within the first 24 hours of the cultivation, but the highest volumetric rate of growth was observed between day 1 and day 3, where growth increased from approximately 0.35 $g.L^{-1}$ to 3.58 $g.L^{-1}$. Growth of the organism slowed beyond day 3, reaching a maximum CDW of 4.02 ± 0.10 $g.L^{-1}$ after approximately 100 hours of cultivation. Growth on a lower substrate concentration, in comparison to MESP medium, resulted in reduced biomass production and, as such, oxygen demand of the culture was lower than observed in the base case cultivation. The result was that adjustment of the agitation speed during cultivation was not necessary to maintain the dO_2 concentration at or above 35 %, and agitation was, therefore, maintained at 300 rpm throughout the cultivation. The aeration rate was adjusted from 1 vvm to 0.75 vvm, and further to 0.5 vvm, after approximately 100 hours of cultivation in order to control foaming.

Pigmentation was first detected on day 3 of the cultivation, with absorbance at 500 nm of approximately 6.8 OD units recorded at the 72-hour sampling point. Extrapolating backwards using the linear rate of increase in pigmentation between 72 and 96 hours of cultivation (Figure 6.15), the latest time for initiation of pigment production was calculated to be approximately 63 hours. Product production thus started between 54.5 and 63 hours of cultivation. Over the following 24 hours this value increased to approximately 23.6 OD units, and a maximum A_{500} value of 24.61 ± 0.34 OD units was recorded after approximately 100 hours. Maximum CDW and A_{500} values were observed at the same sampling point.

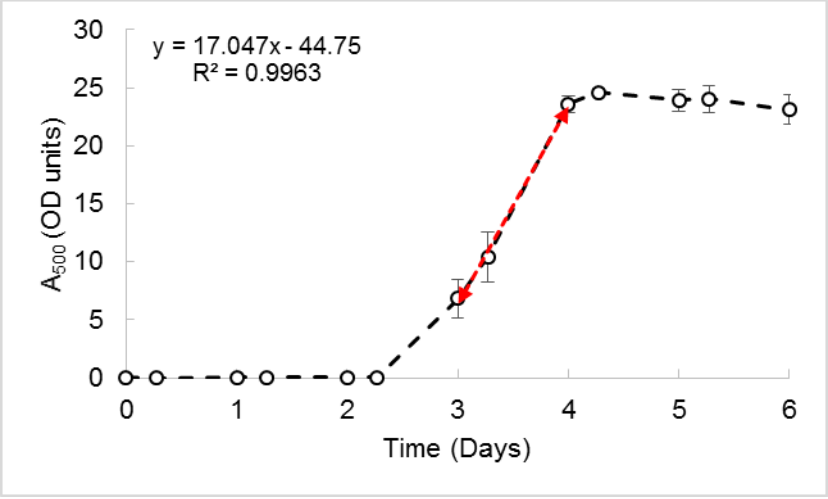


Figure 6.15 Period of linear increase in pigmentation during BioFlo 110 bioreactor cultivation of *P. purpurogenum* using Half MESP medium, denoted by the arrows, with the corresponding trendline equation provided.

Morphology of *P. purpurogenum* DSM 62866 was monitored visually every 24 hours during cultivation on Half MESP medium in the bioreactor, with representative images for each day provided in Figure 6.16.

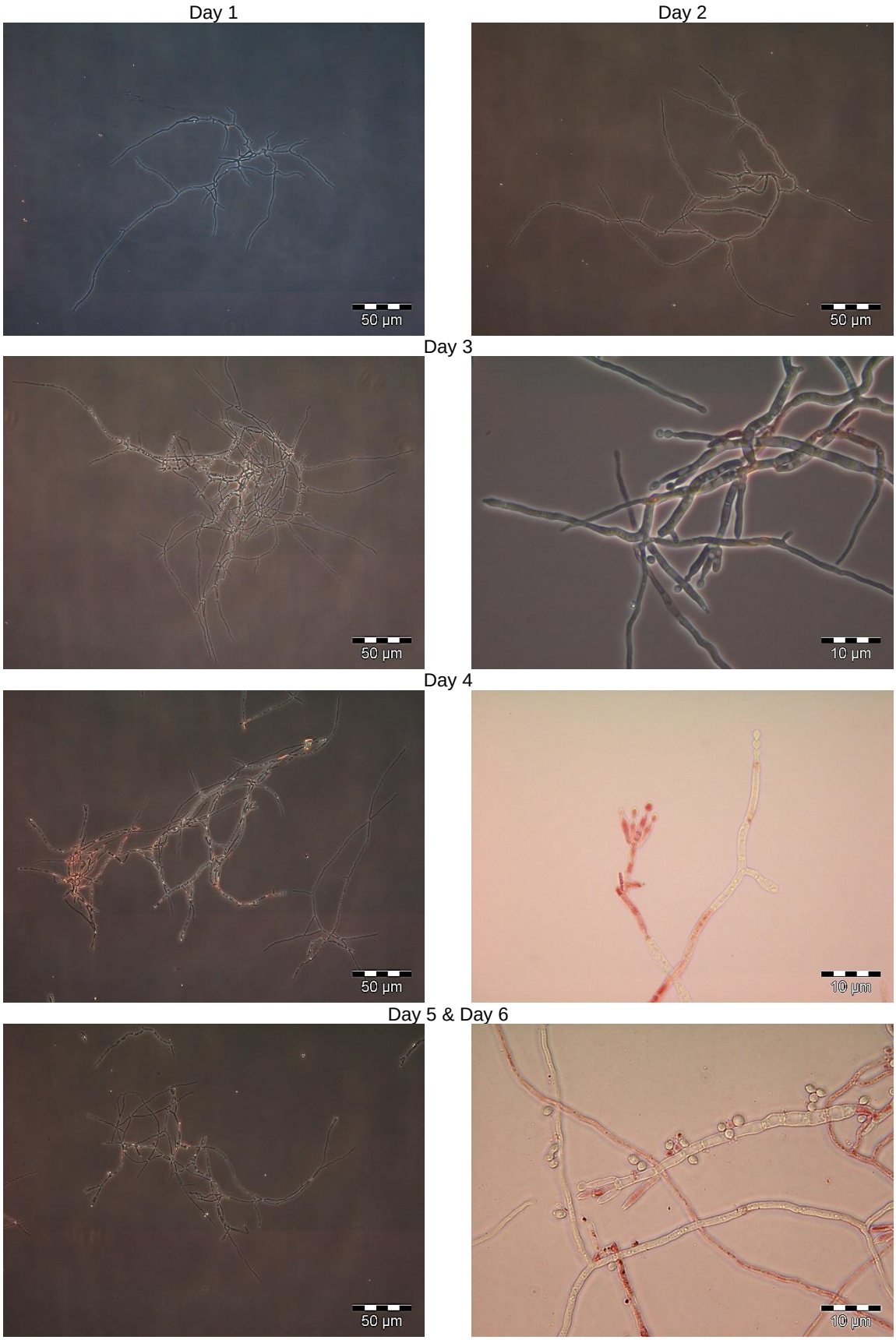


Figure 6.16 Morphology of *P. purpurogenum* DSM 62866 during cultivation on Half MESP medium, pH 5, 50 mM citrate buffer at 30 °C in the BioFlo 110 fermentor.

Simultaneous onset of pigmentation and sporulation was observed during cultivation in MESP medium (Figure 4.15). This was also demonstrated during reactor cultivation using Half MESP medium, with evidence of sporulation by day 3 of the cultivation, coinciding with the onset of pigmentation. Red pigmentation is visible within the fungal hyphae in the images provided for day 4 to day 6 in Figure 6.16.

Evidence of some hyphal breakage was noted in the later stages of the cultivation. This was hypothesised to be related to culture age or sporulation rather than being related to shear experienced by the culture. The maximum agitation speed applied during the cultivation was 400 rpm, but this was decreased back to the starting speed of 300 rpm before hyphal breakage was observed.

6.2.3 Growth kinetics and yields

The growth and pigment production of *P. purpurogenum* on Half MESP medium in the BioFlo 110 fermentor was investigated further through calculation of the growth rate and various yields during the cultivation. Growth of *P. purpurogenum* over the first 24 hours was limited, with no biomass detected through cell dry weight determination by the 6.5-hour sampling point. This was followed by a phase of highest biomass productivity, between approximately 30 and 54 hours of cultivation. As observed when using MESP medium, growth of *P. purpurogenum* during this period exhibited an approximately linear increase, and was defined by a volumetric growth rate of 0.077 ± 0.006 $g.L^{-1}.h^{-1}$, determined through linear regression. This data is presented in Table 6.5. Beyond 72 hours of cultivation, rate of biomass formation began to slow, indicating entry into the stationary phase of growth.

Maximum specific growth rate was once again observed during the early stages of the cultivation. A value of 104 ± 0.004 h^{-1} was recorded between 24 and 30 hours of cultivation (2-point), decreasing to 0.075 ± 0.016 h^{-1} when an additional sampling point was included. This can be attributed to the germination of spores and development into hyphae, as described in Section 4.3.4.

Table 6.5 Biomass productivity during different growth phases and maximum specific growth rate of *P. purpurogenum* DSM 62866 when cultivated in the BioFlo 110 bioreactor using Half MESP medium, pH 5, 50 mM citrate buffer, at 30 °C.

Time (hours)	Average biomass productivity [a]		Specific growth rate [a]	
	$g.L^{-1}.h^{-1}$	R^2 [b]	h^{-1}	R^2 [b]
0-24	0.016 ± 0.005	0.931	-	-
24-30	-	-	0.104 ± 0.004	-
24-48	-	-	0.075 ± 0.016	0.990
30-54	0.077 ± 0.006	0.988	-	-

a Results presented as average ± standard deviation across duplicate cultivations
b Determined through linear regression analysis

Pigment production also showed an approximate linear increase, which occurred between 72 and 96 hours of the cultivation and was defined by a productivity of 0.71 ± 0.11 OD units.h^{-1}. The linear increase in pigmentation was, therefore, observed once growth rate of *P. purpurogenum* had declined. This is in contrast to cultivation using MESP medium, where the period of linear increase in pigmentation began after, and ended before, that of growth.

Growth and pigment production data for reactor cultivation using the two medium types is summarised in Table 6.6, demonstrating that the rate of growth of *P. purpurogenum* DSM 62866 begins to slow much sooner when cultivated on Half MESP medium than on MESP medium, while pigment production is largely unaffected.

Table 6.6 Biomass and pigment productivity achieved during BioFlo 110 reactor cultivation of *P. purpurogenum* using Half MESP or MESP media.

	Half MESP medium		**MESP medium**	
	Time (hours)	Value [a]	Time (hours)	Value [a]
Maximum CDW ($g.L^{-1}$)	102.5	4.02 ± 0.10	120	8.42 ± 0.29
Maximum biomass productivity ($g.L^{-1}.h^{-1}$)	30.5 – 54.5	0.077 ± 0.006	30.5 – 120	0.089 ± 0.007
Maximum A_{500} (OD units)	102.5	24.61 ± 0.34	120	24.07 ± 1.97
Maximum pigment productivity (OD $units.h^{-1}$)	72 – 96	0.71 ± 0.11	78.5 – 102.5	0.72 ± 0.18

a Results presented as average ± standard deviation across duplicate cultivations

Pigment production was shown to be non-growth associated based on the results of the base case bioreactor cultivation in MESP medium (Figure 4.16). Half MESP medium cultivation also showed no trend of increasing pigment production rate with increasing growth rate. The change in volumetric pigment concentration with time was plotted against biomass concentration achieved using Half MESP medium to evaluate the non-growth associated specific rate of pigment production, β (Equation 7), in this medium. A positive correlation was observed between these parameters, with β found to be 0.98 OD $units.g_x^{-1}.h^{-1}$ (SE_m = 0.17). This value represents a 4.3-fold increase over that obtained using MESP medium, which can be attributed to the lower biomass concentration achieved in this medium, while pigment production rate is maintained in comparison to MESP medium.

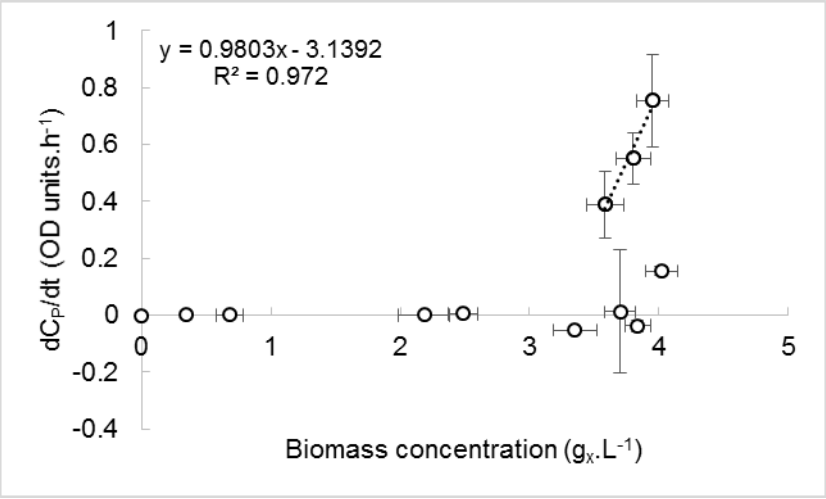


Figure 6.17 Change in pigment concentration (dC_P/dt) as a function of volumetric biomass concentration to evaluate the non-growth associated specific rate of pigment formation during BioFlo 110 reactor cultivation of *P. purpurogenum* DSM 62866 in Half MESP medium. Error bars represent the standard deviation across duplicate cultivations. Trendline equation relates to the period of linear increase in pigmentation.

The biomass and pigment yields ($Y_{X/S}$, $Y_{P/X}$) for this cultivation were calculated in the same way described for MESP medium cultivation (Section 4.3.4). The $Y_{X/S}$ value was calculated over the period of approximately 30 to 78 hours, and $Y_{P/X}$ was calculated at the 102.5 hour sampling point, corresponding to maximum volumetric biomass and pigment concentrations. The yields defining Half MESP medium cultivation in the BioFlo 110 bioreactor system were found to be 0.36 ± 0.04 $g_x.g_s^{-1}$ ($Y_{X/S}$) and 6.13 ± 0.04 OD units.g_x^{-1} ($Y_{P/X}$).

The $Y_{X/S}$ values in the cultivations using the different media did not vary significantly, while the $Y_{P/X}$ value exhibited a 2.1-fold increase when using the Half MESP medium in comparison to the base case cultivation. Specific pigment production by *P. purpurogenum* DSM 62866 is significantly increased when cultivated on Half MESP medium rather than MESP medium. The comparison of the BioFlo 110 bioreactor cultivations using these two media is discussed further in Section 6.2.5.

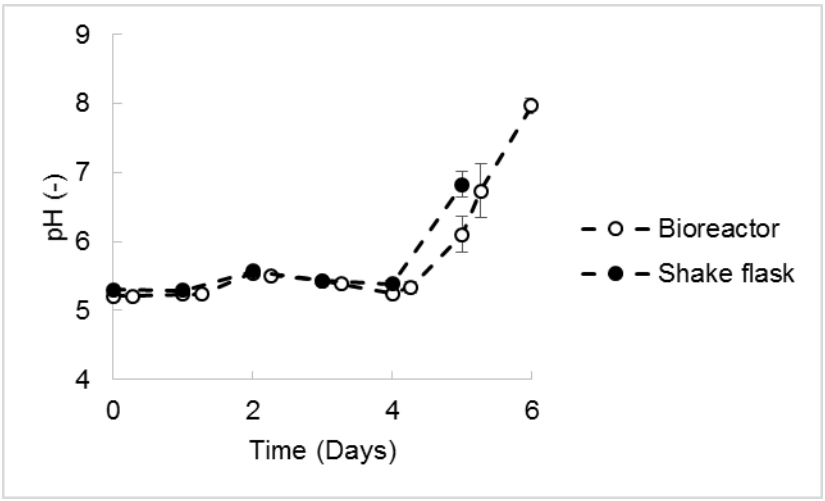
6.2.4 Comparison of shake flask and bioreactor cultivation using Half MESP medium

The results observed during cultivation of *P. purpurogenum* DSM 62866 in the BioFlo 110 bioreactor at 30 °C, using Half MESP medium maintained at a pH of 5 through the application of a 50 mM citrate buffer were compared to shake flask cultivations under the same conditions. The pH, growth and pigment production trends observed during shake flask and bioreactor cultivation are shown in Figure 6.18. The 140 rpm shake flask cultivation was selected for comparison to the bioreactor cultivation as it most closely represents the average growth and pigment production achieved over the range of shaking speeds considered.

The pH, growth and pigment production in the two cultivation systems exhibited the same trends. This is in agreement with the MESP medium cultivations, where growth and pigment production were maintained when scaling up, and cultivation in the bioreactor conferred no benefit in terms of improving overall biomass or product concentration or productivity.

When using Half MESP medium the only differences observed were a slightly higher pH and CDW concentration achieved in the shake flask cultivation after 5 days. Growth in the two systems exhibited the same trend over the first three days of the cultivation, beyond which the biomass concentration in the bioreactor was maintained, while that in the shake flasks continued to increase. Accumulation of pigmentation, however, followed the same trend in the two systems.

a



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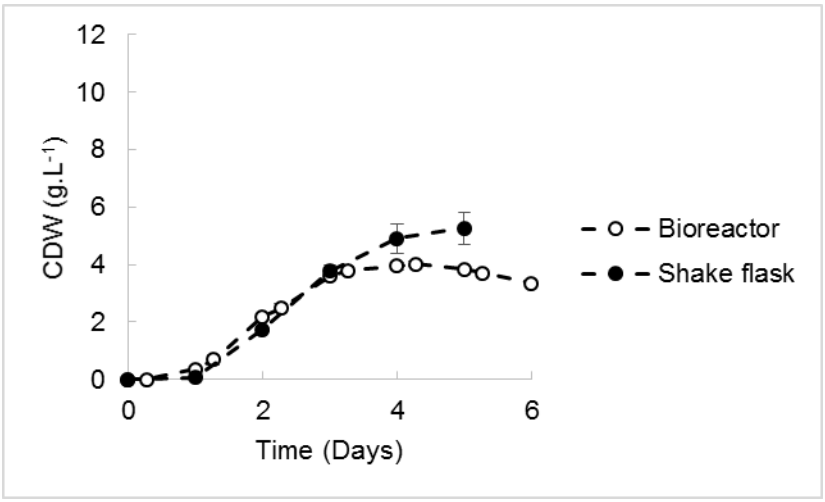

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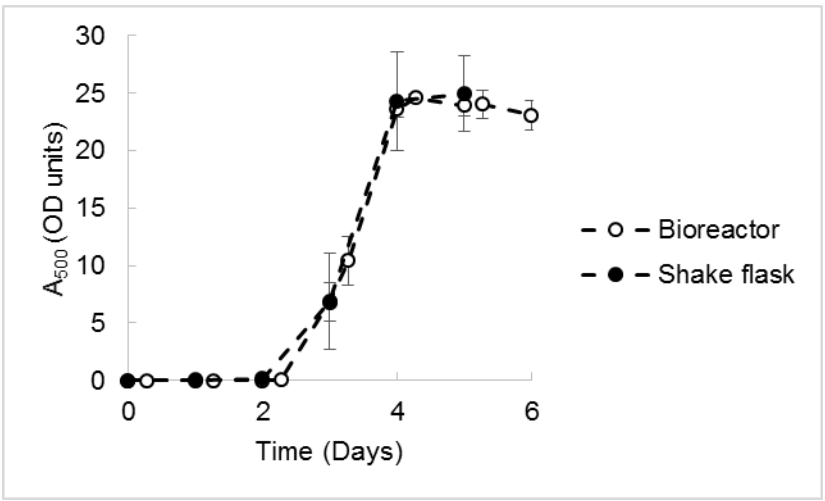


Figure 6.18 Comparison of the pH trends (a), growth (b) and pigment production achieved during cultivation of *P. purpurogenum* in Half MESP medium, pH 5, 50 mM citrate buffer, at 30 °C in the BioFlo 110 fermentor and shake flasks (140 rpm). Bioreactor data represents the average of triplicate measurements performed during duplicate cultivations, while shake flask data represents the average measurement across triplicate flasks. Error bars represent the standard deviation of the data.

6.2.5 Comparison of BioFlo 110 fermentor cultivations using Half MESP and MESP media

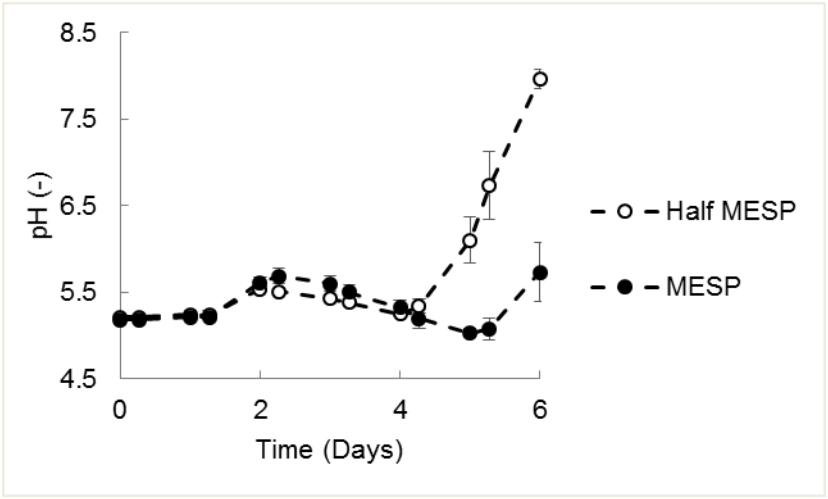
The *P. purpurogenum* DSM 62866 cultivations in the BioFlo 110 fermentor using Half MESP and MESP medium were compared in order to further evaluate the effect of the medium composition on growth and pigment production. This follows on from the calculation of various productivities and yields calculations presented in Sections 4.3.4 and 6.2.3 which revealed notable differences in growth and pigment production when these two medium types were used. Parameters defining the Half MESP and MESP medium cultivations have been summarised in Table 6.7, with the pH trends, growth and pigment production achieved when using the two medium types directly compared in Figure 6.19.

Similar pH trends were observed over the first 4 days of the cultivation with both medium types. Beyond day 4, the Half MESP culture pH began to rise rapidly, while that of the MESP culture only started to increase after day 5. Growth of the cultures was similar over the first three days, after which growth on Half MESP medium began to slow, reaching a maximum CDW of 4.02 ± 0.10 $g.L^{-1}$ on day 4. Growth on MESP medium, however, continued to increase at an approximately constant rate beyond this point, reaching a maximum CDW of 8.42 ± 0.29 $g.L^{-1}$ by day 5.

Considering both the pH and growth trends, it appears that the pH of the culture begins to rise once the culture has achieved its maximum CDW, and the biomass concentration begins to decrease. This decrease in CDW indicates that cell death and lysis is taking place. This is proposed to be a result of nutrient limitation (Ko and Lockwood, 1970) with the increase in pH likely attributed to degradation of proteins in the cell wall with subsequent deamination of released amino acids, as reported in other fungal species (Nanou et al., 2007).

The medium containing half the amount of malt extract supported approximately half the amount of biomass production, in terms of volumetric concentration, explaining the similar $Y_{X/S}$ values observed. Similar maximum growth rates were also observed in the two medium types, with the period of maximum growth, however, reduced from approximately 4 days in MESP medium to 2 days when cultivated on Half MESP medium.

a



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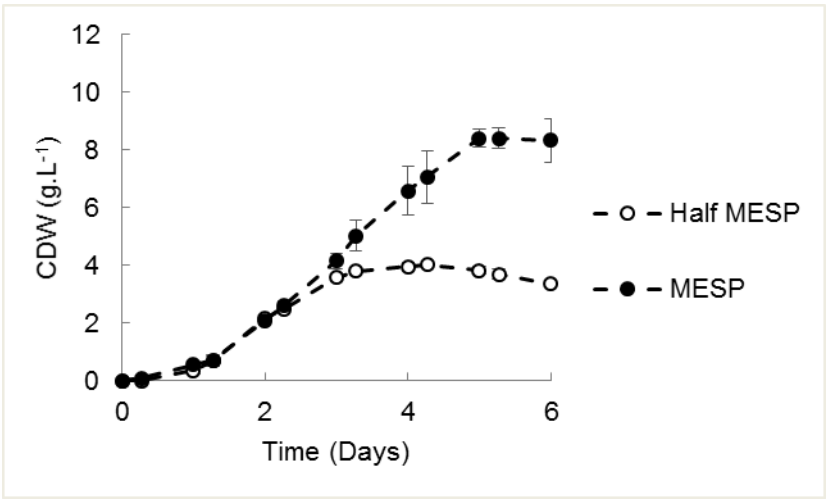

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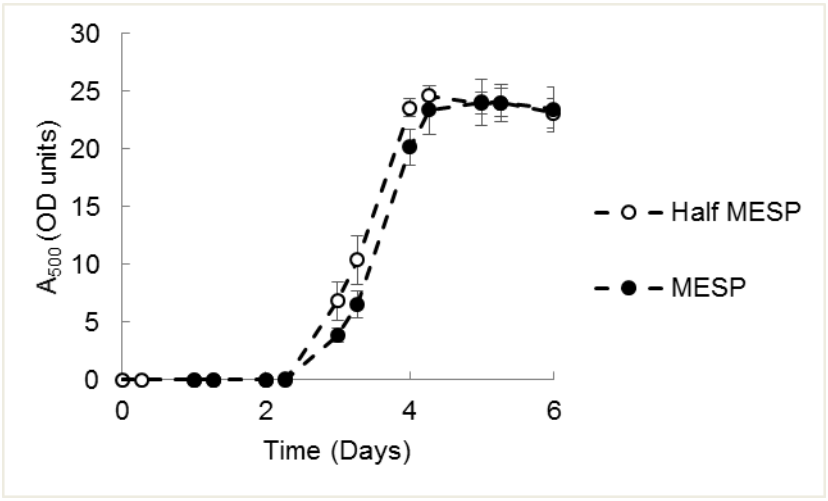


Figure 6.19 Comparison of the pH trends (a), growth (b) and pigment production (c) achieved when cultivating *P. purpurogenum* DSM 62866 in the BioFlo 110 fermentor at 30 °C, using either Half MESP or MESP medium, maintained at a pH of 5 through application of a citrate buffer. Data points represent the average of triplicate measurements during duplicate cultivations, with error bars representing standard deviation of the data.

Table 6.7 Summary of the parameters defining the BioFlo 110 reactor cultivations of *P. purpurogenum* DSM 62866 using Half MESP medium and MESP medium.

Parameter	**Medium**	
	Half MESP	MESP
Maximum CDW ($g.L^{-1}$) [a]	4.02 ± 0.10	8.42 ± 0.29
Maximum average biomass productivity ($g.L^{-1}.h^{-1}$) [a]	0.077 ± 0.006	0.089 ± 0.007
Maximum specific growth rate (h^{-1}) [a]	0.104 ± 0.004	0.099 ± 0.010
$Y_{X/S}$ ($g_X.g_S^{-1}$)	0.36 ± 0.04	0.37 ± 0.01
$Y_{P/X}$ (OD units.g_X^{-1})	6.13 ± 0.04	2.86 ± 0.29
Maximum A_{500} (OD units) [a]	24.61 ± 0.34	24.07 ± 1.97
Maximum pigment productivity (OD units.h^{-1}) [a]	0.71 ± 0.11	0.72 ± 0.18
Non-growth associated specific pigment production rate, β (OD units.$g_x^{-1}.h^{-1}$ ± SE_m)	0.98 ± 0.17	0.23 ± 0.00

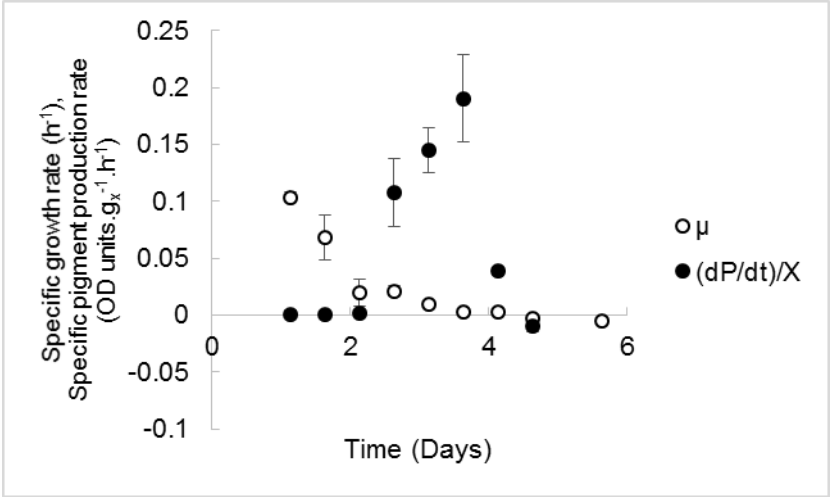
a Results presented as average ± standard deviation across duplicate cultivations

Pigment production trends in both medium types, however, exhibited similarities in terms of both pigment productivity and period of production. Pigment production appeared to start slightly earlier when using Half MESP medium, with the latest possible time for initiation of pigmentation estimated at 63 hours (Figure 6.15), in comparison to 69 hours (Figure 4.14) for the MESP medium cultivation. Both cultivations did, however, accumulate pigmentation at an approximately equal rate, with the maximum pigment productivity observed over a 24 hour period in both cases.

Maximum pigment productivity was determined using linear regression and was found to be 0.71 ± 0.11 OD units.h^{-1} between 72 and 96 hours for the Half MESP medium cultivation, and 0.72 ± 0.18 OD units.h^{-1} between 78.5 and 102.5 hours for the MESP medium cultivation. Maximum A_{500} values achieved were 24.61 ± 0.34 OD units on day 4, and 24.07 ± 1.97 on day 5 of the Half MESP medium and MESP medium cultivations, respectively. There was no significant difference in pigmentation between the two cultivations at all sampling points beyond 96 hours (Table G.3), as confirmed by t-Test, two sample assuming unequal variance.

Specific growth rate and specific pigment production rate were plotted as a function of time in the Half MESP (Figure 6.20a) and MESP (Figure 6.20b) media. These parameters exhibited the same trends in both medium types, with specific growth rate declining as the cultivation proceeded and specific pigment production rate increasing over the production period (approximately 54 to 96 hours). A notable difference was the higher specific pigment production rate in the cultivation medium with reduced total substrate concentration.

a



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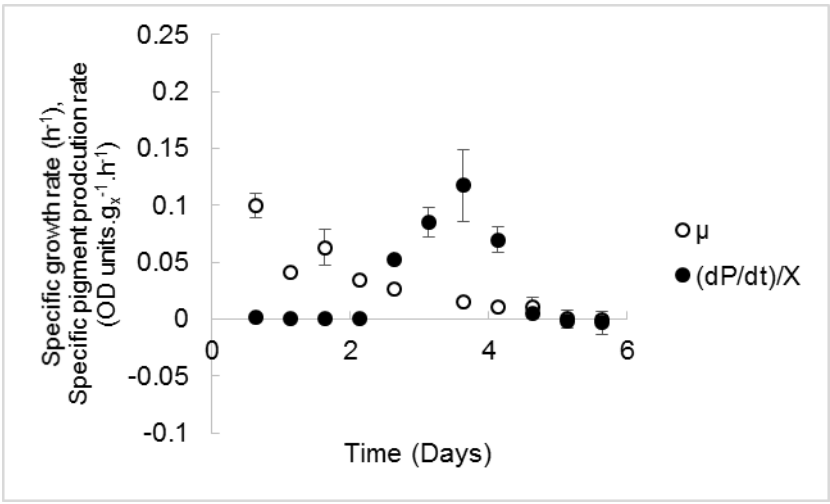


Figure 6.20 Specific growth rate (μ) and specific pigment production rate as a function of time during BioFlo 110 reactor cultivation of *P. purpurogenum* DSM 62866 in Half MESP (a) or MESP medium (b). Error bars represent the standard deviation associated with duplicate cultivations.

When considering malt extract and soya peptone as the major carbon and nitrogen sources, respectively, the adjustment from MESP to Half MESP medium represents a reduction in the C:N ratio of the cultivation medium.

The impact of altering the C:N ratio during the cultivation of *Monascus ruber* to produce red pigments has previously been investigated. Biomass concentration was shown to decrease with decreasing C:N ratio, when the glucose concentration was reduced while maintaining the concentration of monosodium glutamate (MSG), supplied as the nitrogen source (Said et al., 2014). This is consistent with the results of the present study, with Half MESP medium supporting less growth than MESP medium. Volumetric pigment concentration achieved in the *M. ruber* cultivation also declined with the reduction in C:N ratio, but pigment productivity and specific pigment production increased (Said et al., 2014).

These studies demonstrate that reduced C:N ratio affects growth more strongly than pigmentation. Deviation in terms of volumetric pigment concentration achieved could possibly be attributed to the complex nature of the malt extract, soya peptone-based media, where both major components represent a mixture of carbon and nitrogen sources.

The results obtained during bioreactor cultivation of *P. purpurogenum* DSM 62866 show good agreement with those observed in shake flask cultivations. This provides support for the theory, based on shake flask cultivation data (Section 6.2.1), that equivalent volumetric pigment concentrations can be achieved using both MESP medium and Half MESP medium when oxygen is not limiting. This limitation refers to sufficient rate of oxygen supply, rather than residual oxygen concentration, as demonstrated in Section 4.4.

6.3 Impact of replacing main carbohydrate source with a confectionery-based substrate

As discussed in Section 2.3.2.2, medium components represent a major cost in the production of natural colourants through large-scale microbial fermentations. The use of a waste stream as an alternative source of nutrients and replacement for major medium components, therefore, represents a potential means of reducing process costs, contributing towards making the product more economically feasible. This substitution also represents a means of valorising the waste stream, thereby contributing towards waste reduction and the move towards a bio-based circular economy. Confectionery waste was selected for investigation in this study, based on composition being generally dominated by sugars (carbon-based) able to support growth, as well as availability of the waste substrate.

Initial evaluation of two potential substrates was performed using agar plate cultivation. One selected confectionery-based substrate was then used during small-scale liquid cultivation of *P. purpurogenum*, followed by scale-up into the BioFlo 110 bioreactor system. During liquid cultivation, the component was supplied at a concentration of 15 $g.L^{-1}$. This was based on the direct weight substitution of malt extract in the Half MESP medium composition. Half MESP was selected as the basis for this set of experiments as a result of improved specific pigment production observed in this medium in comparison to MESP medium. Growth and pigment production achieved in the confectionery-based medium was compared to that obtained when using Half MESP medium.

6.3.1 Agar plate cultivation using confectionery-based substrates

The potential for utilising a confectionery stream as a substrate for *P. purpurogenum* was first evaluated using agar plate cultivation. Two potential substrates, namely chocolate and marshmallow confectionery, were considered. The confectionery substrate was applied at a concentration of 15 $g.L^{-1}$ with bacteriological agar (15 $g.L^{-1}$) used to solidify the medium. No additional supplementation of the confectionery substrate was considered at this stage. A spore solution of *P. purpurogenum*, harvested from MESP agar plates, was spread onto the agar plate surface and was incubated at 30 °C for 10 days.

Both confectionery substrates supported limited growth of the organism, while pigmentation was only observed on the marshmallow substrate. An image of the chocolate confectionery plate is provided in Figure 6.21, with top and bottom view of the plate shown as a result of the opaque nature of the medium. Development of growth and pigmentation on marshmallow substrate over the 10-day period is represented in Figure 6.22. Deep red pigmentation, which had diffused into the surrounding agar, was observed on the marshmallow medium after 10 days of incubation at 30 °C. This formed the basis of selection of this substrate, over the chocolate confectionery, for further experimentation.

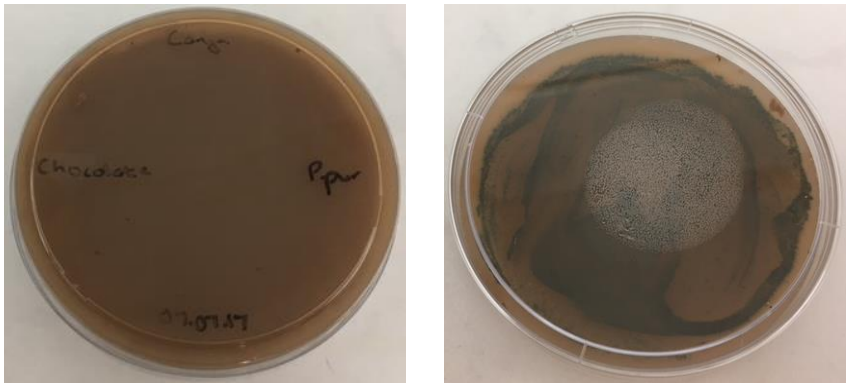
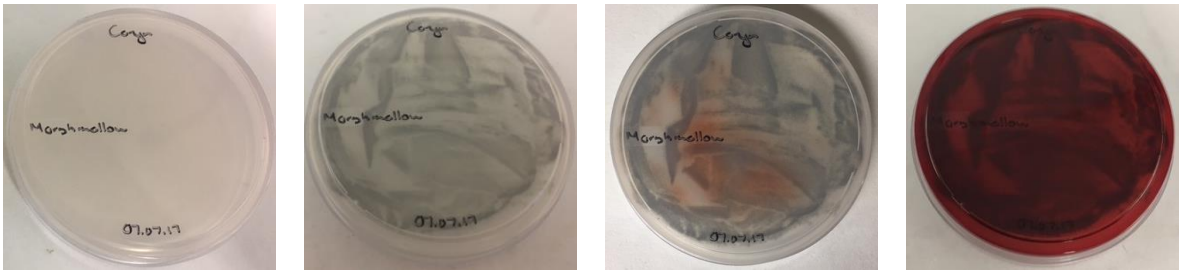


Figure 6.21 Growth of *P. purpurogenum* on chocolate confectionery-based agar plate after incubation at 30 °C for 10 days.



Day 1 Day 4 Day 6 Day 10

Figure 6.22 Growth and pigmentation of *P. purpurogenum* on marshmallow confectionery-based agar plate at various time points during incubation at 30 °C.

6.3.2 Small-scale liquid cultivation on marshmallow confectionery

Submerged liquid cultivation of *P. purpurogenum* using marshmallow confectionery as a substrate was first evaluated using 12-well multiwell plates. This cultivation method was selected to allow evaluation of a number of parameters. Given the carbon-rich nature of this substrate, supplementation with selected nitrogen sources was considered. Similar approaches have been reported for cultivation of *Monascus purpureus* on sugarcane bagasse (Silveira et al., 2013). Application of the citrate buffer previously shown to improve reproducibility of pigment production (Section 4.1.2 and Section 4.1.3) was also investigated.

The marshmallow substrate, applied at 15 $g.L^{-1}$, was evaluated for liquid cultivation with and without the addition of a supplementary nitrogen source. *P. purpurogenum* was shown to be able to grow on this substrate alone during agar plate cultivation, and two supplementary nitrogen sources were selected for investigation. These were soya peptone, a complex organic nitrogen source used previously in this study, and sodium nitrate, an inorganic nitrogen source widely applied in fungal growth media. Both components were added at concentrations of 3, 1.5 or 0.75 $g.L^{-1}$, following on from the work presented in Section 6.1.3. Each medium composition was evaluated with and without the addition of the 50 mM citrate buffer at pH 5, and MESP and Half MESP media (both with buffer addition) were included as controls. This resulted in a total of 16 conditions which were investigated using multiwell plate cultivation. Table 6.8 summarises the composition of the marshmallow-based media investigated.

Table 6.8 Composition of marshmallow confectionery-based media used for cultivation of *P. purpurogenum*.

Medium [a]	**Composition ($g.L^{-1}$)**		
	Marshmallow	Soya peptone	Sodium nitrate
Marsh.	15	-	-
Marsh. + 3 SP	15	3	-
Marsh. + 1.5 SP	15	1.5	-
Marsh. + 0.75 SP	15	0.75	-
Marsh. + 3 $NaNO_3$	15	-	3
Marsh. + 1.5 $NaNO_3$	15	-	1.5
Marsh. + 0.75 $NaNO_3$	15	-	0.75

a Media are used with or without 50 mM citrate buffer, pH 5

The cultivation volume of 2 mL was inoculated at $1x10^5$ spores.mL^{-1} using direct spore inoculation, as described in Appendix B.1. Plates were incubated at 30 °C, with shaking, for 14 days. The pH of individual wells was monitored daily, while CDW was evaluated at the end of the cultivation period, by sacrificing the entire well volume. The average CDW across triplicate wells for each condition is shown in Figure 6.23, with the colour of the bars representing the pigmentation achieved, using the rating system defined in Table 3.4 as a basis. The medium supplemented with sodium nitrate along with citrate buffer addition is excluded from these results as this medium composition was completely inhibitory to growth of *P. purpurogenum* DSM 62866.

Marshmallow medium without nitrogen supplementation or buffer addition (Marsh. w/o buffer) supported growth in the range of that observed in the malt extract-based media. Pigmentation was, however, significantly reduced with an average A_{500} value below 1 OD unit measured in these wells. Use of the citrate buffer along with un-supplemented marshmallow confectionery (Marsh.) had a negative impact on growth, with a 4- to 5-fold decrease in growth recorded in comparison to the same medium without buffer application. The un-supplemented, buffered medium also did not support pigment production. Supplementation of the marshmallow medium with sodium nitrate had a similar effect on cultivation of *P. purpurogenum*, with limited growth and little to no pigmentation observed.

Supplementation with soya peptone, with and without buffer addition, supported growth of *P. purpurogenum* in the range of 5.3 to 7.8 $g.L^{-1}$ and pigmentation was produced in all cases. Both growth and pigmentation were in the range of that observed in the malt extract-based media, with increased growth and improved pigmentation generally observed along with buffer application. Highest biomass and pigment concentrations were recorded for the medium supplemented with 0.75 $g.L^{-1}$ soya peptone along with citrate buffer application.

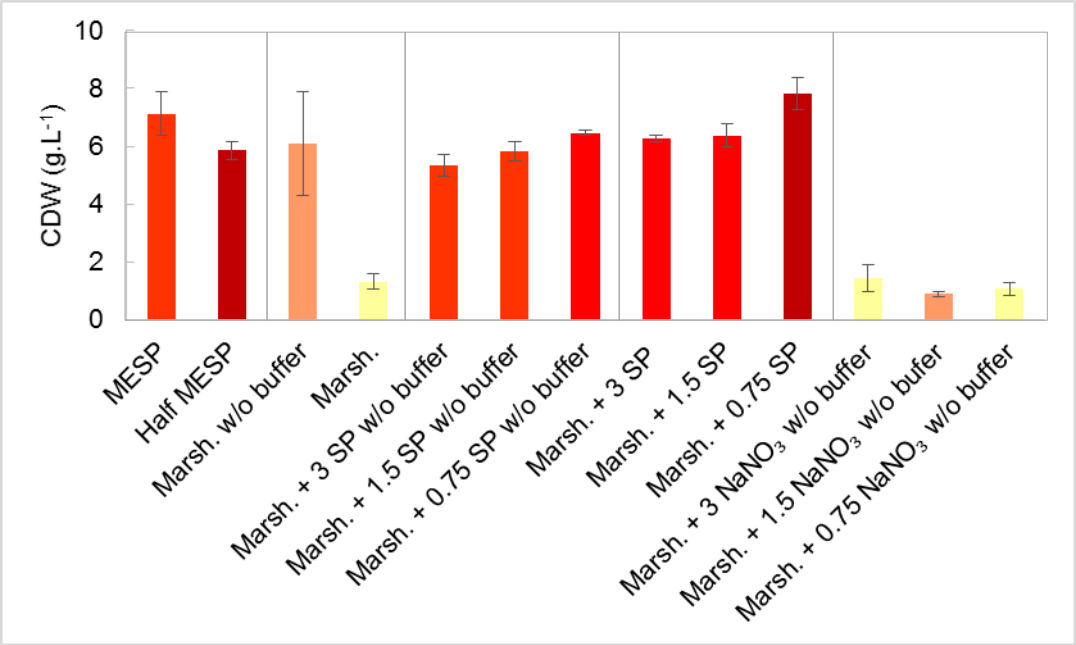
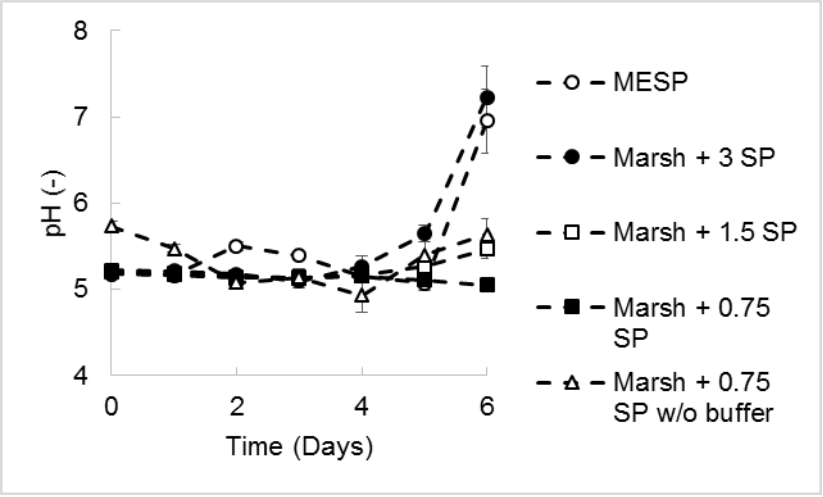


Figure 6.23 Average cell dry weight achieved during multiwell plate liquid cultivation of *P. purpurogenum* in marshmallow confectionery-based media. No supplementation (Marsh.) or supplementation with soya peptone (SP) or $NaNO_3$ at concentrations of 3, 1.5 or 0.75 $g.L^{-1}$. MESP and Half MESP media are included as controls. Citrate buffer (50 mM), pH 5 is applied unless otherwise stated. Error bars represent the standard deviation between triplicate wells. The colour of the bars represents the pigmentation achieved in each medium type.

Given that supplementation of the marshmallow confectionery with soya peptone was shown to be beneficial for growth and pigment production, selected conditions were investigated further using 100 mL shake flask cultivation. Soya peptone was added at 3, 1.5 or 0.75 $g.L^{-1}$ along with buffer addition, while only 0.75 $g.L^{-1}$ supplementation without citrate buffer was considered. Selection of this condition was based on the increased growth and pigmentation observed at this concentration of soya peptone, as well as to confirm the beneficial effect of buffer application observed during multiwell plate cultivation.

Cultivations were performed in 100 mL volume in 500 mL flasks, with MESP medium included as a control for the experiment. The flasks were inoculated with a harvested spore solution to yield a starting concentration of $1x10^5$ $spores.mL^{-1}$ and flasks were incubated at 30 °C, with shaking, for 6 days. The pH, growth and pigmentation of triplicate flasks for each condition was monitored daily. Results are provided in Figure 6.24.

a



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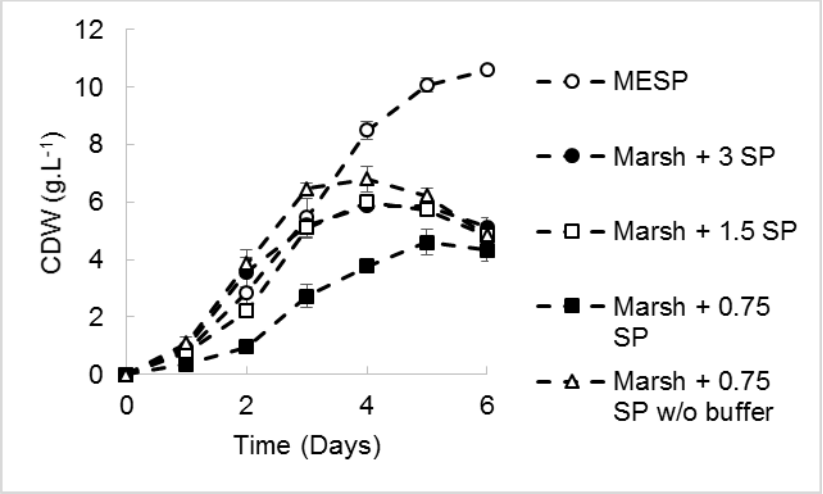

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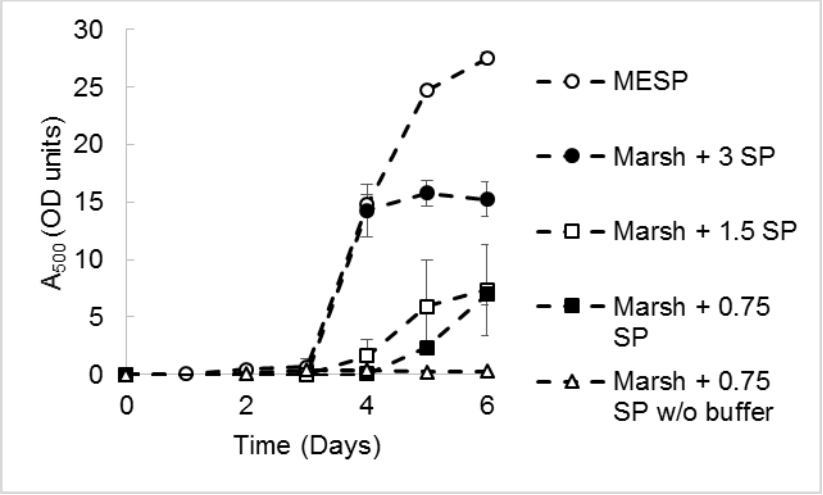


Figure 6.24 pH trends (a), growth (b), and pigmentation (c) observed during shake flask cultivation of *P. purpurogenum* at 30 °C in MESP and marshmallow confectionery-based media, buffered to a pH of 5 using 50 mM citrate buffer, unless otherwise stated. Numbers indicate supplementation with soya peptone (SP) at concentrations of 3, 1.5 or 0.75 $g.L^{-1}$. Results shown are the average values across triplicate flasks, with error bars representing the standard deviation of the data.

The MESP medium control flasks behaved as expected in this experiment. The pH exhibited the usual trend, growth reached approximately 10 $g.L^{-1}$ and pigmentation was defined by an absorbance value of

27.5 OD units. The three marshmallow-based media supplemented with soya peptone, along with buffer application, showed similar pH trends for the first 4 days of the cultivation. Beyond this point, a higher concentration of soya peptone correlated with greater deviation from the desired pH value of 5. Marshmallow medium supplemented at 3 $g.L^{-1}$ soya peptone showed similar pH trends to those observed in the MESP medium towards the end of the cultivation but lacked the characteristic pH peak around day 2.

Marshmallow media buffered to a pH of 5 and supplemented with 3 $g.L^{-1}$ or 1.5 $g.L^{-1}$ soya peptone supported similar growth. Supplementation with 0.75 $g.L^{-1}$ soya peptone, however, resulted in a reduction in maximum biomass achieved, reaching 4.6 $g.L^{-1}$ as opposed to 6 $g.L^{-1}$. This is expected as a higher substrate concentration should support increased growth but is in contrast to trends observed during multiwell plate cultivation. When comparing 0.75 $g.L^{-1}$ SP supplementation with and without buffer addition, it is observed that introducing the buffer has a negative impact on growth, once again in contrast to multiwell plate cultivation results.

In terms of pigment production, the medium without buffer application exhibited no colour development over the 6-day cultivation period. This differs from multiwell plate results in that pigmentation was observed in unbuffered wells but shows agreement in that buffer application was shown to be beneficial for pigment production in both cultivation systems. When considering the three buffered marshmallow media, supplementation with 3 $g.L^{-1}$ soya peptone resulted in the highest volumetric pigment concentration. This was measured at approximately 15 OD units, with other concentrations resulting in pigmentation less than half that value. This result is once again in contrast to that observed in multiwell plate cultivation, where 0.75 $g.L^{-1}$ SP supplementation was shown to result in highest volumetric pigment concentrations.

As discussed in Section 6.1.3.1, differences in oxygen transfer and bulk liquid mixing between multiwell plate and shake flask systems could be responsible for the observed variation in growth and pigmentation. The good correlation observed between shake flask and BioFlo 110 reactor cultivation with other medium types considered (Figure 4.17, Figure 6.18), however, supports the selection of marshmallow + 3 $g.L^{-1}$ soya peptone, with 50 mM citrate buffer at pH 5, for further experimentation.

6.3.3 BioFlo 110 reactor cultivation on marshmallow confectionery

Application of the marshmallow confectionery-based medium was scaled up into 5 L cultivation volume using the New Brunswick Scientific BioFlo 110 bioreactor. Marshmallow was applied at 15 $g.L^{-1}$ with supplementation of 3 $g.L^{-1}$ soya peptone. System parameters were maintained as stated for the base case cultivation in this reactor system (Table 4.8) and the medium was inoculated using a *P. purpurogenum* spore solution to yield an initial concentration of $1x10^5$ $spores.mL^{-1}$ (Appendix C.2). Sampling was performed twice daily with pH, growth, pigmentation and utilisation of medium components evaluated for each time point, as described in Section 3.3.4.4. Individual sugar concentrations were evaluated by HPLC.

6.3.3.1 Overview of the BioFlo 110 reactor cultivation

Duplicate 6-day cultivations were performed in the BioFlo 110 bioreactor using the supplemented marshmallow confectionery medium. The pH, growth, pigmentation and residual sugar concentration values recorded as the cultivation of *P. purpurogenum* proceeded have been plotted in Figure 6.25.

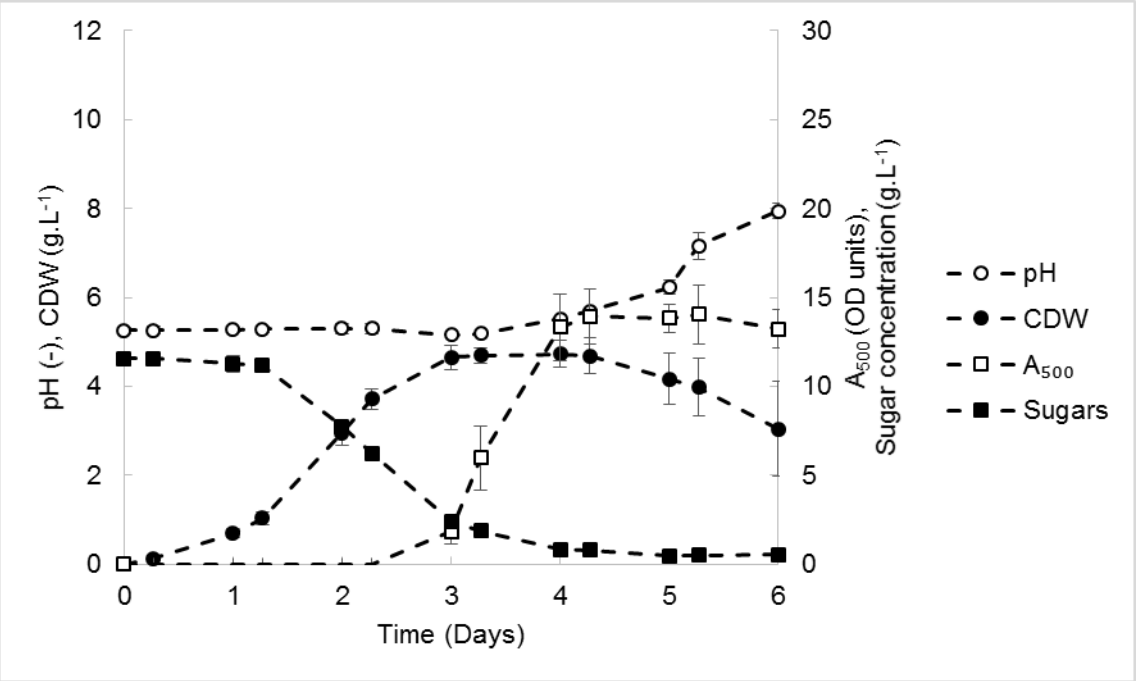


Figure 6.25 Cultivation summary of *P. purpurogenum* DSM 62866 grown in Marshmallow + 3 SP medium, pH 5 (50 mM citrate buffer), at 30 °C, with agitation of 300 to 400 rpm, and aeration of 0.5 to 1 vvm, in the BioFlo 110 modular benchtop fermentor for 6 days. Results show the average pH, growth, pigment production, and sugar concentration for triplicate measurements performed during duplicate cultivations, with error bars representing the standard deviation of the data.

The pH of the culture remained relatively stable over the first 4 days of the cultivation, during which time biomass had reached its maximum volumetric concentration and the culture had entered that stationary phase of growth. Beyond this point, when biomass concentration declined, culture pH began to rise. This is consistent with results observed in shake flasks (Figure 6.24a), as well as previous reactor cultivations with malt-extract based medium (Figure 6.14). The pH reached a maximum value of approximately 8 units by day 6 of the cultivation.

The growth trend of the culture was similar to that observed previously with malt extract-based media, with an approximately linear increase in biomass over the first 30 hours of the cultivation, defined by a low biomass productivity, followed by a period of increased, but still linear, growth rate to approximately 55 hours. Growth rate then began to slow, with a maximum biomass concentration of 4.7 $g.L^{-1}$ achieved by day 3 of the cultivation, and maintained for approximately 24 hours. The final phase of growth was defined by decreasing biomass concentration, with CDW declining to approximately 3 $g.L^{-1}$ by the end of the cultivation.

Pigmentation was first observed on day 3 of the cultivation, once growth rate had begun to decline. Extrapolating backwards using the period of approximately linear increase in pigmentation between 72 and 96 hours of cultivation, as shown in Figure 6.26, the latest time for onset of pigmentation is estimated to be 67 hours. Pigment production thus begins between 54 and 67 hours of cultivation on the marshmallow-based medium, with maximum pigmentation achieved after approximately 100 hours of cultivation defined by an absorbance value of 13.9 OD units. This was within the period of maintenance of maximum biomass concentration.

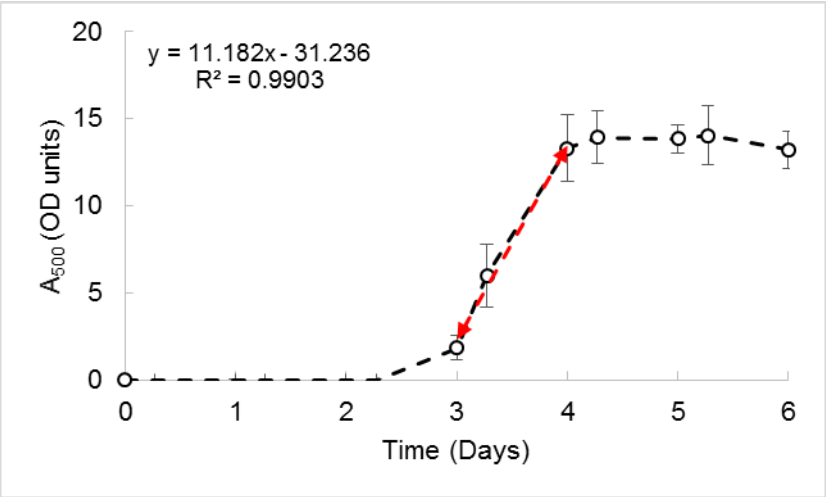


Figure 6.26 Period of linear increase in pigmentation during BioFlo 110 bioreactor cultivation of *P. purpurogenum* using Marsh. + 3 SP medium, denoted by the arrows, with the corresponding trendline equation provided.

Residual sugar concentration at each sampling point was determined using HPLC analysis. This revealed a starting concentration of approximately 11.5 $g.L^{-1}$ total sugars, with little change in the concentration over the first 30 hours of the cultivation. This was followed by a decrease from 11.2 $g.L^{-1}$ to 2.4 $g.L^{-1}$ by the 72 hour time point, which corresponds to the period of maximal growth and the onset of pigmentation. This is in contrast to the MESP and Half MESP medium cultivations, where residual sugar concentration was significantly higher when pigment production was initiated.

The residual sugar concentration continued to decline beyond this time point, with only 0.5 $g.L^{-1}$ total sugars remaining at the end of the cultivation. HPLC analysis also allowed individual sugars to be measured. The chromatogram indicating the respective retention times for sucrose, glucose and fructose has been provided in Appendix E. The utilisation trends for sucrose, glucose and fructose are provided in Figure 6.27. These results show all sugar concentrations declining simultaneously. Fructose is present at the lowest concentration and is the first sugar to be completely utilised, while sucrose is present at the highest concentration and a small amount still remains at the end of the cultivation. This indicates that *P. purpurogenum* DSM 62866 does not preferentially consume any of the sugars, utilising all measured sugars concurrently.

Previously, the inclusion of glucose in the cultivation medium resulted in inhibition of pigment production by *P. purpurogenum* DSM 62866, while in the marshmallow-based medium ability to produce red

pigmentation was retained. This could be attributed to the lower concentration of glucose in this confectionery-based medium, with other sugars also being present in higher concentrations than glucose.

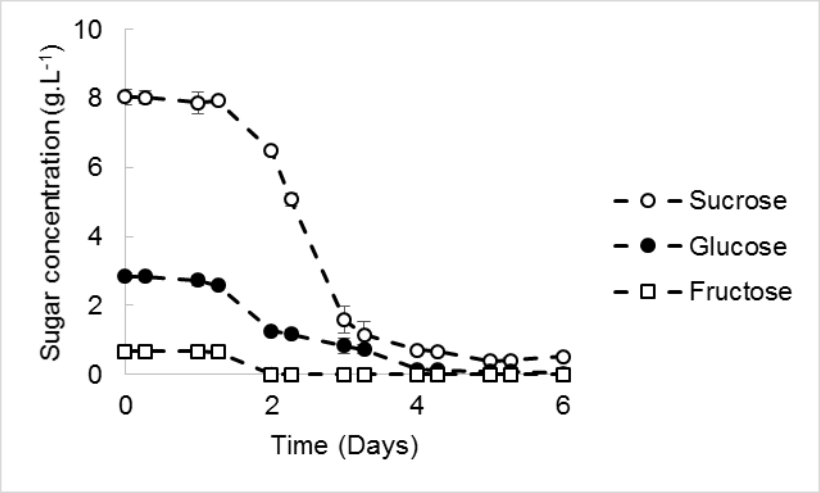


Figure 6.27 Concentration of individual sugars during BioFlo 110 cultivation of *P. purpurogenum* on marshmallow medium supplemented with 3 $g.L^{-1}$ soya peptone, pH 5, 50 mM citrate buffer at 30 °C, determined using HPLC analysis. Error bars represent the standard deviation associated with triplicate measurements across duplicate cultivations.

The growth and pigment production observed in this medium formulated using marshmallow confectionery supports the use of a confectionery-based waste stream as a potential substrate for cultivation of *P. purpurogenum*, with the aim of generating red pigment products. In order to further evaluate the suitability of this substrate, the results were compared to those obtained when using malt extract-based media.

6.3.3.2 Growth kinetics and yields

To further analyse the results and allow comparison of the marshmallow confectionery- and malt extract-based media, the cultivation was evaluated in terms of maximum specific growth rate, biomass and pigment productivity and biomass and pigment yields, as described in Section 3.4.

The growth of the organism on marshmallow confectionery exhibited two periods of approximately linear increase, as observed in the malt extract-based media. The growth rate defining each of these periods was determined using linear regression and is provided in Table 6.9. A significant change in the growth rate of *P. purpurogenum* is observed at the 30-hour sampling point. Although both periods exhibit a linear increase in biomass, the period of maximum growth is clearly defined.

Maximum specific growth rate was observed over the same period recorded for the base case MESP cultivation. A value of 0.097 ± 0.021 h^{-1} was recorded between 6 and 24 hours of cultivation (2-point), decreasing to 0.088 ± 0.021 h^{-1} when an additional sampling point was included. This, once again, indicates that maximum specific growth occurs during germination of spores and development into hyphae, beyond which growth trends are linear, as described in Section 4.3.4.

Table 6.9 Biomass productivity during different growth phases and maximum specific growth rate of *P. purpurogenum* DSM 62866 when cultivated in the BioFlo 110 bioreactor using Marshmallow + 3 SP medium, pH 5, 50 mM citrate buffer, at 30 °C.

Time (hours)	Average biomass productivity [a]		Specific growth rate [a]	
	$g.L^{-1}.h^{-1}$	R^2 [b]	h^{-1}	R^2 [b]
0-30	0.034 ± 0.004	0.985	-	-
6-24	-	-	0.097 ± 0.021	-
6-30	-	-	0.088 ± 0.021	0.992
30-54.5	0.111 ± 0.003	1	-	-

a Results presented as average ± standard deviation across duplicate cultivations
b Determined through linear regression analysis

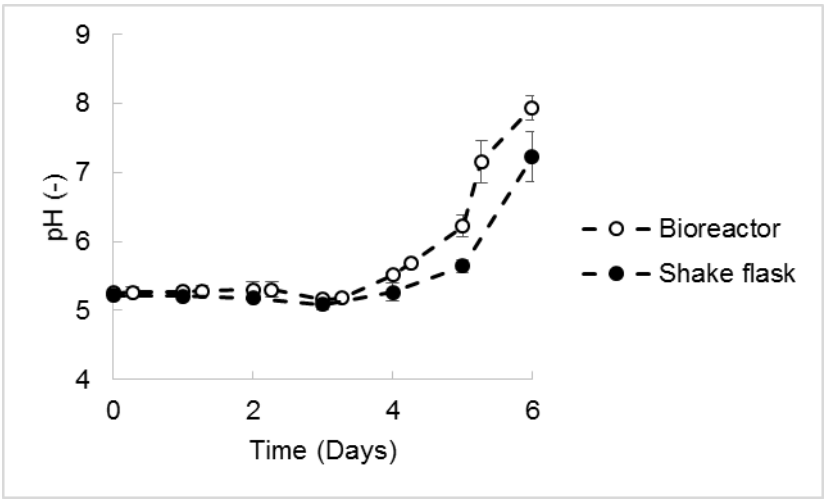
Pigment production also showed an approximate linear trend of production, between 72 and 96 hours of the cultivation, which was defined by productivity of 0.46 ± 0.05 OD units.h^{-1}. Pigment production was observed once growth rate of *P. purpurogenum* had declined, and volumetric biomass concentration was approximately stable. This is in contrast to the trends observed using malt extract-based media, where the biomass concentration continued to increase during pigment production, especially when using the MESP medium. This provides further evidence that pigment production is not growth associated.

Yield of biomass on substrate was calculated for the period of approximately 30 to 78 hours as described in Section 3.4 and was found to be 0.36 ± 0.01 $g_x.g_s^{-1}$, showing agreement with cultivations performed using malt extract-based media. For calculation of the yield of pigment on biomass, the time point of maximum recorded absorbance was considered. In the malt extract-based media, maximum absorbance was recorded along with maximum biomass achieved. In the marshmallow medium, however, these maximum values were recorded at different sampling points. The time point used for calculation of the yield was, therefore, 102.5 hours, when pigmentation had reached maximum volumetric concentration and growth was in a period of maintenance of maximum biomass achieved. The yield of pigment on biomass was calculated to be 3.01 ± 0.68 OD units.g_x^{-1}. These growth rates and yields are compared to those obtained using malt extract-based media in the BioFlo reactor system in Section 6.3.3.4.

6.3.3.3 Comparison of shake flask and bioreactor cultivations using marshmallow confectionery-based medium

The BioFlo 110 bioreactor and shake flask cultivations of *P. purpurogenum* using marshmallow confectionery-based medium were compared to allow the impact of the cultivation system and scale-up to be evaluated, as reported previously for the MESP and Half MESP medium compositions. In both cultivation systems the medium was supplemented with 3 $g.L^{-1}$ soya peptone and maintained at a pH of 5 through the application of 50 mM citrate buffer. The pH, growth and pigment production trends in the shake flask and bioreactor cultivations are provided in Figure 6.28.

a



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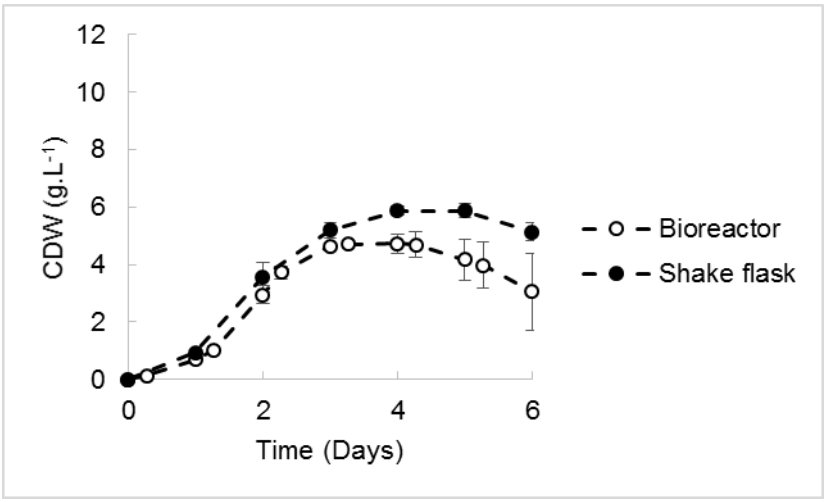

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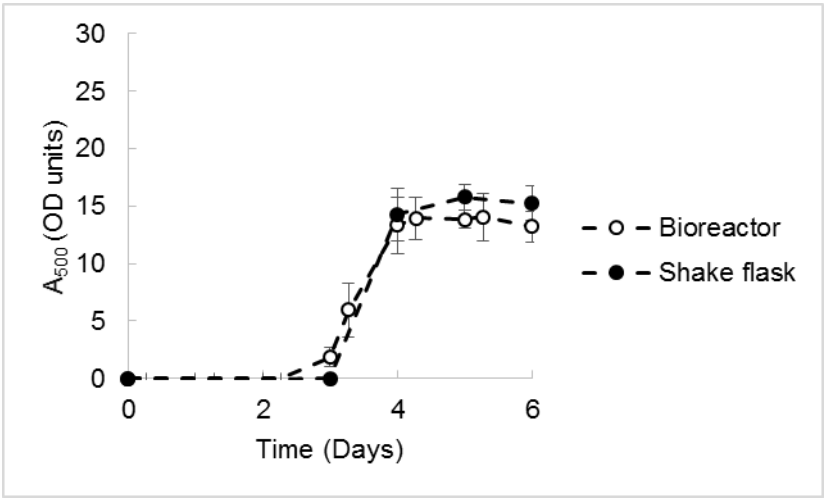


Figure 6.28 Comparison of the pH trends (a), growth (b) and pigment production (c) when cultivating *P. purpurogenum* DSM 62866 on marshmallow confectionery medium supplemented with 3 $g.L^{-1}$ soya peptone, pH 5 maintained using 50 mM citrate buffer, in the BioFlo 110 fermentor or shake flasks at 30 °C. Bioreactor data represents the average of triplicate measurements performed during duplicate cultivations, while shake flask data represents the average measurement across triplicate flasks. Error bars represent the standard deviation of the data.

Similar trends in pH, growth and pigment production were observed in the two cultivation systems over the 6-day period. This is consistent with growth on MESP and Half MESP medium, where the cultivation system had little impact on growth and pigment production of *P. purpurogenum*. The culture pH remained close to the desired value during the first four days of the cultivation. Beyond this a definite increase was observed, with the pH of the bioreactor culture beginning to rise slightly sooner and reaching a final value of approximately 7.9, in comparison to approximately 7.2 in the shake flask cultivation.

Growth in the two systems showed the same trends, with the bioreactor cultivation appearing to proceed through the various phases of growth slightly faster. Maximum CDW of approximately 4.7 $g.L^{-1}$ was achieved in the bioreactor system before the culture entered stationary phase on day 3 of the cultivation. The shake flask cultivation, however, entered stationary phase a day later, after reaching a maximum CDW of almost 5.9 $g.L^{-1}$. Decline in biomass concentration was also greater in the bioreactor culture, reaching a final CDW of 3 $g.L^{-1}$ in comparison to 5.1 $g.L^{-1}$ in the shake flasks.

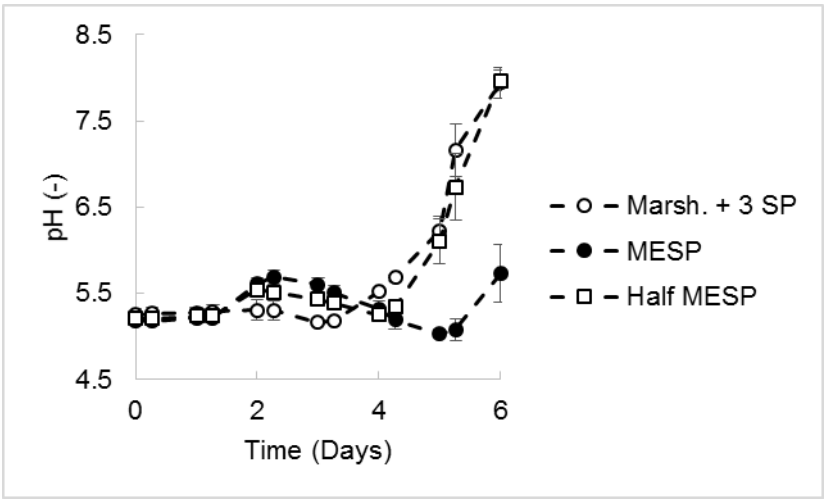
Pigmentation observed in the two systems was also similar. The highest rate of production was observed between day 3 and day 4 of the cultivation, at which point no significant difference in pigmentation existed between the two systems. Results of the t-Test, two sample assuming unequal variance are provided in Table G.4. Beyond day 4, pigmentation in the bioreactor remained relatively stable but that in the shake flasks continued to increase. This resulted in slightly higher volumetric pigment concentrations being recorded in the shake flask cultures. Pigmentation achieved was, however, significantly lower than that observed when *P. purpurogenum* DSM 62866 was cultivated on malt extract-based media, as discussed in Section 6.3.3.4.

6.3.3.4 Comparison of BioFlo 110 bioreactor cultivations using marshmallow confectionery- and malt extract-based media

The BioFlo 110 reactor cultivations using marshmallow- and malt extract-based media were compared to further evaluate the suitability of the confectionery as a substrate for growth and pigment production of *P. purpurogenum*. The pH, growth and pigment production trends recorded during these cultivations have been plotted in Figure 6.29, with important cultivation parameters provided in Table 6.10.

Over the first 3 days of the cultivation, the citrate buffer was able to maintain the pH of the Marsh. + 3 SP medium more effectively than observed previously in the malt extract-based media. This was despite the higher growth rate observed in the confectionery-based medium at this early stage of the cultivation. Beyond day 3 the culture, pH showed a rapid increase, similar to that observed in the Half MESP medium. Overall, a similar growth pattern to that achieved with Half MESP medium was observed, reaching maximum recorded CDW by day 3 of the cultivation and entering the stationary phase of growth before exhibiting a decline in biomass concentration. The consistent trend of increasing pH when biomass concentration begins to decline provides support for the observation that cell death and lysis result in an increase in culture pH (see Section 6.2.5).

a



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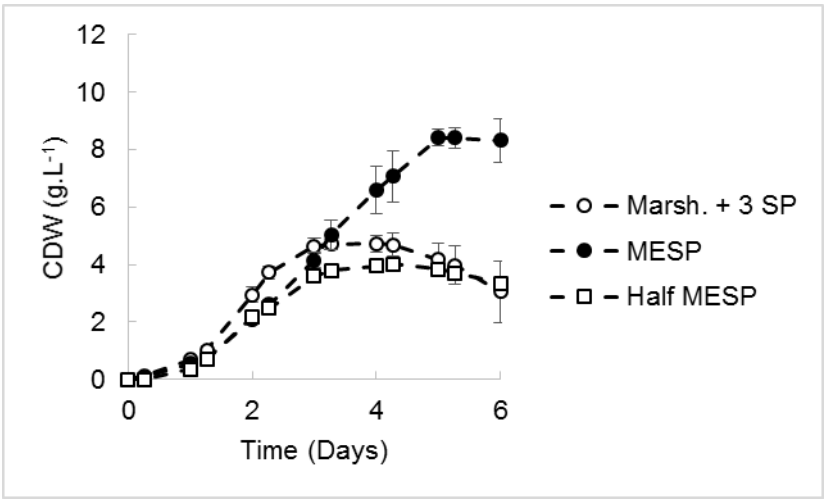

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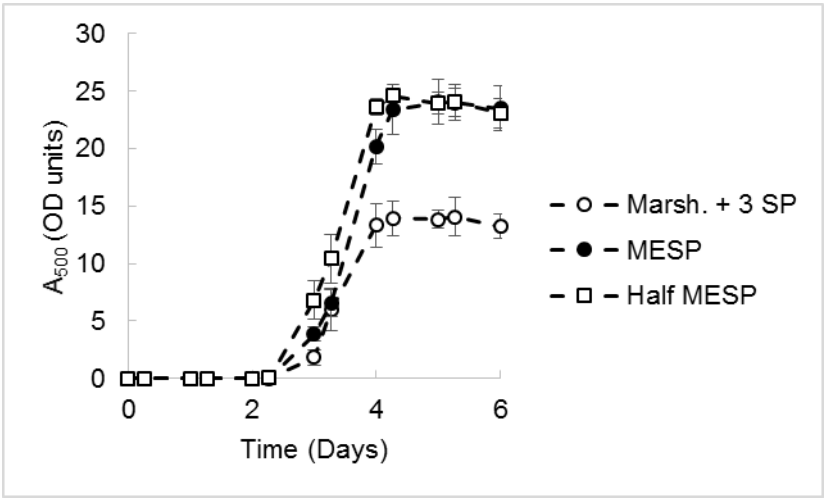

Figure 6.29 Comparison of the pH trends (a), growth (b) and pigment production (c) achieved when cultivating *P. purpurogenum* in the BioFlo 110 fermentor at 30 °C, using either marshmallow confectionery medium supplemented with 3 g.L^{-1} soya peptone, MESP or Half MESP medium, all maintained at a pH of 5 through application of a 50 mM citrate buffer. Results plotted are the average of triplicate measurements during duplicate cultivations, with error bars representing standard deviation of the data.

Table 6.10 Summary of the parameters defining the BioFlo 110 reactor cultivations of *P. purpurogenum* DSM 62866 using marshmallow confectionery- and malt extract-based cultivation media.

Parameter	Medium		
	Marsh. + 3 SP	MESP	Half MESP
Maximum CDW ($g.L^{-1}$) [a]	4.73 ± 0.29	8.42 ± 0.29	4.02 ± 0.10
Period of maximum growth (h)	30.5 – 54.5	30.5 – 120	30.5 – 54.5
Maximum biomass productivity ($g.L^{-1}.h^{-1}$) [a]	0.111 ± 0.003	0.089 ± 0.007	0.077 ± 0.006
Maximum specific growth rate (h^{-1}) [a]	0.097 ± 0.021	0.099 ± 0.010	0.104 ± 0.004
$Y_{X/S}$ ($g_X.g_S^{-1}$)	0.36 ± 0.01	0.37 ± 0.01	0.36 ± 0.04
$Y_{P/X}$ (OD units.g_X^{-1})	3.01 ± 0.68	2.86 ± 0.29	6.13 ± 0.04
Maximum A_{500} (OD units) [a]	13.94 ± 1.54	24.07 ± 1.97	24.61 ± 0.34
Period of maximum pigment production (h)	72 – 96	78.5 – 102.5	72 – 96
Maximum pigment productivity (OD units.h^{-1}) [a]	0.46 ± 0.05	0.72 ± 0.18	0.71 ± 0.11

a Results presented as average ± standard deviation across duplicate cultivations

The 2-point maximum specific growth rates recorded across the 3 medium types were similar. This is expected, given the early stage of cultivation during which these were recorded, where the initial spore concentrations applied were all the same and nutrient limitation would not yet be a contributing factor.

Maximum biomass concentration achieved did, however, vary between these medium types. Maximum concentration achieved with Marsh. + 3 SP medium was slightly higher than that obtained using Half MESP medium: approximately 4.7 $g.L^{-1}$ compared to 4 $g.L^{-1}$. Both of these values are, however, significantly lower than that observed when using MESP medium. This is expected, based on the reduced available substrate, as shown in Figure 6.30 where total sugar concentration over the cultivation period is plotted for each medium type. Yield of biomass on substrate was, however, comparable across all three medium types.

Although the Marsh. + 3 SP and Half MESP media cultivations showed similarities in terms of pH, growth and sugar utilisation trends, recorded pigmentation differed significantly. Reduced substrate availability in the marshmallow-based medium in comparison to the MESP medium resulted in lower biomass concentration as well as a reduction in volumetric pigment concentration and maximum pigment productivity. This is in contrast to the Half MESP medium cultivation, where only growth was affected by halving the malt extract concentration. This results in a yield of pigment on biomass value for the Marsh. + 3 SP medium which is approximately half that obtained in the Half MESP medium and thus similar to that of the MESP medium cultivation.

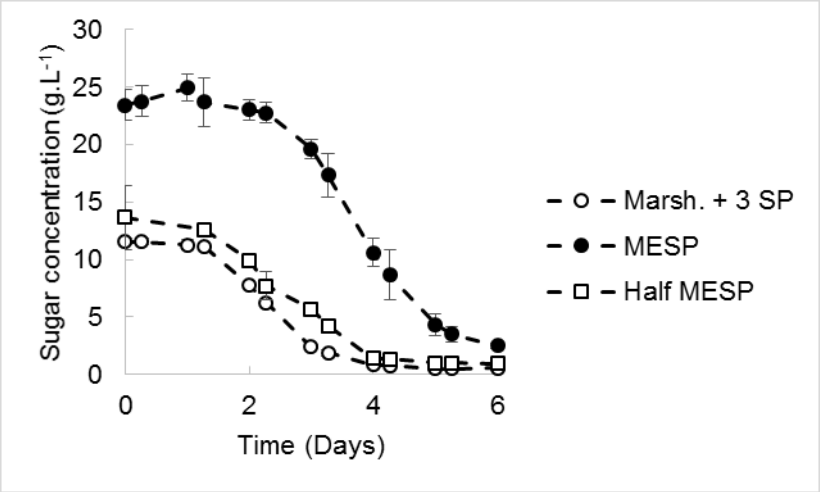

Figure 6.30 Comparison of sugar utilization trends when cultivating *P. purpurogenum* in the BioFlo 110 fermentor using either marshmallow- or malt extract-based cultivation media. Results are the average of triplicate measurements during duplicate cultivations, with error bars representing the standard deviation of the data.

When considering maximum biomass concentration and period of maximum growth across the three medium types, an association between growth and sugar concentration is observed. Growth is seen to decline as the sugars approach depletion. In all three medium types, however, period of maximum pigment production is similar, occurring between approximately day 3 and day 4. Pigment production was estimated to start between approximately 54 and 67 hours of cultivation in the Marsh. + 3 SP medium, which falls within the range observed for Half MESP (54 – 63 hours) and MESP (54 – 69 hours) media. This indicates that initiation of pigmentation is likely not a function of total substrate concentration, or even available sugar concentration. The same is likely true of cessation of pigment production, given that residual sugar concentration is high in MESP medium (approximately 10 $g.L^{-1}$) when volumetric pigment concentration stops increasing.

An overall conclusion which can be drawn from the bioreactor cultivations conducted using these three medium compositions is that while total substrate availability, or sugar concentration, is a determining factor for maximum biomass concentration, it is not so for volumetric pigment concentration. The composition of the medium plays a determining role in pigment production by *P. purpurogenum*, with the substitution of malt extract with marshmallow confectionery reducing maximum volumetric pigment concentration achieved and the yield of pigment on biomass (to approximately that observed in the base case MESP medium).

These experiments demonstrated red pigment production by *P. purpurogenum* DSM 62866 on a medium based on marshmallow confectionery. This was performed across various growth scales, with nitrogen supplementation using an organic source, namely soya peptone, shown to improve pigment production. Marshmallow confectionery has been shown to be a suitable substrate for cultivation of *P. purpurogenum*, with further supplementation studies having the potential to improve pigment productivity on this substrate and improve comparability to production observed when using malt extract-based media.

When considering the implications of this work in terms of applying this process for valorisation of a true confectionery waste stream it is important to remember that marshmallow confectionery was selected as one important confectionery component which allowed the experiments to be conducted in a consistent way.

Although marshmallow consists of a complex mix of sugars and other substances, a true confectionery waste stream would have the potential to be far more complex in terms of its composition (Harrison et al., 2019). Depending on the source or sources of the waste, it could contain a single confectionery source or a mixture of multiple confectionery types, including sugar-based, chocolate, and even starch-based confectionery. The composition of these would vary considerably from that of the marshmallow confectionery used in this study.

Successful growth and pigment production of *P. purpurogenum* DSM 62866 represents proof of concept, in a simplified system, for the potential use of confectionery waste streams to cultivate fungi for the production of pigments. Before scaling this process, a more complex system would need to be evaluated.

7 Conclusions and Recommendations

The aim of this study was to investigate red pigment production by *Penicillium purpurogenum* DSM 62866, determine the response to changes in cultivation conditions and medium composition and characterise the pigments produced. This involved cultivating the organism on a complex medium composed of malt extract and soya peptone; determining the growth and pigment production response to changes in cultivation parameters such as temperature, pH and oxygen availability and scaling up the cultivation into a 7 L stirred tank benchtop bioreactor system. Investigation of the impact of medium composition included altering the ratio of the major medium components as well as investigating the effect of replacing malt extract – the main carbohydrate source in the medium – with a sugar-rich confectionery waste stream, simulated here by marshmallow. Through investigation of these parameters, it was possible to analyse process potential through the comparison of yields and growth rates under varying conditions as well as identify aspects of the cultivation responsible for improvement of pigment production by this filamentous fungus.

7.1 Conclusions

Red pigment production by *P. purpurogenum* DSM 62866 was confirmed on a complex medium composed of malt extract and soya peptone, recommended by the culture collection from which it was obtained (DSMZ, 2014). The extracellular pigmentation was characterised by a peak in absorbance at 500 nm, with a maximum absorbance value of approximately 24 – 25 OD units recorded consistently during cultivation on malt extract-based media. The findings of this study, as they relate to the objectives presented in Section 2.5.2, are detailed below.

In respect of Objective 1, cultivation temperature and medium pH were varied in order to monitor the growth and pigment production response of *P. purpurogenum*. A cultivation temperature of 30 °C was shown to result in improved growth and pigment production during agar plate cultivation, where pigmentation was shown to be secreted into the surrounding medium. This increase in pigment production can be attributed to the influence of this cultivation temperature on overall metabolism. A culture pH of 5, achieved through the application of a citric acid monohydrate – trisodium citrate dihydrate buffer (50 mM), resulted in increased volumetric pigment concentrations in comparison to an unbuffered control medium during shake flask cultivation. The A_{500} value achieved was approximately 25 OD units, with the unbuffered medium exhibiting a value below 1 OD unit (Figure 4.5). The citrate buffer was also shown to improve reproducibility of pigment production by this organism, as reported previously (Ogihara et al., 2000b).

Overall, growth in the buffered medium was reduced, showing both a lower growth rate and final biomass concentration (Table 4.3). This was the first indication that pigment production is not growth-associated, but this was investigated further through evaluation of reactor cultivation kinetics

(Objective 5). The changes in observed pigmentation during agar plate and submerged liquid cultivation indicate that pigment production can be modulated by altering incubation temperature and culture pH.

A further aspect investigated using small-scale submerged liquid cultivation was the impact of shaking platform speed during incubation of shake flask cultures. Pigment production was found to increase with increasing shaking speed to a maximum volumetric pigment concentration defined by an A_{500} value of approximately 25 OD units. This increase in pigment production was speculated to be an effect of either oxygen availability or shear. Given the relationship observed between pigmentation and predicted k_La value at the specified shaking speeds (Figure 4.9), however, an oxygen effect appeared to be more likely. Based on the data collected and analysed, it was postulated that oxygen limitation at lower shaking speeds affected product metabolism, as presented in Table 4.6.

To address Objective 2, cultivation of the organism was scaled up into a 7 L benchtop stirred tank bioreactor using a 5 L working volume. This system offered improved control of cultivation parameters and greater scope for culture monitoring. Growth and pigment production in this system was found to be comparable to that observed during shake flask cultivation when the same conditions of temperature and pH were applied, once again obtaining culture pigmentation defined by an A_{500} value of approximately 25 OD units. Oxygen concentration was prevented from becoming limiting during the base case cultivation, with agitation rate ranging between 300 and 400 rpm to maintain the dissolved oxygen concentration at or above 35 %.

Further investigation of oxygen availability in the bioreactor system, through altering agitation rate, demonstrated that pigmentation is a function of oxygen transfer into the cultivation medium, rather than a function of residual oxygen concentration. Pigment production was shown to increase directly with oxygen transfer rate, within a specified range of k_La values (20 – 25 h^{-1}) corresponding to a maximum oxygen transfer rate of approximately 150 to 188 $mg.L^{-1}.h^{-1}$. Outside this range pigmentation is either not produced (below 20 h^{-1}), or no further increase in pigmentation is observed (above 25 h^{-1}). This direct relationship between oxygen transfer rate and pigment production has not previously been reported for this organism.

The comparable growth and pigment production observed in the shake flasks and bioreactor, when the k_La defining the systems is shown to be in the same range, supports the idea that pigmentation is a function of oxygen transfer rate rather than a response to shear. This is based on the different shear effects that would be experienced by the organism when cultivated in these two systems, under the given operating parameters. It is hypothesised that potential antioxidant properties of this fungal product are responsible for the upregulation of its production in response to increasing oxygen transfer rate into the cultivation medium.

The pigment products produced during bioreactor cultivation were subjected to solvent extraction in order to isolate products with similar properties and allow further characterisation, as stated in Objective 3. The pigmentation produced by *P. purpurogenum* was found to be combination of several red, polar pigment compounds postulated to belong to the azaphilone group of pigments. The major

red product extracted into ethyl acetate and isolated through column chromatography using methanol and dichloromethane is suggested to be an alanine derivative of the *Monascus* pigment rubropunctamine, based on similarities in terms of absorbance maxima and solubility trends as well as result of mass spectrometry analysis.

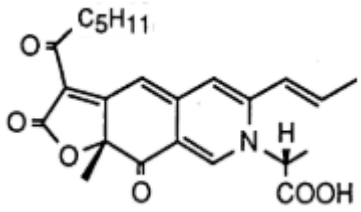

Figure 7.1 Molecular structure of the alanine derivative of rubropunctamine.

Quantification of residual sugar concentration in the medium during bioreactor cultivation revealed that substantial amounts still remain when pigment production begins. This indicates that carbon limitation does not induce pigmentation. Carbon limitation also appears to not regulate cessation of pigment production, given the high residual sugar concentration at day 4 of the MESP medium reactor cultivation, when pigment productivity declines. It was, however, noted that the onset of pigmentation coincided with sporulation. Sporulation has been linked to external factors such as nitrogen limitation or endogenous signals which induce conidiation (Roncal and Ugalde, 2003), which could be investigated further as potential triggers for initiation of pigment production.

Two main groups of experiments were undertaken to investigate the impact of medium composition on growth and pigment production, as outlined in Objective 4. The first considered the effect of altering the composition of the malt extract, soya peptone medium recommended for cultivation of *P. purpurogenum* (DSMZ, 2007, 2014), through substitution or addition of medium components, or altering the concentration ratio of the original components. One such altered medium resulted in a significant increase in specific pigment production during shake flask cultivation and was, therefore, scaled up into the benchtop bioreactor system to allow comparison to the original MESP medium. This cultivation medium contained 15 $g.L^{-1}$ malt extract and 3 $g.L^{-1}$ soya peptone (Half MESP medium). Cultivation parameters including temperature, pH, agitation speed and aeration rate were consistent with those applied during bioreactor cultivation when using the MESP medium.

Halving the malt extract supplied resulted in a reduction in biomass formation, with the maximum biomass concentration achieved being approximately half that obtained when using the MESP medium, while volumetric pigment concentration was not affected. These results are summarised in Table 7.1, and once again show that pigment production is not growth-associated. It does, however, indicate that through altering the composition of the cultivation medium, or more specifically reducing the malt extract concentration, thereby changing the ratio of malt extract (the main carbon source) to soya peptone (the main nitrogen source), an increase in specific pigment production can be achieved.

The kinetics defining the bioreactor cultivations were evaluated as stated in Objective 5, with the higher sampling frequency and monitoring of residual sugar concentration allowing the calculation of various yields and productivities, also shown in Table 7.1. Plotting the change in pigment concentration against either change in biomass concentration or average biomass concentration for the period allowed the growth-associated and biomass-associated (non-growth) specific pigment production rates, α and β, to be determined for the cultivation, based on the Luedeking-Piret model describing product formation, $dC_P/dt = \alpha.dC_X/dt + \beta.C_X$ (Luedeking and Piret, 1959). In both MESP and Half MESP medium cultivations no trend of increasing rate of pigment production with increasing growth rate was observed. A direct correlation between rate of pigment production and volumetric biomass concentration in both medium types revealed β values of 0.23 and 0.98 OD units.$g_X^{-1}.h^{-1}$ in MESP and Half MESP medium, respectively. This demonstrates that pigment production is not growth associated, but rather biomass associated, with pigmentation being produced when the culture is actively growing but showing no relationship with the growth rate of the culture. It is also noted that specific pigment production was higher in the medium with lower total substrate availability.

Biomass concentration (C_X) was shown to be directly affected by substrate availability, and specific pigment production rate (β) was elevated in response to changes in medium composition. Rather than maximising pigment production, the net effect of these changes was that equivalent volumetric pigment concentrations were achieved in both medium types. In order to maximise productivity, a means of retaining elevated specific pigment production, while also increasing the biomass concentration, needs to be elucidated. A potential route to be investigated is fed-batch cultivation of *P. purpurogenum* DSM 62866 using the Half MESP medium composition.

Table 7.1 Summary of growth and pigment production achieved during cultivation of *P. purpurogenum* DSM 62866 on MESP or Half MESP medium in the BioFlo 110 reactor.

Parameter	**Medium**	
	MESP	Half MESP
Maximum CDW ($g.L^{-1}$) [a]	8.42 ± 0.29	4.02 ± 0.10
Maximum average biomass productivity ($g.L^{-1}.h^{-1}$) [a]	0.09 ± 0.01	0.08 ± 0.01
Maximum A_{500} (OD units) [a]	24.07 ± 1.97	24.61 ± 0.34
Maximum pigment productivity (OD units.h^{-1})	0.72 ± 0.18	0.71 ± 0.11
Non-growth associated specific pigment production rate, β (OD units.$g_X^{-1}.h^{-1}$)	0.23	0.98
Specific pigment production, $Y_{P/X}$ (OD units.g_X^{-1})	2.86 ± 0.29	6.13 ± 0.04
$Y_{X/S}$ ($g_X.g_S^{-1}$)	0.37 ± 0.01	0.36 ± 0.04

a Results presented as average ± standard deviation

Even without maximising pigment production, aspects of cultivation in the Half MESP medium are advantageous when considering large-scale application. These include reduced medium cost as a result of the lower concentration of malt extract, as well as a number of implications of lower biomass concentration, such as ease of culture mixing and processing, and lower energy requirements.

The other set of experiments investigating the effect of medium composition (Objective 4) was also related to the aspect of cost reduction in large-scale processes, as well as waste reduction and resource efficiency. This set of experiments considered whether it is possible to replace specific medium components with suitable waste streams. The case study selected was a sugar-rich confectionery waste stream, simulated by marshmallow, as a replacement for malt extract. The impact of the citrate buffer on improving reproducibility of pigment production was confirmed using this substrate, and supplementation with an organic or inorganic nitrogen source was also considered.

Following agar plate and small-scale submerged liquid cultivation, supplementation of 15 $g.L^{-1}$ marshmallow with 3 $g.L^{-1}$ soya peptone, while maintaining the medium pH at a value of 5 through the application of the 50 mM citrate buffer, was selected for further investigation in the BioFlo 110 bioreactor. Soya peptone was consistently shown to be a favourable nitrogen source for pigment production, over the range of others considered. The cultivation in marshmallow-based medium showed similar pH, growth and sugar utilisation trends to those observed during cultivation in the Half MESP medium. Pigmentation was, however, defined by an A_{500} value of approximately 14 OD units, representing a decline in maximum volumetric pigment concentration of greater than 40 % when compared to malt-extract based media. Growth and pigmentation data for cultivation in the three medium types is provided in Table 7.2.

Table 7.2 Summary of growth and pigment production achieved during cultivation of *P. purpurogenum* DSM 62866 on malt extract- or marshmallow confectionery-based media in the BioFlo 110 reactor.

Parameter	**Medium**		
	MESP	Half MESP	Marsh. + 3 SP
Starting sugar concentration ($g.L^{-1}$)	23.42 ± 1.33	13.67 ± 2.80	11.57 ± 0.31
Maximum biomass concentration ($g.L^{-1}$) [a]	8.42 ± 0.29	4.02 ± 0.10	4.73 ± 0.29
Maximum A_{500} (OD units) [a]	24.07 ± 1.97	24.61 ± 0.34	13.94 ± 1.54
$Y_{P/X}$ (OD units.g_X^{-1})	2.86 ± 0.29	6.13 ± 0.04	3.01 ± 0.68

a Results presented as average ± standard deviation

This indicates that although substrate availability, or total sugar concentration, in the medium may directly affect biomass production, pigment production is influenced by additional aspects of the medium. This could potentially include the presence of trace elements in malt extract, not found in the marshmallow confectionery stream. This study did, however, demonstrate red pigment production by *P. purpurogenum* DSM 62866 when grown on a medium based on marshmallow confectionery to simulate a confectionery waste stream, with minimal supplementation. This has not been reported previously for this organism. Further supplementation studies have the potential to increase pigment yield on this substrate, while still maintaining the cost-reduction and waste remediation benefits of utilising a substrate of this nature.

The value and novelty of this study lies in the demonstration of significant red pigment production by *P. purpurogenum* DSM 62866, a strain not previously reported to produce pigmentation, with this organism therefore representing a potentially novel microbial production system for the synthesis of alternative natural colourants.

Current regulation and consumer demand are driving the move toward natural colourant alternatives in consumer products, with microbial pigments receiving increased interest as a result of ease of production through large-scale processes performed under optimised and controlled conditions. Literature provides limited information regarding aspects of these microbial pigments and their synthesis which are relevant to large-scale production. These include investigation of conditions favouring their production; the impact of scale-up; and determining the kinetics of biomass and product formation, which inform process design. Screening studies for microbial pigment producers need to be supplemented with these aspects relevant to production, while also considering pigment-specific investigations which determine application potential, such as toxicity and stability studies.

This study focused on determining whether *P. purpurogenum* DSM 62866 would have potential as a natural pigment producer; identifying conditions which improved pigment production and applying these at various growth scales to optimise production. High yields of red pigmentation were obtained when adjusting cultivation conditions and medium composition to suit the production organism. The use of a novel lost-cost substrate was also considered as a means of improving resource efficiency and process feasibility. Growth and significant pigment production were demonstrated when applying marshmallow confectionery as an alternative carbon source for cultivation of *P. purpurogenum* DSM 62866, demonstrating proof of concept for the use of sugar-rich confectionery waste streams. Another key focus area was determining process-specific data, such as growth rates and biomass and product yields, through conducting benchtop bioreactor-scale fermentations. This data addresses current knowledge gaps and has implications for process design, scale-up and feasibility assessment.

Based on the findings of this study, commercial application of pigment products of *P. purpurogenum* DSM 62866 in food, feed, cosmetic, pharmaceutical, nutraceutical or textile products has been demonstrated as technically feasible. This would require considerable toxicological studies as well as further isolation and characterisation if a single pigment product were to be required for application. It is also possible that a pigment mixture could be applied for colouring purposes if toxicity and stability studies on the colouring mixture yielded favourable results.

7.2 Recommendations

Investigations could be undertaken to further contribute to the findings of this study. The first aspect for investigation is the dependence of pigment production on oxygen transfer rate. Determination of whether the pigment products exhibit antioxidant properties could provide an explanation for the observed trend. Confirmation of the dependence of pigmentation on OTR, and not shear, could be achieved by maintaining the required oxygen transfer rate in the bioreactor system at a lower agitation rate by using an oxygen-enriched air stream.

Investigations with potential to improve volumetric pigment concentrations achieved include fed-batch cultivation and further supplementation studies in marshmallow confectionery-based media. Further investigation of the proposed link between nutrient limitation, sporulation and onset of pigment production could also provide insight into the trigger for pigmentation. This information would inform future process decisions.

Aspects relating to large-scale or industrial production of the pigment include downstream processing and formulation aspects, including stability of the final pigment product. Further isolation and characterisation methods should be investigated for these pigment products as a means to definitively identify the compounds formed under the various conditions tested. Toxicology studies of the pigment products are also required to determine the spectrum of use. This could include screening for known mycotoxins, full genome sequencing, and acute and long-term toxicity testing in animal models.

A further aspect to consider in terms of overall process design, and how it relates to the biorefinery and circular economy concepts, would be the potential for valorisation of the fungal biomass. Techno-economic feasibility studies should be undertaken and could include potential valorisation routes for the biomass, such as energy generation which could be fed back into the process.

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Appendix A Chemicals and suppliers

Table A.1 Media components and chemicals used in this study, and the corresponding suppliers.

Chemical	**Supplier**
Acetic acid (glacial, 100 %)	Merck KGaA, Darmstadt, Germany
Acetone	Merck KGaA, Darmstadt, Germany
Agar	Merck KGaA (biolab), Darmstadt, Germany
Ammonium nitrate	Merck KGaA, Darmstadt, Germany
Antifoam 204	Sigma-Aldrich, St. Louis, United States
Barium hydroxide	Sigma-Aldrich, St. Louis, United States
Butanol (1-butanol)	Merck KGaA, Darmstadt, Germany
C_{18}-reversed phase silica gel (fully endcapped)	Sigma-Aldrich, St. Louis, United States
Chloroform	Merck KGaA, Darmstadt, Germany
Citric acid monohydrate	Merck KGaA (univAR®), Darmstadt, Germany
Dichloromethane	Sigma-Aldrich, St. Louis, United States
Diethyl ether	Merck KGaA, Darmstadt, Germany
Dimethyl sulfoxide	Merck KGaA, Darmstadt, Germany
Disodium hydrogen phosphate	Merck KGaA, Darmstadt, Germany
Ethanol	Merck KGaA, Darmstadt, Germany
Ethyl acetate	Merck KGaA (uniLAB®), Darmstadt, Germany
Glucose monohydrate	Merck KGaA, Darmstadt, Germany
Malt extract	Merck KGaA (biolab), Darmstadt, Germany
Maltose monohydrate	Sigma-Aldrich, St. Louis, United States
Methanol	Kimix, Cape Town, South Africa
Peptone	Merck KGaA (biolab), Darmstadt, Germany
Peptone from soybean, enzymatic digest	Fluka Analytical (Sigma-Aldrich, St. Louis, United States)
Petroleum ether (40-60)	Merck KGaA (univAR®), Darmstadt, Germany
Potato dextrose agar	Merck KGaA (biolab), Darmstadt, Germany
Silica gel	Merck KGaA, Darmstadt, Germany
Sodium acetate	Merck KGaA (univAR®), Darmstadt, Germany
Sodium dihydrogen phosphate	Merck KGaA, Darmstadt, Germany
Soluble starch	Merck KGaA, Darmstadt, Germany
Sulphuric aicd (95-99 %)	Merck KGaA, Darmstadt, Germany
Toluene	Merck KGaA (univAR®), Darmstadt, Germany
Trisodium citrate dihydrate	EMD Millipore Corporation (Merck KGaA, Darmstadt, Germany)
Yeast extract	Merck KGaA (biolab), Darmstadt, Germany
Zinc sulphate heptahydrate	Merck KGaA (univAR®), Darmstadt, Germany

Appendix B Assays

B.1 Direct spore inoculation

Inoculation of liquid cultures with a spore solution to yield a starting spore concentration of $1x10^5$ spores.mL^{-1} was achieved as follows:

- Inoculate freshly prepared MESP agar plates with *P. purpurogenum* spores and incubate at 30 °C for 7 days
- Harvest the spores into sterile deionised water (dH_2O) or cultivation medium by pouring the liquid onto the surface of the agar and disturbing the growth surface with a flamed glass rod
- Mix the harvested spore solution well, then load 10 µL onto a Neubauer Improved counting chamber (Marienfeld)
- Count the number of spores in each of the four corner 0.2 mm x 0.2 mm blocks and one central block
- Repeat this count three times
- Use the average number of spores counted to calculate the number of spores per mL in the spore solution using the formula:

$$\text{Spore concentration (spores.mL}^{-1}) = \text{Average spore count} \times 25 \times 10^4 \qquad \text{Equation 20}$$

- Calculate the volume required for inoculation using the formula:

$$C_1V_1 = C_2V_2 \qquad \text{Equation 21}$$

where C_1 is the calculated spore concentration (spore.mL^{-1})
V_1 is the volume required for inoculation (L)
C_2 is the desired spore concentration ($1x10^5$ spores.mL^{-1}), and
V_2 is the cultivation volume (L)

- Transfer the calculated volume of spore solution into the cultivation medium, under sterile conditions, taking care not to exceed the predetermined cultivation volume
- Incubate as required.

B.2 Cell dry weight (CDW) determination

Growth of *P. purpurogenum* during liquid cultivation was evaluated by determining the cell dry weight value of a known volume of culture as described below:

- Place the 0.45 µm cellulose nitrate membrane filter (Sartorius Stedim Biotech) at 80 °C for 24 hours, before placing in a desiccator to cool and then weighing to 4 decimal places to obtain the starting weight of the filter
- Place the filter on a vacuum filter support and filter the sample volume (at least 1 mL) through the membrane filter, under vacuum
- If required for further analysis, collect the filtrate
- Wash the biomass, now on the membrane filter, with deionised water
- Transfer the filter (and biomass) back to the 80 °C oven for 24 to 48 hours, cool in a desiccator and then weigh, once dried to constant weight
- The difference in mass is attributed to the biomass and is used to calculate the biomass concentration using the formula:

$$\text{Biomass concentration } (g.L^{-1}) = \frac{\text{Final filter weight (g)-Initial filter weight (g)}}{\text{Sample volume (mL)}} \times 1000 \qquad \text{Equation 22}$$

B.3 Utilisation of sugar substrate during bioreactor cultivation

Additional monitoring of the reactor-scale cultivations of *P. purpurogenum* included measuring the residual sugar concentration at each sampling point. The calibration curve used to calculate the sugar concentration in a cultivation sample was generated using maltose (Sigma-Aldrich) solutions of varying concentrations obtained through dilution of a 2 $g.L^{-1}$ stock solution. The residual sugar concentration in assayed samples was, therefore, expressed as equivalent units of maltose ($g.L^{-1}$). This assay was performed in triplicate at each sampling point.

B.3.1 Zinc sulphate-barium hydroxide protein precipitation

Prior to performing the sugar quantification assay, the cultivation samples, a dH_2O sample, and the 2 $g.L^{-1}$ maltose stock solution were subjected to a protein precipitation step in order to prevent interference of protein components with the sugar assay results. This protein precipitation procedure was described by Somogyi (1945) as a means of deproteinising blood samples prior to determining blood sugar concentration and was performed as follows:

- Prepare the barium hydroxide (0.3 $mol.L^{-1}$) and zinc sulphate (50 $g.L^{-1}$ $ZnSO_4 \cdot 7H_2O$) solutions
- Add 0.1 mL sample and 1.5 mL dH_2O to a 2 mL microcentrifuge tube. Mix well
- Add 0.2 mL barium hydroxide solution. Mix well
- Add 0.2 mL zinc sulphate solution. Mix well. This effectively results in a 1/20 dilution of the starting sample (resulting maltose stock solution of 0.1 $g.L^{-1}$)
- Centrifuge the samples at 13000 rpm for 10 minutes
- Remove the required volume of supernatant for further analysis.

B.3.2 Sulphuric acid-UV sugar quantification

Following protein precipitation, the sugar concentration in the sample was determined using an assay based on the method described by Albalasmeh, Berhe and Ghezzehei (2013), and using maltose to generate a calibration curve. The protocol is described below:

- Dilute the protein precipitation supernatant of the maltose stock solution (0.1 $g.L^{-1}$) to yield concentrations of 0.01, 0.02, 0.03, 0.04, and 0.05 $g.L^{-1}$
- Dilute all cultivation samples such that their final absorbance lies within the range of the maltose calibration curve
- Add 0.5 mL of sample (protein precipitation supernatant of dH_2O, maltose, or cultivation sample) to a test tube
- Add 1.5 mL of concentrated sulphuric acid (95-99%, Merck) rapidly to the sample in a direct stream. The test tube will get hot
- Vortex the tube for 30 seconds then allow to cool to room temperature
- Place the hydrolysed sample in a quartz cuvette (High precision cell, Quartz SUPRACIL®, Hellma Analytics) and measure the absorbance over the range of 250 nm to 400 nm (Thermo Scientific Genesys 10S UV-vis Spectrophotometer) in order to confirm the expected peak in absorbance at 316 nm (A_{316}). Absorbance of all samples was blanked against dH_2O. Absorbance was recorded using the Visionlite software
- Plot the A_{316} values for the maltose samples against their concentration in order to generate the calibration curve
- Use the equation describing the calibration curve to calculate the sugar concentration (as maltose-equivalent units, $g.L^{-1}$) in the cultivation sample, remembering to account for the dilution of the sample.

B.4 Thin layer chromatography (TLC)

Thin layer chromatography is used for the separation of components in a solution. This method was applied in this study as a means of achieving separation of the pigment compounds present in the extract obtained after liquid-liquid extraction using water-immiscible solvents. The process is described below:

- Rinse the TLC chamber with the selected mobile phase
- Add the mobile phase to the chamber, to a depth of approximately 5 mm, close the chamber and allow to solvent vapours to saturate the chamber
- Draw the sample load line onto the TLC plate (Macherey-Nagel GmbH & Co. KG, 0.20 mm silica gel 60) in pencil, ensuring this will be above the solvent level in the chamber
- Load the sample onto the TLC plate using a spotter. Add small volumes at a time, allowing the spot to dry before adding more. This will keep the sample spot small (1-2 mm). TLC spotters can be made by pulling heated capillary tubes to create a fine point
- Allow the sample spot to dry, then place the plate into the TLC chamber
- Allow the mobile phase to move up the plate until the solvent front is approximately 5 mm from the end of the plate
- Remove the plate and mark the solvent front immediately
- Measure the distance moved by separated components and calculate the retention factor (R_f) using the formula:

$$R_f = \frac{\text{Distance from load line to component}}{\text{Distance from load line to solvent front}} \qquad \text{Equation 23}$$

- Take a photograph of the resulting separation for later comparison.

NOTE:
To improve separation and the speed of the TLC process, a complete initial run through of the plate can be performed using the selected mobile phase. This will clear the plate of any impurities prior to loading the samples. The plate must, however, be allowed to dry completely before repeating the process with the loaded samples.

Appendix C BioFlo 110 modular benchtop fermentor

C.1 Fermentor setup

The BioFlo 110 fermentor used for cultivation scale-up of *P. purpurogenum* was prepared for inoculation as described below:

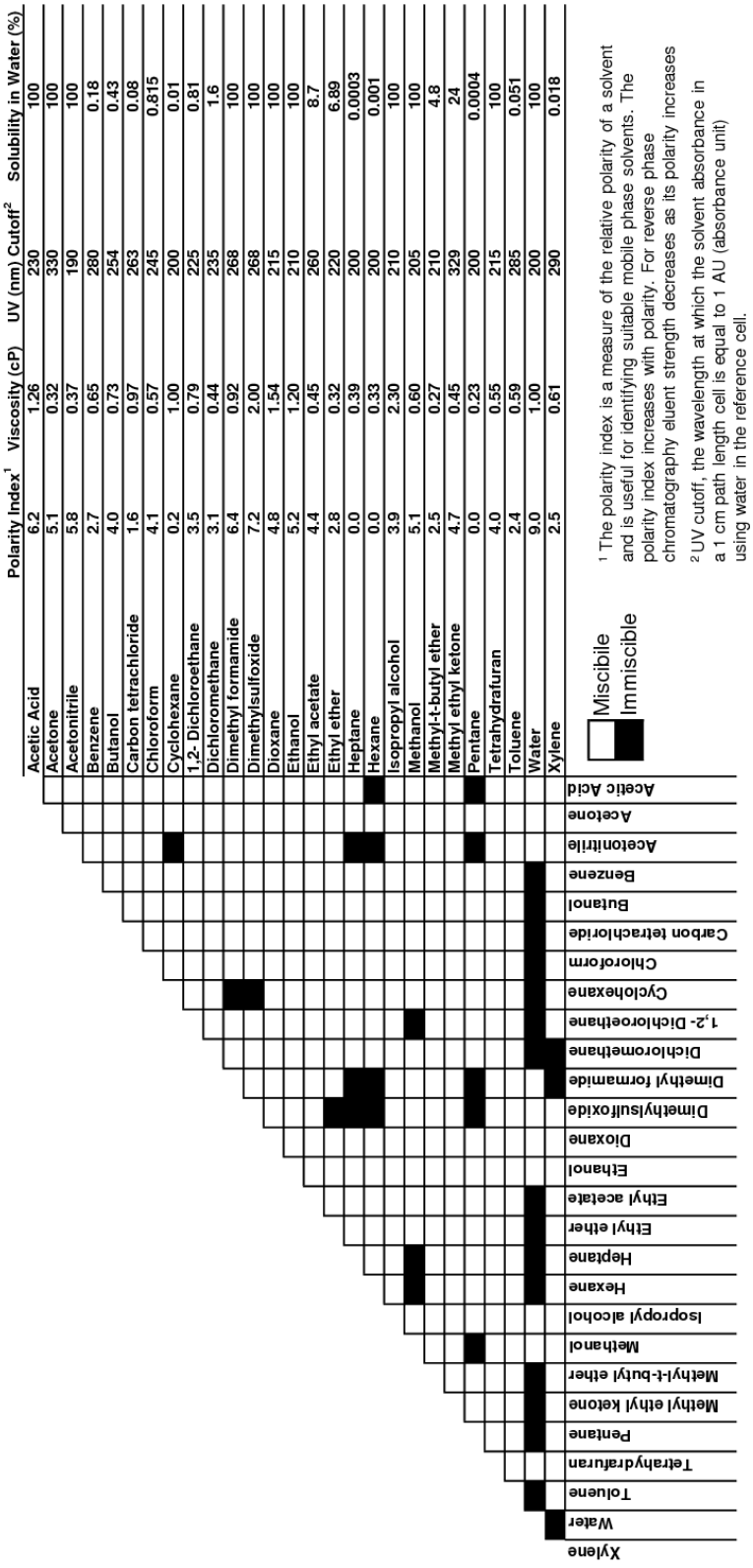
- Clean all reactor components with warm, soapy water. Rinse well
- Assemble the reactor, place the condenser in the correct port, and check that all fittings are secure
- Calibrate the pH probe using solutions of pH 7 and pH 4, using the primary control unit (PCU) to set the zero and span values, respectively
- Add 4.5 L of prepared cultivation medium into the reactor vessel followed by 1.25 mL of antifoam (Antifoam 204, Sigma-Aldrich, used at a concentration of 250 $\mu L.L^{-1}$)
- Replace the electrolyte solution in the dO_2 probe
- Insert the pH and dO_2 probes and the temperature probe thermowell housing
- Connect the sampling assembly
- Attach air filters (0.2 µm, Millipore) at the air inlet and the condenser outlet
- Clamp all air lines and sample tubes to prevent cultivation medium being forced out of the vessel during autoclaving, and cover any sensitive components with aluminium foil
- Place the entire reactor assembly into the autoclave, and sterilise at 121 °C for 20 minutes
- Remove the reactor from the autoclave and allow to cool
- Connect the required probes to the PCU, attach the air supply, connect the cooling water stream to the reactor cooling coil and the condenser, place the heating blanket around the reactor, and place the agitator motor onto the impeller shaft assembly
- Leave the dO_2 probe attached to the PCU for at least 6 hours, or overnight, to polarise
- Once polarised, calibrate the dO_2 probe by sparging with nitrogen and then air, and using the PCU to set the 0 % and 100 % level, respectively, for dissolved oxygen in the medium
- Based on predetermined cultivation conditions, set the temperature and agitation speed using the PCU, and the aeration rate using the rotameter
- Once all parameters have reached the set values, the system is ready for inoculation.

C.2 Fermentor inoculation

The fermentor was inoculated using a spore solution to yield a starting spore concentration of $1x10^5$ spores.mL^{-1}, as for other liquid cultivations, but required a slight modification.

- Follow steps 1 to 7 of direct spore inoculation (Appendix B.1)
- Measure the calculated volume of spore solution required for inoculation and place this in a 500 mL measuring cylinder
- Top up to 500 mL using sterile cultivation medium prepared previously
- Aseptically transfer the entire 500 mL volume into the reactor, bringing the cultivation volume to 5 L
- Monitor and sample the system as required.

Appendix D Solvent miscibility table



	Polarity Index[1]	Viscosity (cP)	UV (nm) Cutoff[2]	Solubility in Water (%)
Acetic Acid	6.2	1.26	230	100
Acetone	5.1	0.32	330	100
Acetonitrile	5.8	0.37	190	100
Benzene	2.7	0.65	280	0.18
Butanol	4.0	0.73	254	0.43
Carbon tetrachloride	1.6	0.97	263	0.08
Chloroform	4.1	0.57	245	0.815
Cyclohexane	0.2	1.00	200	0.01
1,2- Dichloroethane	3.5	0.79	225	0.81
Dichloromethane	3.1	0.44	235	1.6
Dimethyl formamide	6.4	0.92	268	100
Dimethylsulfoxide	7.2	2.00	268	100
Dioxane	4.8	1.54	215	100
Ethanol	5.2	1.20	210	100
Ethyl acetate	4.4	0.45	260	8.7
Ethyl ether	2.8	0.32	220	6.89
Heptane	0.0	0.39	200	0.0003
Hexane	0.0	0.33	200	0.001
Isopropyl alcohol	3.9	2.30	210	100
Methanol	5.1	0.60	205	100
Methyl-t-butyl ether	2.5	0.27	210	4.8
Methyl ethyl ketone	4.7	0.45	329	24
Pentane	0.0	0.23	200	0.0004
Tetrahydrafuran	4.0	0.55	215	100
Toluene	2.4	0.59	285	0.051
Water	9.0	1.00	200	100
Xylene	2.5	0.61	290	0.018

Miscibile
Immiscible

[1] The polarity index is a measure of the relative polarity of a solvent and is useful for identifying suitable mobile phase solvents. The polarity index increases with polarity. For reverse phase chromatography eluent strength decreases as its polarity increases

[2] UV cutoff, the wavelength at which the solvent absorbance in a 1 cm path length cell is equal to 1 AU (absorbance unit) using water in the reference cell.

Solvent Polarity Chart

Relative Polarity	Formula	Group	Solvents
Non-polar	R-H	Alkanes	Petroleum ethers, hexanes, ligroin
	Ar-H	Aromatics	Toluene
	R-O-R	Ethers	Diethyl ether
	R-X	Alkyl halides	Tricholoromethane, chloroform
	R-COOR	Esters	Ethyl acetate
	R-CO-R	Aldehydes and ketones	Acetone, MEK
	R-NH2	Amines	Pyridine, triethylamine
	R-OH	Alcohols	MeOH, EtOH, IPA, Butanol
	R-COHN2	Amides	Dimethylformamide
	R-COOH	Carboxylic Acid	Ethanoic Acid
Polar	H-O-H	Water	

Solvent Miscibility and Viscosity Chart
adapted from Paul Sadek The HPLC Solvent Guide Wiley-Interscience, 2002.

Mobile phases, stationary phase, analyte and samples must be compatible

Appendix E Pigment characterisation data

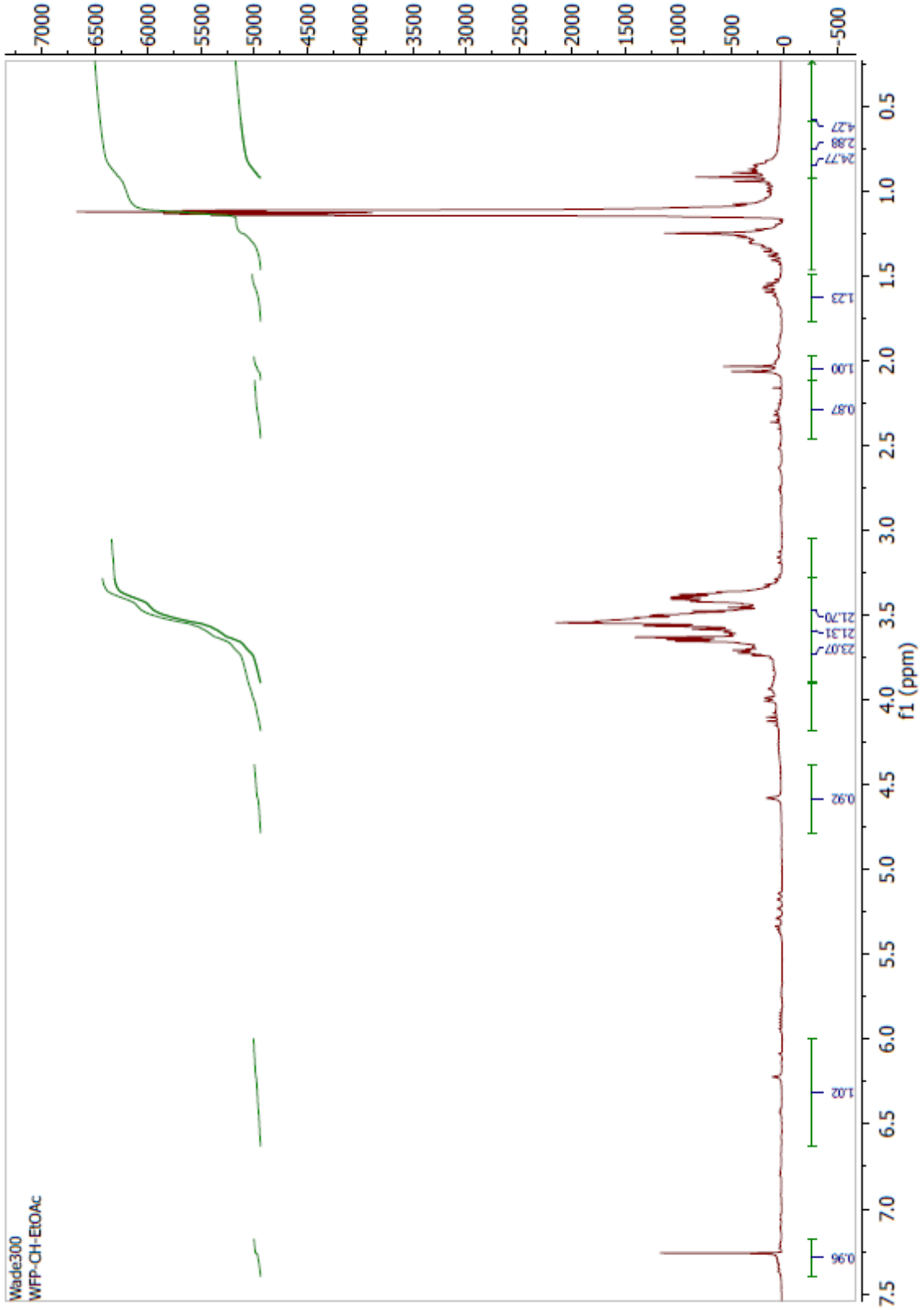


Figure E.1 Spectrum resulting from ^{1}H-NMR analysis of the crude ethyl acetate extract of *P. purpurogenum* DSM 62866 culture.

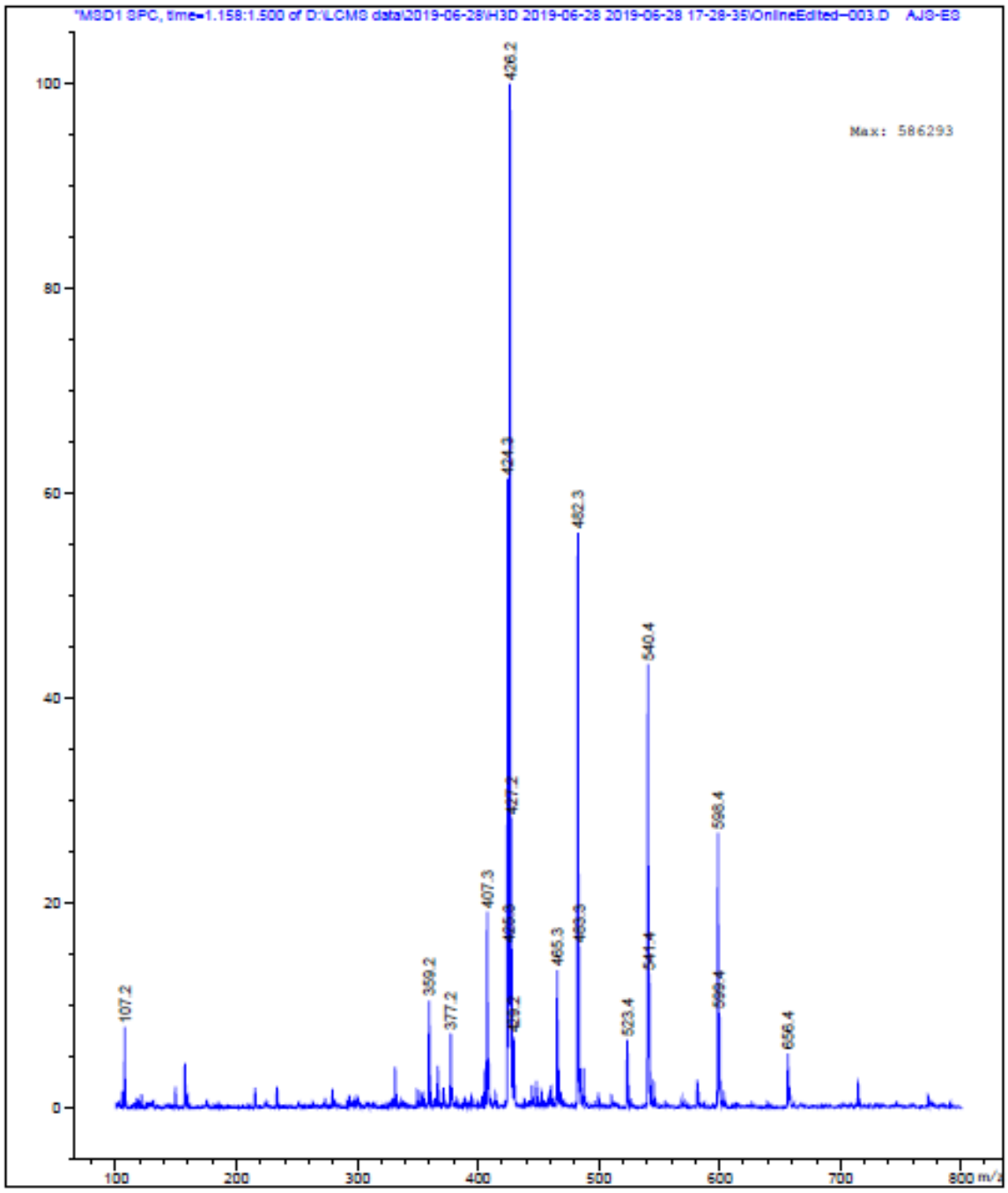


Figure E.2 Mass spectrum of red pigment product extracted from cell-free *P. purpurogenum* culture broth using ethyl acetate and isolated through column chromatography using a mobile phase of methanol and dichloromethane.

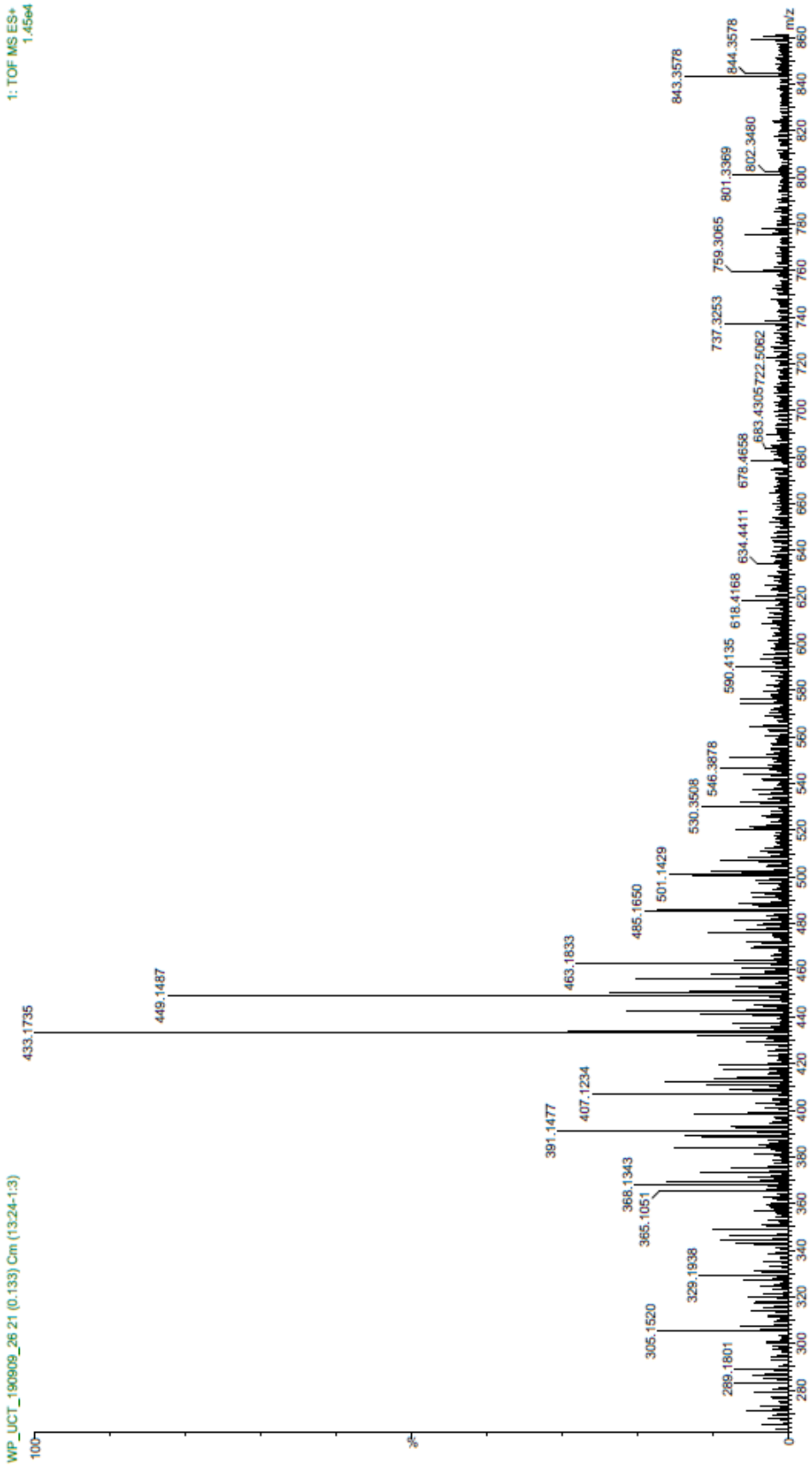


Figure E.3 Mass spectrum of red pigments remaining in the aqueous phase following ethyl acetate extraction of cell-free *P. purpurogenum* culture broth.

Appendix F HPLC

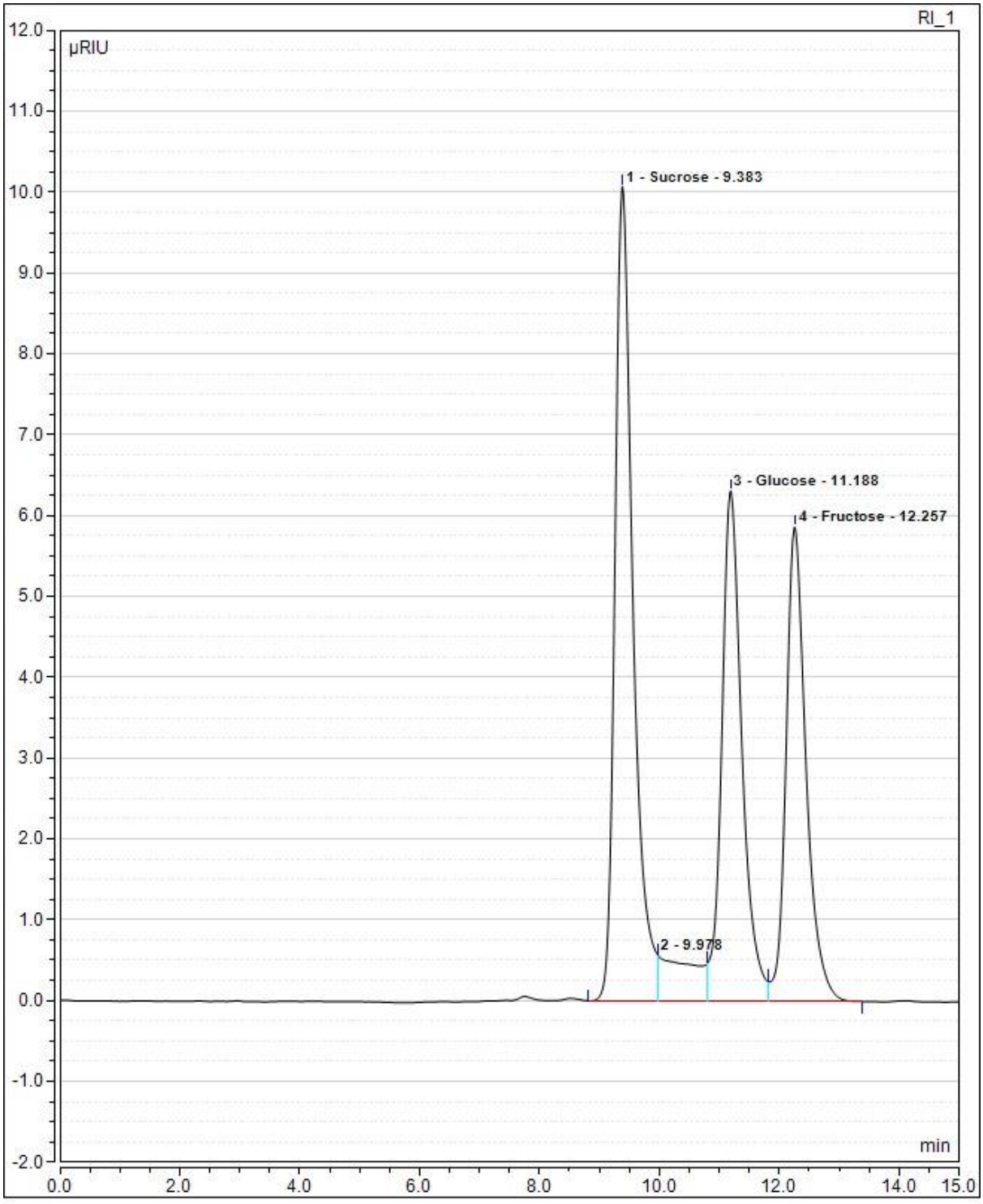


Figure F.1 Chromatogram for the sample containing 1 $g.L^{-1}$ of each of sucrose, glucose and fructose demonstrating their respective retention times.

Appendix G Statistical analyses

Table G.1 t-Test, two sample assuming unequal variance, for maximum A_{500} value achieved during cultivation of *P. purpurogenum* in MESP medium, pH 5, 50 mM citrate buffer, in 100 mL shake flask culture and 5 L bioreactor culture.

	Bioreactor	**Shake flask**
Mean	24.07	25.69
Variance	3.89	2.67
Observations	6	3
Hypothesised mean difference	0	
Degrees of freedom	5	
t_{stat}	-1.31	
$t_{critical}$, one-tail	2.02	
$t_{critical}$, two-tail	2.57	

Table G.2 Results of the t-Test, two sample assuming unequal variance, performed in Microsoft Excel for specific pigment production in Half MESP and Half MESP + Maltose media.

	Half MESP	**Half MESP + Maltose**
Mean	4.62	4.99
Variance	0.25	1.44
Observations	3	3
Hypothesised mean difference	0	
Degrees of freedom	3	
t_{stat}	-0.49	
$t_{critical}$, one-tail	2.35	
$t_{critical}$, two-tail	3.18	

Table G.3 t-Test, two sample assuming unequal variance, for A_{500} values recorded at sequential time points during cultivation of *P. purpurogenum* DSM 62866 in the BioFlo 110 fermentor using either Half MESP medium or MESP medium, pH 5, 50 mM citrate buffer, at 30 °C.

	MESP medium	**Half MESP medium**
102.5 Hours		
Mean	23.40	24.61
Variance	4.59	0.12
Observations	6	6
Hypothesised mean difference	0	
Degrees of freedom	5	
t_{stat}	-1.37	
$t_{critical}$, one-tail	2.02	
$t_{critical}$, two-tail	2.57	
120 Hours		
Mean	24.07	23.96
Variance	3.89	0.89
Observations	6	6
Hypothesised mean difference	0	
Degrees of freedom	7	
t_{stat}	0.13	
$t_{critical}$, one-tail	1.89	
$t_{critical}$, two-tail	2.36	
126.5 Hours		
Mean	23.99	24.03
Variance	2.46	1.44
Observations	6	6
Hypothesised mean difference	0	
Degrees of freedom	9	
t_{stat}	-0.04	
$t_{critical}$, one-tail	1.83	
$t_{critical}$, two-tail	2.26	
144 Hours		
Mean	23.48	23.10
Variance	3.80	1.62
Observations	6	6
Hypothesised mean difference	0	
Degrees of freedom	9	
t_{stat}	0.40	
$t_{critical}$, one-tail	1.83	
$t_{critical}$, two-tail	2.26	

Table G.4 t-Test, two sample assuming unequal variance, for A_{500} value recorded on Day 4 of the cultivation of *P. purpurogenum* in Marshmallow + 3 SP medium, pH 5, 50 mM citrate buffer, in 100 mL shake flask culture and 5 L bioreactor culture

	Bioreactor	**Shake flask**
Mean	13.32	14.27
Variance	3.54	5.33
Observations	6	3
Hypothesised mean difference	0	
Degrees of freedom	3	
tstat	0.62	
tcritical, one-tail	2.35	
tcritical, two-tail	3.18	