STUDIES ON GROWTH, MODELLING AND PIGMENT PRODUCTION BY THE YEAST *Phaffia rhodozyma*

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SUMMARY

Within the aquaculture industry a potential has been identified for the pigment astaxanthin. Astaxanthin is the carotenoid responsible for the distinctive coloration of salmonids, crustaceans and certain birds. Due to the fact that animals cannot synthesize carotenoids themselves, it is necessary for these pigments to be present in their food source. In the case of farm-raised salmonids and crustaceans, supplementation of their food with astaxanthin is required. The chemical synthesis of astaxanthin is very costly and complicated. As a result natural, microbial sources of astaxanthin are being investigated. *Phaffia rhodozyma* is the only yeast known to synthesize astaxanthin as its principle carotenoid. The aim of this dissertation is to present a study investigating the growth and pigmentation of *P. rhodozyma*, with a view to its commercial production.

A *P. rhodozyma* mutant (UCT-1N-3693) with a 50% increased total carotenoid content was selected after NTG mutagenesis of the wild strain. Shake flask assessment of this mutant revealed a total carotenoid content of 455 μg/g.

The mutant was subsequently cultivated in the laboratory in batch, continuous and fed-batch reactor configurations at a temperature of 22°C and a pH of 5.0. Carotenoid formation in *P. rhodozyma* was found to be growth-associated. During batch cultivation a final biomass concentration of 6.1 g/l, a specific growth rate of 0.11 h^{-1} and a total carotenoid content of 416 μg/g was obtained.

A drop in the biomass and carotenoid concentrations with a concurrent increase in the residual substrate concentration was observed during continuous cultivation at dilution rates greater than 0.09 h^{-1}. Increasing the oxygen concentration by supplementation of the air flow with pure oxygen during continuous cultivation at $D=0.1$ h^{-1}, did not induce carotenoid formation in *P. rhodozyma*.

The potential of molasses as an inexpensive growth substrate was successfully demonstrated during shake flask, batch and fed-batch cultivation of *P. rhodozyma*.

By means of a mass balance approach, a feed regime was established for the fed-batch cultivation of *P. rhodozyma*. Good correlation was shown between experimental data obtained during batch and fed-batch cultivation of *P. rhodozyma* and mathematical equations modelling the growth and pigment formation in *P. rhodozyma* on both glucose and molasses medium. The mathematical model was based on exponential growth, Monod substrate kinetics and growth-associated product formation.

P. rhodozyma was shown to exhibit the Crabtree effect. During fed-batch and continuous cultivation at high residual substrate concentrations yeast fermentation products such as ethanol and acetic acid were detected under fully aerobic growth conditions. The Crabtree effect was overcome during fed-batch cultivation of *P. rhodozyma* at residual substrate concentrations less than 0.5 g/l.

In conclusion, the findings of this research project indicate the potential of using a fed-batch system with molasses as a growth substrate for large scale production of *P. rhodozyma*.

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NOMENCLATURE

Abbreviations:

DO - Dissolved oxygen
EMS - Ethyl methanesulphonate
HPLC - High performance liquid chromatography
MS - Mass spectroscopy
NTG - *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
OTR - Oxygen transfer rate
OUR - Oxygen utilization rate
rpm - Revolutions per minute
TLC - Thin layer chromatography
v/v.m - Volume air flow per volume culture per minute
YM - Yeast/malt extract broth
YNB - Yeast nitrogen base broth

Symbols:

A_{480} - Absorbance at a specified wavelength
C - Dissolved oxygen concentration
C^* - Saturated dissolved oxygen concentration
D - Dilution rate
D_c - Critical dilution rate
F - Volumetric feed flow rate
k_d - Death rate constant
k_La - Mass transfer coefficient
K_s - Saturation constant for substrate
m_s - Maintenance coefficient
q_p - Specific rate of product formation
P - Product (total carotenoid) concentration
P_o - Initial product concentration (time = 0)
P^T - Total culture product
P_o^T - Initial total culture product (time = 0)
r_x - Rate of biomass formation
S - Concentration of growth limiting substrate

S_o	-	Initial concentration of growth limiting substrate
S_f	-	Concentration of growth limiting substrate in feed stream
t	-	Time
V	-	Culture volume in culture vessel
V_o	-	Initial culture volume in culture vessel
X	-	Biomass concentration
X_f	-	Biomass concentration in the feed stream
X_o	-	Initial biomass concentration (time = 0)
X^T	-	Total culture biomass
X_o^T	-	Initial total culture biomass (time = 0)
Y	-	Biomass yield (biomass formed/substrate consumed)
Y^m	-	Maximum biomass yield (no endogenous metabolism)
$Y_{(P/S)}$	-	Product yield (product formed/substrate consumed)
$Y_{(P/X)}$	-	Product yield (product formed/biomass formed)
μ	-	Specific growth rate
μ_A	-	Apparent specific growth rate
μ_{max}	-	Maximum specific growth rate
μ_T	-	Total specific growth rate

CHAPTER 1

INTRODUCTION

Astaxanthin (3,3'-dihydroxy-ß,ß,-carotene-4,4'-dione) is the principal carotenoid pigment responsible for the distinctive orange-red pigmentation of marine invertebrates (lobsters, crabs and shrimps), fish (salmon and trout) and birds (flamingoes) (Johnson *et al.* 1977; Christophersen *et al.* 1989).

Consumer acceptance of cultivated salmonids and crustaceans is affected by the colour of their flesh (Ostrander *et al.* 1976). Since animals are unable to synthesize carotenoids themselves (Goodwin 1976), it is necessary for these pigments to be present in their food sources. While this is usually supplied through algae and crustaceans in the natural habitats (Christophersen *et al.* 1989), astaxanthin supplementation of feeds is required for salmonids raised in fish farms. This is accomplished with considerable cost to the producer, in the region of 10% to 15% of total feed costs (Torrissen *et al.* 1989). The world-wide production of farm raised salmon increased substantially during the 1980s and had already exceeded 200 000 t.p.a. in 1990 (Johnson and An 1991). Based on these production rates, it is estimated that in excess of 15 000 kg per annum of carotenoid pigments will be used as feed additives for farmed salmonids in the 1990s, with a possible 100 000 kg per annum being required by the year 2000 (Johnson and An 1991). This means that the demand for a cheaper and more effective source of astaxanthin will increase significantly before the turn of the century.

The chemical synthesis of astaxanthin is very complex and costly due to the presence of chiral centers in its molecular structure. Considerable interest is being generated within the aquaculture industry for cheaper, natural and microbial sources of astaxanthin (Johnson and An 1991). *Phaffia* is the only genus of yeast known to synthesize astaxanthin as its principle carotenoid. The aim of this research project was to study the production of astaxanthin by *Phaffia rhodozyma* with a view to its commercial production by local industry.

Both the protein and the astaxanthin content of *P. rhodozyma* are important when considering the possible use of the yeast as an animal feed. A compromise may therefore be required between *P. rhodozyma* strains with a high carotenoid content and a high growth rate. Another important consideration for the industrial production of astaxanthin by *P. rhodozyma* is

the utilization of an inexpensive growth substrate (Johnson and An 1991). As a consequence, the growth and pigment formation of *P. rhodozyma* on molasses medium is extensively reported and discussed in this dissertation.

Mathematical models for a given biochemical process are essential tools for the design and scale up of that process. By means of an unstructured and nonsegregated approach, mathematical equations predicting both the biomass and product formation of *P. rhodozyma* are presented in this dissertation.

A literature review, discussing the potential of *P. rhodozyma* as a source of astaxanthin and as a nutrient in fish feeds, is provided in Chapter 2. Yeast strain improvement methods for obtaining higher levels of pigmentation in *P. rhodozyma* wild stains and the general considerations for the production of astaxanthin by *P. rhodozyma*, including downstream processing requirements, are also discussed. Finally, analytical methods for the quantification of carotenoids present in *P. rhodozyma* are introduced.

A strain improvement programme was initiated in order to select for an improved astaxanthin producer by mutagenesis of the *P. rhodozyma* wild strain. Evaluation of the *P. rhodozyma* mutants selected, alongside preliminary experimental work aimed at identifying the optimal culture conditions for *P. rhodozyma* growth and pigmentation during shake flask cultivation, is reported and discussed in Chapter 3.

In addition to the nutritional and environmental considerations for the possible large-scale production of astaxanthin from *P. rhodozyma*, thought must also be given to the choice of reactor configuration. Knowledge of the kinetic parameters for the growth and pigmentation of *P. rhodozyma* is also essential. Chapter 4 reports and discusses the results obtained during batch and continuous cultivation of *P. rhodozyma* in a laboratory scale bio-reactor.

A batch reactor with substrate feeds (fed-batch system) is commonly employed for the commercial production of yeasts (Bailey and Ollis 1986; Schuler and Kargi 1992). Using a mass balance approach and the kinetic data obtained during chemostat studies (Chapter 4), a mathematical model predicting the final biomass and pigment levels during the fed-batch

cultivation of *P. rhodozyma* was generated. The applicability of this model to pertinent culture conditions is discussed in Chapter 5.

The phenomena known as the Crabtree effect has been reported in a number of yeasts (De Deken 1965; Wöhrer *et al.* 1981; Berry 1989). The Crabtree effect occurs as a result of substrate inhibition or catabolite repression. The Crabtree effect results in low cell yields and the formation of fermentation products (*eg.* ethanol and acetic acid), due to substrate metabolism proceeding mainly via aerobic fermentation and not via respiration. As a consequence, the Crabtree effect should be avoided when the primary objective is yeast biomass production. The Crabtree effect is briefly introduced in Chapter 3, when discussing the growth of *P. rhodozyma* on medium containing high sugar concentrations. A detailed investigation for the presence of the Crabtree effect in *P. rhodozyma* is discussed and reported in Chapter 6.

Chapter 7 concludes this dissertation by presenting the findings of the research project.

CHAPTER 2

LITERATURE REVIEW

2.1. INTRODUCTION

2.1.1. Astaxanthin, a member of the carotenoids

At least 600 different naturally occurring carotenoids have been studied and characterized (Britton 1985). Carotenoids are a class of hydrocarbons with a basic C_{40} structure. The polyene skeleton is either acyclic or terminated by one or two cyclic end groups. A series of conjugated double bonds in the carotenoid structure results in their yellow to red colours. Two main groups of carotenoids can be described (Lea 1988), "carotenes" which are essentially hydrocarbon in nature, and xanthophylls which are oxygenated derivatives of carotenes containing a polar end group (Britton 1985).

Astaxanthin (Fig. 2.1) is a member of the xanthophylls. Isolated astaxanthin, in its crystalline form, has the appearance of a fine, dark violet-brown powder with a melting point of approximately 224°C (Davis 1976).

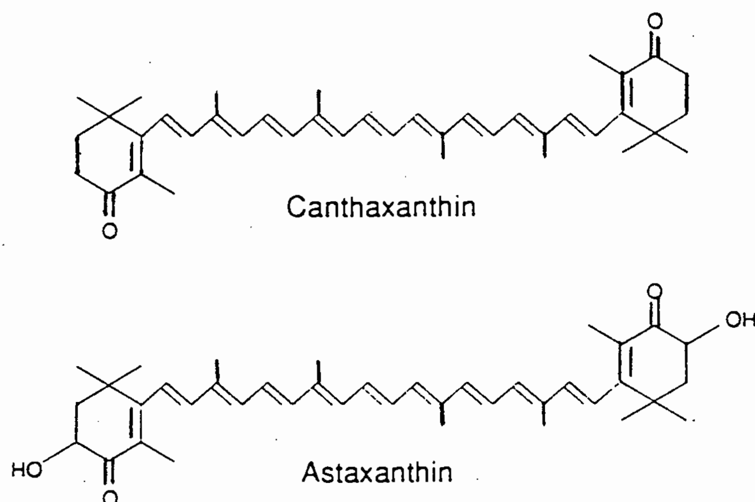


FIGURE 2.1. Chemical structures of astaxanthin and canthaxanthin (Johnson and An 1991).

A chiral center contained within the structure of astaxanthin allows for three optical isomers (Johnson and An 1991). These are the *cis-* (3S,3'S), *trans-* (3R,3'R) and *meso*-astaxanthin (3R,3'S or 3S,3'R) isomers (Fig. 2.2).

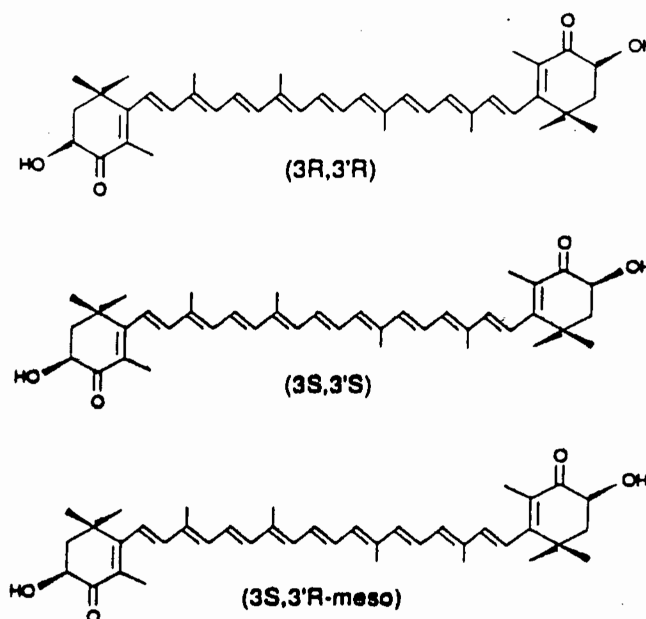


FIGURE 2.2. Structure of configurational isomers of astaxanthin (Johnson and An 1991).

2.1.2. Synthetic sources of astaxanthin

At present synthetic sources of astaxanthin dominate the market. Pigmentation of salmon flesh has been accomplished using the synthetic carotenoid canthaxanthin (ß,ß-carotene-4,4'-dione), which is structurally closely related to astaxanthin (Fig. 2.1), as a feed additive (Schmidt and Baker 1969). Canthaxanthin has been manufactured by Hoffmann - La Roche, Basel, Switzerland since 1964. The commercial price for canthaxanthin in 1990 was approximately $1200/kg (Johnson and An 1991). Synthetic astaxanthin, also manufactured by Roche, has also been successfully used as an additive to fish feeds. In salmon feeds, synthetic astaxanthin is preferred over canthaxanthin as a pigmenter due to the fact that it is more efficiently absorbed by the fish and gives the fish a more

natural colour (Torrissen *et al.* 1989). Chemically synthesized astaxanthin sells for $2000 to $2500/kg (dry weight basis). The product takes the form of stabilized beadlets containing 5% astaxanthin (Johnson and An 1991).

2.1.3. Natural and microbial sources of astaxanthin

Torrissen *et al.* (1989) reviewed the investigation of the use of crustaceans and crustacean by-products as pigment sources. Addition of shrimp waste to fish diets has traditionally been used as a pigment source in Norway (Hildingstam 1976). However crustacean shells are low in protein, and extensive processing is required in order to improve their nutritional quality. Their inclusion in fish diets as a pigment source has limited potential (Torrissen *et al.* 1989).

Astaxanthin is also known to be synthesized by various bacteria and fungi (excluding yeast). Commercial astaxanthin production by these organisms has not been considered due to their low carotenoid levels and slow growth rates (Johnson and An 1991). In contrast certain algae accumulating very high levels of astaxanthin (up to 2% on a dry weight basis) have been found (Johnson and An 1991). A good example of such an algae is *Haematococcus pluvialis* (Goodwin and Jamikorn 1954). Unfortunately the astaxanthin produced by algae may not be suitable for incorporation into animal feeds, due to the fact that most of the astaxanthin occurs in an esterified form, with the result that deposition and metabolism could be affected in certain animals (Johnson and An 1991). *Phaffia rhodozyma* is the only yeast known to synthesize astaxanthin as its principal carotenoid. It has already created considerable interest within the aquaculture industry as a potential cheap source of astaxanthin. Initial animal feeding studies have shown the potential of *Phaffia rhodozyma* as a supplement in salmonids and trout (Johnson 1977; Johnson *et al.* 1980; Gentles and Haard 1991). Various investigations have also been completed in order to study astaxanthin formation in *Phaffia rhodozyma*, with a view to optimizing its pigment production (Johnson and Lewis 1979; An *et al.* 1989; An and Johnson 1990; Haard 1988; Martin *et al.* 1993; Meyer and Du Preez 1993; Fang and Cheng 1993; Meyer and Du Preez 1994).

2.2. MICROBIAL PRODUCTION OF ASTAXANTHIN BY THE YEAST *Phaffia rhodozyma*

2.2.1. The Microorganism

Phaffia rhodozyma, a red yeast, was first described by Miller and co-workers (Miller *et al.* 1976). The yeast was isolated from exudates of deciduous trees in mountainous regions of Japan and Alaska (Phaff *et al.* 1972) and was originally named *"Rhodozyma montanae"*. However a Latin diagnosis is required by the International Code of Botanical Nomenclature. In recognition of the work of Herman J. Phaff, the genus of the yeast was changed to *Phaffia* (Miller *et al.* 1976). The genus *Phaffia* possesses only one species, *Phaffia rhodozyma*.

Phaffia rhodozyma exist only as vegetative cells, with a multilayered cell wall. The cells are ellipsoidal in shape. The cell size varies from 3.8 to 7.5 μm by 5.5 to 10.5 μm, and the cells occur singly, in pairs, or occasionally in short chains. (Miller *et al.* 1976) There are two distinct characteristics of *P. rhodozyma* that make it unique when compared to other carotenoid producing yeasts. These are the ability of the yeast to aerobically metabolise sugars and the fact that its principal carotenoid (85%) is astaxanthin (Miller *et al.* 1976; Andrewes *et al.* 1976).

2.2.2 Astaxanthin biosynthetic pathway

Numerous studies have been completed regarding the chemistry of carotenoids (Moss and Weedon 1976; Andrewes and Starr 1976). However, in contrast, very little work has been done concerning the actual biosynthesis of carotenoids, specifically the xanthophylls. Most naturally occurring carotenoids are tetraterpenes (Goodwin 1976). The starting point of tetraterpenoid biosynthesis is the mevalonate pathway (Britton 1976; Johnson and An 1991). The pathway begins at acetyl CoA, the condensation product of coenzyme A and acetic acid (Fig. 2.3).

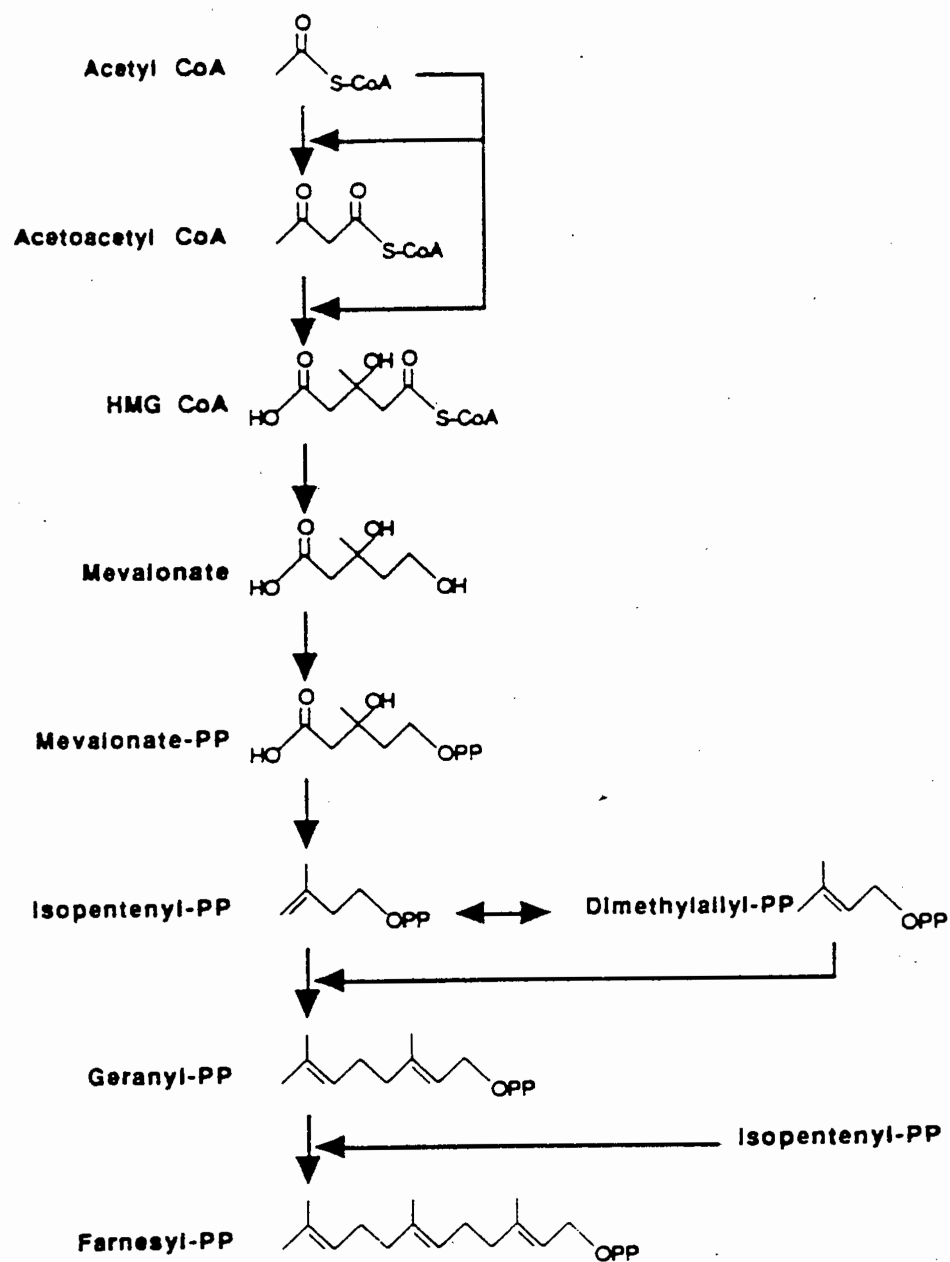


FIGURE 2.3. The Mevolonate pathway to farnesyl-pyrophosphate (Johnson and An 1991).

Two acetyl CoA molecules react to form acetoacetyl CoA, which then reacts with a third acetyl CoA molecule in the presence of the enzyme HMG-CoA synthetase, to form the compound 3-hydroxy-3-methylglutaryl coenzyme A

(HMG-CoA). Mevalonic acid (MVA) is formed by the conversion of HMG-CoA in a two-step reaction. By means of an enzyme catalyzed reaction, MVA is converted to mevalonate-5-pyrophosphate (MVAPP). In the presence of ATP, MVAPP is converted into the important C_5 intermediate isopentyl pyrophosphate (IPP). IPP is the fundamental C_5 biosynthetic unit from which all carotenoids are formed (Britton 1976).

Before chain elongation can begin, isomeration of IPP into dimethylallyl pyrophosphate (DMAPP) is required. DMAPP then condenses with a molecule of IPP to form a C_{10} compound geranyl pyrophosphate (GPP). Reaction with two more IPP molecules successively produces farnesyl pyrophosphate (FPP, the C_{15} precursor of tri-terpenes), and geranyl geranyl pyrophosphate (GGPP, the C_{20} precursor to tetraterpenes) (Britton 1976).

The final stage in the pathway before the actual carotenoids are formed is the formation of phytoene (Fig. 2.4). The intermediate between GGPP and phytoene is prephytoene pyrophosphate (prephytoene-PP). Prephytoene-PP is formed from two GGPP molecules in the absence of NADPH. A hydrogen atom is lost from each of the GGPP molecules in the process (Britton 1976). Prephytoene-PP is then converted into phytoene. The mechanism for this reaction takes into account the stereo-chemistry of the hydrogen loss (Britton 1976).

From phytoene, the formation of all carotenoids is based on a standard logical sequence of biosynthetic events, namely: desaturation, cyclization and oxygenation (Johnson and An 1991). Desaturation involves the conversion of the saturated phytoene precursor into various unsaturated carotenes. The sequence involves pairs of hydrogen atoms being removed from the two sides of the phytoene molecule alternately in order to successively produce the carotenes phytofluene, ζ-carotene, neurosporene and finally lycopene (Johnson and An 1991). Lycopene is the acyclic precursor of the cyclic carotenoids (Britton 1976). By means of two cyclization reactions, lycopene is converted to ß-carotene via the intermediate carotene, γ-carotene. Oxygenation then occurs in a step wise process in order to produce the final carotenoid pigment.

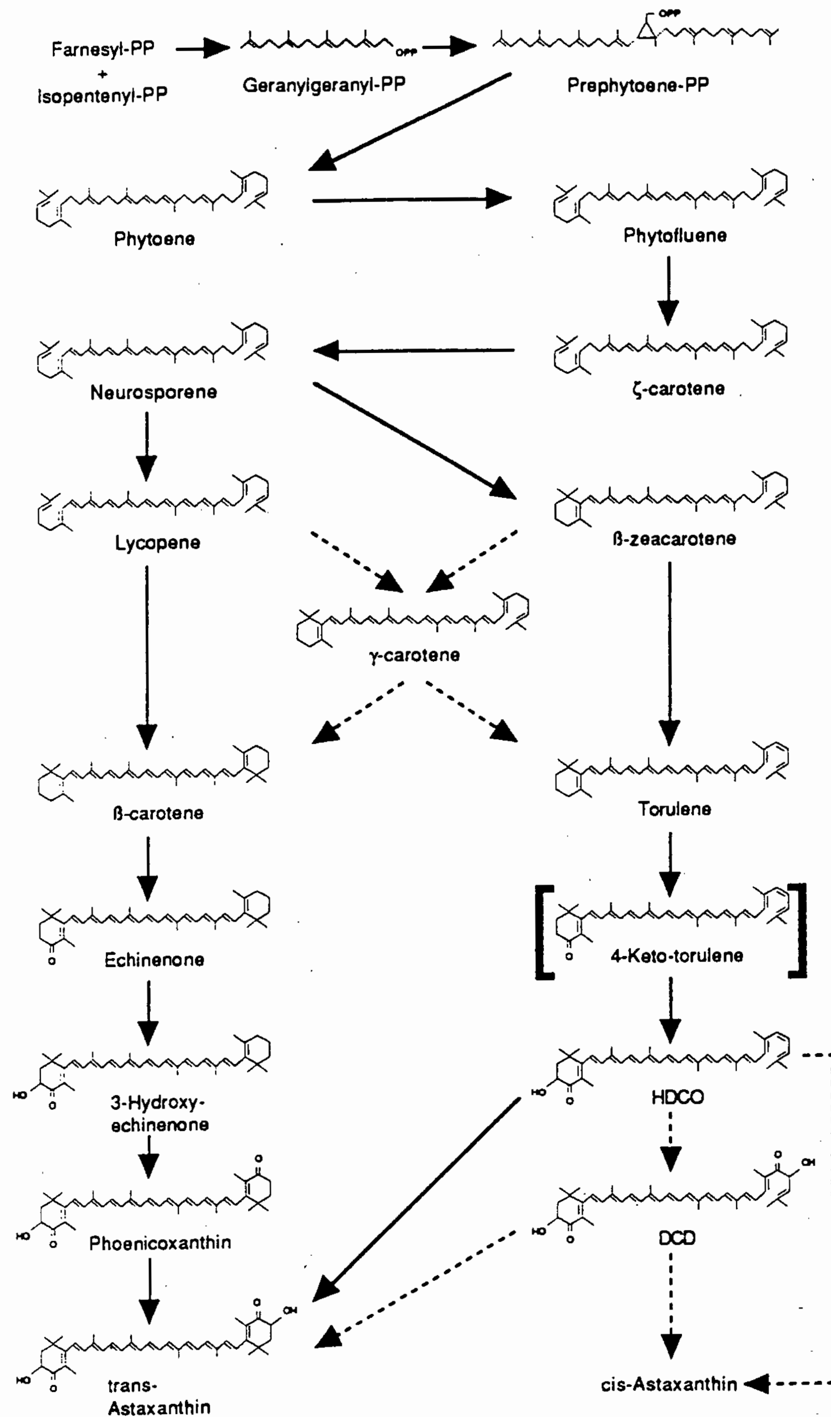


FIGURE 2.4. The two astaxanthin biosynthetic pathways proposed for *Phaffia rhodozyma* (Johnson and An 1991).

Biosynthetic pathways for the formation of astaxanthin by *P. rhodozyma* have not yet been established accurately (Johnson and An 1991). The pathways proposed above have been put forward based on the isolation and chemical identification of various carotenoids in the yeast. The first study investigating the carotenoids produced by *P. rhodozyma* was carried out by Andrewes and collaborators (1976). They suggested a pathway for astaxanthin formation beginning at neurosporene. Neurosporene is converted to ß-carotene through a well known series of reactions. ß-Carotene is, in turn, converted directly to echinenone. A specific enzymatic reaction converts echinenone to 3-hydroxy-echinenone. Phoenicoxanthin is then formed by a direct keto-insertion reaction on 3-hydroxy-echinenone. Astaxanthin formation is the final step in the pathway. This occurs by hydroxylation at the C-3' site of phoenicoxanthin (Andrewes *et al.* 1976).

This biosynthetic pathway as suggested by Andrewes (1976) differs from previous ones proposed for astaxanthin biosynthesis in crustaceans and birds. This is mainly due to the fact that certain intermediates, such as canthaxanthin and isocryptoxanthin, were not isolated during their study of carotenoid formation in *P. rhodozyma* (Andrewes *et al.* 1976; Andrewes and Starr 1976). It was therefore assumed that these carotenoids do not necessarily form part of the astaxanthin biosynthetic pathway in *P. rhodozyma*.

A new carotenoid 3-hydroxy-3'-4'-didehydro-ß-Ψ-carotene-4-one (HDCO) was detected in *P. rhodozyma* by Andrewes *et al.* (1976). However, its biosynthesis could not be explained in their proposed pathway. Furthermore, in a study by Johnson and Lewis (1979), the carotenoid ß-zeacarotene was also detected and a later study by An et al. (1989) disclosed the carotenoids 3-3'-dihydroxy-ß-γ-carotene-4,4'-dione (DCD) and torulene in *P. rhodozyma*. Isolation of these previously undetected carotenoids allowed Johnson and An (1991) to propose an alternative pathway for astaxanthin formation in *P. rhodozyma* (namely, ß-zeacarotene → torulene → 4-keto-torulene → HDCO → astaxanthin (Fig. 2.4). An *et al.* (1989) detected both ß-carotene and torulene in *Phaffia* mutants, allowing Johnson and An (1991) to suggest the

possibility of two biosynthetic pathways for astaxanthin formation being present in *P. rhodozyma*. The one has a bi-cyclic precursor (Andrewes *et al.* 1976) and the other a mono-cyclic precursor (Johnson and An 1991). Much more work is required in order to define the complex pathway of astaxanthin formation in *P. rhodozyma* comprehensively (Johnson and An 1991).

Due to the fact that monoterpenes and astaxanthin arise biosynthetically through the condensation of isoprene units (Johnson and An 1991), a study was conducted by Meyer *et al.* (1994a) in order to investigate the effect of monoterpenes on astaxanthin production by *P. rhodozyma*. Meyer *et al.* (1994a) concluded that the addition of acyclic monoterpenes to *P. rhodozyma* growth medium, at a concentration above 100 μl/l, resulted in growth inhibition of the yeast. However, Meyer and co-workers (1994a) also found that the total pigment and astaxanthin content of *P. rhodozyma* did increase with the addition of 500 μl/l of α-pinene (a di-cyclic monoterpene). Meyer *et al.* (1994a) were unable to ascertain whether α-pinene had a stimulatory effect on astaxanthin production in *P. rhodozyma* or whether it was actually incorporated into the astaxanthin biosynthetic pathway. Further investigation into this effect would be required (Meyer *et al.* 1994a).

2.2.3. Strain improvement

It is very rare to use wild strains of a microorganism isolated directly from nature in commercial fermentation processes. In general the metabolite concentrations produced by wild strains are too low for direct and economical industrial processing (Crueger 1989). Industrial astaxanthin production by *P. rhodozyma* is limited by the unavailability of stable astaxanthin hyperproducing strains and the difficulty of maintaining a selection pressure in the process. Natural isolates of *P. rhodozyma* contain 200-300 μg astaxanthin per g yeast (An *et al.* 1989). In order to improve the prospects for industrial astaxanthin production by *P. rhodozyma*, strain development of the yeast is essential (An *et al.* 1989; Johnson and An 1991). It is possible for genetic improvement of asexual yeasts, such as *P. rhodozyma*, to be accomplished by three different approaches, namely

mutagenesis, recombination of mutants and gene cloning (Johnson and An 1991).

Mutagenesis of *P. rhodozyma* wild strains was carried out by An *et al.* (1989), Fang and Cheng (1993), Meyer *et al.* (1993) and Adrio *et al.* (1993). In work conducted by An *et al.* (1989), the effectiveness of ethyl methanesulphonate (EMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and UV light as mutagens for generating *P. rhodozyma* mutants with increased astaxanthin levels was tested. NTG was found to be the best mutagen, producing stable clones with a high carotenoid content (>1500 μg/g yeast) (An *et al.* 1989). In most cases mutagenesis caused by EMS and UV light yielded unstable mutants with a low astaxanthin content (An *et al.* 1989). Consecutive use of the mutagens increased the level of instability in the yeast strains, making the selection of hyperproducing mutants very difficult (Johnson and An 1991). Adrio and co-workers (1993) confirm the difficulties associated with the use of UV light and EMS as mutagenic agents for the isolation of astaxanthin hyperproducing mutants of *P. rhodozyma*.

Visual examination of the yeast colonies is not always effective as a method for finding more highly pigmented yeast strains (Johnson and An 1991; Adrio *et al.* 1993). The rate of mutation is often low and differences in carotenoid levels between the mutants and their parents cannot be observed visually with ease (Johnson and An 1991). Development of a selection procedure for hyperproducing mutants is therefore required (An *et al.* 1989). Inhibitors of carotenogenesis, such as antimycin A and ß-ionone, have been used to isolate *P. rhodozyma* mutants with a higher carotenoid content successfully (Okagbue and Lewis 1984b; An *et al.* 1989; Lewis *et al.* 1990; Johnson and An 1991; Fang an Cheng 1993; Adrio *et al.* 1993; Meyer *et al.* 1993). In work done by An *et al.* (1989), a selection procedure using antimycin A was developed. The exact mechanism by which antimycin selection increases astaxanthin formation in *P. rhodozyma* is not known. An *et al.* (1989) suggest that antimycin alters reactions that are associated with the respiratory chain. These reactions appear to control the rate of astaxanthin biosynthesis in *P. rhodozyma*, making the identification of hyperproducing mutants possible by their darker pink/red coloration on

antimycin containing plates. Lewis *et al.* (1990) used ß-ionone to isolate and select astaxanthin overproducing mutants of *P. rhodozyma*. It appears that ß-ionone blocks the astaxanthin biosynthetic pathway at the ß-carotene level (Lewis *et al.* 1990). Astaxanthin overproducing mutants are easily identified on YM plates containing ß-ionone (10^{-4}M concentration), also as a result of their darker pink/red colour when compared to parent strains. The use of NTG as a mutagen and ß-ionone as a selection agent for the identification of stable astaxanthin over-producing mutants was successfully employed by Fang and Cheng (1993) as well as Meyer *et al.* (1993). Fang and Cheng (1993) isolated a mutant producing 1515 μg of astaxanthin per g yeast, while Meyer and co-workers (1993) isolated a *P. rhodozyma* strain producing 1688 μg astaxanthin per g yeast (Table 2.1).

Flow cytometry and cell sorting (FCCS) was also used to identify and isolate hyperproducing mutants of *P. rhodozyma* successfully (An *et al.* 1991). Cell cytometers simultaneously analyze cells for forward scatter (function of cell size), side scatter (function of cell granularity) and for fluorescent emissions at different wavelengths (Johnson and An 1991). Modern instruments can analyze and sort cells at a speed of 400-1000 cells/second (Johnson and An 1991). Carotenoid hyperproducing mutants of *P. rhodozyma* showed considerable variation in forward scatter and autofluorescence when compared to wild-type strains of *P. rhodozyma* (An *et al.* 1991). Cells possessing a higher fluorescence were sorted, resulting in a 10 000-fold enrichment of carotenoid hyperproducing mutants. Possible future technical developments in FCCS could further improve this method as a way of selecting and characterizing astaxanthin hyperproducing mutants of *P. rhodozyma* (Johnson and An 1991).

According to Adrio *et al.* (1993), the absence of suitable genetically marked strains of *P. rhodozyma* limits the progress being made towards the development of stable astaxanthin over-producing strains. In general, auxotrophic mutants isolated from a specific microorganism can be used as genetic markers (Adrio *et al.* 1993). The successful isolation of auxotrophic mutants of *P. rhodozyma* was accomplished by Adrio *et al.* (1993), using the polyene antibiotic nystatin.

Meyer *et al.* (1993) report the use of a technique known as randomly amplified polymorphic DNAs (RAPDs) for the detection of single base changes in the genomic DNA of *P. rhodozyma*. These base changes are introduced during mutagenesis by NTG. A single primer, of arbitrary sequence, is used to amplify genomic DNA segments from *P. rhodozyma*, and the RAPDs technique results in a method whereby DNA differences induced by NTG in astaxanthin over-producing mutants are detected. Thus DNA fingerprinting of *P. rhodozyma* mutants, for strain identification, can be accomplished (Meyer *et al.* 1993).

Recombination of mutant *P. rhodozyma* strains by protoplast fusion was accomplished by Chun *et al.* (1992). About 10% of the hybrids obtained by cell fusion produced more carotenoid than their parents (>2000 μg/g yeast). Meyer *et al.* (1993) reported a decrease in the maximum specific growth rate, from 0.18 h^{-1} to 0.12 h^{-1}, for *P. rhodozyma* mutants with an increased carotenoid content of 287 and 2289 μg/g respectively. The possibility exists of using protoplast fusion to combine astaxanthin hyperproducing *P. rhodozyma* strains with yeast strains having a high growth rate. The use of protoplast fusion depends on the availability of *P. rhodozyma* strains with differing qualities.

At present there appears to be no literature available regarding the use of gene cloning as a technique to increase astaxanthin production in *P. rhodozyma*. Cloning and overexpression of genes encoding astaxanthin formation in *P. rhodozyma* would be difficult due to the fact that several genes are involved in carotenoid biosynthesis (Johnson and An 1991). The exact pathway and genes involved are unknown and this further complicates the gene cloning approach. If rate limiting enzymes or rate determining precursors in the astaxanthin biosynthetic pathway could be identified, then gene cloning as a strain improvement technique would have the potential to become very useful (Johnson and An 1991). Furthermore a comprehensive understanding of the biosynthetic pathway for astaxanthin formation in *P. rhodozyma* could also allow for the isolation of stable astaxanthin hyperproducing strains.

Phaffia rhodozyma strain	Carbon source and conc. in medium		Yeast Yield		Astaxanthin Yield		Reference
	Type	(g/l)	(g/l)*	(g/g)**	(ug/ml)	(ug/g)	
UCD 67-210	Molasses	10	1.90	0.32	0.80	444	Haard (1988)
UCD 67-210	Molasses	21	3.10	0.28	2.10	670	
UCD 67-210	Molasses	40	7.40	0.31	6.50	876	
UCD 67-210	Molasses	100	14.10	0.20	15.30	1086	
UCD 67-210	Glucose	4	3.85	0.96	1.62	421	Johnson and Lewis (1979)
UCD 67-210	Glucose	16	6.46	0.40	1.11	171	
UCD 67-210	D-Maltose	4	3.63	0.91	1.88	512	
UCD 67-210	D-Cellobiose	4	3.48	0.87	2.27	652	
UCD 67-210	Sucrose	4	3.72	0.93	1.89	508	
UCD 67-210	D-Xylose	4	1.12	0.21	0.58	479	
UCD 67-385	Glucose	2	2.57	1.29	0.75	290	An et al. (1989)
UCD 67-385	D-Cellobiose	2	3.92	1.96	1.06	270	
ant-1	Glucose	2	2.01	1.01	1.35	670	
ant-1	D-Cellobiose	2	4.11	2.06	1.23	300	
ant-1-4	Glucose	2	1.82	0.91	1.91	1050	
ant-1-4	D-Cellobiose	2	1.78	0.89	2.24	1260	
NCHU-FS301	Glucose	10	4.78	0.30	7.81	1633	Fang and Cheng (1993)
NCHU-FS301	D-Maltose	10	4.05	0.33	5.34	1317	
NCHU-FS301	Fructose	10	4.93	0.31	7.15	1451	
NCHU-FS301	D-Cellobiose	10	2.27	0.23	4.10	1811	
NCHU-FS301	Sucrose	10	4.93	0.29	7.75	1689	
NCHU-FS301	D-Xylose	10	1.80	0.19	1.47	819	
J4-3	Glucose	10	4.71	0.47	6.56	1392	Meyer et al. (1993)
J4-3	D-Maltose	10	4.85	0.49	8.01	1652	
J4-3	Fructose	10	4.75	0.48	6.23	1311	
J4-3	D-Cellobiose	10	5.01	0.50	8.05	1607	
J4-3	Sucrose	10	4.90	0.49	7.54	1539	
J4-3	D-Xylose	10	4.11	0.41	5.50	1339	
CBS 5905T	Glucose	10	5.50	0.55	1.12	204	Meyer et al. (1993)
M4	Glucose	10	5.90	0.59	3.35	567	
B334	Glucose	10	5.30	0.53	4.10	773	
E329	Glucose	10	4.90	0.49	5.38	1097	
H1-27	Glucose	10	5.10	0.51	6.00	1176	
J4-3	Glucose	10	4.70	0.47	6.54	1392	
N9	Glucose	10	4.60	0.46	8.44	1834	
N9	Gluc/Acetic acid	0:0.5	1.25	2.50	1.70	1340	Meyer and Du Preez (1993a)
N9	Gluc/Acetic acid	0:1.0	1.42	1.42	1.70	123	
N9	Gluc/Acetic acid	0:1.5	1.73	1.15	1.80	810	
N9	Gluc/Acetic acid	10:0	3.25	0.33	5.10	1570	
N9	Gluc/Acetic acid	10:0.5	3.46	0.33	4.60	1340	
N9	Gluc/Acetic acid	10:1.0	3.70	0.34	4.20	1140	
N9	Gluc/Acetic acid	10:1.5	4.09	0.36	3.80	930	
ATCC#24202	Peat hydrolysate	15	4.60	0.31	7.21	1567	Martin et al. (1993)
N9	Grape juice (W)	46	12.03	0.26	14	1170	Meyer and Du Preez (1994a)
N9	Grape juice (W)	68	16.10	0.24	10.3	640	
N9	Grape juice (W)	92	20.46	0.22	5.2	260	
N9	Grape juice (R)	41	11.09	0.27	13.8	1240	
N9	Grape juice (R)	65	15.02	0.23	6.4	560	
N9	Grape juice (R)	90	19.92	0.23	3.4	190	

* Dry mass basis
** g biomass per g substrate utilized

TABLE 2.1. Summary of yeast biomass and astaxanthin production for *P. rhodozyma* strains evaluated on various substrates.

2.3. CONSIDERATIONS FOR THE PRODUCTION OF ASTAXANTHIN BY *Phaffia rhodozyma*

2.3.1. Effect of culture conditions.

Astaxanthin biosynthesis in *P. rhodozyma* is regulated by various environmental factors, such as light, pH, temperature, carbon source, dissolved oxygen concentration *etc.* In order to make effective use of the yeast as an animal feed additive both the biomass and the pigment is of value. Hence, it is necessary to investigate thoroughly the effect of these factors on astaxanthin formation and biomass production. A cross-comparison of the various studies reported the literature is difficult owing to the use of different mutants with varied pigment levels and growth rates.

2.3.1.1. Temperature and pH

The growth and pigmentation of *P. rhodozyma* as a function of temperature has been studied. The maximum temperature for growth was found to be 27.5 °C (Meyer and Du Preez 1994c; Johnson and Lewis 1979). During batch cultivation of *P. rhodozyma* by Meyer and Du Preez (1994c), the optimum growth temperature was found to be 22°C (μ=0.12 h^{-1}). This supports the findings of Johnson and Lewis (1979). Fang and Cheng (1993), reported the optimum temperature for growth and pigmentation of their *P. rhodozyma* strain to be 15 to 20°C, with growth decreasing considerably below 15°C and above 20 °C.

Johnson and Lewis (1979) report an optimal pH of 4.5 to 5.0. Meyer and Du Preez (1994c) report little effect of pH on growth and pigmentation of *P. rhodozyma* in the range 4.5 to 5.5. However, results of work done by Harvey and Larsen (1995) under similar growth conditions differ from these results by showing that pH has a major influence on both cell growth and pigment production in *P. rhodozyma*. They found the optimum for cell growth and pigment production to be pH 4.0.

2.3.1.2. Nutrients for growth.

A series of experiments was carried out by Johnson and Lewis (1979), in order to investigate the effect of different carbon substrates (D-glucose, D-maltose, sucrose, D-mannitol, D-xylose D-cellobiose and succinate), on both yeast growth and pigment formation. From the results of their studies, D-cellobiose was found to induce the greatest level of pigmentation (652 μg/g yeast) in *P. rhodozyma* strain UCD 67-210. Sucrose and glucose supported the highest specific growth rates of 0.19 h^{-1} and 0.21 h^{-1} respectively (*cf.* 0.1 h^{-1} for cellobiose). Astaxanthin levels of yeast grown on sucrose and glucose were in excess of 400 μg/g yeast (Table 2.1).

Fang and Cheng (1993) carried out a study in order to investigate the effect of various carbon sources (glucose, sucrose, maltose, fructose, lactose, L-arabinose, D-raffinose, D-cellobiose, D- mannitol, D-sorbitol and xylose) and nitrogen sources (peptone, beef extract, soytone, yeast extract, urea, ammonium sulphate, potassium nitrate and ammonium nitrate) on growth and pigment production of *P. rhodozyma* mutant NCHU-FS301 (Table 2.1). Their study showed glucose to support the highest level of pigment formation (1600 μg/g yeast), while yeast extract was the nitrogen source supporting the highest level of pigment formation (1300 μg/g yeast).

Optimization of microbial carotenoid biosynthesis by controlling the substrate concentration has been reported by Yun *et al.* (1990). Their studies showed the effect of controlling the initial sucrose concentration on carotenoid formation in the cells of *Daucus carota* (carrot). From the results of their work, they concluded that it was necessary to cultivate the cells at a low sucrose concentration initially in order to maximize the cell growth rate, and then to increase the sucrose concentration to maximize the biosynthesis of carotenoids. Haard (1988) demonstrated the potential of molasses as a substrate for *P. rhodozyma* production (Table 2.1). The sugar analyses for the grade of molasses used by Haard (1988) were 49.8% sucrose, 4.4% glucose and 4.6% fructose. Both the biomass concentration and the astaxanthin content were found to be a function of molasses concentration. A biomass concentration of 14.1 g/l and an astaxanthin yield in excess of 1000 μg/g yeast was reported for a molasses

concentration of 100 mg/ml, using the same yeast strain (UCD 67-210) as Johnson and Lewis (1979). This high astaxanthin level shows that suppression of astaxanthin synthesis, as noted by Johnson and Lewis (1979) for glucose concentrations in excess of 40 mg/ml, does not occur with molasses sugar. This serves to further highlight the potential of molasses as a substrate for astaxanthin formation by *P. rhodozyma*.

Grape juice has also been shown to be a suitable substrate for *P. rhodozyma* (Longo *et al.* 1992; Meyer and Du Preez 1994a). Supplementation of the grape juice with vitamins or yeast extract is essential in order to produce high levels of biomass and astaxanthin (Meyer and Du Preez 1994a). A maximum specific growth rate (μ_{max}) of 0.08 h^{-1}, a cell yield coefficient of 0.24 to 0.27 g biomass formed per g substrate utilized and an astaxanthin content of 1170 to 1240 μg/g yeast were reported by Meyer *et al.* (1994a) for *P. rhodozyma* (mutant N9) grown on grape juice, supplemented with YM medium and having a total sugars concentration of 40 to 50 g/l (Table 2.1). Longo and co-workers (1992) suggest grape juice as a possible cheap raw material for astaxanthin production from *P. rhodozyma* in wine growing regions.

Alfalfa residual juice (ARJ) was found to support high yeast growth rates (Okagbue and Lewis 1984a). However astaxanthin formation was inhibited, and the use of ARJ as a substrate for the growth and pigmentation of *P. rhodozyma* has limited potential. Peat hydrolysates have been used as substrates for the growth of a variety of microorganisms (Martin *et al.* 1993). Growth of *P. rhodozyma* (strain ATCC #24202) on peat hydrolysates at optimum conditions produced 4.6 g/l dry biomass, with a pigment content of 1500 μg/g yeast (Martin *et al.* 1993).

Meyer and Du Preez (1993) investigated the effect of acetic acid on astaxanthin production in *P. rhodozyma* (mutant N9). A maximum specific growth rate (μ_{max}) of 0.12 h^{-1} and an astaxanthin content of 1340 μg/g yeast was reported for *P. rhodozyma* N9 grown on medium containing 10 g/l glucose and 0.5 g/l acetic acid (Table 2.1). *P. rhodozyma* growth is completely inhibited at acetic acid concentrations greater than 2 g/l, with and without any glucose in the media (Meyer and Du Preez 1993). Due to

the inhibitory effect of acetic acid on microbial growth (Meyer and Du Preez 1993), an investigation was carried out using fed-batch cultures in order to maintain a low acetic acid concentration. Regulating the pH of the culture with acetic acid after the exponential growth phase resulted in an increase in the biomass concentration (from 5.53 g/l to 8.43 g/l) as well as an increase in the astaxanthin content (from 470 µg/g to 1430 µg/g). Extending the cultivation by titration with acetic acid can increase the biomass substantially while maintaining a high astaxanthin content (Meyer and Du Preez 1993).

In a mixed culture system of *Bacillus circulans* WL-12 and *P. rhodozyma*, the effect of different carbon sources on astaxanthin production in *P. rhodozyma* (strain UCD 67-210) was investigated by Okagbue and Lewis (1983). The advantage of a mixed culture system lies in its ability to extract and process *P. rhodozyma* in a single-step operation, thus providing a possible cost effective approach for the use of *P. rhodozyma* as a dietary source of astaxanthin (Johnson *et al.* 1978; Okagbue and Lewis 1983). In the mixed culture system, results showed that the monosaccharide carbon sources (glucose, fructose, sucrose and maltose) produced high yields of astaxanthin (1.4 µg/ml) in the yeast (Okagbue and Lewis 1983). *P. rhodozyma* grown on the substrates cellobiose, xylose and starch showed little to no growth. This contrasts with the mono-culture system of Johnson and Lewis (1979), where cellobiose provided satisfactory results for both yeast growth rate and astaxanthin formation. It must also be noted from the results of Okagbue and Lewis (1983) that even the best carbon source tested in their study (sucrose), produced a much lower astaxanthin yield than the best carbon sources used in a mono-culture system (Johnson and Lewis 1979).

The effect of aeration on growth was investigated by Johnson and Lewis (1979). They found the yields of astaxanthin and biomass to be independent of oxygen dissolution above a dissolved oxygen concentration of 4.0 mg/l (±50% saturation concentration). Below this level both yields were reduced significantly. Under microaerophilic conditions the level of astaxanthin produced was extremely low. Astaxanthin made up only 26% of total carotenoids compared to 90% under aerobic conditions (Johnson

and Lewis 1979). The primary carotenoid produced under anaerobic conditions was ß-carotene. In certain organisms nitrogen limitation plays an important part in the accumulation of carotenoids in the organism. There is no substantial evidence to show the effect of nitrogen limitation on astaxanthin formation in *P. rhodozyma* (Johnson and An 1991). Furthermore there is also a limited knowledge of the effects of micronutrients on carotenogenesis in *P. rhodozyma* (Johnson and An 1991).

2.3.1.3. The effect of light

Initially it was reported that light did not stimulate astaxanthin formation in *P. rhodozyma* (Johnson and Lewis 1979). However, in later studies by An and Johnson (1990) and Meyer and Du Preez (1994b), the distinct influence of light on growth and carotenoid formation in *P. rhodozyma* was shown. An and Johnson (1990) found that the exposure of *P. rhodozyma* to white and red light at high intensities resulted in decreased astaxanthin synthesis, and growth inhibition. Blue light was found to induce higher levels of pigmentation in yeast cells grown on YM agar (An and Johnson 1990). The combined effect of light and antimycin A was also investigated (An and Johnson 1990). Results showed a definite increase in yeast carotenoid formation, indicating that carotenoids could be formed as a result of intracellular oxygen radical formation in order to protect the cells against oxidative damage (Johnson and An 1991). Results from a study carried out by Schroeder and Johnson (1993) indicate that light-regulated carotenoid biosynthesis in *P. rhodozyma* could be induced by a mechanism involving an activated oxygen species. This in turn suggests that the carotenoids in *P. rhodozyma* may play role of antioxidant protection (Schroeder and Johnson 1993).

Meyer and Du Preez (1994b) show that there is a distinct difference in the growth and pigmentation of *P. rhodozyma* mutants grown in the dark and cultures grown in the light. Higher pigmentation levels were obtained when the cells were illuminated during the exponential growth phase. There is agreement with the work of Johnson and An (1990), in that blue light was also found to be primarily responsible for photo-induced astaxanthin production by Meyer and Du Preez (1994b).

2.3.2. Kinetics of growth and astaxanthin formation

Johnson and Lewis (1979) found that carotenoid formation in *P. rhodozyma* is growth associated. In their study astaxanthin was found to be the primary carotenoid and was produced mainly during the exponential growth period. Cessation of growth coincided with glucose exhaustion from the medium. It was observed that the astaxanthin level increased slightly after biomass growth had terminated, Johnson and An (1991) speculated that *P. rhodozyma* excretes a carbon intermediate during growth and this intermediate is then utilized as a carbon source to stimulate carotenogenesis once growth has stopped. The slight increase in *P. rhodozyma* pigment content (μg per g yeast) during stationary phase was also observed by Meyer and Du Preez (1994c). They ascribe this increase in pigment level to a decrease in the biomass and not to prolonged pigment biosynthesis in the stationary phase. During continuous cultivation of *P. rhodozyma* (mutant J4-3) a decrease in the biomass yield ($Y_{x/s}$), as well as the biomass and pigment concentration was reported above a dilution rate of 0.07 h^{-1}. A concomitant increase in the level of residual glucose concentration in the steady state culture at this dilution rate was observed (Meyer and Du Preez 1994c). Industrial production of astaxanthin by *P. rhodozyma* by continuous cultivation would not be viable due to its very low maximum specific growth rate (0.12 h^{-1}), as well as its low astaxanthin content at higher dilution rates (Meyer and Du Preez 1994c).

2.4. DOWNSTREAM PROCESSING CONSIDERATIONS

2.4.1. Carotenoid instabilities

In general, carotenoids are inherently unstable. They are especially sensitive to light, heat, oxygen, acids and, in the case of astaxanthin, alkali (Davies 1976). When isolating carotenoids from their various sources, precautions must be taken in order to prevent possible losses and low overall recovery of the carotenoid. Davies (1976) suggests that carotenoids be protected from heat damage by selecting low boiling point solvents for extraction

purposes, as the solvent ultimately has to be removed. The solvents should also be purified in order to avoid impurities from having any detrimental effect on the carotenoid. Furthermore, additional precautions need to be taken in order to protect the carotenoids against the effects of light and oxygen. Taking all the necessary precautions into account, various methods have been proposed for the extraction and isolation of astaxanthin from *P. rhodozyma* (Johnson and Lewis 1979; Okagbue and Lewis 1984c; Haard 1988; An *et al.* 1989; Sedmak *et al.* 1990). In addition to this various methods have also been proposed for the extraction and analysis of carotenoids present in salmonids (Johnson *et al.* 1980; Christophersen *et al.* 1989; Gentles and Haard 1991).

2.4.2. Disruption of *Phaffia rhodozyma*

Proper preparation of *P. rhodozyma* yeast cells is required for the successful transfer and deposition of astaxanthin in trout flesh (Johnson *et al.* 1977; Gentles and Haard 1991). The yeast has a cell wall and capsule that appears to be indigestible by fish (Okagbue and Lewis 1983). Initially it was thought that the astaxanthin present in *P. rhodozyma* was only biologically available to fish if the cells were mechanically broken (Johnson *et al.* 1980). Various studies have been conducted in order to investigate the degree of cell disruption required for adequate carotenoid release from *P. rhodozyma* (Gentles and Haard 1991; Sedmak *et al.* 1990; Johnson and Lewis 1978; Johnson *et al.* 1980; Okagbue and Lewis 1983)

A cost effective, large-scale process for the extraction and purification of astaxanthin from *P. rhodozyma* does not exist (Johnson and An 1991). Cell disruption techniques such as high pressure homogenisation, hydrodynamic cavitation, bead milling and enzymatic processes could be considered. Johnson *et al.* (1977) suggest that the most efficient deposition of astaxanthin in salmonids occurred when the cell wall of *P. rhodozyma* was partially removed by enzymatic digestion. Limited discussion regarding the use of large-scale cell disruption techniques for *P. rhodozyma* is available in the literature. Extracellular enzymes produced by the bacterium *Bacillus circulans* WL-12 partially digest the cell wall of

P. rhodozyma, rendering the carotenoids extractable in a mixed culture of *B. circulans* and *P. rhodozyma* (Johnson *et al.* 1978; Okagbue and Lewis 1983). The mixed culture system worked most efficiently at pH 6.5 and low yeast concentration (Johnson *et al.* 1978). A study conducted by Gentles and Haard (1991) showed high levels of flesh coloration in trout fed a diet containing spray dried *P. rhodozyma* yeast cells. This finding has significance with regard to the development of an economically viable commercial feed using *P. rhodozyma* as a source of astaxanthin, as the use of mechanical milling or a complex mixed culture system for the industrial production of astaxanthin can be avoided.

2.5. QUANTIFICATION OF CAROTENOIDS

2.5.1. Methods for laboratory scale cell disruption and carotenoid extraction

In order to isolate and quantify the carotenoids present in *P. rhodozyma* it is necessary to fully disrupt the yeast cells so that the carotenoids can be released. Mechanical cell disruption can be accomplished by means of high speed bead mills (Johnson and Lewis 1979; An *et al.* 1989), a mortar and pestle (Haard 1988) or a french pressure cell.

A method for carotenoid extraction from freeze dried *P. rhodozyma* cells, using a mortar and pestle, is described by Haard (1988). Yeast cells are ground with fine sand and 60% methanol. The homogenate is then filtered with the aid of 60% methanol. The filter cake is ground again and the homogenate is filtered, this time with acetone. The acetone extracts are collected and petroleum ether is added in order to achieve a phase separation. The carotenoid containing phase is then analyzed by means of measuring its absorbance at a specified wavelength on a spectrophotometer. An *et al.* (1989) describe a similar method, with the exception that cell disruption is achieved by means of a bead beater. Approximately 13 ml of cell suspension, harvested from a *P. rhodozyma* culture, is successfully disrupted by the same volume of 0.5 mm glass beads. The potential of autolysis as a tool for the extraction of astaxanthin

from *P. rhodozyma* is shown by Okagbue and Lewis (1984c). Distilled water and 0.02 M citrate buffer pH 7.0 were found to be suitable autolysing systems.

A different approach for the effective release of carotenoids from *P. rhodozyma* is reported by Sedmak *et al.* (1990). Washed yeast cells are ruptured by addition of hot (55°C) dimethyl sulphoxide (DMSO) which solubilises the yeast cell wall. The carotenoids are then extracted into an organic solvent consisting of hexane and ethyl acetate (50:50, v/v). Only a small volume (0.1-2 ml) of culture is required for complete pigment extraction. Quantitative analysis of the carotenoid containing solution is then carried out by means of absorption spectroscopy. Methods involving the mechanical disruption of *P. rhodozyma* cells with subsequent extraction of the carotenoids into an organic solvent are reported to be slow, impractical and not very reproducible (Sedmak *et al.* 1990). In contrast, *P. rhodozyma* cell disruption with dimethyl sulphoxide is rapid (10 samples can be processed in an hour), reproducible (standard deviation of less than 5% from the mean), and very practical as only small volumes of culture are required for analytical purposes (Sedmak *et al.* 1990).

2.5.2. Measurement and determination of carotenoids

Carotenoids, along with chlorophylls were among the first compounds ever to be separated by chromatography (Lea 1988). Thin layer chromatography (TLC) can be effectively used for the separation and identification of carotenoids (Britton 1985). Although TLC separates carotenoids easily and rapidly, accurate quantitative and qualitative analysis is difficult due to the fact that the compounds begin to bleach before the support layer can be scraped off (Ruddat and Will 1985). The inherent thermal instability of carotenoids makes analysis by gas chromatography impractical (Ruddat and Will 1985). These difficulties are avoided by using high-performance liquid chromatography (HPLC) for carotenoid identification.

All carotenoids absorb light strongly in the visible, or in some cases the UV region of the spectrum, and it is standard practice to use light spectroscopy

for the quantitative determination of carotenoids (Britton 1985). HPLC, employing detection by visible light absorption, is also used for the quantitative determination of carotenoids. This method is the most sensitive and accurate means of quantitative analysis of carotenoids (Britton 1985).

2.5.3. Methods for the determination and measurement of the carotenoids present in *Phaffia rhodozyma*

2.5.3.1. TLC and absorption spectroscopy

Identification and quantification of the individual carotenoids present in *P. rhodozyma* and salmonids can be accomplished by means of TLC (Johnson and Lewis 1979; An *et al.* 1989; Christophersen *et al.* 1989). An *et al.* (1989) chromatographed carotenoids extracted from *P. rhodozyma* by TLC on silica gel plates with 20% acetone-80% petroleum ether. Pigments are identified by their absorbance maxima and also by co-chromatography with standards. Carotenoid concentrations are calculated by their respective extinction coefficients.

Quantification of the total carotenoid concentration present in *P. rhodozyma* extracts can be carried out by means of absorption spectroscopy (Johnson and Lewis 1979; Haard 1988; An *et al.* 1989; Sedmak *et al.* 1990). The total amount of carotenoid present in the extracting solvent can be calculated by the following formula, based on the Beer-Lambert law (Davies 1976):

$$\text{Total Carotenoid (g)} = \frac{A \times V}{E^{1\%}_{1cm} \times 100}$$

where: A = Absorbance at specified wavelength

V = Volume of extracted pigment in solvent (ml)

$E^{1\%}_{1cm}$ = 1% Extinction coefficient

The wavelength at which the absorbance is to be measured is dependent on the solvent used during the extraction procedure. These, and corresponding extinction coefficients are given in Table 2.2.

Solvent System	(nm)	Extinction Coefficients		References
		Total Caro.	Astaxanthin	
Petroleum ether	474	2100	1600	Johnson and Lewis (1979) An et al. (1989)
Acetone	478	2100	----	Okagbue and Lewis (1984c)
Hexane:ethyl acetate	480	2150	----	Sedmak et al. (1990)
Methanol	480	2100	----	Meyer et al. (1993)

TABLE 2.2. Summary of the light absorption maxima and extinction coefficients for carotenoids extraxted from *P. rhodozyma* cells.

2.5.2.2. High Performance Liquid Chromatography

HPLC analysis using a silica column with a hexane:ethyl acetate (50:50 by volume) eluent is reported to give good resolution of the carotenoids extracted from *P. rhodozyma* (Sedmak *et al.* 1990). Depending on the stage of growth and the growth medium, results of the analysis indicate that the astaxanthin content ranges from 65%-95% of the total amount of carotenoids present in *P. rhodozyma*. Meyer *et al.* (1993) report the successful determination of astaxanthin in *P. rhodozyma* extract, by HPLC using a stainless steel, reversed phase column and a mobile phase of methanol:acetonitrile (9:1 v/v). HPLC was also used successfully for the quantitative analysis of carotenoid pigments isolated from salmonids using a C_{18} column with a solvent of ethyl acetate and methanol/water (9:1), and visible spectrophotometric analysis in the 400 to 500 nm region (Christophersen *et al.* 1989).

CHAPTER 3

YEAST STRAIN IMPROVEMENT AND SHAKE FLASK CULTIVATION OF *Phaffia rhodozyma*

3.1. INTRODUCTION

The potential of *Phaffia rhodozyma* as a source of astaxanthin and as a nutrient in fish feeds, has already been discussed. A local yeast manufacturer wished to broaden their product range. They supplied a wild strain of *Phaffia rhodozyma* for the purposes of investigating possible commercial production of astaxanthin. Work reported in this chapter centers around a preliminary experimental programme, firstly, to select an improved astaxanthin producer by mutagenesis, and secondly to identify optimal culture conditions in terms of pH, temperature, nitrogen source and carbon source. The latter studies were performed as shake flask studies.

3.2. MATERIALS AND METHODS

3.2.1. Yeast strain and standard culture conditions

A *Phaffia rhodozyma* wild strain (No. Y1194) was obtained as a gift from Anchor Yeast, Cape Town, R.S.A. The strain was sourced from the culture collection of the CSIR. It was maintained on YM agar plates at 4°C, and subcultured monthly.

The wild strain as well as the mutants produced in this study were evaluated in 500 ml Erlenmeyer flasks containing 50 ml yeast/malt extract broth (YM broth), consisting of (per liter) 10 g glucose, 5 g peptone, 3 g yeast extract and 3 g malt extract, as growth medium. Where required, the media was buffered with a 0.1 M potassium hydrogen phthalate buffer. Shake flask culture inoculum was grown for 24 hours at 20 to 22°C on 50 ml YM broth in 500 ml flasks, inoculated from a fresh plate. Shake flasks were inoculated with 5% (v/v) *P. rhodozyma* inoculum culture. The YM broth was supplemented with 0.1 % (v/v) Antifoam (Sigma Chemical Co. St. Louis MO.) The shake flask cultures were grown at 20 to 22°C on a reciprocating shaker (Labotec (Pty) Ltd., Johannesburg, R.S.A.) at 150 r.p.m. for 120 h.

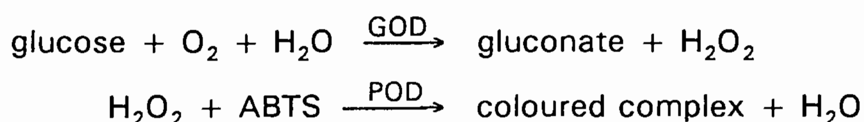
3.2.2. Analytical methods used

3.2.2.1. Biomass determination

Biomass formation was measured as dry cell mass. The biomass pellets collected from fixed volume samples were washed and dried pre-weighed eppendorf tubes at 105°C for 48 hours.

3.2.2.2. Residual sugar determination

Residual glucose concentration was monitored spectrophotometrically at 620 nm following its conversion to a coloured complex by the following reactions, involving the enzymes glucose oxidase (GOD) and peroxidase (POD):



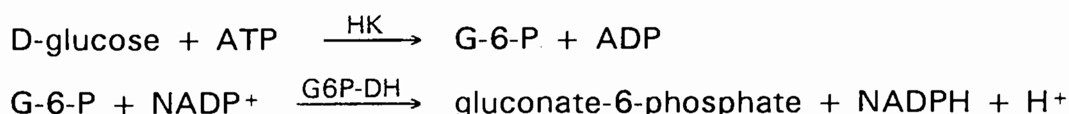
$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{POD}} \text{coloured complex} + H_2O$$

where ABTS is di-ammonium 2,2 - azino - bis(3-ethylbenzothiazoline-6-sulphonate). The assay was conducted using culture supernatant and a Boehringer Mannheim analysis kit (Cat. No. 124 036). The linearity of the assay was confirmed for a glucose concentration of 0 to 0.5 g/l.

Residual sugar concentration in the supernatant of molasses cultures was also monitored by enzymic reaction, using a Boehringer Mannheim kit (Cat. No. 716 260). The assay determines the concentrations of sucrose, D-glucose and D-fructose separately. The D-glucose concentration is determined before and after the enzymic hydrolysis of sucrose. Sucrose content is then calculated from the difference of the D-glucose concentrations. D-fructose is determined subsequent to the determination of D-glucose. The enzymic reactions are as follows:

(i) Determination of D-glucose before inversion, using hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH):

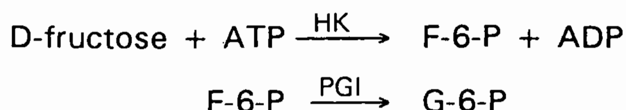


$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{gluconate-6-phosphate} + \text{NADPH} + H^+$$

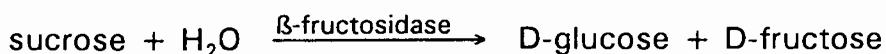
The NADPH formed by this reaction is stoichiometric with the amount of D-glucose and is measured by absorbance at 340 nm.

(ii) Determination of D-fructose, using HK and phospho-glucose isomerase (PGI):



G-6-P (glucose-6-phosphate) again reacts with NADP, and now the amount of NADPH is stoichiometric with the amount of D-fructose.

(iii) Enzymatic inversion of sucrose by ß-fructosidase:



The determination of D-glucose after inversion is carried out according to the principle outlined above. The linearity of the assay was confirmed for sugar concentrations of 0 to 0.8 g/l.

3.2.2.3. Carotenoid determination

The dimethyl sulphoxide (DMSO) method as described by Sedmak *et al.* (1990) was used for carotenoid release from *P. rhodozyma* followed by extraction into a hexane/ethyl acetate (Sedmak *et al.* 1990) mixture or methanol (Meyer *et al.* 1993). Spectrophotometric analysis of the carotenoid extract allows for determination of the total amount of carotenoid present in *P. rhodozyma* cells. The procedure for analysis is described below.

P. rhodozyma (0.5 to 1 ml) culture was pipetted into sealable test tubes. After harvesting the cells and discarding the supernatant, the pink coloured cells were washed twice with 4 ml of distilled water. The supernatant was again carefully discarded and the tubes were inverted to drain the water. The insides of the tubes were wiped with tissue paper to dry off any excess water. To each tube 0.5 ml DMSO (American Burdick & Jackson, Muskegon, MI), preheated to 55°C, was added. The tubes were agitated by a vortex mixer for 30 seconds. The organic solvent (1 ml) was then

added to the DMSO/cell mixture, and 0.1 ml of 0.01 M sodium phosphate buffer (pH 7.0) was added in order to partition the carotenoids into the organic solvent. Initially a 50:50 mixture of hexane and ethyl acetate (v/v), (Sedmak *et al.* 1990) was used as an organic solvent for carotenoid extraction. In later analyses high purity methanol (American Burdick & Jackson, Muskegon, MI) was used as organic solvent (Meyer *et al.* 1993). The test tubes were again agitated by a vortex mixer for a further 30 seconds. After centrifugation of the test tubes, the absorbance of the supernatant at 480 nm was recorded on a UV spectrophotometer (Cary 1E UV-Vis Spectrophotometer). The total carotenoid was calculated using the following formulae:

$$\text{Total carotenoid } (\mu g/ml) = P = \frac{A_{480} \times V_t}{E \times V_s} \times 10000$$

$$\text{Total carotenoid } (\mu g/g) = \frac{P\ (\mu g/ml)}{X\ (g/ml)}$$

where A_{480} is the absorbance at 480 nm, V_t the total volume (1.6 ml), E the 1% extinction coefficient (Table 2.2), V_s the sample volume (ml) and X is the sample dry mass (g/ml).

This method was found to be less cumbersome when compared to the mechanical cell disruption of *P. rhodozyma* described by Johnson and Lewis (1979), Haard (1988) and An *et al.* (1989).

3.2.3. NTG mutagenesis

Mutagenesis was performed on freshly grown cells using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Sigma Chemical Co. St. Louis MO.) according to a method described by An *et al.* (1989). For NTG mutagenesis, approximately 50 ml of freshly grown (overnight) culture was poured into a sterile centrifuge tube. Yeast cells were washed twice with 5 ml 0.1 M sodium citrate. Washed cells were then resuspended in sodium citrate to an optical density of 2 at 660 nm. A 1 ml NTG solution of concentration 1 g/l was made up by addition of sodium citrate. The required volume of NTG

solution was then pipetted into the cell suspension in order to obtain the desired final concentration. NTG and cell suspension were mixed for 30 seconds using a vortex agitator and left to incubate at room temperature for 20 minutes. Cells were washed with 1 ml of 0.1 M potassium phosphate (pH 7.0) and resuspended in the same buffer before transferring to YM broth. The inoculated YM broths were incubated overnight to allow expression of the mutation before plating.

Plate counts were carried out once the yeast colonies were visible. A kill curve of the fractional survival of the washed cells as a function of the NTG concentration was established. Mutated cultures showing in excess of 90% kill were plated in order to screen visually for astaxanthin hyperproducing mutants.

Astaxanthin-overproducing mutants were also screened by using ß-ionone (Sigma Chemical Co. St. Louis MO.) as an astaxanthin synthesis inhibitor. In this selection ß-ionone was dissolved in ethanol and added to YM agar immediately before pouring plates, giving a final concentration of 10^{-4} M ß-ionone. After 1 week incubation, the colonies appearing the darkest pink were selected and transferred to YM plates and incubated for a further week before evaluation in shake flasks as detailed in Section 3.2.1.

3.2.4. Shake flask culture for assessment of culture conditions

The effect of varying initial glucose concentration on growth and pigmentation of *P. rhodozyma* was investigated. A minimal medium consisting 1.7 g/l yeast nitrogen base (YNB) and 5 g/l $(NH_4)_2SO_4$ was used for this investigation. Shake flasks containing YNB with initial glucose concentrations ranging from 0 to 40 g/l were inoculated with a *P. rhodozyma* mutant.

The shake flask cultures were grown under standard conditions as described in Section 3.2.1. The culture biomass and residual glucose content were monitored for periods of 12, 24 and 72 hours after inoculation.

3.2.5. Development of molasses medium

To investigate molasses as a crude carbon source, cane molasses (supplied by Pure Cane Molasses, Durban, R.S.A.) was obtained as a gift from Anchor Yeast, Cape Town, R.S.A. The molasses syrup was diluted to the required total sugar concentration with redistilled water, and then clarified by centrifugation.

Due to the complex nature of molasses, and its deficiency in certain essential nutrients for the growth of microorganisms, scouting experiments were conducted in order to determine the additional nutrient requirements for adequate growth and pigmentation of *P. rhodozyma* on molasses at a standard growth temperature of 20 to 22°C (Section 3.2.1). Supplementation of the molasses medium with various nitrogen sources and yeast extract was investigated. As a control experiment, *P. rhodozyma* was also grown on molasses to which no additional nutrients had been added.

3.3. RESULTS AND DISCUSSION

3.3.1. Growth of *Phaffia rhodozyma* under standard conditions

Shake flask cultivation of the *P. rhodozyma* wild strain under standard culture conditions (Section 3.2.1) for 120 hours resulted in a final biomass concentration of 5.97 g/l. This value represented the mean of three determinations with a standard deviation of 2.3% from the mean. The total carotenoid content of the *P. rhodozyma* wild strain was 318 μg carotenoid per g yeast produced (dry weight basis). A biomass yield (g biomass mass formed per g substrate consumed) and carotenoid yield (μg carotenoid formed per g substrate consumed) of 0.50 g/g and 190 μg/g were obtained respectively.

3.3.2. Analytical procedures

3.3.2.1. Biomass determination

Gravimetric determination of *P. rhodozyma* dry cell mass proved to be reproducible and accurate. A constant biomass was achieved after drying the cells at 105°C for a period of 24 hours. Molasses samples were washed at least twice before drying in order to eliminate possible inaccuracies caused by the presence of noncellular solids in molasses.

A standard deviation of less than 3.5% and 5.0% of the mean was calculated for the analysis of dry biomass in YM broth cultures and molasses cultures respectively.

3.3.2.2. Sugar analysis

The use of enzymic analysis kits for the determination of residual substrate in both YM and molasses culture supernatant also proved to be consistent and accurate. A standard deviation of less than 3% of the mean was calculated for both assays.

For the glucose assay, samples having a glucose concentration of more than 0.5 g/l were diluted with distilled water, and in the case of molasses, samples containing more than 0.8 g/l residual sugar were diluted prior to analysis, to ensure the measured absorbance fell within the range of linearity for the assay. Although the absorbance was found to remain constant over an incubation period of 10 to 50 minutes, at 20°C, absorbance readings were taken after 20 minutes as a matter of consistency.

3.3.2.3. Carotenoid determination

Using the DMSO method for *P. rhodozyma* cell disruption followed by extraction in an organic solvent, a linear relationship between the mass of cells and the absorbance at 480 nm was reported by Sedmak *et al.* (1990). Cultures containing high biomass concentrations were therefore diluted prior

to extraction in order to ensure that the absorbance would fall within the linear range. Complete cell disruption using DMSO was confirmed by microscopic analysis. Sedmak *et al.* (1990) extracted carotenoids into an organic mixture of hexane and ethyl acetate (50:50 v/v). This however results in a two phase solution, with the carotenoids being found in the top phase, making the transfer of carotenoid extract to cuvettes for spectrophotometric analysis difficult. Meyer *et al.* (1993) report the successful extraction *of P. rhodozyma* carotenoids into either acetone or methanol, after cell disruption with DMSO. The standard deviation of the total carotenoid content in *P. rhodozyma* cells as determined by the DMSO method was found to be 5.2% of the mean value.

3.3.3. Yeast strain improvement

Typically a mutagen is used under conditions causing approximately 90% kill, as this allows for a good mutation frequency. Hence, scouting experiments were performed in order to find suitable mutation conditions.

A kill curve of the fractional survival of cells as a function of NTG concentration was established (Fig 3.1). A NTG concentration of 0.09 mg/ml was required in order to achieve a 90% kill. Mutated cultures showing in excess of 90% kill were plated on YM agar plates in order to screen for astaxanthin hyperproducing mutants. Approximately 3000 mutant colonies were screened. *P. rhodozyma* mutant UCT-1N-3693 was selected by visual screening of yeast colonies appearing darker in coloration. *P. rhodozyma* wild strain and mutant (UCT-1N-3693) cultures grown on YM agar plates are shown in Fig. 3.2 and Fig. 3.3 respectively. *P. rhodozyma* UCT-1N-3693 was subjected to a further NTG mutation cycle. Generation of the kill curve for this mutation showed an increased resistance of mutant UCT-1N-3693 to NTG treatment, as a higher concentration was required in order to achieve a kill of greater than 90% (Fig. 3.1).
P. rhodozyma strains UCT-2N-8793-1 and UCT-2N-8793-2, obtained by mutation of UCT-1N-3693, were again selected by visual screening.

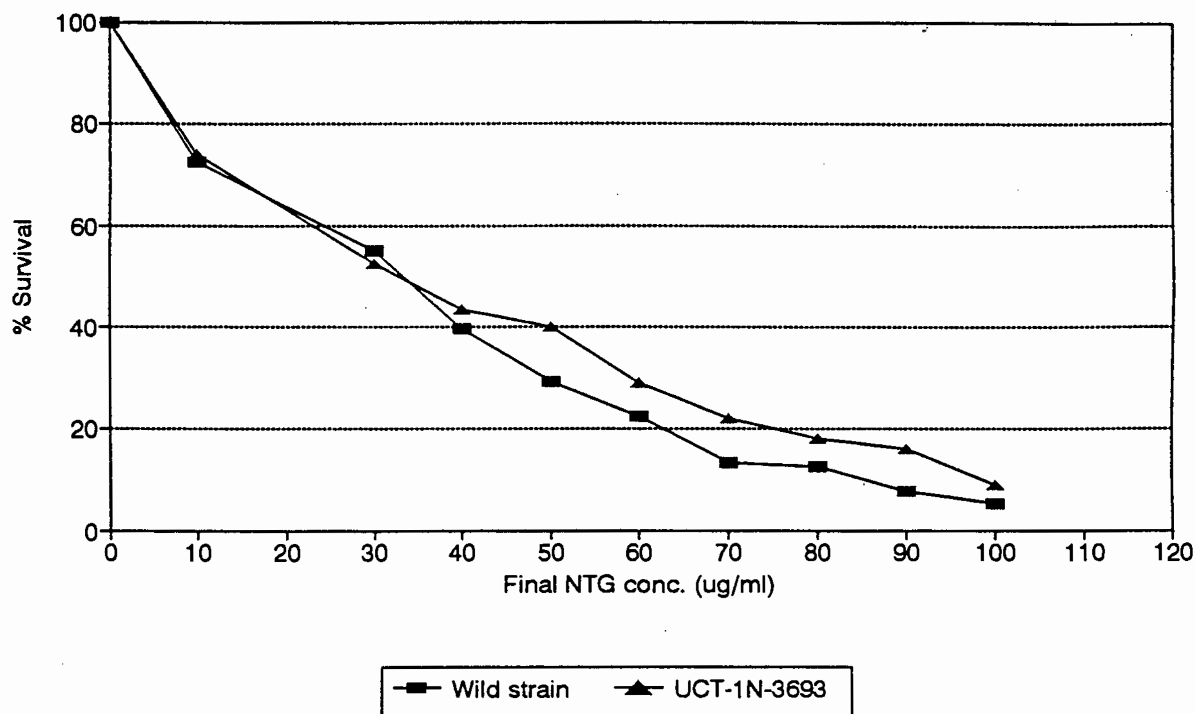


FIGURE 3.1. Kill curve generated by NTG mutation of *P. rhodozyma* strains.

Shake flask evaluation of the *P. rhodozyma* wild strain in YM broth showed the strain to produce a maximum of 318 µg carotenoid per g yeast (dry weight). This value corresponds to the values reported in the literature for natural isolates of *P. rhodozyma* (An *et al.* 1989). Shake flask evaluation of *P. rhodozyma* strains UCT-1N-3693, UCT-2N-8793-1 and UCT-2N-8793-2, showed a 45% increase in the total carotenoid content compared with the wild strain. However there was no increase shown in the total carotenoid content of strains UCT-2N-8793-1 and UCT-2N-8793-2 over UCT-1N-3693 (Table 3.1). These results showed visual screening of mutant colonies to be an unsatisfactory method for the detection and isolation of carotenoid-overproducing mutants of *P. rhodozyma*. This highlighted the need for a specific selection agent for carotenoid-overproducing mutants (An *et al.* 1989). ß-Ionone was therefore used as a selection agent for astaxanthin-overproducing mutants.

FIGURE 3.2. *P. rhodozyma* wild strain culture grown on YM agar medium.

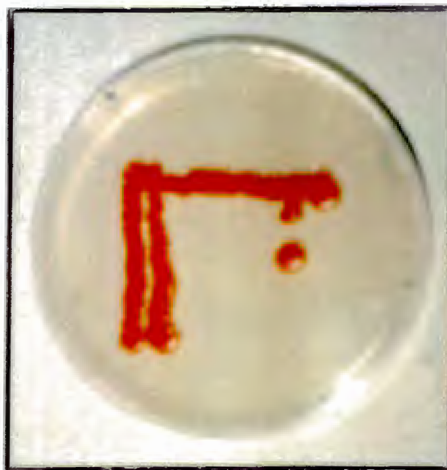


FIGURE 3.3. *P. rhodozyma* mutant (UCT-1N-3693) culture grown on YM agar medium.

Shake flask evaluation of colonies selected (UCT-2N-30394-1 and UCT-2N-30394-2) as a result of their dark pink coloration on ß-ionone plates showed no change in total carotenoid content when compared to other mutant strains (Table 3.1). This indicated that tuning of the selection procedure for the use of ß-ionone was required in order to select for astaxanthin-overproducing mutants of *P. rhodozyma* effectively.

Phaffia rhodozyma strain	Dry mass* (g/l)	Total Carotenoid** (ug/ml)	Total Carotenoid** (ug/g)	Yields*** Biomass (g/g)	Yields*** Carotenoid (ug/g)
Wild-type	5.97	1.90	318	0.50	190
UCT-1N-3693	5.87	2.67	455	0.49	267
UCT-2N-8793-1	5.50	2.49	453	0.45	249
UCT-2N-8793-2	5.33	2.36	443	0.43	236
UCT-2N-30394-1	5.63	2.38	423	0.46	238
UCT-2N-30394-2	5.53	2.51	454	0.45	251

* Dry mass data represents the mean of three determinations, with a standard deviationon of less than 2.8% from the mean.

** Values for total carotenoid represent the mean of two determinations, with a standard deviation of less than 4.2% from the mean.

*** Yields are calculated on a g biomass or ug pigment, per g of carbon substrate utilized.

TABLE 3.1. Shake flask evaluation of *P. rhodozyma* mutants at standard conditions (Section 3.2.1).

Although the carotenoid content of mutant UCT-1N-3693 was considerably lower than values reported for astaxanthin-overproducing mutants of *P. rhodozyma* (An *et al.* 1989; Fang and Cheng 1993; Meyer *et al.* 1993), further NTG mutations of *P. rhodozyma* strains and fine tuning of the selection procedure was not initiated. An extensive strain improvement programme was not deemed essential by the sponsors at this stage of the research project for several reasons. Firstly, a yeast strain with a high growth rate is essential for use of the currently available plant for astaxanthin production by *P. rhodozyma*. Secondly this study centers around the establishment of fed-batch culture conditions and the kinetics for

P. rhodozyma growth and pigment formation. Finally, if deemed necessary there existed the possibility of obtaining an yeast strain with an improved astaxanthin content from other sources.

3.3.4. Shake flask culture for the assessment of culture conditions

In shake flask experiments the effect of varying the initial glucose concentration in YM broth on growth and pigmentation of *P. rhodozyma* UCT-1N-3693, under standard conditions, was investigated. After a 12 hour period of growth no residual glucose was observed in cultures having an initial glucose concentration of less than 1 g/l. Complete glucose utilization of cultures containing more than 10 g/l glucose was observed after 72 hours. Biomass formation (on a dry mass basis), total pigment formation and residual glucose levels in the culture were monitored. An increase in the initial glucose concentration resulted in an increase in the total biomass produced (Table 3.2). The specific amount of carotenoid formed remained relatively constant for glucose concentrations between 10 and 40 g/l. However, due to the fact that the total biomass of the yeast increased substantially in high glucose media, volumetrically (μg/ml) more pigment was produced (Table 3.2). At glucose concentrations less than 10 g/l, the total amount of carotenoid produced decreased both on a volumetric and mass basis. A decrease in the pigment yield (μg carotenoid produced per g glucose utilized) and the cell yield (g biomass formed per g glucose utilized) was observed with an increase in glucose concentration. Johnson and Lewis (1979) also report the effect of initial glucose concentration on biomass and pigment formation yields in shake flask cultures of *P. rhodozyma*. By varying the medium volume and shaking rates of shake flask cultures, the effect of relative aeration on growth and pigmentation was investigated. They showed that a high glucose concentration and a low aeration rate in the growth medium caused a significant reduction in the efficiency of pigment and biomass production of *P. rhodozyma*. This strongly indicates that the fermentative metabolism of *P. rhodozyma* is affected by glucose and dissolved oxygen (DO) concentrations.

Shake Flask Experiment	Initial glucose (g/l)	Glucose concentration 12 hrs (g/l)	24 hrs (g/l)	72 hrs (g/l)	Dry mass* (g/l)	Total Carotenoid** (ug/ml)	(ug/g)	Yields*** Biomass (g/g)	Carotenoid (ug/g)
1	40.0	35.5	11.6	0.0	12.5	4.01	321	0.29	100
2	26.0	22.0	3.1	0.0	11.0	3.76	342	0.39	145
3	15.7	11.3	0.9	0.0	8.8	3.45	392	0.50	220
4	8.8	5.0	0.0	0.0	5.5	2.15	391	0.52	244
5	0.9	0.0	0.0	0.0	2.0	0.55	275	1.11	611
6	0.5	0.0	0.0	0.0	1.8	0.40	222	1.60	800
7	0.2	0.0	0.0	0.0	1.5	0.29	193	2.50	1450
8	0.0	0.0	0.0	0.0	0.8	0.16	200	---	---

* Dry mass data represents the mean of three determinations, with a standard deviation of less than 3.2% from the mean.

** Values for total carotenoid represent the mean of two determinations, with a standard deviation of less than 6.5% from the mean.

*** Yields are calculated on a basis of g biomass or ug pigment, per g of carbon substrate utilized.

TABLE 3.2. Effect of varying the initial glucose concentration in shake flask experiments at standard conditions (Section 3.2.1).

Glucose degradation by *Saccharomyces cerevisiae* was observed to proceed via respiration at low glucose concentrations (De Deken 1965). However, for *S. cerevisiae* growing exponentially on high concentrations of glucose, in the presence of air, the glucose degradation was observed to proceed mainly via aerobic fermentation. Inhibition of the synthesis of respiratory enzymes by high fermentation rates, which occur at high glucose concentrations, is known as the 'Crabtree Effect' (De Deken 1965). The Crabtree effect has been reported for a number of different yeasts besides *S. cerevisiae* (Wöhrer *et al.* 1981), and could affect the levels of biomass and pigment production in *P. rhodozyma*.

During industrial scale cultivation of *P. rhodozyma* for astaxanthin production, the effect of sugar and DO concentrations will be critical on both yeast biomass and pigmentation yields. These effects have been investigated rigorously, using laboratory scale fermentation equipment. The

results will be presented and discussed in Chapters 4, 5 and 6 of this dissertation.

3.3.5. Development of molasses medium for growth and pigmentation of *Phaffia rhodozyma*

3.3.5.1. Effect of nutrient supplementation in molasses medium

Since cane molasses is readily available in South Africa and is currently used as an industrial substrate for the production of various yeasts including *Saccharomyces cerevisiae* (Bakers' yeast), the potential of using molasses as an inexpensive substrate for the growth and pigmentation of *P. rhodozyma* was investigated in a series of shake flask experiments.

Cane molasses is a by-product of the sugar refining process. Being agriculturally based, its composition is affected by the variety and maturity of the cane as well as the climatic and soil conditions (Soffiantini *et al.* 1971; Steg and Van der Meer 1985). As such, it is not possible to establish an absolute analysis of the molasses composition. Typically the total sugar concentration of molasses varies from 50 to 60%. Molasses constitutes 30 to 40% sucrose, 15 to 20 % reducing sugars such as glucose and fructose, and 2 to 4% unfermentable sugars. Inorganic constituents of molasses include phosphorous, calcium, potassium, chlorine and sodium, all constituting less than 1% (except potassium, 1.5 to 5%) of the total molasses composition. Nitrogen compounds are relatively low (2 to 3%). Non-sugar organic matter is also present in molasses (9 to 12%) in the form of soluble gums, organic acids and small amounts of waxes, sterols and vitamins (Soffiantini *et al.* 1971; Steg and Van der Meer 1985).

Based on protein content (*i.e.* N x 6.25) the equivalent nitrogen composition of standard YM broth was calculated to be approximately 1.0 g/l . The nitrogen compounds in cane molasses are relatively low and in general the equivalent nitrogen value of molasses medium diluted to a sugars concentration of 40 g/l is less than 0.1 g/l (Soffiantini *et al.* 1971). Hence the supplementation of the shake flask medium containing molasses at a

sugar concentration of 10 g/l, to an equivalent nitrogen composition of 1.0 g/l was investigated. Ammonium sulphate (inorganic) and peptone (organic) were used as nitrogen sources.

An increase in the cell dry mass and pigment content of *P. rhodozyma* mutant UCT-1N-3693, as well as an increase in the respective yield of biomass and carotenoid was observed in cultures with media containing ammonium sulphate and peptone (Table 3.3).

Shake Flask No.	Peptone (g/l)	Malt Extract (g/l)	Yeast Extract (g/l)	Ammonium Sulphate (g/l)	Equiv. Nitrogen (g/l)	Dry mass* (g/l)	Total Carotenoid** (ug/ml)	Total Carotenoid** (ug/g)	Yields*** Biomass (g/g)	Yields*** Carotenoid (ug/g)
1	---	---	---	---	0.05	3.2	0.97	303	0.25	97
2	5.0	---	---	---	1.0	4.6	1.44	313	0.39	144
3	---	---	---	4.3	1.0	5.4	1.83	339	0.47	183
4	---	---	---	6.5	1.5	5.5	1.82	331	0.48	182
5	---	---	---	2.2	0.5	5.2	1.77	340	0.45	177
6	---	3.0	---	4.3	1.02	4.9	1.66	339	0.42	166
7	---	---	3.0	4.3	1.3	5.8	2.06	355	0.51	206

* Dry mass data represents the mean of three determinations, with a standard deviation of less than 4.0% from the mean.

** Values for total carotenoid represent the mean of two determinations, with a standard deviation of less than 6.0% from the mean.

*** Yields are calculated on a basis of g biomass or ug pigment, per g of carbon substrate utilized.

TABLE 3.3. Growth of *P. rhodozyma* (UCT-1N-3693) on molasses medium containing various nutrients under standard conditions (Section 3.2.1).

This result, as well as further results investigating the combined effect of carbon and nitrogen sources (Table 3.4), indicates that nitrogen supplementation of molasses media is essential. Use of ammonium sulphate as a nitrogen source yielded a significantly better result when compared to peptone (Table 3.3). Its use is favoured, since a cheap inorganic nitrogen source is generally preferred for the industrial production of yeasts. The concentration of ammonium sulphate in molasses media, in the range 0.5 to 1.5 g/l equivalent nitrogen, had little effect on the growth and pigmentation

of *P. rhodozyma*. Johnson and Lewis (1979) report a similar result for the growth of *P. rhodozyma* on yeast nitrogen base (YNB) broth with varied concentrations of ammonium sulphate. Addition of 3 g/l yeast extract to the molasses medium increased the biomass and pigment yields (Table 3.3). Yeast extract provides micronutrients and vitamins favouring the growth and pigmentation of *P. rhodozyma* in a micronutrient deficient medium such as molasses. Johnson and Lewis (1979) report a similar increase in pigmentation for *P. rhodozyma* grown on yeast nitrogen base (YNB) broth with addition of 0.1 to 10 g/l yeast extract.

3.3.5.2. Effect of pH

The effect of pH on growth and pigmentation of *P. rhodozyma* grown on buffered molasses medium with a sugar concentration of 10 g/l and a concentration of 4.3 g/l ammonium sulphate as a nitrogen source was investigated (Fig. 3.2).

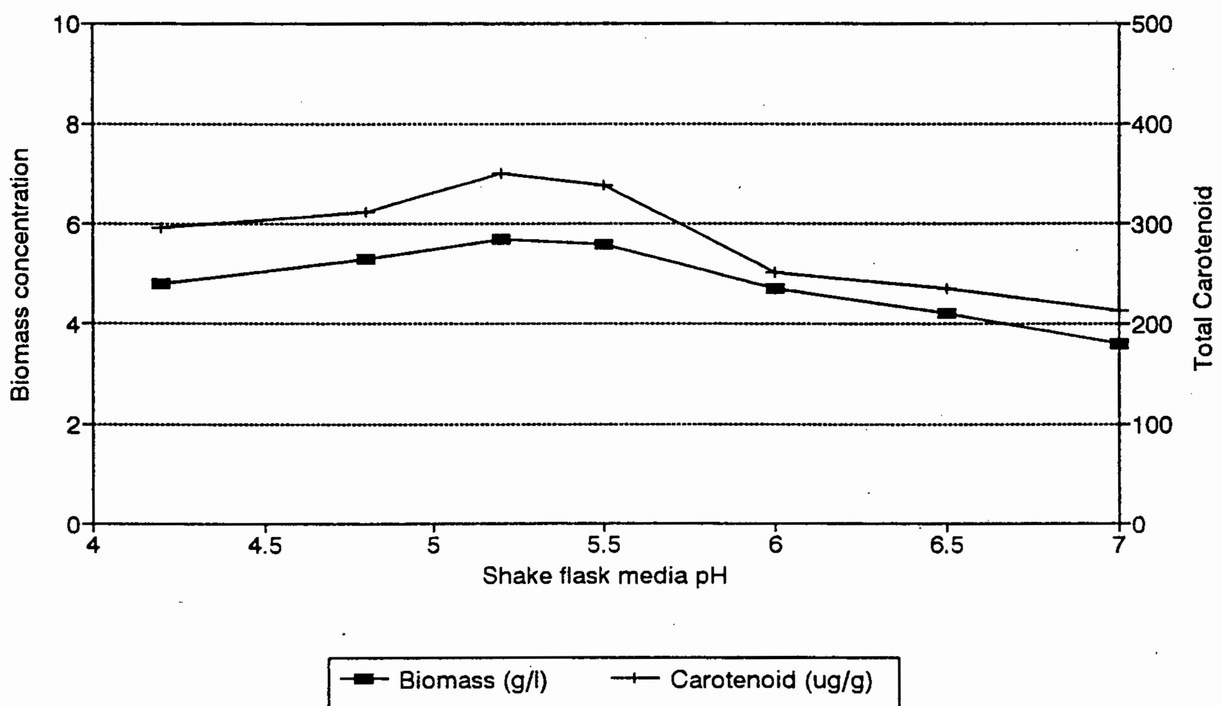


FIGURE 3.2. Effect of pH on *P. rhodozyma* (UCT-1N-3693) growth and pigmentation, using molasses as a growth medium.

Experiments conducted at pH 4.5 to 5.5 were buffered using a 0.1 M potassium hydrogen phthalate buffer, while experiments conducted at a pH of 5.5 to 7.0 were buffered using a 0.1 M sodium phosphate buffer. Similar results were obtained at pH 5.5 for separate cultures buffered with both potassium hydrogen phthalate and sodium phosphate buffers. The total biomass and carotenoid formed by *P. rhodozyma* decreased significantly as the pH increased above a value of 5.5 (Fig. 3.4). The optimum pH was found to be between 5.0 and 5.5. This optimum is comparable with the optimum pH values reported for *P. rhodozyma* growth on glucose (Johnson and Lewis 1979; Fang and Cheng 1993; Meyer and Du Preez 1994c).

3.3.5.3. Effect of molasses sugar concentration

An increase in the concentration of fermentable sugars in the molasses media supplemented with $(NH_4)_2SO_4$ as a nitrogen source, resulted in an increase in biomass and volumetric carotenoid production (Table 3.4). However, as occurred with an increase in glucose concentration, a corresponding decrease in biomass and pigment yields was seen with increasing sugar concentration. Similarly, Haard (1988) reported a decrease in the biomass yield with an increase in sugar concentration for *P. rhodozyma* grown on molasses medium supplemented with yeast nitrogen base and peptone. Although in contrast, there was an approximate 10 fold increase in carotenoid yield (Table 2.1). The opposite trend in carotenoid yield was observed in our study for both increasing molasses (Table 3.4) and glucose concentrations (Table 3.2). This is in agreement with the glucose results of Johnson and Lewis (1979). A possible explanation for the observation of Haard (1988) may center on the molasses source which contained a higher percentage of sucrose (50%). An increased astaxanthin content was also observed by Haard (1988) for *P. rhodozyma* grown on a medium consisting of sucrose, glucose and fructose (10:1:1 w/w//w), indicating possible induction of astaxanthin synthesis by sucrose. In addition, other substances enhancing astaxanthin biosynthesis may have been present in the molasses used by Haard (1988).

Shake Flask Experiment	Molasses sugars (g/l)	Ammonium sulphate (g/l)	Dry mass* (g/l)	Total Carotenoid** (ug/ml)	Total Carotenoid** (ug/g)	Yields*** Biomass (g/g)	Yields*** Carotenoid (ug/g)
1a	30	0.0	7.6	2.56	336	0.23	85
1b	30	4.3	9.7	2.96	305	0.30	99
2a	20	0.0	5.4	1.79	351	0.27	90
2b	20	4.3	6.7	2.05	308	0.34	103
3a	10	0.0	3.9	1.16	298	0.33	116
3b	10	4.3	5.4	1.86	344	0.48	186
4a	1.0	0.0	1.3	0.29	233	0.7	290
4b	1.0	4.3	1.8	0.48	266	1.2	480

* Dry mass data represents the mean of three determinations, with a standard deviation of less than 5.0% from the mean.

** Values for total carotenoid represent the mean of two determinations, with a standard deviation of less than 6.0% from the mean.

*** Yields are calculated on a basis of g biomass or ug pigment, per g of carbon substrate utilized.

TABLE 3.4. Effect of sugar concentration on *P. rhodozyma* (UCT-1N-3693) grown on molasses medium in shake flask culture under standard conditions (Section 3.2.1). The media was supplemented with ammonium sulphate as a nitrogen source.

3.4. CONCLUSIONS

The total carotenoid content of a *P. rhodozyma* wild strain was increased 1.5 times after mutation with NTG. An *et al.* (1989) reported that carotenoid hyperproducing mutants, produced by successive mutation of *P. rhodozyma* wild strains, can become unstable and revert to production of lower levels of carotenoid than the parent strain. Such instability has not been observed for this mutant over a period of 2 years in the laboratory.

While higher levels of astaxanthin production have been obtained by mutation, this is usually accompanied by a decrease in the specific growth rate of the mutant. Economic feasibility of lower astaxanthin producers with a higher growth rate, could result in *P. rhodozyma* being used as a

valuable protein source as well as a source of pigmentation in animal feeds. Clearly a compromise between carotenoid production and the growth rate of *P. rhodozyma* mutants must be considered.

Increased glucose concentration (over the range 0.2 to 40 g/l), as well as the initial sugar concentration of molasses in *P. rhodozyma* shake flask cultures has an effect of decreasing both the biomass and pigment yields. Johnson and Lewis (1979) report a similar effect of glucose concentration on the growth and pigmentation of *P. rhodozyma* in shake flask cultures. These results suggest that the phenomenon known as the 'Crabtree effect' (De Deken 1965) may be exhibited by *P. rhodozyma* when grown aerobically at high initial glucose concentrations and that further experimentation (reported in Chapter 6) was required for confirmation of the Crabtree effect in *P. rhodozyma*.

Shake flask cultivation of *P. rhodozyma* on South African cane molasses medium was confirmed. The requirement of additional nutrients for enhanced growth and pigmentation on molasses medium was demonstrated. Ammonium sulphate proved to be a suitable nitrogen source, while the addition of yeast extract complemented the growth and pigmentation of *P. rhodozyma* by providing the molasses medium with additional micronutrients. A comparison of an elemental analysis of *P. rhodozyma* by Meyer *et al*. 1993 with laboratory media indicated the need for phosphate, magnesium and calcium as inorganic nutrients. The inorganic salts (KH_2PO_4; $MgSO_4.7H_2O$; $CaCl_2.2H_2O$) will be included in the medium for fermenter culture studies.

CHAPTER 4

GROWTH OF *Phaffia rhodozyma* IN BATCH AND CONTINUOUS CULTURE

4.1. INTRODUCTION

Where both microbial and chemical synthesis routes to a product exist, the choice between these commonly depends on the economics of the process (Atlas 1984). In order to optimise the microbial synthesis of astaxanthin by *P. rhodozyma*, it is desirable to maximize astaxanthin formation and process economics (Johnson and An 1991). In most microbial processes, the effects of temperature, pH, aeration, growth medium and maximal growth rate of the microorganism are of paramount importance to growth yield, product yield and process economics (Schuler and Kargi 1992; Atlas 1984).

In addition to the nutritional and environmental parameters for a given microbial process, consideration must also be given to the reactor design and configuration. Two main reactor configurations exist, namely, batch and continuous cultivation schemes. During batch cultivation, the microorganism is introduced to a fixed initial amount of substrate and growth continues until the nutrient source is depleted. In continuous flow processes, a steady state is reached where the nutrient, product and cell biomass concentrations are constant. A constant culture volume is essential to maintain this steady state. A continuous process such as this is generally referred to as a chemostat (Schuler and Kargi 1992).

Operability, reliability and process economics are important factors governing the choice of reactor configuration. Batch cultures can suffer variability from one run to another resulting in variation in the respective biomass and product concentrations, ratios and qualities. On the other hand, factors such as the maintenance of sterility and the occurrence of genetic instabilities over long time periods, favours the choice of a batch system over continuous cultivation. Furthermore batch operation provides a degree of flexibility in processes where the product demand varies and small product volumes are required. Continuous systems are used mainly used for the production of large volume, growth-associated bioproducts *e.g.* ethanol (Schuler and Kargi 1992).

Modifications of batch and continuous reactor systems are sometimes employed in order to have better control over microbial growth and product

formation. One such modification of major importance is the fed-batch reactor system. During fed-batch culture, nutrients are continuously fed, while the effluent is removed discontinuously. Variation of the culture volume for fed-batch operation distinguishes it from batch and chemostat operation. Fed-batch culture is used to overcome the effects of substrate inhibition and catabolite repression. This results in an increased culture biomass yield. Industrial systems such as the production of penicillin and bakers' yeast are based on fed-batch cultivation (Bailey and Ollis 1986).

In addition to selecting the appropriate reactor configuration, knowledge of biological reaction kinetics is essential in understanding how biological reactions work. Kinetic parameters associated with microbial growth and product formation can be established by experimentation. With a view to gaining an understanding of the basic kinetics for growth and pigment formation in *P. rhodozyma*, studies were conducted in both batch and continuous culture.

Batch cultivation studies, using glucose as a growth limiting substrate, were used to determine biomass and pigment yields, as well the type of product formation (growth or non-growth associated). In addition the kinetics for the batch cultivation of *P. rhodozyma* on molasses medium was investigated.

During continuous cultivation the effect of varying the substrate dilution rate was investigated in order to determine the kinetic constants μ_{max} (maximum specific growth rate) and K_s (saturation constant). The effect of varying the feed glucose concentration at a fixed dilution rate in chemostat operation was investigated in order to monitor biomass and pigment yields with respect to possible substrate inhibition as a result of the Crabtree effect. A study observing the effect of varying the total oxygen concentration supplied to the chemostat, at a fixed dilution rate, was also completed. This was done in order to investigate the possibility of inducing carotenoid biosynthesis at high oxygen concentrations.

The results of these studies in batch and chemostat culture are reported and discussed in this chapter. Chapter 5 reports and discuses the results for the fed-batch cultivation of *P. rhodozyma*.

4.2. MATERIALS AND METHODS

4.2.1. Yeast strain and inoculum

P. rhodozyma mutant UCT-1N-3693, obtained by NTG mutation (Section 3.3.2) was used in the evaluation of growth and pigment formation in both batch and continuous cultivation. The inoculum for these cultures was prepared in shake flasks.

Pre-inoculum culture was grown on 50 ml YM broth in 500 ml Erlenmeyer flasks for 48 hours at 20 to 22°C on a reciprocating shaker at 150 r.p.m. By sterile transfer, 5 ml of pre-inoculum culture was transferred to a 1 liter Erlenmeyer flask containing 70 ml YM broth. The culture was grown for 24 hours, as described above, before transfer to the reactor.

4.2.2. Laboratory scale bioreactor design.

The bioreactor system used for these investigations was designed in the Department of Chemical Engineering at the University of Cape Town (UCT) (Fig. 4.1).

The experiments were conducted under mono-culture conditions in a 2 l Quickfit culture vessel with a working volume of 1.5 l. The top of the vessel was fitted with a stainless steel lid, which was clamped and sealed by means of a polyurethane rubber O-ring. Agitation of the reactor contents was achieved by means of a six blade rushton impeller with a diameter of 79 mm. The impeller was fitted to a Rexxon 14" drill press motor (Model No. RDM-100A), with variable rotation speeds. The stirrer speed was set at 540 r.p.m.

FIGURE 4.1. UCT-designed laboratory scale bioreactor for the cultivation of *P. rhodozyma*

The temperature in the vessel was controlled by continuous circulation of warm water (24°C) through a stainless steel heating coil. The temperature was monitored by a LM35 precision centigrade temperature sensor. A thermostat and solenoid valve allowed for the intermittent flow of cold water (10°C) from a refrigerated waterbath through a separate coil in order to control the culture temperature at 22°C.

The pH was monitored and controlled using a sterilizable pH electrode (Phoenix Electrode Co., Houston, TX, U.S.A.) coupled to a UCT designed pH controller. Control at pH 5.0 was maintained by the automatic addition of
2 M NaOH and 1 M HCl using a peristaltic pump.

The dissolved oxygen concentration (DO) was monitored with a glass sterilizable oxygen electrode (Phoenix Electrode Co., Houston, TX, U.S.A.) coupled to a UCT-designed DO monitor. The DO was maintained at a level of above 2.0 mg/l by supplying sterile air through a 2 μm air filter (Pall Process Filtration Ltd., Portsmouth, U.K.), at a flowrate equivalent to 2 v/v.m. For the experiments investigating the effect of oxygen concentration on pigment formation, pure medical oxygen (Afrox, Cape Town, R.S.A.) was mixed with the sterile air prior to being introduced into the bioreactor. The fraction of pure oxygen supplied to the culture was monitored volumetrically by means of a rotameter.

4.2.3. Batch cultivation

For *P. rhodozyma* cultivation in batch mode, the medium contained (per liter): 2 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.1% Antifoam (Sigma, St. Louis, MO, U.S.A. Cat. No. A-5551) and 1 g yeast extract (Meyer and Du Preez 1994c). In addition, the medium contained 1 g/l potassium hydrogen phthalate and 0.25 g/l NaOH as an initial buffer (pH 5.0). D-glucose was used as a carbon source at a concentration of 10 g/l, and was sterilized separately from the other nutrients at 121°C. All nutrients were combined after cooling, prior to inoculation. For the batch cultivation of *P. rhodozyma* on molasses, the above nutrients were added to a molasses solution (10 g/l total sugars), after sterilization and cooling. Culture samples were taken by means of a 5 ml syringe, attached to a stainless steel pipe entering the culture vessel through a sample port in the reactor lid. In order to prevent contamination of the culture or the samples, a Bunsen burner was used to flame the stainless steel pipe prior to taking a sample.

4.2.4. Continuous cultivation

After inoculation, batch growth of *P. rhodozyma* was monitored by taking samples at regular time intervals. The culture was allowed to grow in batch mode to stationary phase after which sterile medium addition by peristaltic pump was started for chemostat operation. A constant volume was maintained in the vessel by pumping off excess culture through an outlet stainless steel pipe continuously.

A period of at least three residence times was allowed for culture conditions to reach steady state after a parameter had been changed (Pirt 1975). Steady state for each setting was confirmed by ensuring that the biomass concentration (dry mass basis) did not vary by more than 5% from the mean during three consecutive determinations over a 6 h period, after a period of three residence times had been reached. The dilution rate (D), defined as the flowrate of fresh sterile medium divided by the culture volume, was checked at regular intervals.

4.2.5. Analytical methods

Dry mass, residual sugar concentration and total carotenoid content were determined as described in Section 3.2.2. Samples taken during batch and continuous cultivation were immediately microfuged to separate cells from supernatant for dry mass analysis. After centrifugation the supernatant was stored at -20°C until analysed for residual sugar content.

4.3. RESULTS AND DISCUSSION

4.3.1. Batch cultivation

A profile for the batch cultivation of *P. rhodozyma* on a medium containing 10 g/l glucose medium is shown in Fig. 4.2.

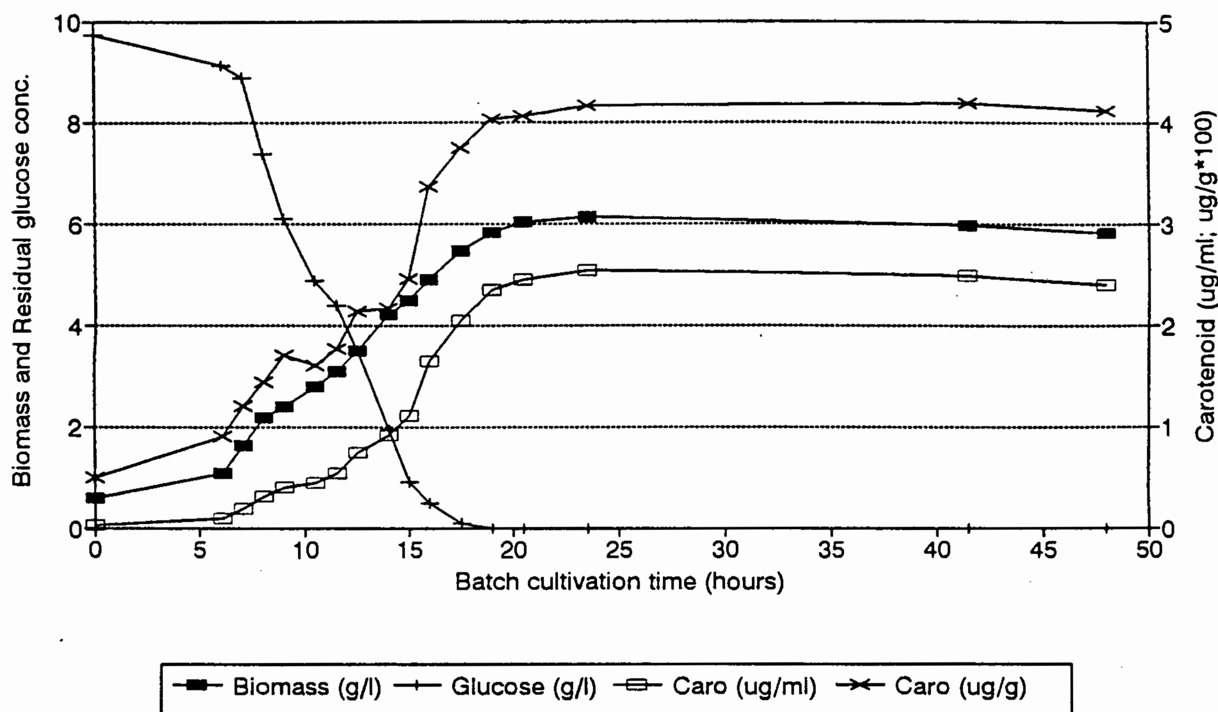


FIGURE 4.2. Batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and medium containing 10 g/l glucose.

A six hour lag phase was observed. Biomass formation occurred over the ensuing 16 hours. A constant dry weight of 6.1 g/l was reached after 22 h. The termination of cell growth was observed approximately 3 to 4 hours after glucose exhaustion from the medium. Pigment production was clearly growth associated (Fig 4.3). The intracellular concentration increased from 50 to 416 µg/g during the exponential growth phase. A maximum specific growth rate of 0.11 h^{-1} was calculated for batch growth on glucose medium.

At a constant agitation rate of 540 r.p.m. and an air flow rate of 2 v/v.m, the dissolved oxygen (DO) concentration decreased from 8.5 to 2.1 mg/l (25% saturation) during the exponential growth phase (Fig. 4.4). This indicates an increase in the oxygen utilization rate (OUR). As the culture enters the stationary phase the DO level increased to the saturation DO concentration. The yield factor relating grams cells formed per gram O_2 consumed (Y_{X/O_2}) was calculated to be 1.14 g/g. This compares well with a value of 0.97 g/g for *S. cerevisiae* grown aerobically on glucose medium (Bailey and Ollis 1986).

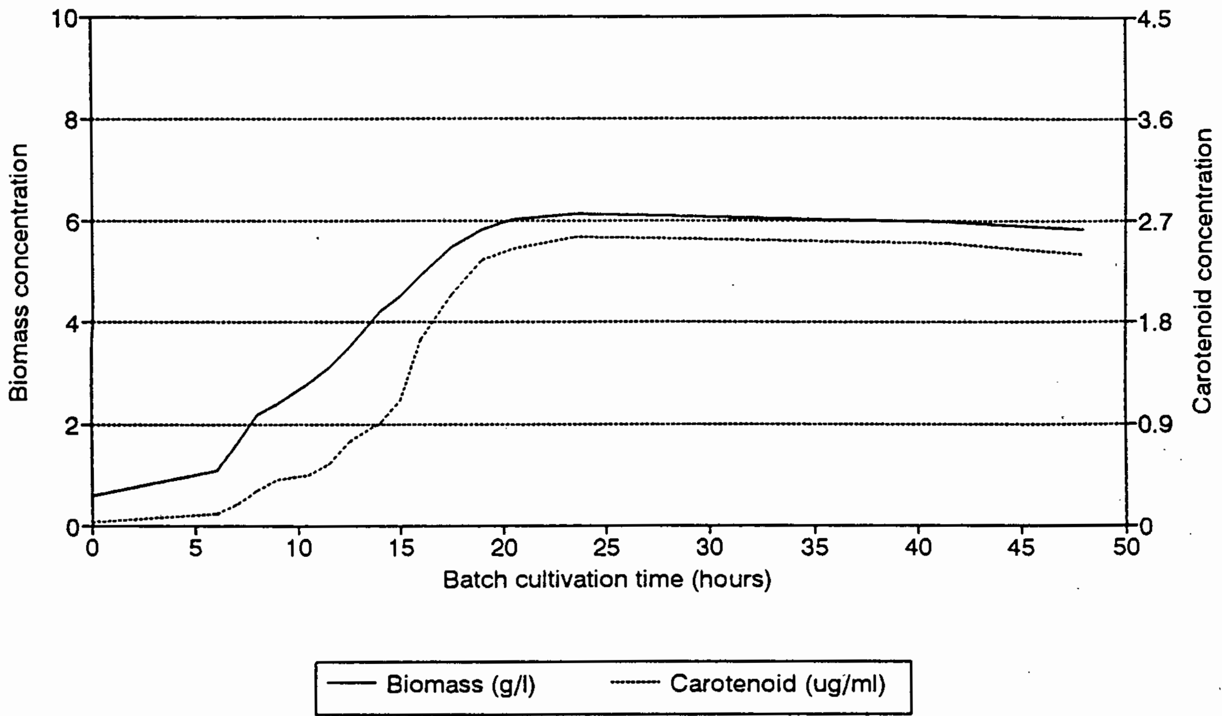


FIGURE 4.3. Demonstration of growth association for carotenoid formation in *P. rhodozyma* (UCT-1N-3693).

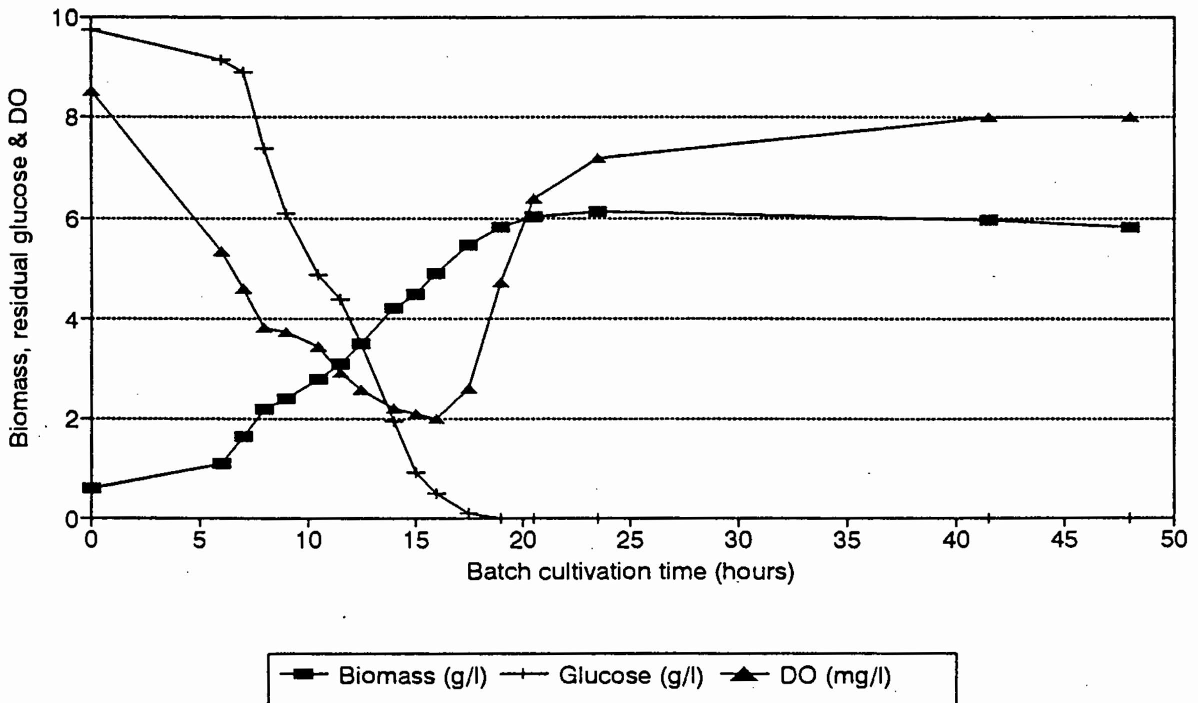


FIGURE 4.4. Variation of the dissolved oxygen concentration during batch cultivation of *P. rhodozyma* (UCT-1N-3693) on glucose medium.

The profile for batch cultivation of *P. rhodozyma* on molasses medium is shown by Fig. 4.5. Although a lower total *P. rhodozyma* biomass was produced for growth on molasses compared to growth on glucose medium (5.4 g/l *cf.* 6.1 g/l, dry mass basis), the level of total pigment produced (μg/g) was the same on molasses as it was for glucose *i.e.* ±410 μg/g. Pigment production is also clearly growth associated for *P. rhodozyma* grown on molasses medium. A slower rate of growth and pigment formation was observed for *P. rhodozyma* grown on molasses medium (Fig. 4.5) when compared to growth on glucose medium (Fig 4.2). This is reflected by the culture entering stationary phase after approximately 42 hours when grown on molasses medium (*cf.* 22 hours for growth on glucose medium). Furthermore a lower maximum specific growth rate of 0.095 h^{-1} was recorded.

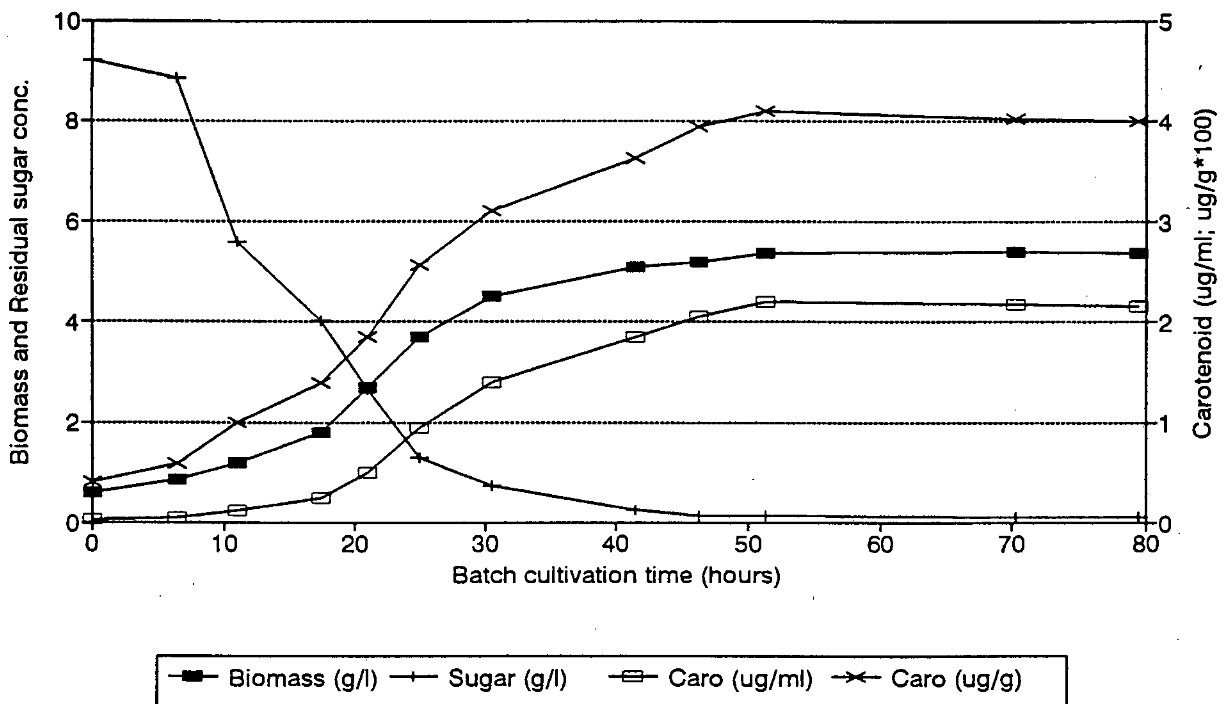


FIGURE 4.5. Batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C and pH 5.0 on molasses medium with a sugar concentration of 10 g/l.

Fig. 4.6 shows the utilization of the individual sugars present in molasses. Glucose and sucrose are consumed at the same rate. Fructose consumption began upon exhaustion of glucose from the medium. Final biomass and

product yields (per g of substrate utilized) of 0.56 g/g and 265 μg/g were obtained respectively. These values compare favourably with the biomass and product yields of 0.55 g/g and 259 μg/g respectively, for *P. rhodozyma* grown on glucose medium.

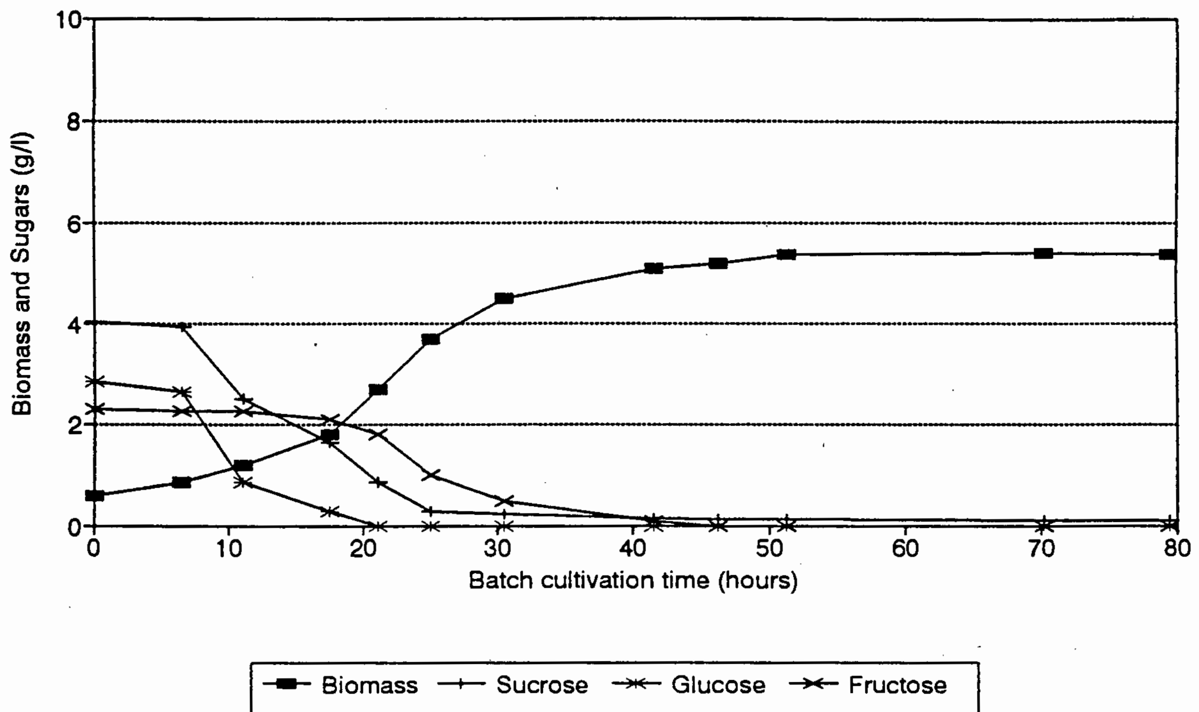


FIGURE 4.6. Utilization of individual sugars during batch cultivation of *P. rhodozyma* (UCT-1N-3693) on molasses medium.

4.3.2 Chemostat: Variation of the dilution rate

For the growth of *P. rhodozyma* in continuous culture the cell biomass remained constant up to a dilution rate of 0.08 h^{-1}, after which the biomass decreased with a corresponding increase of the residual glucose concentration in the culture at steady state (Fig. 4.7). The total pigment content on a dry mass basis also remained relatively constant up to a dilution rate of 0.09 h^{-1} after which it decreased substantially with an increase in the dilution rate. Meyer and Du Preez (1994c) also found that the highest total pigment and astaxanthin levels were found at lower dilution rates where no residual glucose was detected in the steady state culture.

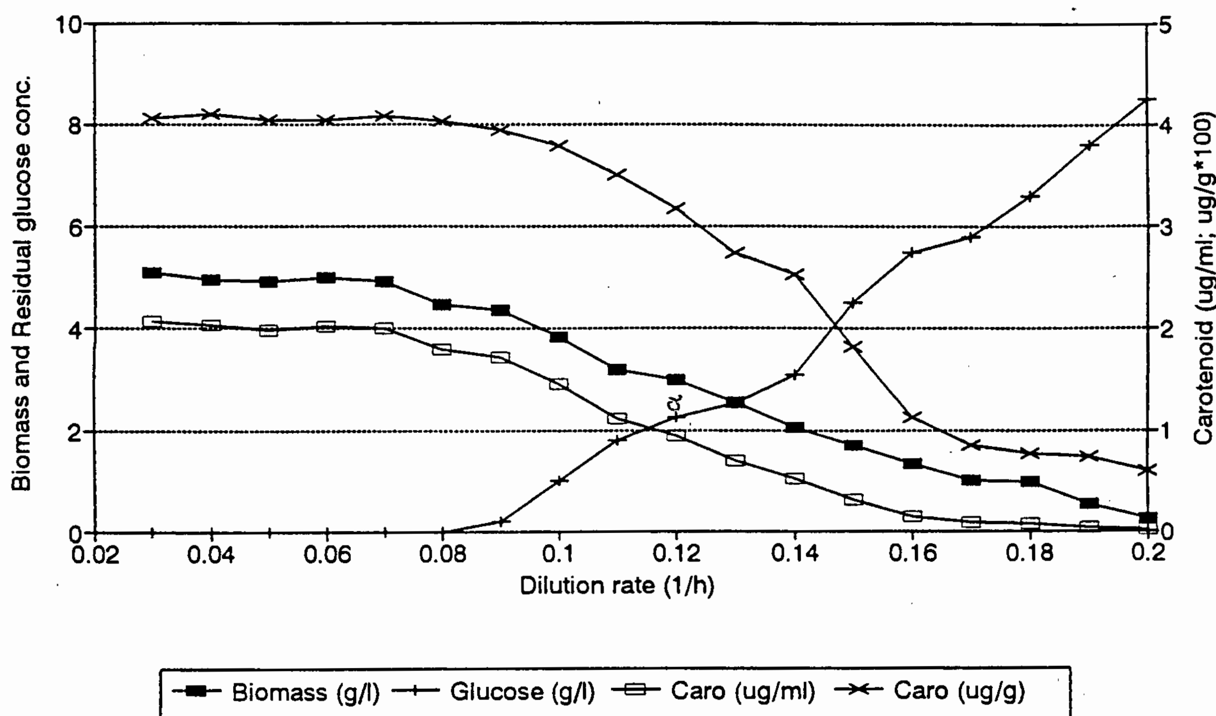


FIGURE 4.7. Effect of varying the dilution rate during continuous cultivation of *P. rhodozyma* (UCT-1N-3693) on medium containing 10 g/l glucose.

The medium used during continuous cultivation (Section 4.2.3) was of the same composition as used by Meyer and Du Preez (1994c) for *P. rhodozyma*. Carbon limitation of the medium was confirmed by Meyer and Du Preez (1994c), by the pulse addition of 2 g/l glucose to a steady state culture of *P. rhodozyma*. This triggered an immediate increase in the biomass concentration.

4.3.2.1. Determination of μ_{max} and K_s values in the Monod equation

The Monod equation describes the functional relationship between the specific growth rate (μ) of a microorganism and the concentration of a single

limiting nutrient (S) in the growth medium. It is a saturated function and is of the same form as the Langmuir adsorption isotherm and the standard rate equation for enzyme-catalysed reactions with a single substrate (Michaelis-Menten). The Monod equation is given by:

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{4.1}$$

where μ_{max} is the maximum achievable growth rate and K_s is the saturation constant. K_s is equal to the concentration of the rate-limiting substrate when the specific rate of growth is equal to one-half of the maximum.

In continuous operation the steady state material balance for biomass is given by:

$$DX_f = (D - \mu)X \tag{4.2}$$

where X_f is the biomass in the liquid feed stream. For sterile feed $X_f = 0$, resulting in:

$$\mu = D \tag{4.3}$$

Linearisation of Equation 4.1 gives:

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}} \frac{1}{S} + \frac{1}{\mu_{max}} \tag{4.4}$$

Hence a plot of 1/D (=$1/\mu$) as a function of 1/S (Lineweaver-Burk plot) yields a straight line . This allows for the determination of values for μ_{max} and K_s from the slope and the intercept of the line.

A Lineweaver-Burk plot was constructed using the experimental data shown in Fig. 4.7. The values obtained graphically for μ_{max} and K_s are 0.22 h^{-1} and 1.9 g/l respectively (Fig 4.8). This was repeated for three independent chemostat runs and these values were found to be in good agreement. Average values μ_{max} and K_s resulting are 0.21 $\pm$ 0.1 h^{-1} and 1.9 $\pm$ 0.2 g/l, representing a standard deviation from the mean of less than 5%.

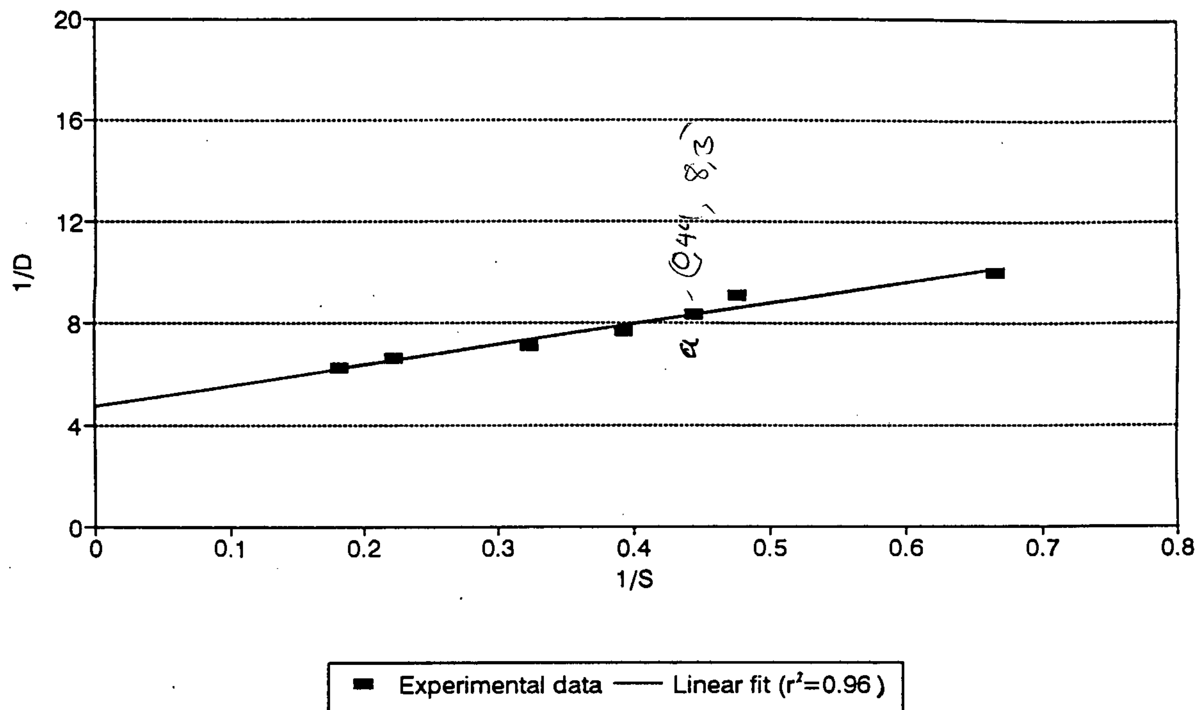


FIGURE 4.8. Lineweaver-Burk plot of data obtained during the continuous cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C and pH 5.0.

4.3.2.2. Determination of μ_{max} by washout

As the dilution rate increases it is observed that the cell biomass decreases. Eventually a dilution rate will be reached at which the steady state biomass concentration is zero due to cell wash-out (Pirt 1975). This is known as the critical dilution rate (D_c). The maximum growth rate (μ_{max}) of a culture can therefore be determined by following the decrease in biomass concentration at known dilution rates. It then follows that $D_c \approx \mu_{max}$ (Pirt 1975). Although complete wash-out of cells was not observed during the continuous cultivation of *P. rhodozyma*, very low biomass concentrations (< 0.15 g/l) were recorded at dilution rates greater than 0.2 h^{-1}. This indicates that the value of μ_{max} could be in the region of 0.2 to 0.23 h^{-1}.

It has been shown that an increase in the total pigment content of *P. rhodozyma* mutants results in a decrease in their maximum specific growth rates (Meyer *et al.* 1993). A maximum specific growth rate of

0.2 h^{-1} was reported for *P. rhodozyma* strain UCD 67-210 (total pigment, 421 μg/g) grown on glucose medium by Johnson and Lewis (1979). This is similar to a value of μ_{max}=0.21 h^{-1} obtained for strain UCT-1N-3693 with a total pigment content of 400 ±20 μg/g. This value of μ_{max} is much higher than the values (μ_{max}=0.12 h^{-1}) reported by Meyer and Du Preez (1994c) for *P. rhodozyma* growth in continuous culture. This most probably is due to the fact that the mutant used for their studies (*P. rhodozyma* J4-3) is a high carotenoid producer (1688 μg/g *cf.* 420 μg/g for UCT-1N-3693). This demonstrates the requirement for a compromise between *P. rhodozyma* strains with a high carotenoid content and those with a high specific growth rate. This is necessary in order to maximize the level of *P. rhodozyma* biomass production during large scale cultivation, because the astaxanthin as well as the protein content of the yeast is important for its use as an animal feed.

4.3.2.3. Discussion of mixing effects in the bioreactor

Two main functions of agitation in microbial cultures are: (i) the assistance of mass transfer between the gas, liquid and solid phases present in the culture, and (ii) mixing of the culture in order to maintain homogeneous chemical and physical conditions in the culture (Pirt 1975). Mixing is a particularly important parameter during continuous cultivation as it is assumed that a feed droplet entering the reactor is instantaneously mixed with the reactor contents.

The effects of micromixing and eddy size in a bioreactor are also important considerations. Dunlop and Ye (1990) showed the decrease in cell yield (g biomass formed per g substrate utilized), during continuous cultivation of *S. cerevisiae* at high dilution rates, to be less pronounced under conditions where the measured eddy size was greater than 100 μm.

Mixing time is defined as the period of time needed for a liquid droplet passing into an agitated vessel, filled with a fluid with the same physical properties as the droplet, to be completely mixed with the bulk of the fluid (Aiba *et al.*. 1965). The mixing time in the reaction vessel, used for batch and continuous cultivation of *P. rhodozyma*, was determined by addition of

a droplet of 1M KCl solution to the vessel containing 1.5 l of distilled water. The conductivity of the water was monitored, and the time taken for the conductivity to reach a new steady state value after the addition of a droplet of KCl solution was recorded. The mixing time for the reactor configuration described in Section 4.3.2 operated at and an impeller speed of 540 r.p.m. was found to be to be between 3 and 5 seconds. This compares favourably with a mixing time of 9 seconds reported by Aiba *et al.* (1965) for a 3 liter vessel with an impeller speed of 300 r.p.m.

4.3.3. Chemostat: Variation of the feed substrate concentration

The concentration of glucose in the substrate feed was altered from 5 to 25 g/l during continuous, aerobic cultivation of *P. rhodozyma* at a fixed dilution rate of 0.1 h^{-1}. Standard culture conditions for temperature (22°C) and pH (5.0) were used. Based on the conversion of glucose to biomass for *S. cerevisiae* (Bailey and Ollis 1986), Appendix A shows calculations confirming carbon limitation of the growth medium. Aerobic growth conditions in the bioreactor were also confirmed by the procedure detailed in Section 6.2.2.

The biomass concentration increased from 2.5 g/l at a feed glucose concentration of 5 g/l, to a maximum of 5.0 g/l at an feed glucose concentration of 10 g/l (Fig 4.9). Further increase in glucose feed concentration resulted in a decrease in the biomass concentration. The biomass yield (g cell dry mass/g glucose utilized) decreased significantly from 0.54 to 0.20 g/g above a glucose concentration of 10 g/l in the feed. Hence an optimum glucose feed concentration for biomass formation of 8 to 10 g/l is reported (Fig. 4.10). The total pigment production (μg/g) also showed an optimum around a feed concentration of 10 g/l glucose, decreasing notably above a feed glucose concentration of 12 g/l.

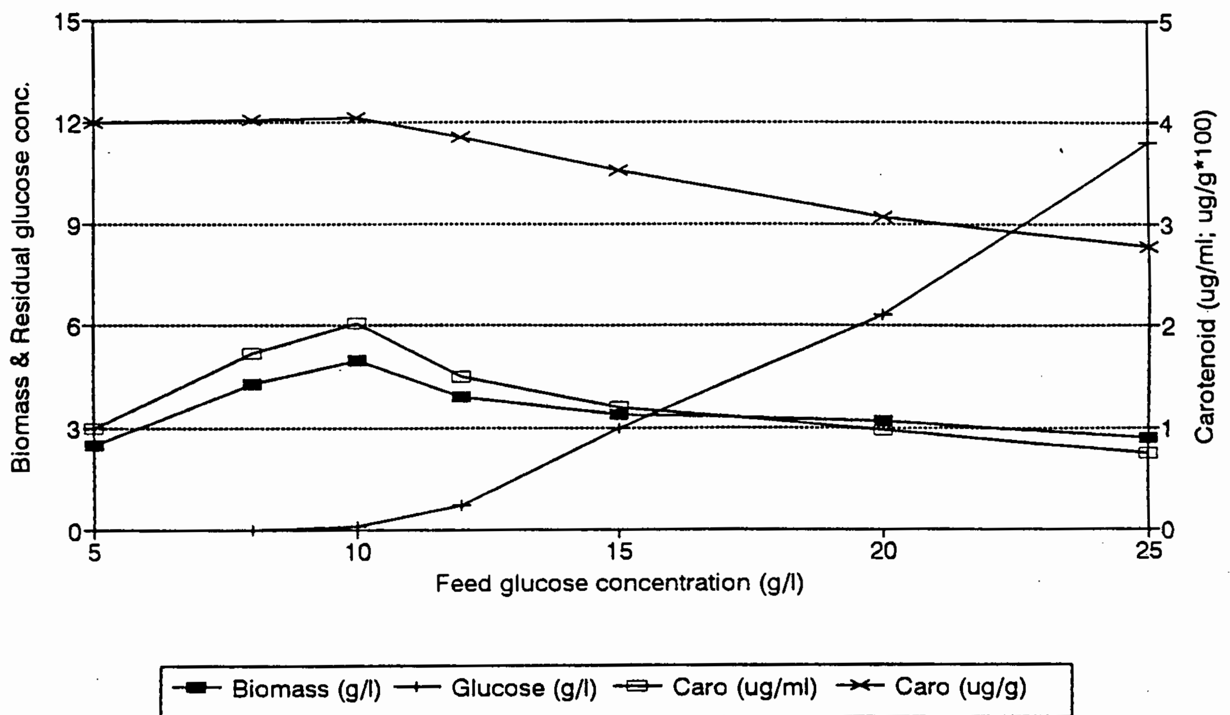


FIGURE 4.9. Effect of varying the feed glucose concentration during continuous cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and D=0.1 h^{-1}.

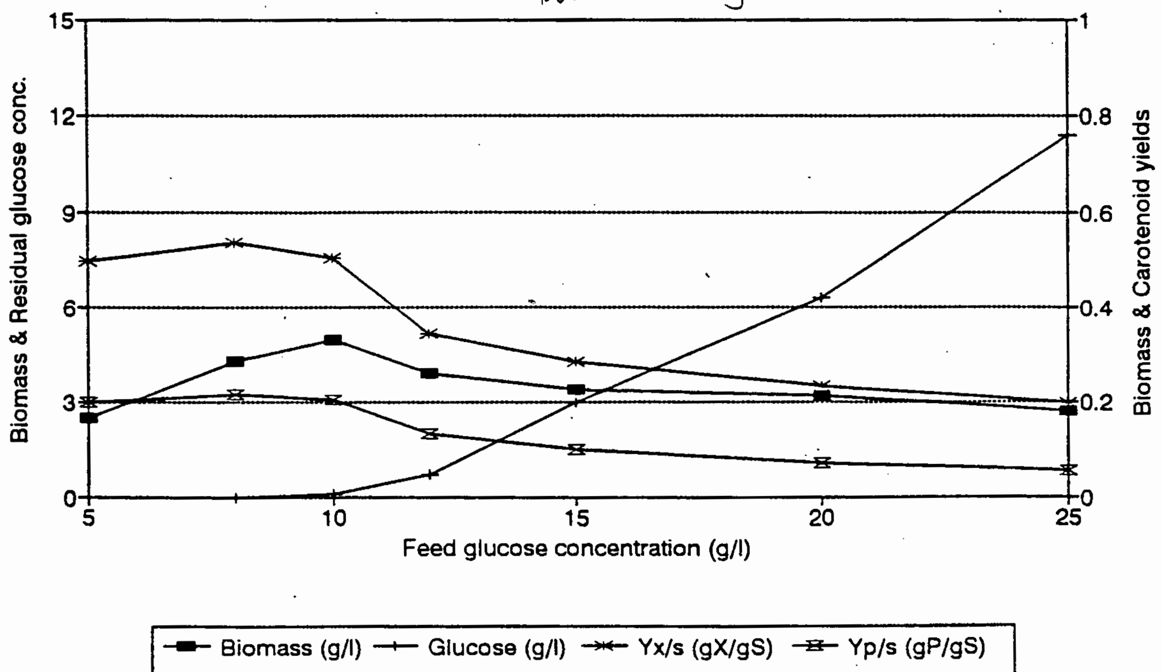


FIGURE 4.10. The effect of varying the feed glucose concentration on biomass and pigment yields during continuous cultivation of *P. rhodozyma* (UCT-1N-3693) as described above.

As the glucose concentration increased above 10 g/l, residual glucose was detected in the culture supernatant. The level of residual glucose detected increased form 0.15 g/l to 11.4 g/l with a corresponding increase in the glucose concentration of the feed from 10 g/l to 25 g/l (Fig. 4.9). A corresponding decrease in the biomass yield at steady state conditions was observed (Fig. 4.10). This result suggests that the aerobic metabolism of *P. rhodozyma* may be subject to glucose repression when grown aerobically at high residual glucose concentrations. This indicates that the Crabtree effect (Section 3.3.3) could be a significant consideration for the industrial production *P. rhodozyma* as a source of astaxanthin.

4.3.4. Chemostat: Variation of the oxygen concentration

A very low total pigment content (<100 μg/g) was observed in a batch culture of *P. rhodozyma* after 120 h aerobic cultivation at an air flowrate of 0.2 v/v.m compared to a pigment content of 400 μg/g at an air flowrate of 2 v/v.m. Since astaxanthin is an oxygenated compound (Fig. 2.1), and the final step in the proposed astaxanthin biosynthetic pathway (Fig. 2.4) is an oxidative step, the effect of increasing the total oxygen concentration in the bioreactor during continuous cultivation ($D=0.1$ h^{-1}) was investigated.

Increasing the total oxygen supplied to the culture resulted in an increase in the residual glucose concentration (Fig. 4.11). The residual glucose concentration increased markedly above a total oxygen concentration of 29% and this coincided with a decrease in the biomass and pigment concentrations (Fig. 4.11). The decrease in biomass concentration at high total oxygen levels could be ascribed to cell death and washout as a result of oxygen toxicity.

An increased oxygen concentration did not stimulate carotenogenesis in *P. rhodozyma*. This is reflected by a decrease in the pigment content of the cells (Fig 4.11) and a decrease in the carotenoid yield ($Y_{p/x}$) in terms of μg pigment produced per g of glucose utilized (Fig 4.12). The decrease in pigment concentration is explained by the fact that carotenoid formation in

P. rhodozyma is growth associated, and the low biomass concentration results in a reduced pigment concentration.

The biomass yield (g cell dry mass/g glucose utilized) remained relatively constant with a change in the oxygen concentration in the bioreactor and a concurrent increase in the residual glucose concentration (Fig 4.12). During chemostat operation, with an increase in the glucose concentration of the feed (Section 4.3.3), the biomass yield was observed to decrease with a paralleled increase in the residual glucose concentration.

Oxygen accepts electrons and is also readily reduced because its two outer electrons are unpaired (Prescott *et al.* 1993). Acceptance of electrons by oxygen results in it being able to exist in a number of energetic states. Triplet oxygen ($^3O_2$) is the most common form. Singlet oxygen ($^1O_2$) has a higher energy level, with the result that it is chemically reactive and extremely toxic to living organisms (Atlas 1984). Oxygen reduction results in the formation of hydrogen peroxide (H_2O_2) and oxygen radicals such as superoxide radicals ($O_2^{-}\cdot$) and hydroxyl radicals ($OH\cdot$). These products of oxygen reduction are also extremely toxic as they are powerful oxidising agents and easily destroy cellular contents (Prescott *et al.* 1993).

Carotenoids are known to quench singlet oxygen effectively, *i.e.* they absorb energy from singlet oxygen and convert it back into its unexcited ground state (Prescott *et al.* 1993). Schroeder and Johnson (1993), investigated the antioxidant role of carotenoids in *P. rhodozyma*. They found that the carotenoid content of *P. rhodozyma* changed in conditions that altered the level of intracellular activated oxygen species. Singlet oxygen and oxygen radicals do occur spontaneously in oxygenated environments and it follows that an increase in the oxygen concentration of the culture could result in an increase in the number of oxygen radicals present in the culture. The decrease in pigment concentration observed in Fig. 4.12 indicates that total oxygen concentration *per se* does not necessarily stimulate carotenoid formation in *P. rhodozyma*.

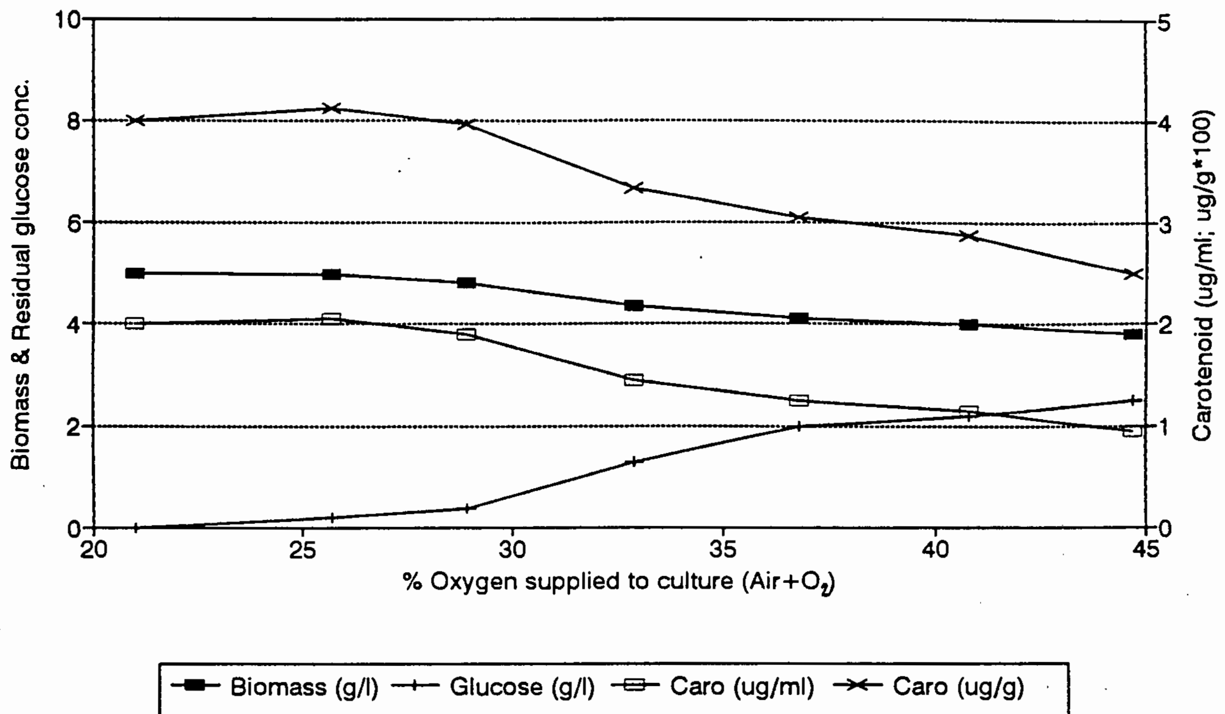


FIGURE 4.11. The effect of increasing the oxygen concentration during continuous cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0, a glucose feed concentration of 10 g/l and D=0.1 h^{-1}.

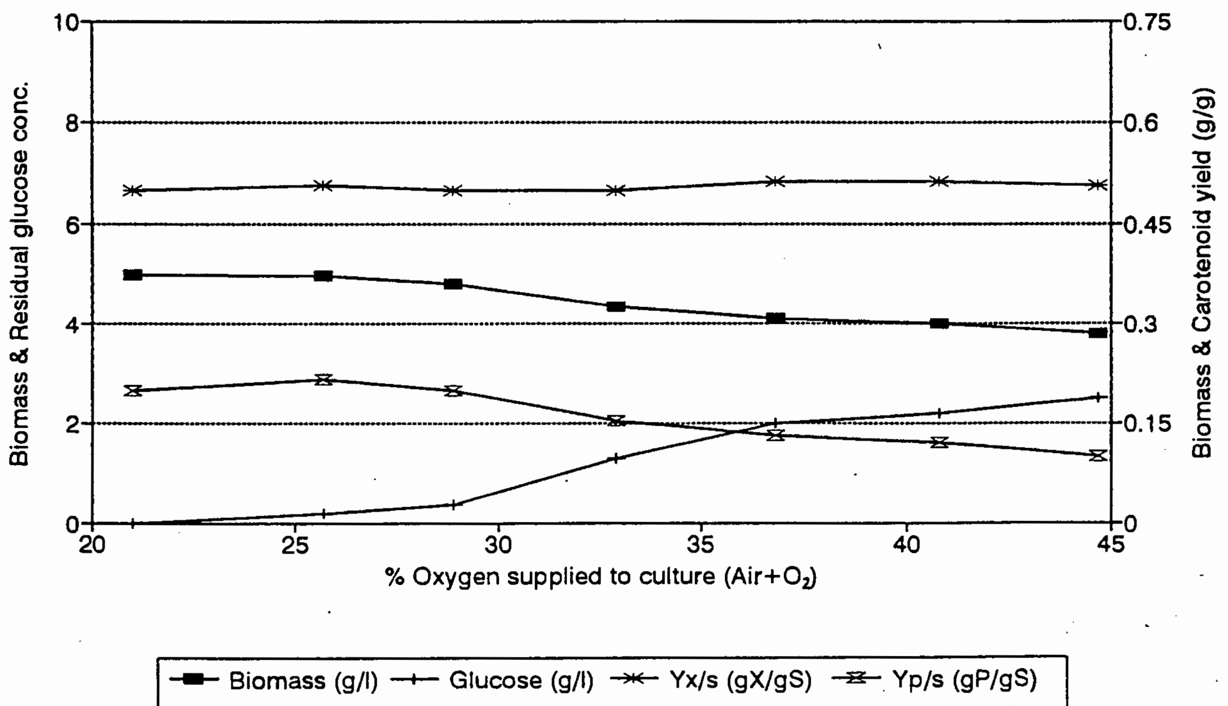


FIGURE 4.12. The effect of increasing the oxygen concentration on biomass and pigment yields during continuous cultivation of *P. rhodozyma* (UCT-1N-3693) as described above.

4.4. CONCLUSIONS

Batch growth of *P. rhodozyma* strain UCT-1N-3693 on glucose medium resulted in a maximum cell dry mass of 6.1 g/l and a total carotenoid content of 416 μg/g. Carotenoid formation in *P. rhodozyma* was growth associated. *P. rhodozyma* was also successfully grown in a batch cultivation using molasses medium supplemented with yeast extract and ammonium sulphate, as a carbon source. A final cell dry mass of 5.4 g/l and a pigment content of 410 μg/g was obtained. Furthermore the biomass and pigment yields (per g substrate utilized) showed good agreement for *P. rhodozyma* growth on both glucose and molasses medium.

The profile for the growth of *P. rhodozyma* in continuous culture, at varying dilution rates (Fig. 4.3), showed a decrease in biomass formation above dilution rates of 0.09 h^{-1}. Increasing the initial glucose concentration lead to a decrease in the conversion of glucose to cell mass with a concurrent increase in residual glucose concentration. This suggests the presence of the Crabtree effect for aerobic glucose fermentation by *P. rhodozyma*. Increased oxygen levels had no effect on carotenoid biosynthesis by *P. rhodozyma*. An increase in the oxygen concentration of the culture did not induce carotenoid formation in *P. rhodozyma*.

High biomass and pigment yields are achieved by *P. rhodozyma* at low residual sugar levels. These low residual levels can be achieved by use of either a continuous or a fed-batch culture system. Large scale industrial astaxanthin production by continuous cultivation of *P. rhodozyma* will not be feasible as a result of its low μ_{max} (0.21 h^{-1}) and concomitant high risk of contamination. The use of fed-batch cultivation of *P. rhodozyma* for commercial production will therefore have to be considered.

CHAPTER 5

GROWTH AND PIGMENTATION MODELLING OF *Phaffia rhodozyma* IN BATCH AND FED-BATCH CULTURE

5.1. INTRODUCTION

Modelling of biochemical reaction kinetics is essential for the design and scale up of any biochemical process. The applicability and complexity of the model depends on the process being modelled.

Complete description of the growth and product formation kinetics by considering the functioning of discrete units (cells) in the culture at a mechanistic level, results in a structured and segregated approach to modelling. Although structured and segregated models are the most realistic, frequently they require numerical solution due to their complexity (Bailey and Ollis 1986).

For many systems segregation is not a critical component of culture responses, resulting in the satisfactory use of nonsegregated models (Schuler and Kargi 1992). Further simplification of process modelling is achieved by an unstructured approach to the model. An unstructured model assumes fixed cell composition, which is valid primarily for steady state continuous culture and the exponential phase in batch culture. Limitations in the use of unstructured nonsegregated models must be recognized. For instance, the response to large or rapid perturbations cannot be described satisfactorily by unstructured models nor can an unstructured approach be used to predict the growth responses of plasmid-containing cultures (Schuler and Kargi 1992). Having considered the general exceptions for an unstructured and nonsegregated approach to modelling, it must be noted that this approach is still applicable to many situations involving microbial growth and product formation, and in most cases it allows for an analytical solution.

Due to the fact that very little is known about the biosynthetic pathway for astaxanthin formation in *P. rhodozyma* and the enzymes regulating its formation (Johnson and An 1991), a structured approach to the modelling of cell growth and carotenoid production is not possible. Hence an unstructured approach, using simple Monod kinetics, was taken. The adverse effect of high sugar levels on the biomass and pigment yields of *P. rhodozyma* in shake flask and continuous culture (Section 3.3.3 and

Section 4.3.3 respectively) led to fed-batch cultivation being considered a better alternative for the commercial production of astaxanthin. Standard mathematical equations, describing both a batch and fed-batch process, were used to evaluate the growth and pigmentation of *P. rhodozyma*. Substitution of kinetic data discussed in Section 4.3.2 into these equations resulted in a model that was used to predict final biomass and pigment yields for the fed-batch cultivation of *P. rhodozyma* on both glucose and molasses medium.

5.2. MICROBIAL GROWTH AND PRODUCT FORMATION MODELLING

5.2.1. Unstructured batch growth model

When microorganisms are introduced to fresh culture medium a lag phase, with no immediate increase in biomass or cell number, is usually observed. After this period of adaption of the cells to their new environment, they begin to multiply rapidly and the cell mass and cell density increase exponentially with time. This is known as the exponential phase. During this phase each microorganism divides after a constant average interval. The resulting exponential cell population increase can be described by the Malthus equation:

$$\frac{dX}{dt} = \mu X \qquad (5.1)$$

where μ is the specific growth rate of the microorganism and X is the biomass formed. Exponential growth is unsustainable and population growth eventually ceases. This period is known as the stationary phase. Hence a typical batch growth curve assumes a sigmoidal shape. Microbial populations enter the stationary phase for several reasons including nutrient depletion, restricted oxygen transfer and the accumulation of toxic waste products. Equation 5.1 can be modified to describe this stationary phase population by the inclusion of an inhibiting factor proportional to the square of the active biomass concentration, giving:

$$\frac{dX}{dt} = \mu X\,(1 - X/X_{max}) \qquad (5.2)$$

where X_{max} is the stationary phase biomass concentration. The effect of substrate limitation is taken into account by the value of X_{max}. Equation 5.2 is known as the logistic equation and its integrated form (Equation 5.3) represents the sigmoidal shape describing a typical batch growth curve:

$$X = \frac{X_o\, e^{\mu t}}{1 - X_o(1 - e^{\mu t})/X_{max}} \qquad (5.3)$$

The logistic equation is one unstructured approach to the modelling of microbial growth. The second approach implies that microbial growth is limited by the amount of substrate supplied in the culture medium. A simple mathematical model predicting the growth curve for a batch culture which is limited by a single substrate (S) can be derived from the Monod equation (Section 4.3.2.1):

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad (5.4)$$

and the following equation which describes the biomass growth yield in terms of the increase in cell mass consequent to utilization of substrate in the medium:

$$Y = \frac{(X - X_o)}{(S_o - S)} \qquad (5.5)$$

where S_o is the initial value of growth limiting substrate, and Y is the cell yield (g biomass/g substrate utilized).

Substitution for μ and S in Equation 5.1 results in:

$$\frac{dX}{dt} = \frac{\mu_{max} X(YS_o + X_o - X)}{K_s Y + YS_o + X_o - X} \qquad (5.6)$$

Integration of the above equation with respect to X and t gives:

$$\mu_{max} t = \ln(X/X_o)\frac{(K_s Y + YS_o + X_o)}{(YS_o + X_o)} - \ln\{(YS_o + X_o - X)YS_o\}\frac{K_s Y}{(YS_o + X_o)} \qquad (5.7)$$

This equation describes the sigmoidal-shaped batch growth curve.

The above equations do not take into account the fact that the cells catabolize cellular reserves during the stationary phase for new building blocks and for energy-producing monomers (Schuler and Kargi 1992). This is termed endogenous metabolism. Considering the effect of endogenous metabolism on cell growth gives:

$$\mu_A = \mu_T - k_d \qquad (5.8)$$

where μ_A is the apparent specific growth rate and μ_T is the total specific growth rate. In the presence of endogenous metabolism the Monod equation (Equation 5.4) can be re-written as:

$$\mu_A = \frac{\mu_{max} S}{(K_s + S) - k_d} \qquad (5.9)$$

and biomass formation (X) is described by:

$$X = Y^m[S_o - S] \cdot \frac{\mu_A}{\mu_A + k_d} \qquad (5.10)$$

where, Y^m denotes the maximum biomass yield coefficient (no endogenous metabolism or maintenance energy). Y^m has a single constant value and is independent of growth rate.

The energy required for various essential metabolic functions, such as endogenous metabolism, is termed maintenance energy. A maintenance coefficient (m_s), defined as the measure of the amount of substrate consumed by a cell for functions other than biomass production (Abbott and Clamen 1973), is given by the following equation:

$$m_s = \frac{k_d}{Y^m} \qquad (5.11)$$

5.2.2 Unstructured product formation model

Formation of microbial products is classified into three main categories:

(i) Growth associated products are produced simultaneous to microbial growth and can be described by the relationship:

$$q_p = \frac{1}{X}\frac{dP}{dt} = Y_{(p/x)}\,\mu \qquad (5.12)$$

where q_p is defined as the specific rate of product formation and $Y_{(p/x)}$ is the final product yield (g product formed/g biomass formed).

(ii) Non-growth associated product formation takes place during the stationary phase when the cell growth rate is zero:

$$q_p = \beta \qquad (5.13)$$

where ß is a constant.

(iii) Mixed-growth associated product formation takes place during both the exponential phase and the stationary phase of cell growth. The specific rate of product formation is given by the Luedeking-Piret equation:

$$q_p = \alpha\mu + \beta \quad (5.14)$$

If $\alpha = 0$, the product is non-growth associated, and if $\beta = 0$ the product would be only growth associated with α being equal to $Y_{(p/x)}$.

Now, in the case of growth associated product formation, Equation 5.12 can be written as:

$$dP = q_p\, X\, dt \quad (5.15)$$

in order to describe the product formed in a batch culture over an infinitely small time interval (dt). If q_p is constant, the product concentration after time t, is given by:

$$P = Po + q_p \int_0^t X\, dt \quad (5.16)$$

Integrating Equation 5.12 for X and assuming exponential growth (*i.e.* $X = X_o e^{\mu t}$), gives the expression:

$$P = P_o + q_p X_o (e^{\mu t} - 1)/\mu \quad (5.17)$$

where q_p is dependent on the type of microbial product formation (Equations 5.12, 5.13 or 5.14).

Growth associated product formation may also be described by the logistic equation in the form:

$$P = \frac{P_o\, e^{\mu t}}{1 - P_o(1 - e^{\mu t})/P_{max}} \quad (5.18)$$

where P_o and P_{max} are the initial product concentration and the stationary phase product concentration respectively. Furthermore, for growth associated product formation, the value of P_{max} is related to the maximum biomass produced during the stationary phase.

5.2.3. Fed-batch cultivation model

In fed-batch culture, substrate is fed into the reactor continuously. This method of culture overcomes the problem of substrate inhibition by maintaining a low substrate concentration in the reactor. Due to the fact that there is a substrate feed stream entering the reactor, the culture volume is also altered and this must be taken into account in the equations used to describe the reactor. A simple model for microbial growth in fed-batch cultivation can be developed using a mass balance approach.

The mass balance equation for biomass formation in a fed-batch reactor is given by:

$$\frac{d}{dt}[V \cdot X] = Vr_x + F(t)X_f \qquad (5.19)$$

where F(t) is the volumetric feed flow rate at time t, V is the culture volume r_x is the rate of biomass formation ($r_x = \mu X$) and X_f is the concentration of biomass in the feed stream. Assuming that the density of the culture fluid does not change substantially with time during microbial cultivation, then:

$$\frac{dV}{dt} = F(t) \qquad (5.20)$$

Differentiation of the left-hand side of Equation 5.19 and substituting for dV/dt in Equation 5.20, leads to the following equation:

$$\frac{dX}{dt} = \frac{F(t)}{V}(X_f - X) + r_x \qquad (5.21)$$

Substituting $X_f = 0$ (sterile feed) and $r_x = \mu X$ into Equation 5.21 gives:

$$\frac{dX}{dt} = \mu X - \frac{F(t)}{V} X \qquad (5.22)$$

Similarly, a material balance for the growth-limiting substrate in the culture can be written as:

$$\frac{dS}{dt} = \frac{\mu X}{Y} - \frac{F(t)}{V}(S_f - S) \qquad (5.23)$$

where S_f is the concentration of growth-limiting substrate in the feed stream. Using Monod kinetics (Equation 5.4), substitution can be made for μ into the above two equations. This results in expressions that can be used to model the rates of biomass formation and substrate consumption in a fed-batch reactor.

A fed-batch reactor may be operated at a constant feed rate (resulting in varying culture conditions) or at an exponential feed rate, resulting in a constant substrate concentration and hence constant growth rate in the reactor. In the latter, the substrate is maintained at a low level such that $S \ll S_f$. Then $X = YS_o$, $dX/dt = 0$ and $dS/dt = 0$. The fed-batch system is then said to operating in a quasi-steady state, hence Equation 5.23 becomes:

$$\frac{F(t)}{V} S_f = \frac{\mu X}{Y} \qquad (5.24)$$

Numerical values for kinetic parameters, as well as the feed substrate concentration, can be substituted into the equation in order to calculate the feed rate required for a constant substrate concentration to be maintained while taking into account the volume changes in the reactor.

Now, at quasi-steady state the total biomass formed (X_T) can be defined as $X_T = X_m V$, where X_m is the maximum biomass concentration ($X_m \approx YS_f$ at quasi-steady state). The rate of change in total biomass concentration can then be written as:

$$\frac{dX_T}{dt} = \frac{X_T}{V} F(t) = X_m F(t) = F(t) Y S_f \qquad (5.25)$$

Integration of Equation 5.25 yields:

$$X_T = X_o^T + F(t) Y S_f \qquad (5.26)$$

Equation 5.26 can be used to predict a final biomass yield for a fed-batch reaction under specified initial conditions.

A microbial product profile can be obtained by using the definition of specific rate of product formation (q_p). When q_p is constant then:

$$\frac{dP_T}{dt} = q_p X_m [V_o + F(t)t] \qquad (5.27)$$

where P_T is the total amount of product in the culture ($P_T = PV$). Integration of Equation 5.27 gives:

$$P_T = P_o^T + q_p X_m [Vo + (F(t)t/2)]t \qquad (5.28)$$

In a similar way Equation 2.28 can be used to predict the final amount of product formed.

5.3. EXPERIMENTAL MATERIALS AND METHODS

5.3.1.. Batch cultivation of *P. rhodozyma*

P. rhodozyma mutant UCT-1N-3693, was grown under aerobic conditions in batch culture using the fermentation equipment and method described in Section 4.2.2. The culture medium was exclusively carbon limited and contained glucose at a concentration of 10 g/l. The other components of the culture medium are described in Section 4.2.3.

5.3.2. Fed-batch cultivation of *P. rhodozyma*

The same equipment and method as described in Section 4.2.2. was used for the fed-batch cultivation of *P. rhodozyma* on both glucose and molasses medium. After inoculation of the medium with *P. rhodozyma* mutant UCT-1N-3693, the culture was allowed to grow to stationary phase.

Fed-batch operation was started prior to complete glucose exhaustion from the medium (*i.e.* a detected residual glucose concentration of less than 0.3 g/l). This was accomplished by pumping the feed medium into the reactor using a variable speed peristaltic pump. Nitrogen and phosphate levels in the feed medium (Section 4.2.3) were increased with increasing glucose concentration in order to ensure that carbon was the only limiting nutrient (Appendix A). The feed rate of the medium was adjusted, according to the feed regime calculated by the mathematical model. The reactor was controlled by allowing the fed-batch system to operate at quasi-steady state, with the nutrient consumption rate being nearly equal to the nutrient feed rate (*i.e.* $S \approx 0$ and $dS/dt = 0$). The medium feed rate was checked against the increase in culture volume with respect to time.

5.3.3. Analytical Methods

Analytical methods as described in Section 3.2.2 were used for dry mass, residual substrate and total carotenoid analysis.

5.4. RESULTS AND DISCUSSION

5.4.1 Modelling batch growth and product formation of *P. rhodozyma*

During batch cultivation of *P. rhodozyma*, a typical sigmoidal-shaped curve is obtained for cell growth and carotenoid formation and carotenoid formation appears to be solely growth associated (Section 4.3.1). Reasonable correlation with experimental batch data was found using the integrated forms of Equations 5.1 (Malthus equation for exponential growth) and 5.13 (growth associated product formation assuming exponential growth) in order to describe the exponential growth phase and growth associated product formation respectively (Fig. 5.1 and Fig. 5.2). However, use of these equations neglects the fact that there is a limit to the biomass and carotenoid concentration as a result of the culture entering the stationary phase.

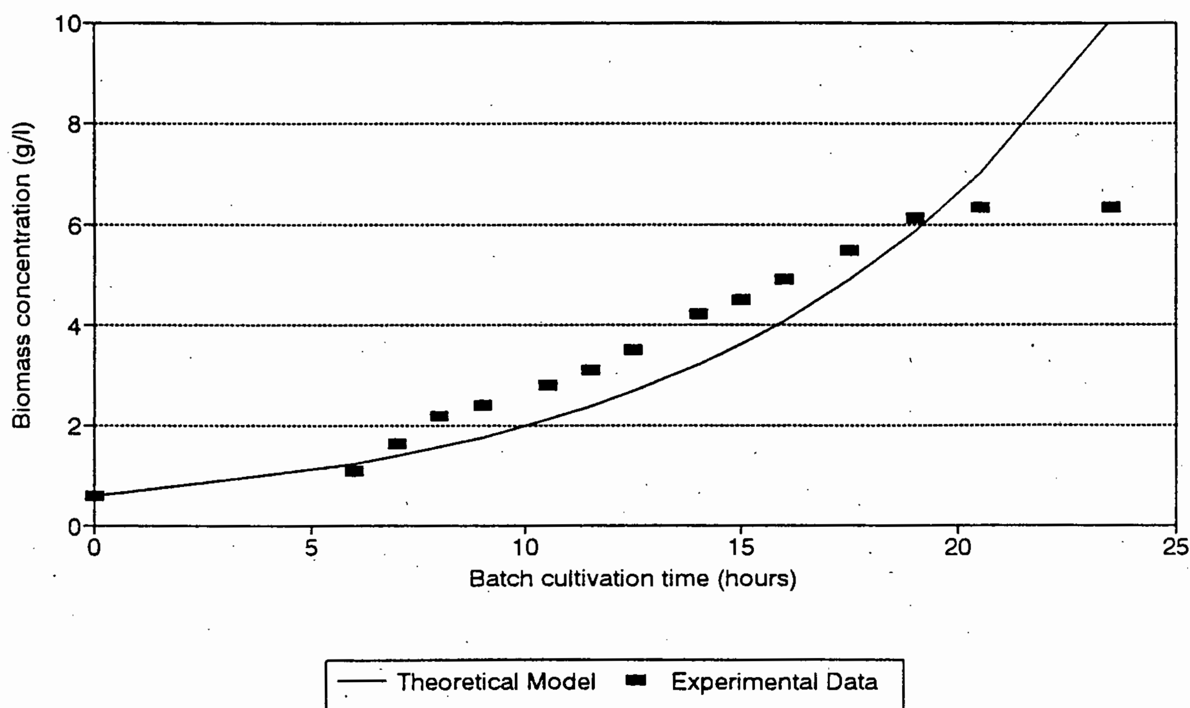


FIGURE 5.1. Biomass formation during batch cultivation of *P. rhodozyma*. Theoretical data is based on the Malthus equation.

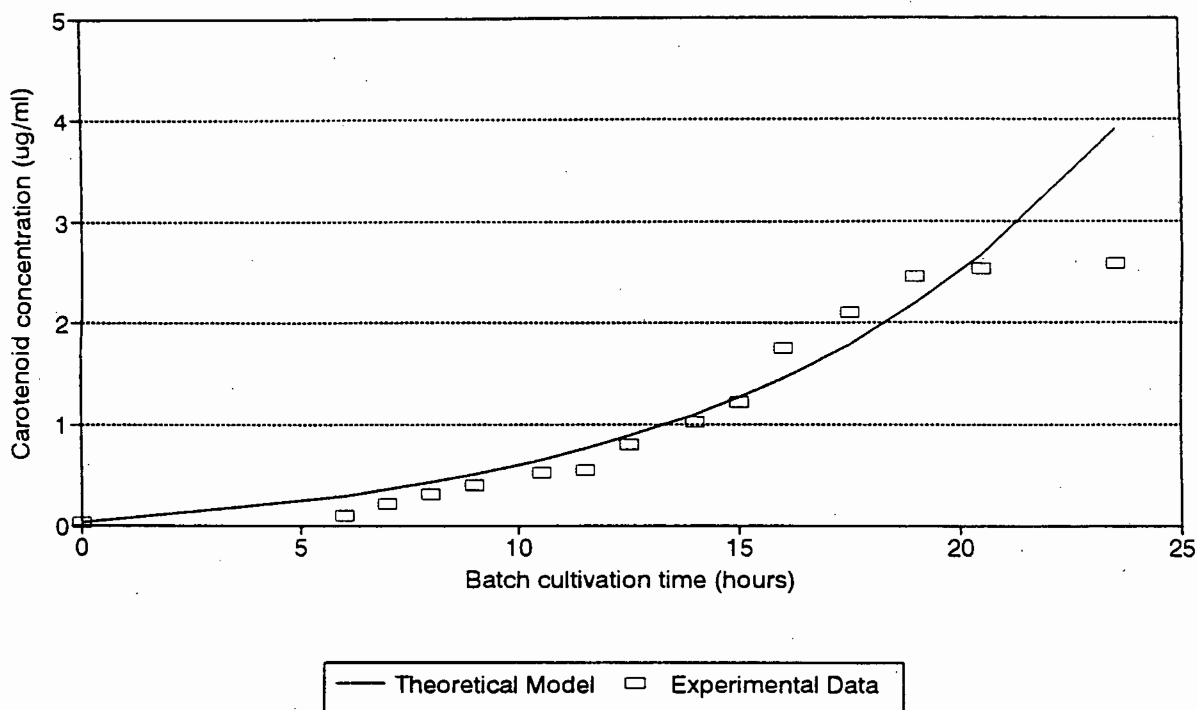


FIGURE 5.2. Carotenoid formation during batch cultivation of *P. rhodozyma*. Theoretical data is based on growth associated product formation.

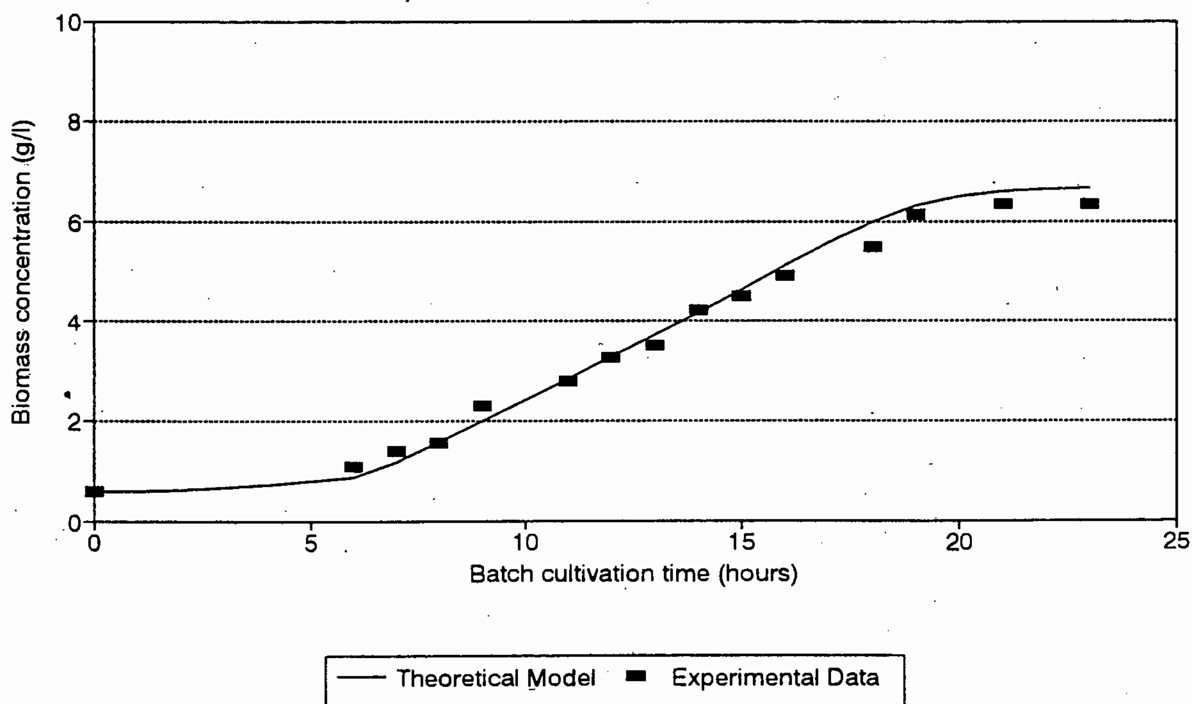


FIGURE 5.3. Biomass formation during batch cultivation of *P. rhodozyma*. Theoretical data is based on a mathematical model assuming exponential growth and Monod kinetics.

Good correlation with experimental data for the batch growth of *P. rhodozyma* , was found using the simple mathematical model given by Equation 5.7 (Fig 5.3). This model is based on exponential cell growth (Equation 5.1), Monod kinetics (Equation 5.4) and the definition of biomass yield (Equation 5.5), and takes the stationary phase into account by assuming that microbial growth is limited by the amount of substrate supplied to the culture.

The logistic equation also takes the stationary phase into account, as it assumes a limiting concentration for both biomass and product. A plot of experimental biomass data substituted into the linearised form of the logistic equation (Fig. 5.4) yielded a better fit ($r^2=0.96$). This indicated that the logistic model can describe biomass formation. Similarly, application of the logistic model to carotenoid formation (Fig. 5.5) also gave a good fit ($r^2=0.94$). The deviation of the data from the fit in both cases showed a trend, as opposed to being scatter. Similar trends have been seen by other researchers when applying the logistic equation to microbial growth and product formation in different organisms.

The maintenance coefficient for *P. rhodozyma* could not be determined by Equation 5.11 owing to a varying biomass yield, possibly as a result of the Crabtree effect and formation of fermentation products such as ethanol and acetic acid (Section 6.3.2 and Section 6.3.3). Furthermore during batch cultivation of *P. rhodozyma*, it was observed that glucose is exhausted before biomass formation ceases. This indicates the metabolism of an intermediate substrate, making determination of the maintenance coefficient very difficult. Meyer an Du Preez (1994c), also report that the maintenance coefficient could not be determined via standard equations. The formation of carotenoids by *P. rhodozyma* is an energy consuming process with the result that changes in astaxanthin content will have a great influence on the maintenance coefficient (Meyer an Du Preez 1994c).

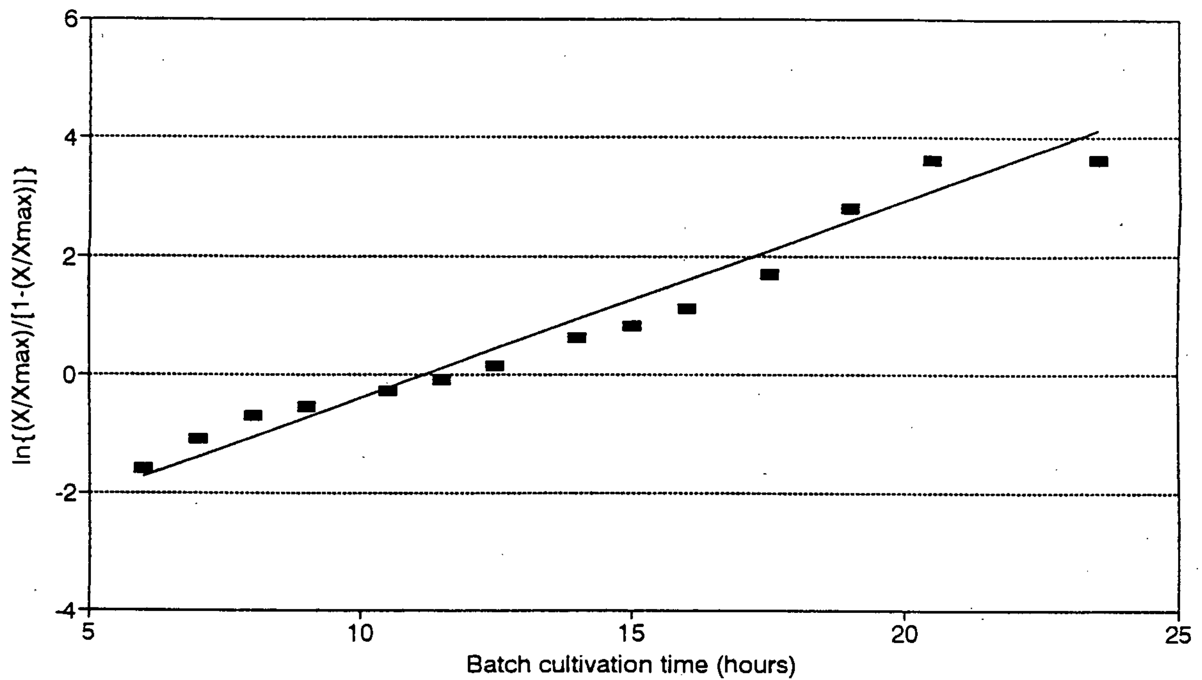


FIGURE 5.4. Linearised form of the Logistic equation applied to biomass data for batch cultivation of *P. rhodozyma*.

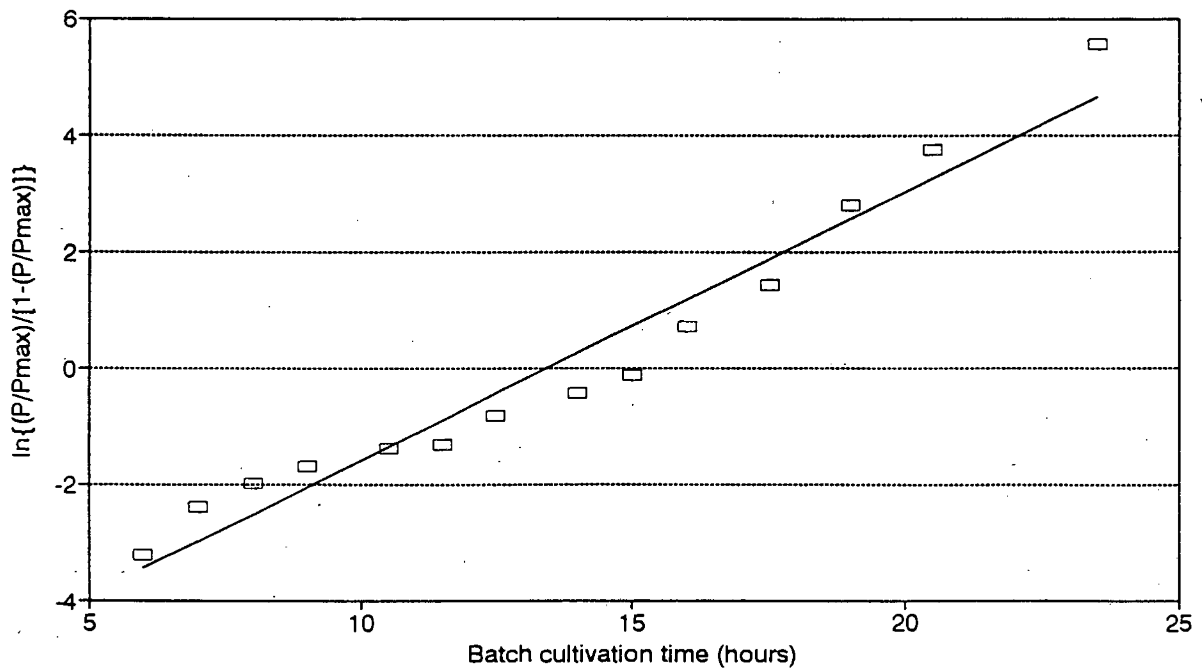


FIGURE 5.5. Linearised form of the Logistic equation applied to carotenoid formation data for batch cultivation of *P. rhodozyma*.

5.4.2. Modelling fed-batch cultivation of *P. rhodozyma*

5.4.2.1. Experimental fed-batch operation and output

Based on an elemental composition and a typical stoichiometric equation for the conversion of nutrients to biomass by *S. cerevisiae* (Appendix A), the limiting levels of both phosphate and nitrogen were calculated and used to approximate required levels of these nutrient for *P. rhodozyma*. The concentration of KH_2PO_4 and $(NH_4)_2SO_4$ in the nutrient feed were increased in proportion to the increase in glucose concentration in the feed in the feed to ensure that the fed-batch feed medium was solely carbon limited.

The first two fed-batch cultures were controlled by varying the substrate flowrate in order to maintain a "constant" glucose concentration in the reactor (data not shown). A glucose concentration in the reactor of 0.5 g/l was assumed. Two factors influenced the choice of this concentration: namely, feed pump constraints and the desire to avoid substrate inhibition at high glucose concentrations. Using a feed glucose concentration of 40 g/l, a final biomass concentration of 13.5 g/l was obtained (dry weight basis). This corresponded to a total biomass yield of 0.52 g biomass formed per g glucose utilized. No residual glucose (within the detection limit of the glucose assay), was observed in the culture at any point during the fed-batch period of operation. The data obtained from these two trial fed-batch cultures was used as a starting point for development of a fed-batch model.

Equation 5.23 was used to determine a feed regime. In order to achieve a quasi-steady state, the residual substrate concentration and the growth rate (μ) must be constant and hence $dS/dt=0$. Thus manipulating Equation 5.23:

$$\frac{F(t)}{V} = \frac{\mu X/Y}{(S_f - S)} \qquad (5.29)$$

If a very low residual substrate concentration is maintained, $S_f \gg S$ and the above equation then equates Equation 5.24.

From the Monod equation (Equation 5.4) substitution can be made for μ in Equation 5.29, resulting in:

$$\frac{F(t)}{V} = \frac{(\mu_{max}S)X/(Ks+S)Y}{(S_f - S)} \qquad (5.30)$$

Substitution was made for values of μ_{max} and K_s (ex. Section 4.3.2.1) in Equation 5.30. An estimate for the value of S in Equation 5.30 was made based on the initial rate of substrate feed to the reactor, a reactor volume of 1 l (Table 5.1). Values for X and Y in Equation 5.30 (Table 5.1) were chosen based on data obtained from the trial fed-batch cultures. Substitution of all of the above values as well as the glucose concentration in the feed (S_f), resulted in the calculation of a feed regime for the fed-batch cultivation of *P. rhodozyma*.

Table 5.1 shows the results of predicted values used in Equation 5.30 versus actual experimental data obtained during fed-batch cultivation of *P. rhodozyma* at feed glucose concentrations of 27, 40 and 55 g/l respectively. Operation of the fed-batch system at quasi-steady was confirmed by the constant value observed for residual glucose concentration. The detection limit for the glucose assay was 0.10 g/l, with the result that a very low culture residual glucose concentration was not accurately detected. By adjusting the feed flowrate a constant growth rate was achieved.

	Substrate conc: 27 g/l		Substrate conc: 40 g/l		Substrate conc: 55 g/l	
Parameters	Predicted data	Experiment data	Predicted data	Experiment data	Predicted data	Experiment data
Residual substrate (S, g/l)	0.29	<0.10	0.33	<0.10	0.39	<0.10
Final biomass (X, g/l)	11.00	10.97	14.00	13.70	19.50	19.00
Yield coefficient (Y, g/g)	0.50	0.57	0.50	0.52	0.50	0.55

TABLE 5.1. Predicted data versus experimental data obtained using a mass balance approach for the calculation of a feed regime during the fed-batch cultivation of *P. rhodozyma*.

5.4.2.2. Mathematical modelling of fed-batch performance

Establishment of a suitable model to enable the calculation of a fed-batch feed regime (Equations 5.24 and 5.30) necessitated the generation of a mathematical model to describe *P. rhodozyma* biomass and carotenoid formation. These are given by Equations 5.26 and 5.28 respectively.

Hence, a theoretical biomass profile based on an initial volume (V_o) of 1 l, a cell growth yield (Y) of 0.5 and the specified feed glucose concentration (S_f) for the particular experiment, was established. Due to the manual adjustment of the feed pump and culture volume losses from sampling, the actual experimental culture volumes and feed rates during the fermentation varied slightly from the theoretical values predicted by the feed regime calculation. Experimental values of F(t) and V_o at each sampling point were substituted into Equation 5.26. In Figs. 5.6 to 5.8, the total biomass content of the culture at various glucose feed concentrations, predicted theoretically from the calculated feeding profile, predicted from the experimental feeding profile and obtained in experiments are compared.

Similarly, Equation 5.28 was used to predict the total carotenoid formation profile of *P. rhodozyma* during fed-batch cultivation. The value of q_p in Equation 5.28 is based on the definition of growth associated product formation given by Equation 5.12. A fed-batch system operates at quasi-steady state when nutrient consumption rate is equal to nutrient feed rate. Since $dX/dt=0$ at quasi steady state then, from Equation 5.22, $\mu=F(t)/V$, *i.e.* $\mu=D$. The value of μ in Equation 5.12 can therefore be estimated by the dilution rate during the fed-batch reaction. From batch data the value of 400 μg/g can be assumed for $Y_{(p/x)}$. The average fed-batch dilution rate was calculated to be 0.025 hr^{-1}, resulting in a value of q_p of 10 μg/g.hr. A value for X_m in the same equation is given by $X_m=YS_f$ and therefore $X_m=20$ and 27.5 g/l for feed substrate concentrations of 40 and 55 g/l respectively. In Figs. 5.9 and 5.10, the total carotenoid content of the culture at various glucose feed concentrations, predicted theoretically from the calculated feeding profile, predicted from the experimental feeding profile and obtained in experiments are compared.

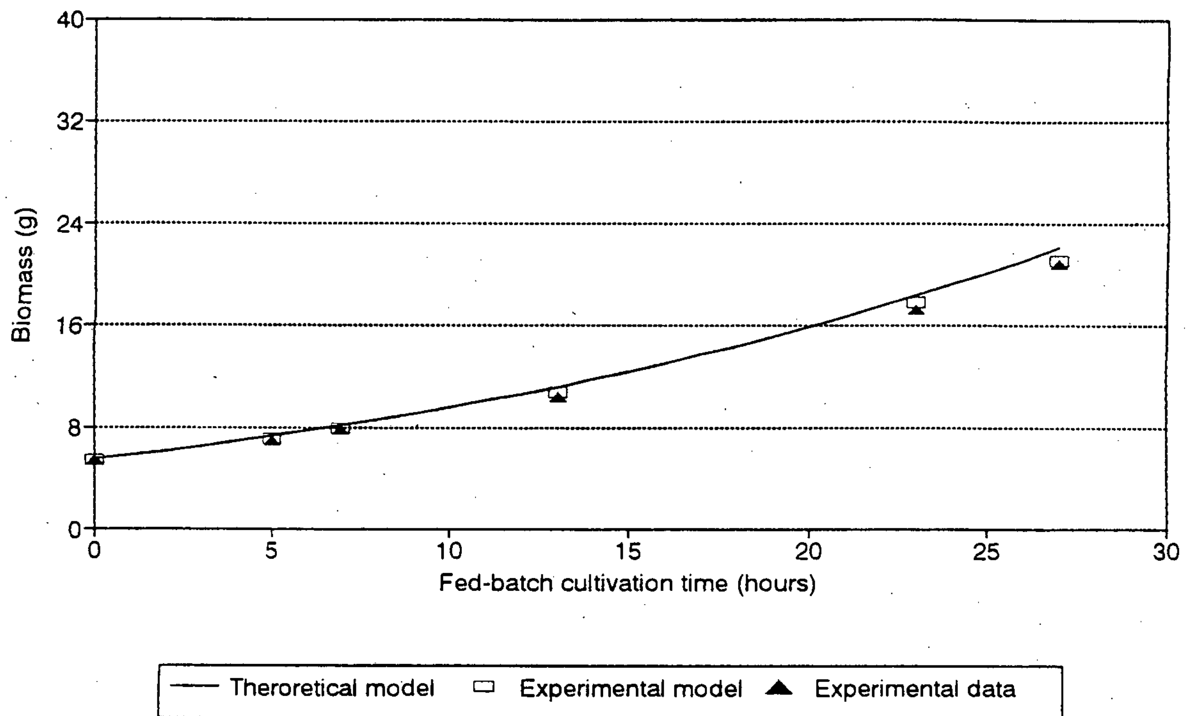


FIGURE 5.6. Model for biomass formation during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 27 g/l.

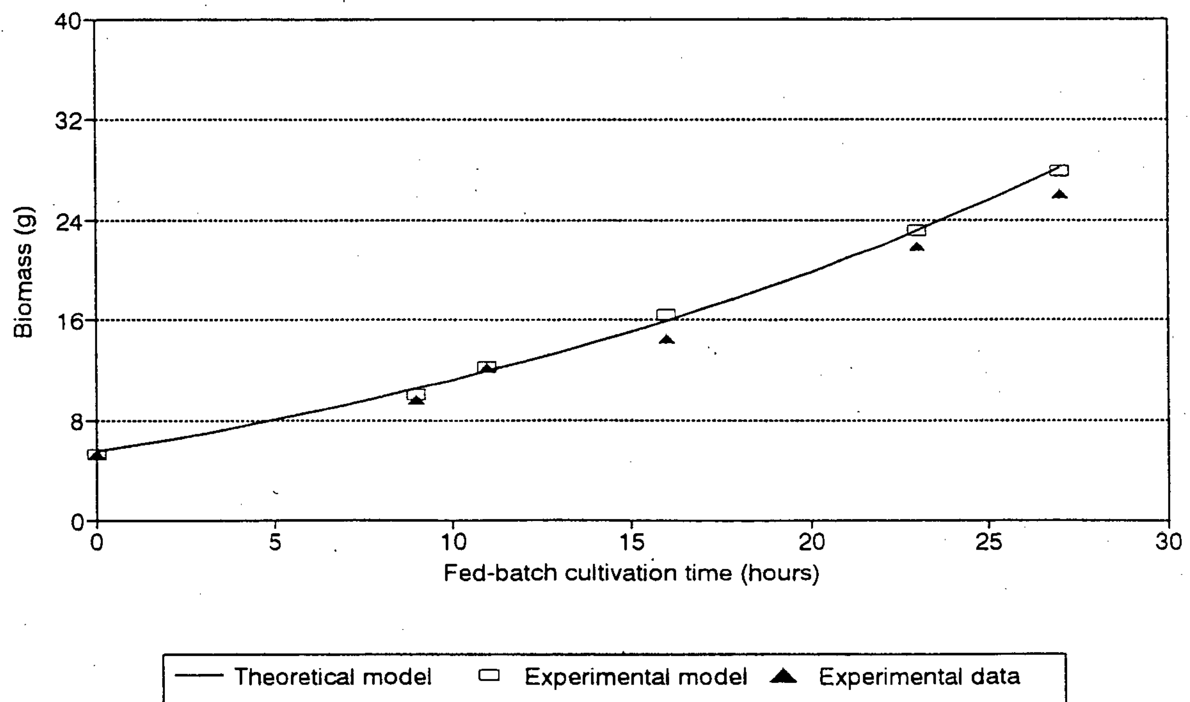


FIGURE 5.7. Model for biomass formation during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 40 g/l.

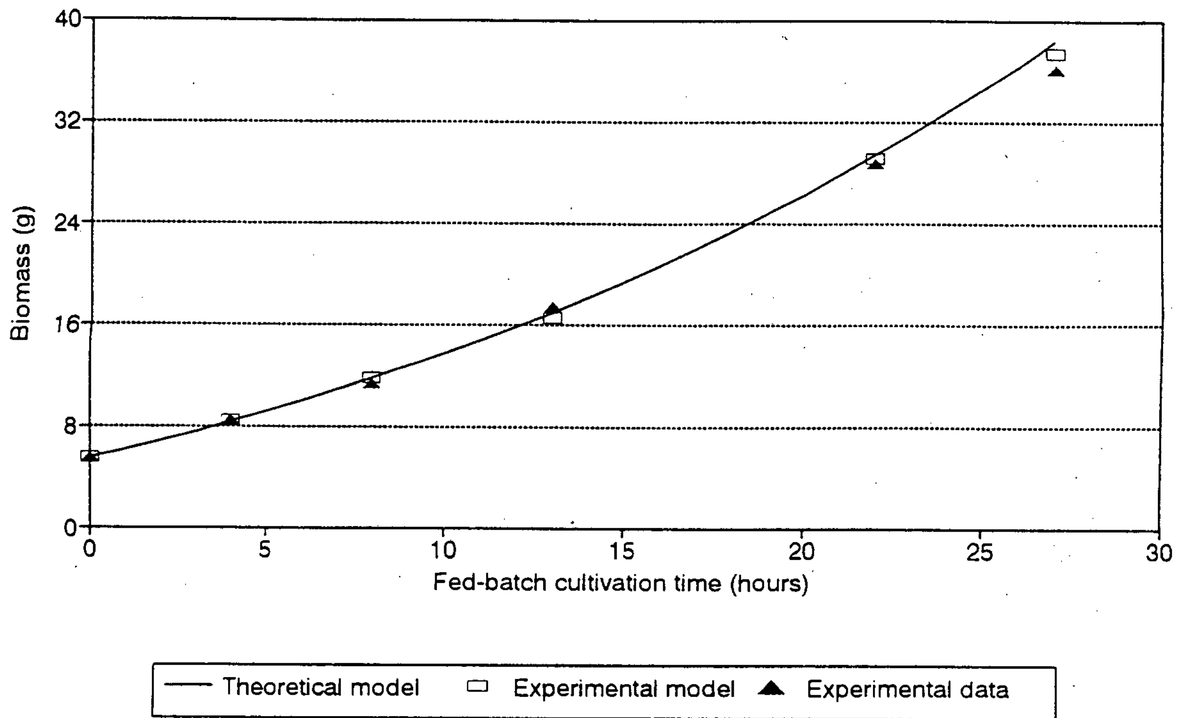


FIGURE 5.8. Model for biomass formation during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 55 g/l.

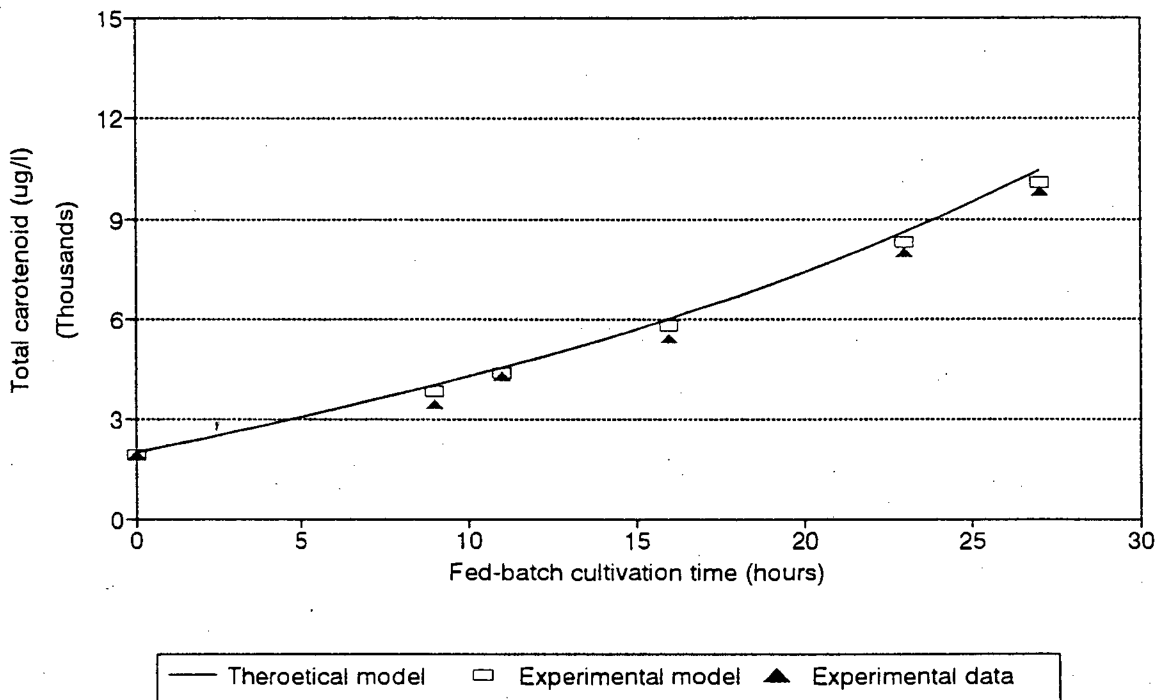


FIGURE 5.9. Model for carotenoid formation during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 40 g/l.

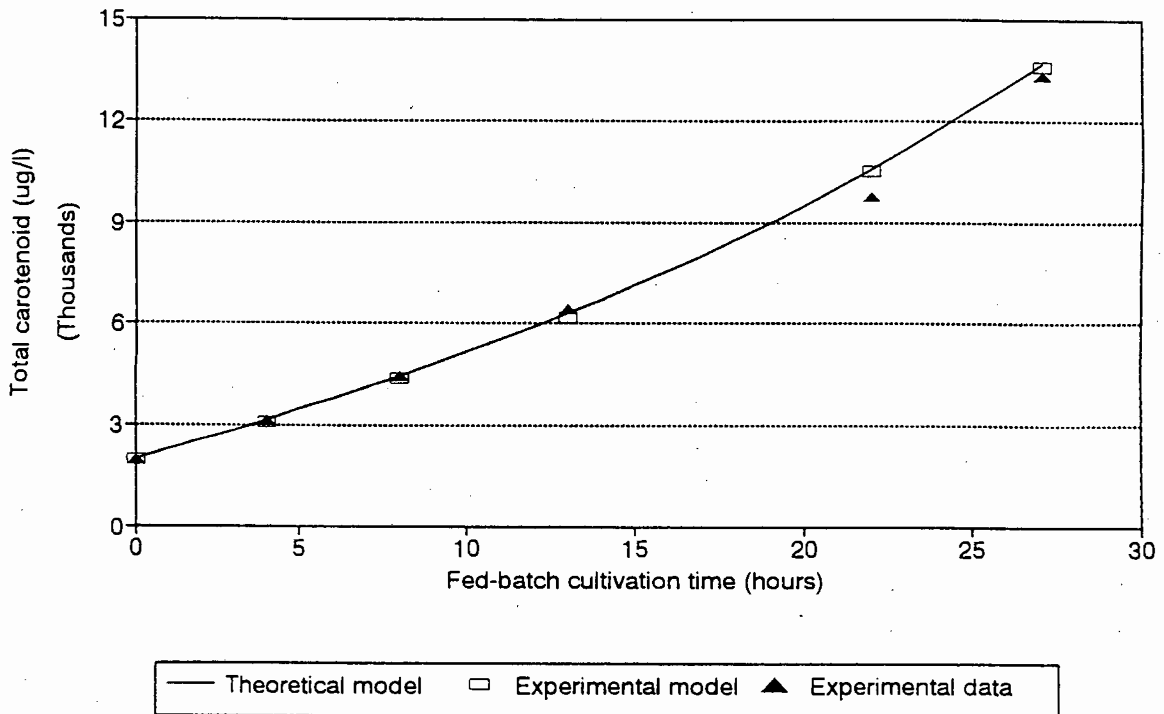


FIGURE 5.10. Model for carotenoid formation during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 55 g/l.

5.4.2.3. Fed-batch cultivation of *P. rhodozyma* on molasses medium

Shake flask experiments and a batch experiment at a 2 l scale confirmed the growth and pigmentation of *P. rhodozyma* on molasses medium (Sections 3.3.4 and 4.3.1 respectively). As a result of the requirement of a cheap substrate for economic large-scale production of astaxanthin from *P. rhodozyma*, fed-batch cultivation of *P. rhodozyma* on molasses medium was investigated.

A feed concentration of fermentable sugars of 40 g/l was chosen for the medium due to the fact that for the industrial cultivation of *S. cerevisiae* on molasses, the sugar concentration is approximately 40 g/l. The concentration of $(NH_4)_2SO_4$ was adjusted in order to ensure carbon limitation of the medium (Appendix A). Using the same kinetic constants and the same approach as detailed above for glucose medium, the experimental data for *P. rhodozyma* grown on molasses medium showed good agreement with the predictions for total biomass and carotenoid concentrations (Figs. 5.11 and 5.12).

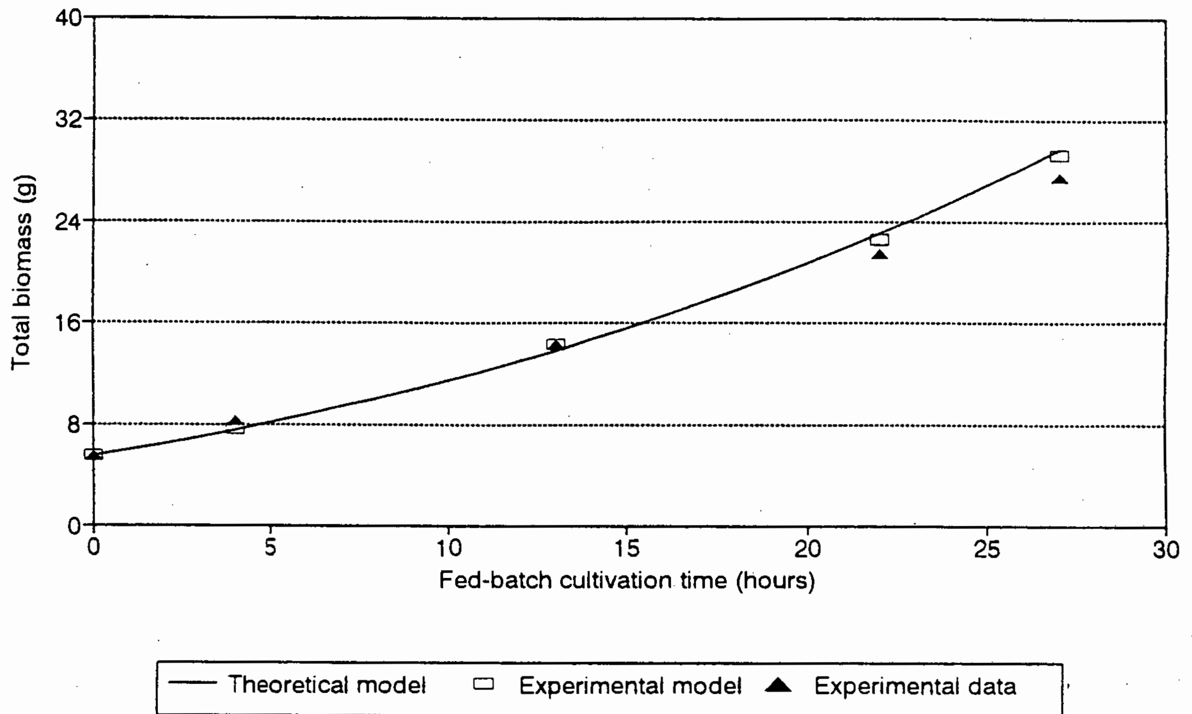


FIGURE 5.11. Model for biomass formation during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, ph 5.0 and on

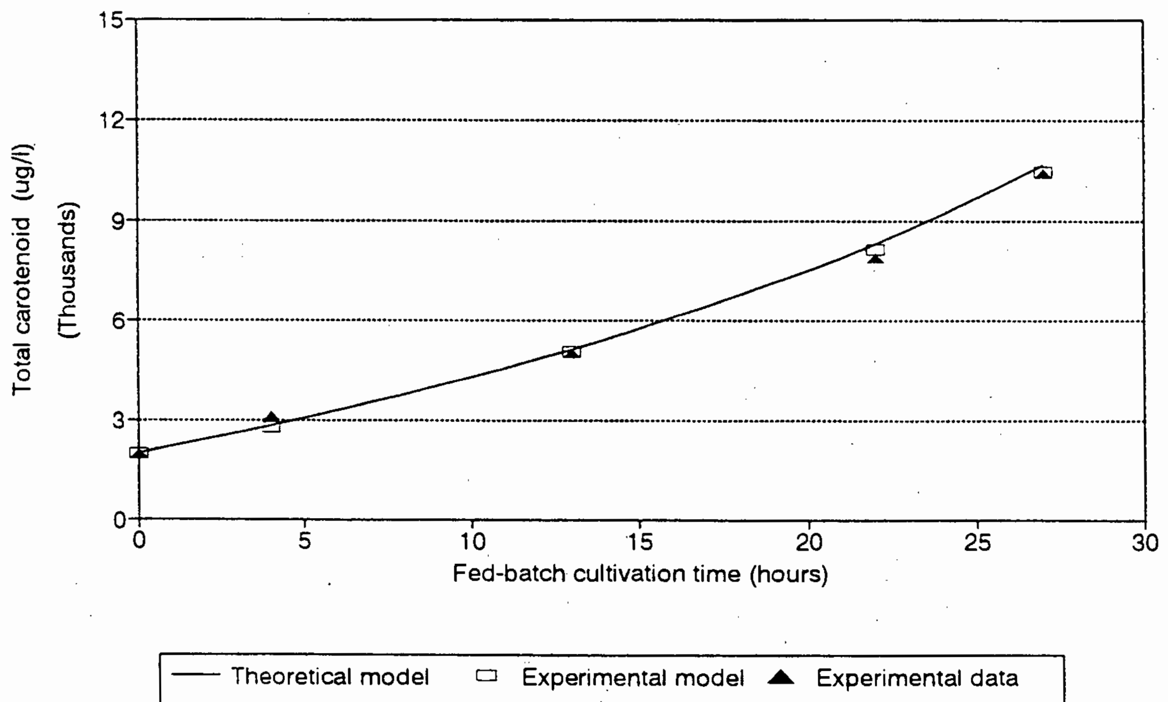


FIGURE 5.12. Model for carotenoid formation during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C pH 5.0 and on molasses medium with a feed sugar concentration of 40 g/l.

Residual sugar analysis of the culture detected a sucrose concentration of less than 0.2 g/l. No other sugars were detected. The assay detection limit for both glucose and fructose was 0.1 g/l. A comparison of the biomass yields and pigment levels for *P. rhodozyma* grown on molasses medium with glucose medium at the same sugar concentration (40 g/l) shows good agreement of the data (Fig. 5.13).

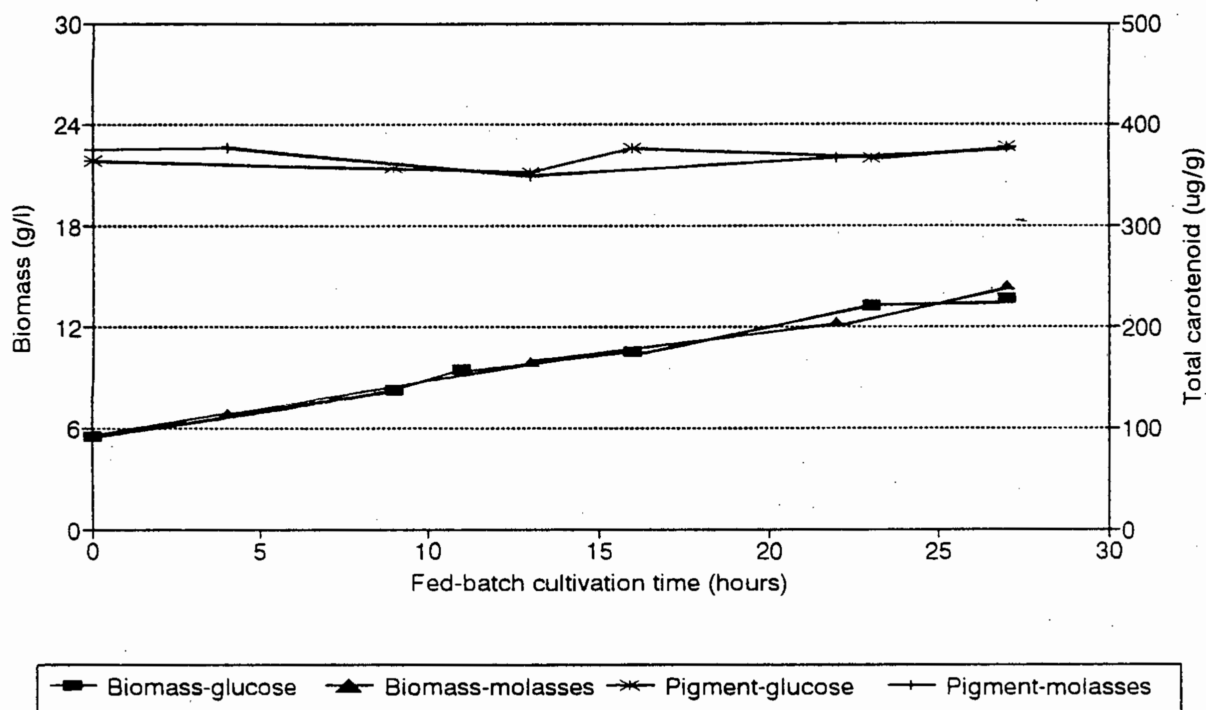


FIGURE 5.13. Comparison of biomass and carotenoid formation during fed-batch cultivation of *P. rhodozyma* on glucose and molasses medium respectively.

5.5. CONCLUSIONS

Simple mathematical equations can be used to predict growth and carotenoid formation of *P. rhodozyma* during batch cultivation. Reasonable correlation with experimental data for the growth and pigmentation of *P. rhodozyma* in batch culture, using the Malthus equation for exponential cell growth and the equation for growth associated product formation assuming exponential growth.

A simple mathematical model using the Malthus equation, the Monod equation and the definition of biomass yield (increase in cell mass as a result of substrate utilization) gave a better fit to experimental data. This was due to the fact the model takes the stationary phase into account during batch cultivation of *P. rhodozyma*. Similarly, use of the logistic model provided an improved description of *P. rhodozyma* growth and pigmentation, due to the fact that it assumes a limit for both biomass and carotenoid formation.

Confirmation of the growth association for carotenoid formation in *P. rhodozyma* was demonstrated by the accuracy of the fit between predictions and the batch data using the logistic equation.

Modelling of *P. rhodozyma* growth and pigmentation during fed-batch cultivation was accomplished by the mass balance approach using simple Monod kinetics. The results indicate that a feed pattern can be established, using kinetic data generated in continuous and batch cultivation, in order to obtain desired biomass and carotenoid concentrations.

Successful application of this model for the fed-batch cultivation of *P. rhodozyma* on molasses medium was demonstrated. Similar growth and pigmentation yields are obtained when *P. rhodozyma* is grown on glucose and molasses medium. This result indicates that it should be possible to use molasses as a cheap substrate for the industrial production of astaxanthin.

CHAPTER 6

DEMONSTRATION OF THE CRABTREE EFFECT IN *Phaffia rhodozyma*

6.1. INTRODUCTION

Glucose catabolism in yeasts can occur in several different ways: namely, (i) Aerobic respiration occurs in the presence of molecular oxygen and results in a higher yield of yeast cells at the expense of the production of organic compounds (*e.g.* ethanol); (ii) Anaerobic fermentation occurs in the absence of oxygen and results in a lower ATP yield, a lower yield of yeast biomass and the formation of ethanol and organic acids as fermentation products; (iii) Aerobic fermentation occurs under fully aerobic culture conditions in the presence of high levels of readily metabolizable sugars, resulting in a reduced biomass yield and the formation of fermentation products.

Biomass and growth associated product formation are favoured by aerobic respiration (Bailey and Ollis 1986). The phenomena, involving the suppression of aerobic respiration, is known as the Crabtree effect (De Deken 1965; Wöhrer *et al.* 1981; Berry 1989). The exact regulatory mechanism involved is not clearly understood, however the Crabtree effect is often interpreted as catabolic repression because of the reduced activities of respiratory enzymes in the presence of glucose (Wöhrer *et al.* 1981). Above a critical glucose concentration, the presence of excess glucose can trigger a switch in the mode of yeast metabolism. Dunlop and Ye (1990) postulated that with better micromixing the glucose is transported more effectively to the cell surface. Under conditions of poor micromixing (eddy size $>100\mu m$), failure to transfer adequate glucose to the cell resulted in the onset of catabolite repression at lower dilution rates during the continuous cultivation of *S. cerevisiae*. Therefore it must be noted that mass transfer effects within a can influence the mode of yeast metabolism.

The Crabtree effect is not displayed by all yeasts. In studies conducted by De deken (1965) and Wöhrer *et al.* (1981), the following yeasts were identified as exhibiting the Crabtree effect (Crabtree positive yeasts): *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces fragilis, Debaryomyces globosus, Torulopsis dattila, Brettanomyces lambicus, Nematospora coryli* and *Nadsonia fluvescens*.. Of these yeasts only *S. cerevisiae* and *K. fragilis* were found able to utilize ethanol, produced during aerobic fermentation, for cell growth (Wöhrer *et al.* 1981). Crabtree

negative yeasts included *Saccharomyces fragilis, Candida utilis, Candida tropicalis, Torulopsis sphaerica, Debaryomyces hansenii* and *Pichia fermentans.* Identification of the presence of the Crabtree effect is accomplished by the various methods detailed below.

De Deken (1965) describes a method whereby Crabtree positive yeasts were identified during cultivation of yeast strains on medium containing 30 g/l hexose, by the ratio of O_2 consumed to CO_2 evolved exceeding 1. This ratio is known as the respiratory quotient (RQ) and was obtained by manometric determinations (De deken 1965). Wöhrer *et al.* (1981) classified yeast strains of different genera as either Crabtree positive or Crabtree negative by the ability of the yeasts to metabolise glucose via aerobic fermentation during batch and continuous cultivation. Aerobic fermentation was confirmed by analysis for the formation ethanol and acetic acid as fermentation products under defined aerobic culture conditions. Furthermore, the existence of the Crabtree effect can be identified by a drop in the yeast biomass yield (Berry 1989). This signifies the suppression of aerobic respiration.

Gas-liquid mass transfer of oxygen to the culture is another important consideration as the dissolved oxygen (DO) concentration is a very important parameter during the aerobic cultivation of microorganisms. Since oxygen is sparingly soluble in water it is possible that the rate of oxygen utilization (OUR), at high cell concentrations, could exceed the rate of oxygen transfer (OTR). This leads to oxygen limitation (Pirt 1975; Bailey and Ollis 1986; Schuler and Kargi 1992). The critical oxygen concentration (below which oxygen-limitation occurs) is about 5% to 10% of the saturated DO concentration for bacteria or yeast (Schuler and Kargi 1992). The saturated DO concentration in water at 25°C and 1 atm pressure is about 8.1 to 8.4 mg/l (Pirt 1975). The presence of dissolved salts can alter the saturation value, while increasingly high temperatures decrease the DO saturation concentration. In order to ensure aerobic culture conditions it is necessary to verify that the OTR exceeds the OUR for a given microbial culture.

The Crabtree effect was briefly introduced in Section 3.3.3, when discussing the growth of *P. rhodozyma* on media containing high sugar concentrations. For the successful use of *P. rhodozyma* as an animal feed, both high biomass yields and high astaxanthin levels are required. The purpose of this study is to investigate *P. rhodozyma* growth and pigmentation as a function of glucose concentration and to ascertain whether the Crabtree Effect is in fact exhibited in *P. rhodozyma*. Evaluation of the Crabtree effect in *P. rhodozyma* is accomplished by monitoring for the formation of ethanol and acetic acid, as fermentation products, during continuous and fed-batch cultivation of *P. rhodozyma* under defined aerobic conditions. The effect of increased residual glucose concentration on biomass yield, is also monitored as confirmation of the Crabtree effect in *P. rhodozyma*.

6.2. MATERIALS AND METHODS

6.2.1. Shake flask, continuous and fed-batch cultivation of *Phaffia rhodozyma*

As a control experiment, *P. rhodozyma* was grown under anaerobic conditions in shake flask culture. After inoculation of mutant UCT-1N-3693 in a 500 ml Erlenmeyer flask containing 50 ml YM broth, the contents of the flask was sparged with nitrogen and the flask was sealed with a rubber stopper. Shake flask cultivation was then carried out by the same procedure as described in Section 3.2.1.

P. rhodozyma mutant UCT-1N-3693 was grown in continuous and fed-batch culture as described in Section 4.2.2 and Section 5.3.2 respectively.

During continuous cultivation, the dilution rate of fresh feed medium containing glucose as a carbon source (10 g/l) was varied from 0.09 h^{-1} to 0.2 h^{-1}. For fed-batch cultures, the concentration of glucose in the feed medium was increased from 55 g/l to 125 g/l. Phosphate and nitrogen levels in the feed medium were also increased proportionately in order to ensure that the medium was solely carbon limited (Appendix A).

Fermentation samples were qualitatively analysed for the fermentation products, ethanol and acetic acid (Section 6.2.3).

6.2.2. Establishment of defined aerobic culture conditions

In order to ensure defined aerobic conditions it was necessary to determine the oxygen transfer rate (OTR) in the reactor as well as the oxygen utilization rate (OUR) of the culture. For unicellular microorganisms, the OTR from gas bubbles introduced into the broth by sparging, is usually limited by oxygen transfer through the liquid film surrounding the gas bubbles. The rate of oxygen transfer from the gas to liquid phase is given by:

$$OTR = \frac{dC}{dt} = k_La(C^* - C) \qquad (6.1)$$

where, k_La is the volumetric oxygen transfer coefficient, C^* is the saturated dissolved oxygen concentration and C is the actual dissolved oxygen concentration in the culture broth.

Values for k_La were determined by sparging the contents of the reactor with nitrogen until a dissolved oxygen (DO) concentration of less than 0.1 mg/l was measured by the polargraphic DO electrode. Air was then sparged into the vessel at a flow rate equivalent to 2 v/v.m. The impeller speed was set at 540 r.p.m. and the temperature of the culture medium was maintained at 22°C (Section 4.2.2). The increase in DO with respect to time was recorded. Integration of Equation 6.1 yields Equation 6.2:

$$\ln(C^* - C) = k_La\ t \qquad (6.2)$$

This allows for the determination of k_La from experimental data, by plotting $\ln(C^* - C)$ versus time. The value of k_La is given by the slope of the line. Using this procedure the OTR in distilled water, fresh media prior to inoculation, and the culture broth at the end of a fermentation was determined.

The OUR for *P. rhodozyma* was calculated during exponential batch growth by switching off the air supply to the culture and recording the decrease in

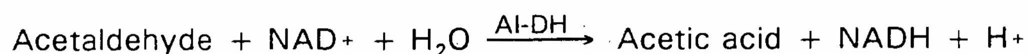
DO with respect to time. The OUR is equivalent to the rate of decrease in DO as a function of time.

6.2.3. Analytical methods

Analytical methods described in Section 3.2.2. were used to monitor cell dry mass, total carotenoid content and residual glucose determination.

Culture supernatants were prepared by microcentrifugation of samples for 5 minutes. The supernatant was stored at -20°C. The supernatant was analysed qualitatively for fermentation products by means of mass spectroscopy in order to determine the abundance of ions characteristic of ethanol and acetic acid. A 15 m long, 0.22 mm I.D. deactivated fused silica capillary column was used for detection of the ions. Typically, only a small fraction (1/100 to 1/250) of the sample in the carrier gas enters the column. The data was recorded using a Hewlett Packard 5971A Mass Selective detector attached to a Hewlett Packard QS/16S personal computer. The data was recorded in the Selected Ion Mode (SIM).

In order to quantify the levels of ethanol produced during continuous cultivation of *P. rhodozyma* at high dilution rates, use was made of a Boeringer Mannheim analysis kit (Cat. No. 176290). This operates on the principle that ethanol is oxidized by nicotinamide-adenine dinucleotide (NAD) to acetaldehyde and reduced nicotinamide-adenine dinucleotide (NADH), in the presence of the enzyme alcohol dehydrogenase (ADH). The acetaldehyde is then oxidized to acetic acid in the presence of aldehyde-dehydrogenase (Al-DH) *i.e.*:



$$\text{Acetaldehyde} + \text{NAD}^+ + H_2O \xrightarrow{\text{Al-DH}} \text{Acetic acid} + \text{NADH} + H^+$$

NADH formed by both reactions is determined by means of its absorbance at 365 nm. The amount of NADH formed is stoichiometric with half the concentration of ethanol in the sample.

6.3. RESULTS AND DISCUSSION

6.3.1. OTR and OUR determinations

Determination of the volumetric oxygen transfer coefficient (k_La) at the given fermenter conditions resulted in the following values:

Distilled water : $k_La = 0.040\ s^{-1}$
Media before inoculation : $k_La = 0.042\ s^{-1}$
Fermentation broth : $k_La = 0.037\ s^{-1}$.

At 22°C the saturated DO concentration is 8.8 mg/l in water at 1 atm (Bailey and Ollis 1986). Salts present in the culture medium lowered the saturation concentration to 8.5 mg/l at 22°C.

During the exponential growth phase of *P. rhodozyma* in batch culture, the average DO concentration was measured to be 3.3 mg/l. Using Equation 6.1 and $k_La = 0.037\ s^{-1}$, the OTR was calculated to be 0.192 mg/l.s. Based on the stoichiometric equation for the formation of yeast biomass (Appendix A) a theoretical OUR of 0.135 mg/l.s was calculated. The OUR of *P. rhodozyma* during the exponential growth phase in batch culture was found to be 0.124 mg/l.s. This value therefore was in good agreement with the theoretical OUR. Noting that the OUR is a function of both biomass concentration (X) and growth rate (μ), the OUR during continuous cultivation with increased dilution rates (i.e. increased values for μ at steady state), and the OUR during fed-batch cultivation with increased biomass concentrations were calculated. The maximum OUR values during continuous and fed-batch cultivation of *P. rhodozyma* were calculated to be 0.129 and 0.113 mg/l.s respectively. Comparison of OTR with the respective OUR values shows that the OTR was significantly higher. This confirms fully aerobic growth conditions for both continuous and fed-batch cultivation of *P. rhodozyma* under the conditions described in Section 4.2.2 and Section 5.3.2 respectively. This confirmation is necessary in order to be able to ascribe changes in cell biomass to substrate inhibition effects (*i.e.* Crabtree effect) and not to an oxygen limitation effect with confidence.

6.3.2. Confirmation of the Crabtree effect in *Phaffia rhodozyma* during fed-batch cultivation

6.3.2.1. The effect of residual glucose concentration on biomass yield

For the fed-batch cultivation of *P. rhodozyma* on medium containing feed glucose concentrations of 27, 40 and 55 g/l respectively (Section 5.3.2), the reactor was controlled by keeping the growth rate constant and adjusting the feed rate of carbon limited medium to the reactor. The residual glucose concentration was found to be lower than the assay detection limit of 0.1 g/l (Figs. 6.1 to 6.3). At these feed glucose concentrations it was also shown that the total biomass measured in the culture followed theoretical predictions, made using a mass balance approach, with good agreement (Figs. 5.5 to 5.7).

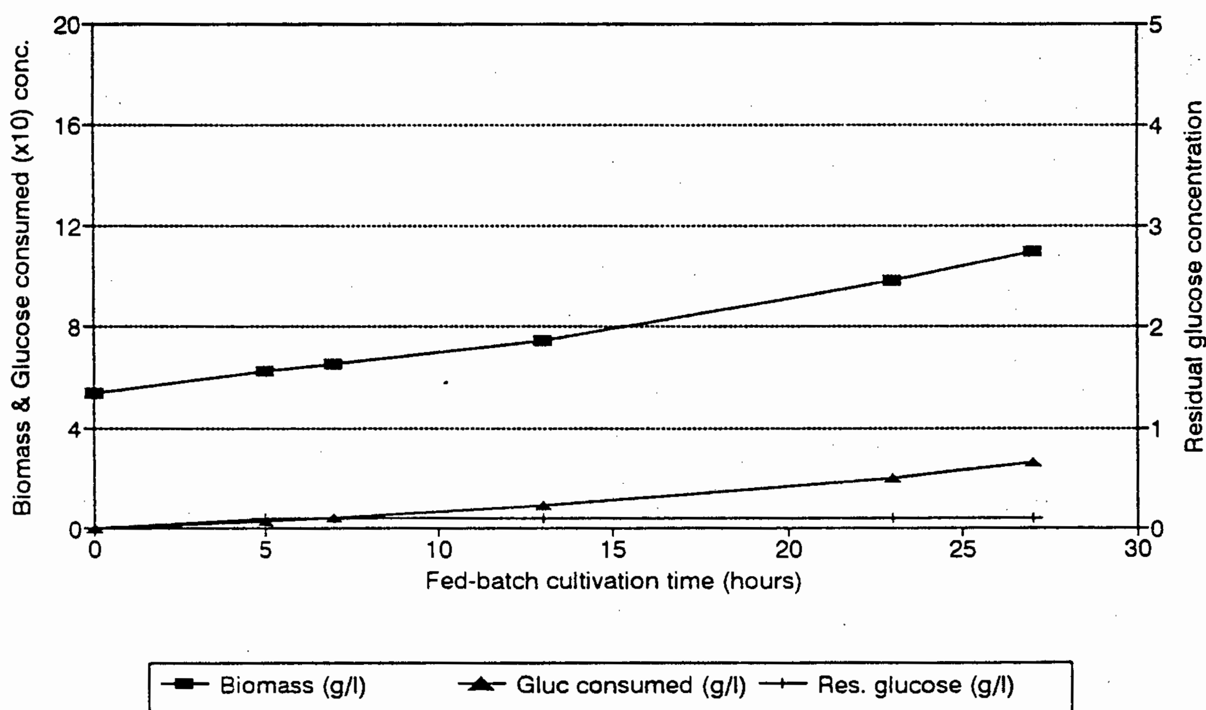


FIGURE 6.1. Fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 27 g/l.

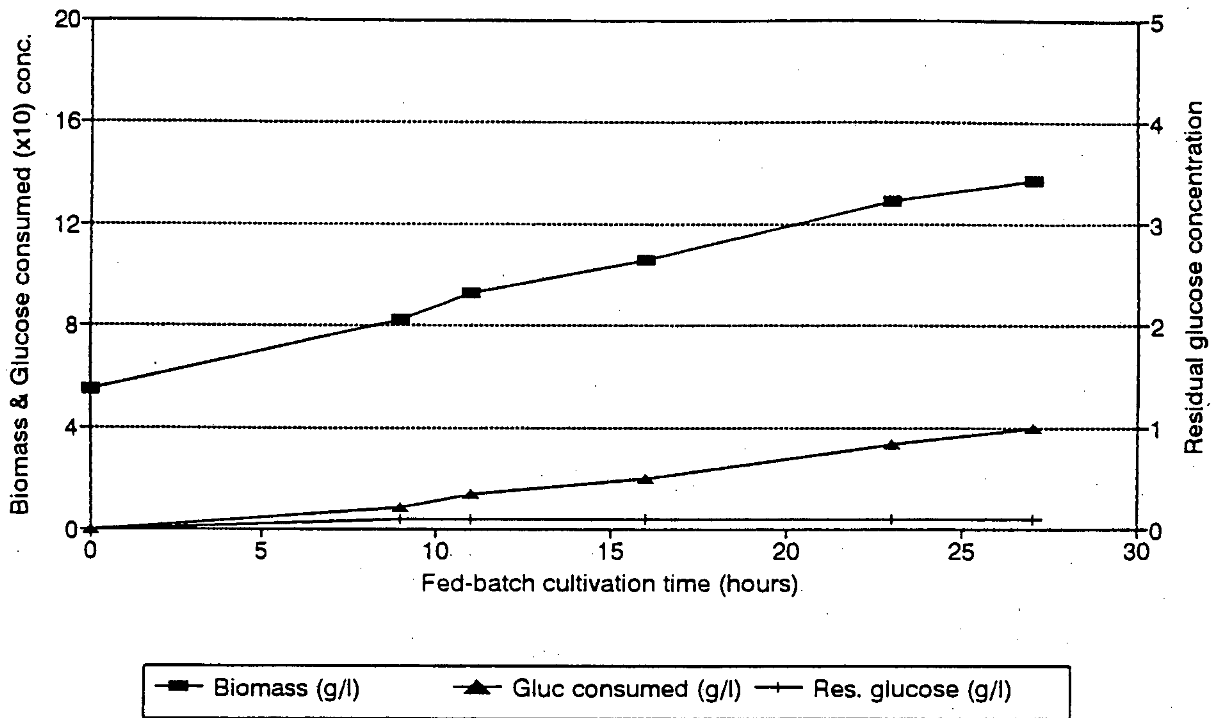


FIGURE 6.2. Fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 40 g/l.

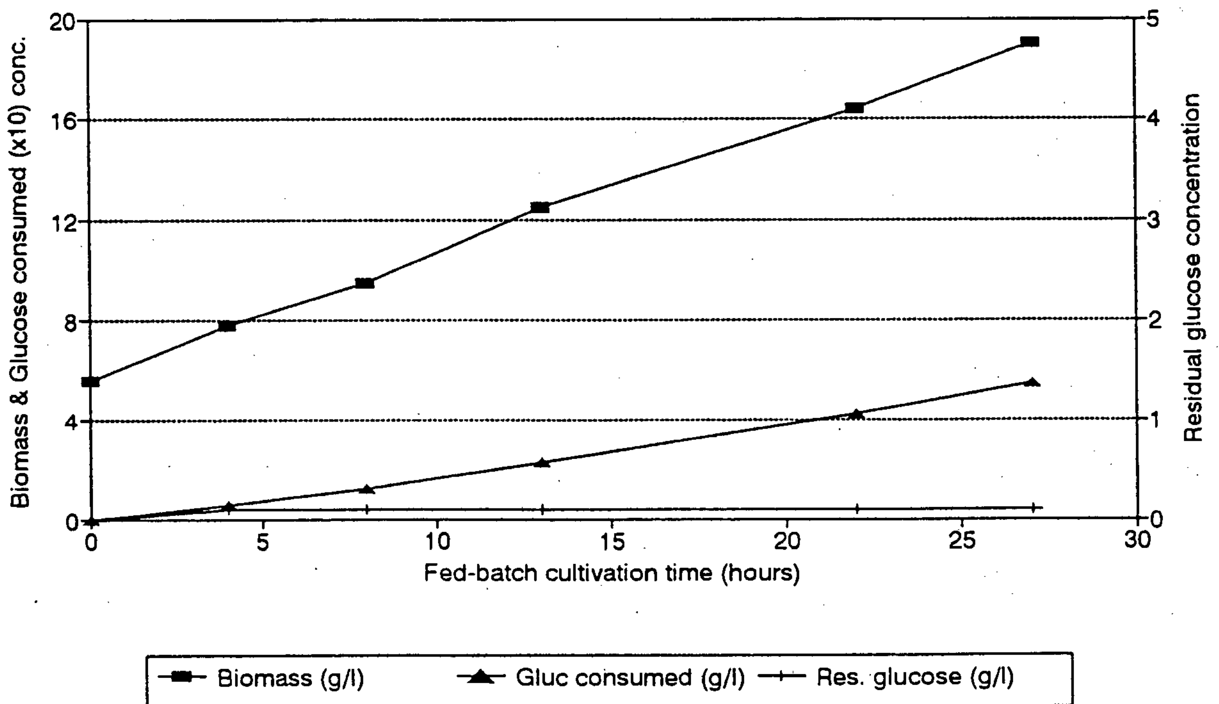


FIGURE 6.3. Fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 55 g/l.

Increasing the glucose concentration of the feed to 125 g/l, resulted in the detection of residual glucose in the culture supernatant (Fig 6.4). Glucose accumulated in the culture, indicating incomplete glucose metabolism. A breakdown of the model predicting biomass formation at a feed glucose concentration of 125 g/l is clearly seen (Fig. 6.5). A decrease in the biomass yield (per g glucose utilized) was also observed with increased residual substrate concentration (Fig 6.6). In contrast, a relatively constant biomass yield (0.5 $\pm$0.05 g/g) was calculated for the fed-batch cultivation of *P. rhodozyma* at glucose feed concentrations of 27,40 and 55 g/l respectively (Fig 6.7). These observations tend to suggest that the Crabtree effect is observed during the cultivation of *P. rhodozyma* at high residual glucose concentrations.

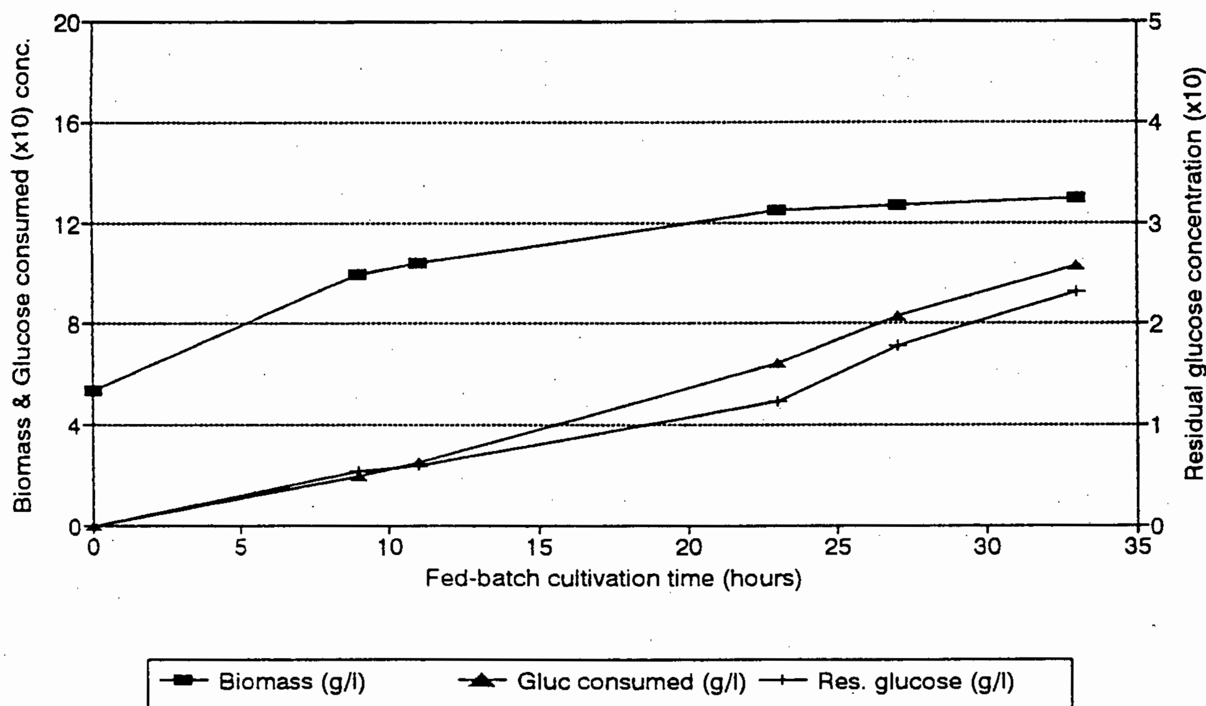


FIGURE 6.4. Fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 125 g/l.

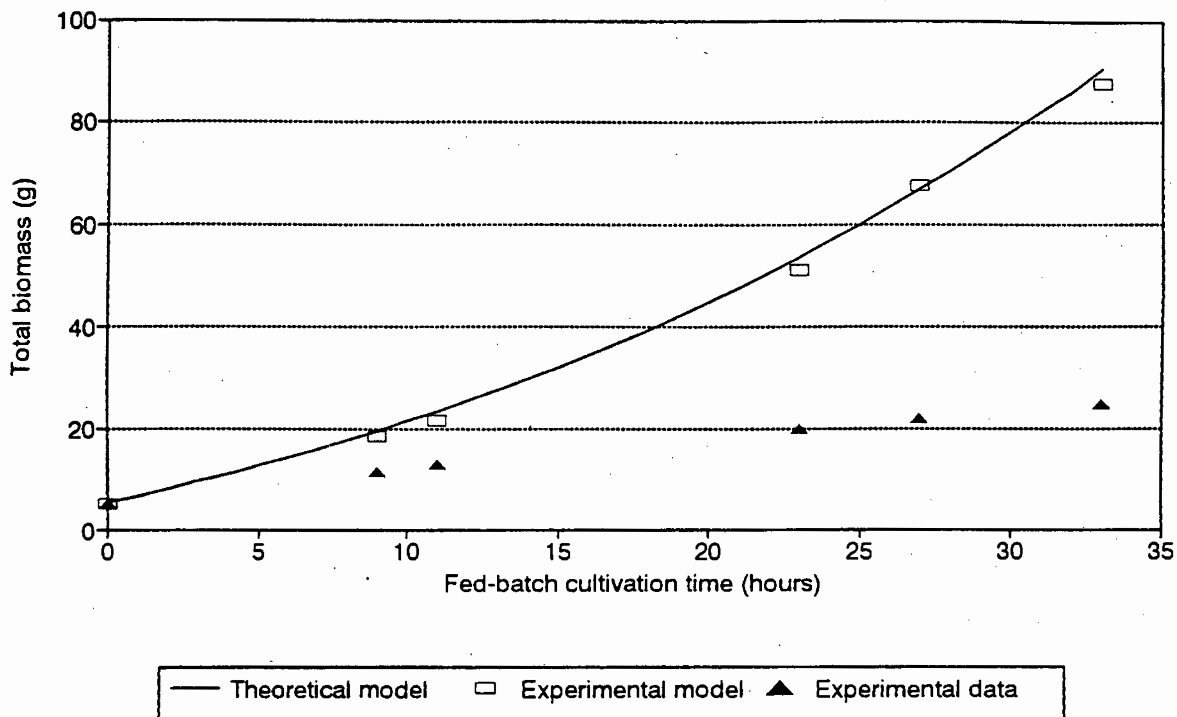


FIGURE 6.5. Breakdown of the model during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) with a feed glucose concentration of 125 g/l.

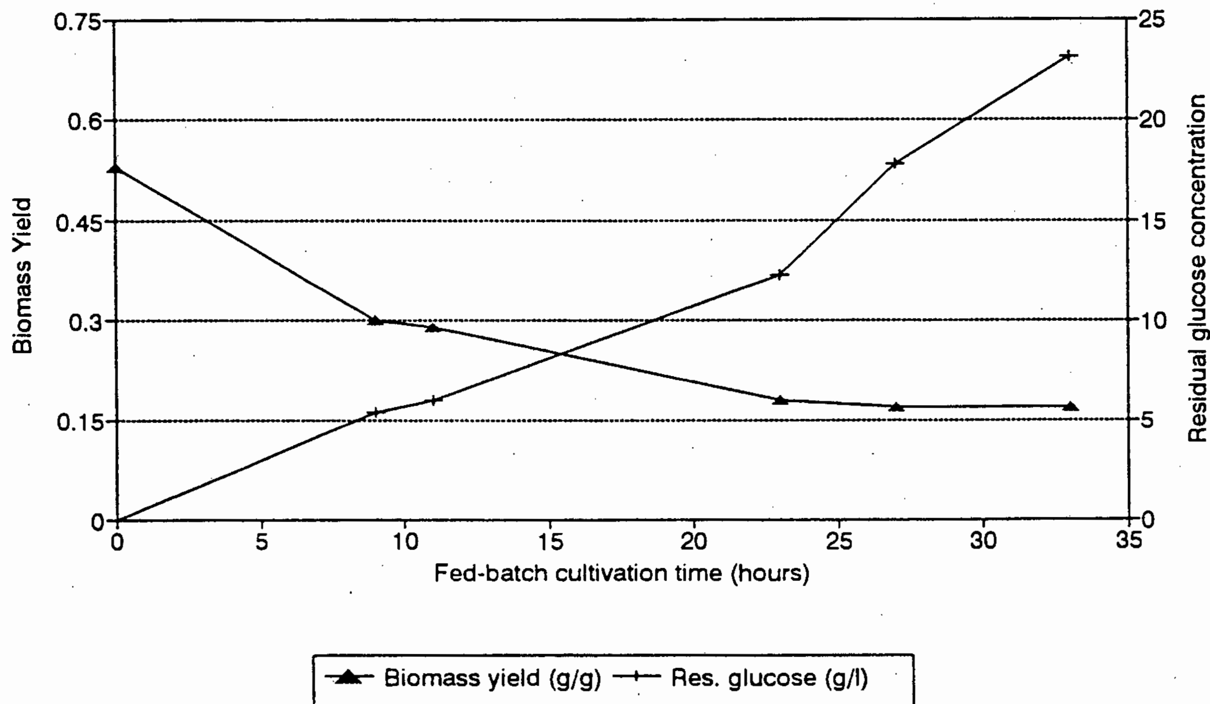


FIGURE 6.6. Effect of increased residual glucose concentration on biomass yield during fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) with a feed glucose concentration of 125 g/l.

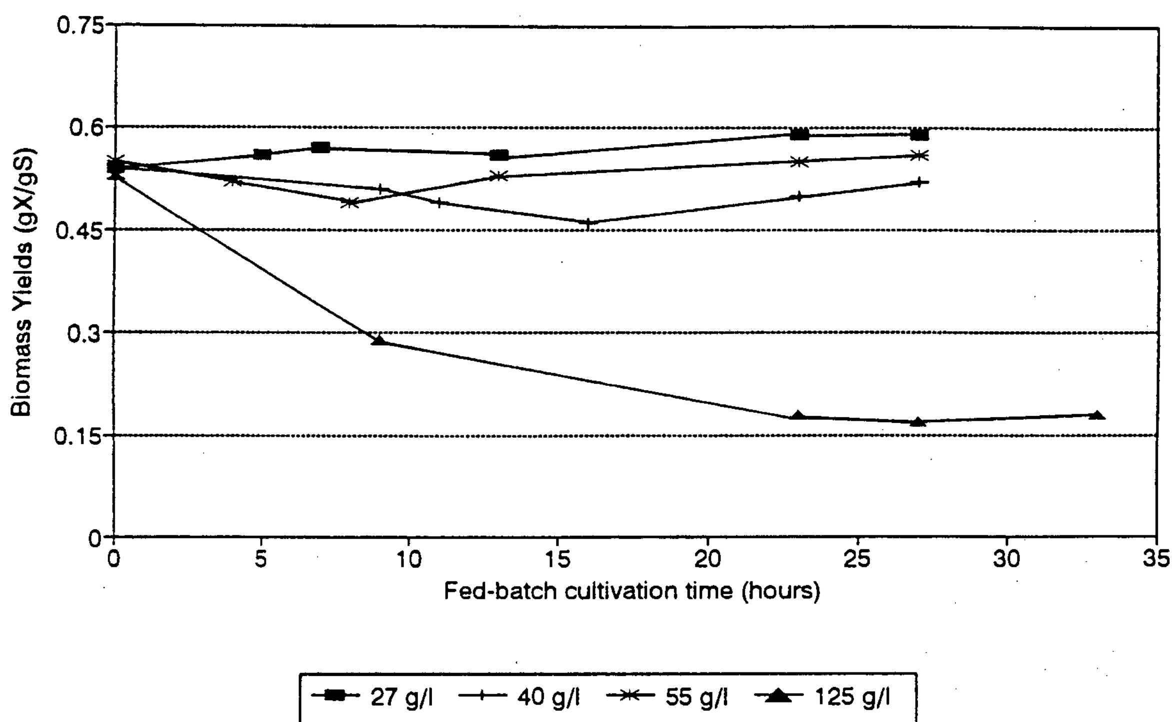


FIGURE 6.7. *P. ,hodozyma* (UCT-1N-3693) biomass yields (gX/gS) for the fed-batch cultivation at various feed glucose concentrations.

6.3.2.2. Detection of fermentation products in culture samples

In order to further validate the possible presence of the Crabtree effect, the supernatant of fed-batch samples at glucose concentrations of 125 g/l and 40 g/l respectively, was qualitatively analyzed by means of mass spectroscopy (MS). As a control, the supernatant of *a P. rhodozyma* culture grown under anaerobic conditions in a shake flask was also analysed by MS. Samples were injected into the MS unit at various time intervals over a 24 minute period. Due to the fact that water is the major constituent of the culture supernatant, its spectrum was initially identified in order to locate the time interval that characterized each sample peak. The results for the identification of water at a mass to charge ratio of 18.0 are shown in Fig. 6.8. Sample peak A-0 is that of culture supernatant grown under anaerobic conditions. Peaks B-0 to B-6 (B-7 is a duplicate of B-6) and C-0 to C-6 are for samples from fed-batch cultivation with a feed glucose concentration of 125 and 40 g/l respectively. In each case the size of the peak bears not significance to the concentration of water in the samples as

the peak size is merely a function of the sample volume injected into the MS unit.

Identification of ethanol and acetic acid as fermentation products present in the culture supernatant was accomplished by selecting characteristic mass to charge ratios of 31.0 and 45.0 for ethanol, and 45.0 and 60.0 for acetic acid. The sample peaks shown in Fig. 6.9 reflect the cumulative values for mass to charge ratios of 31.0, 45.0 and 60.0 detected in the culture samples. A significant level of ethanol and acetic acid is observed in anaerobic culture supernatant (peak A-0). Detection of ethanol and acetic acid during anaerobic shake flask cultivation indicated that *P. rhodozyma* does indeed have the ability to metabolize glucose for the synthesis of compounds other than biomass. The peaks B-0, C-0 and B-1, C-1 are of samples taken at the start and end of the batch growth period respectively and their size indicates only a possible presence of these compounds. Furthermore the small size of peaks B-1 and C-1 tends to indicate that any fermentation products formed during the batch growth period are utilized by the cells as no significant level was detected. In contrast peaks B-2 to B-7 indicate the detection of significant levels of ethanol and acetic acid. This implies that these compounds are formed as fermentation products by *P. rhodozyma* at high residual glucose concentrations. No significant fermentation products were detected for the fed-batch fermentation at 40 g/l (peaks C-2 to C-6). The peaks at B-2 to B-6 coincide with the data where residual glucose was detected in the culture for the fed-batch cultivation with a feed glucose concentration of 125 g/l. Figs. 6.10 and 6.11 (plotted on the same scale) show values for the cumulative abundance of ions (characteristic for ethanol and acetic acid) corresponding to values for residual glucose detected in the same samples for the fed-batch cultivation at feed glucose concentrations of 125 and 40 g/l respectively. These results show that fermentation products are formed at high residual glucose concentrations under aerobic conditions. Hence the Crabtree effect exists for the growth of *P. rhodozyma* on glucose medium at a high concentration.

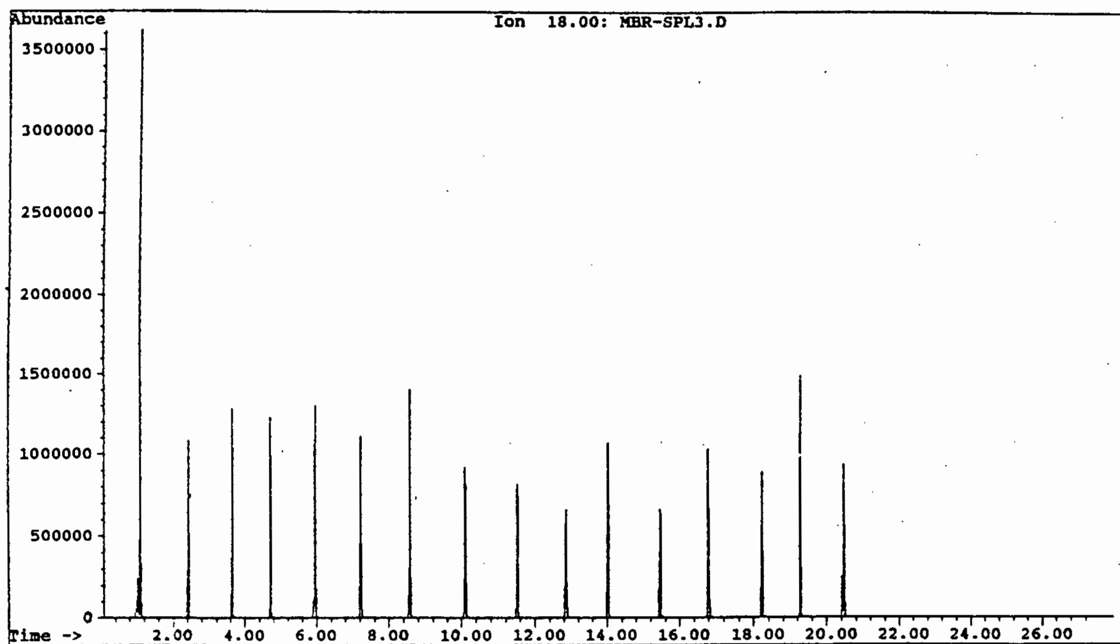


FIGURE 6.8. Mass spectral analysis of water in culture samples during fed-batch cultivation of *P. rhodozyma* with a feed glucose concentration of 125 g/l.

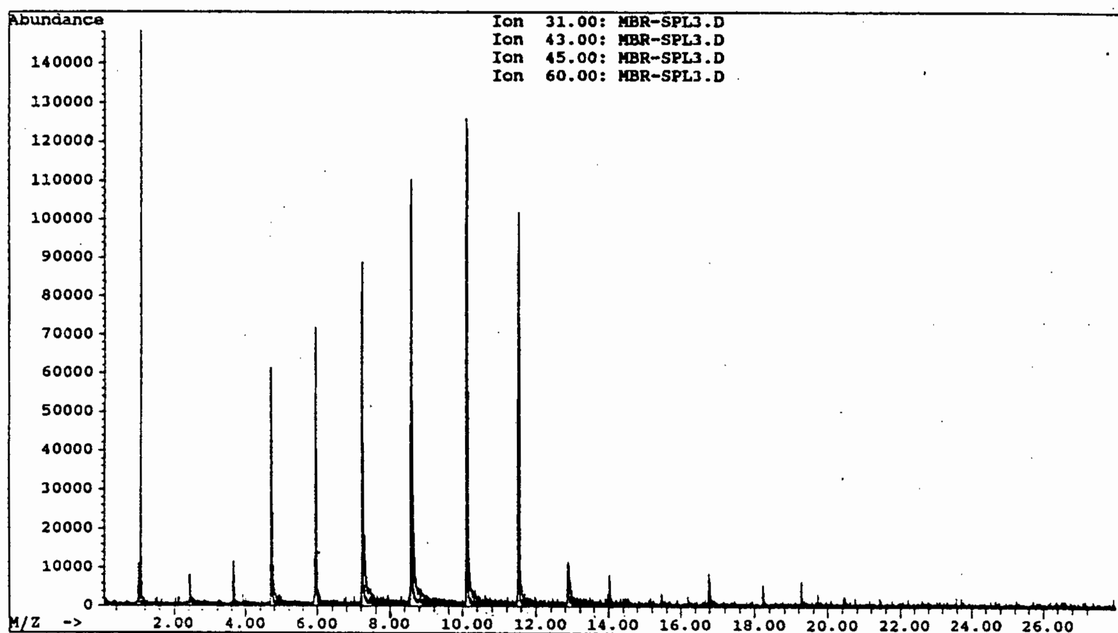


FIGURE 6.9. Mass spectral analysis of ethanol and acetic acid in culture samples during fed-batch cultivation of *P. rhodozyma* with a feed glucose concentration of 125 g/l.

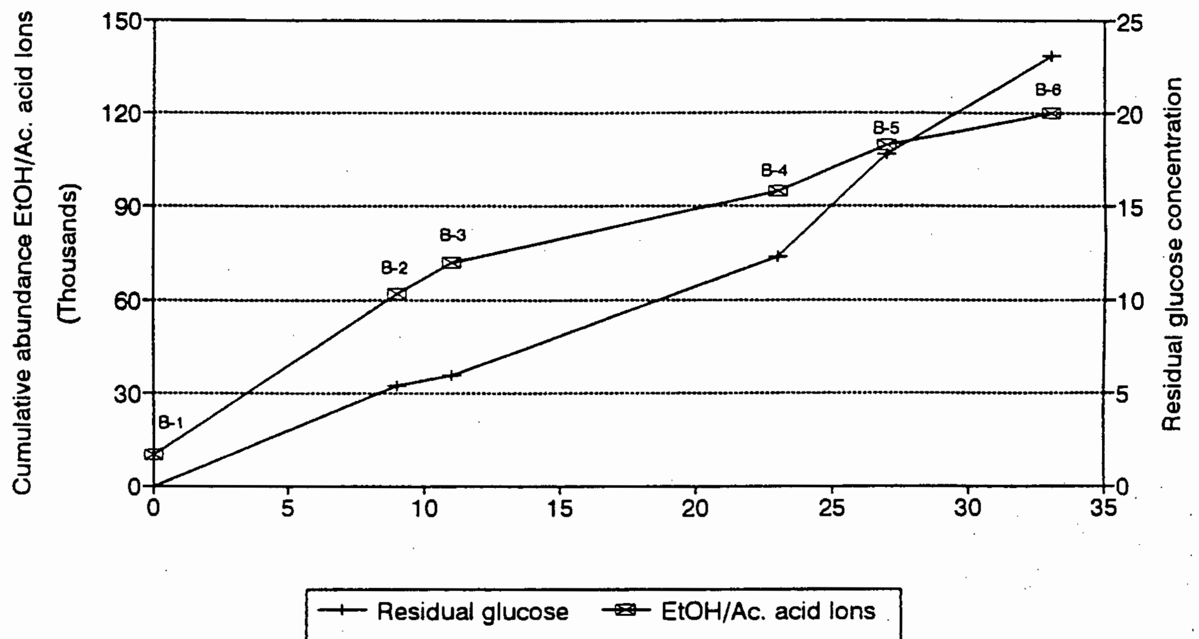


FIGURE 6.10. Cumulative abundance of ethanol/acetic acid ions and residual glucose detected during fed-batch cultivation of *P. rhodozyma* with a feed glucose concentration of 125 g/l.

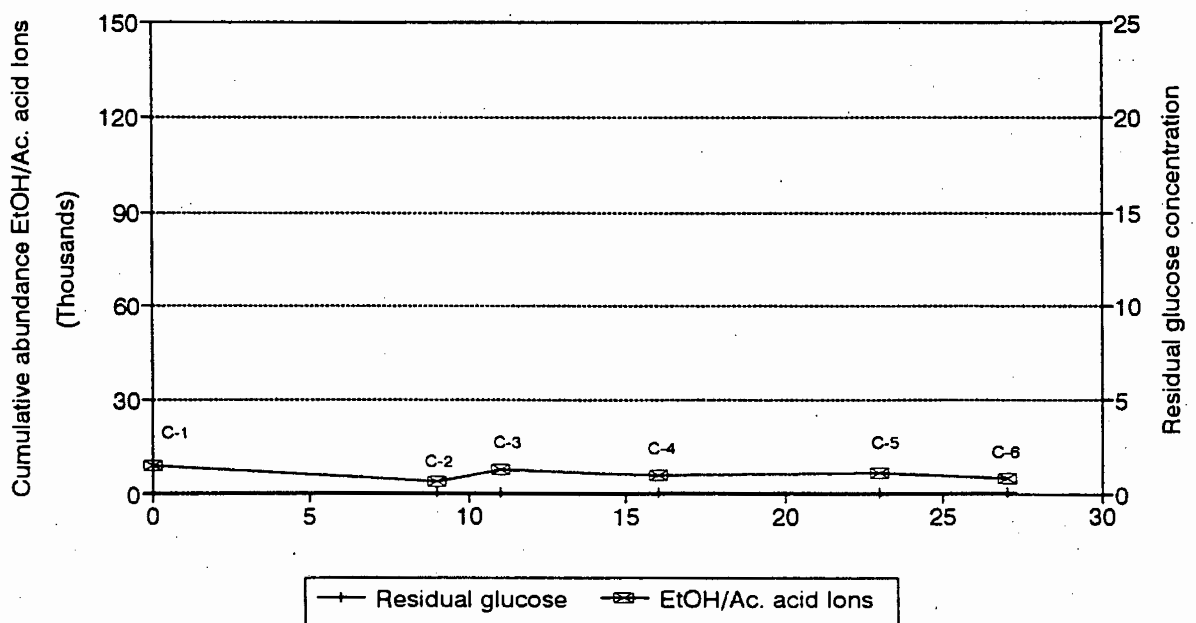


FIGURE 6.11. Cumulative abundance of ethanol/acetic acid ions and residual glucose detected during fed-batch cultivation of *P. rhodozyma* with a feed glucose concentration of 40 g/l.

The effect of increased feed glucose concentration on the product yield of *P. rhodozyma*, in terms of mass carotenoid produced per unit mass of substrate utilized, is shown in Fig. 6.12. As with the biomass yield, the carotenoid yield at a feed glucose concentration of 125 g/l decreased significantly. It remained relatively constant at feed concentrations of 40 and 55 g/l respectively. The carotenoid yield in terms of μg carotenoid per g biomass however remains constant regardless of the feed glucose concentration (Fig. 6.13). This indicates that the decrease in carotenoid yield is directly linked with the decrease in biomass yield and ties in with carotenoid formation in *P. rhodozyma* being growth associated (Section 4.3.1).

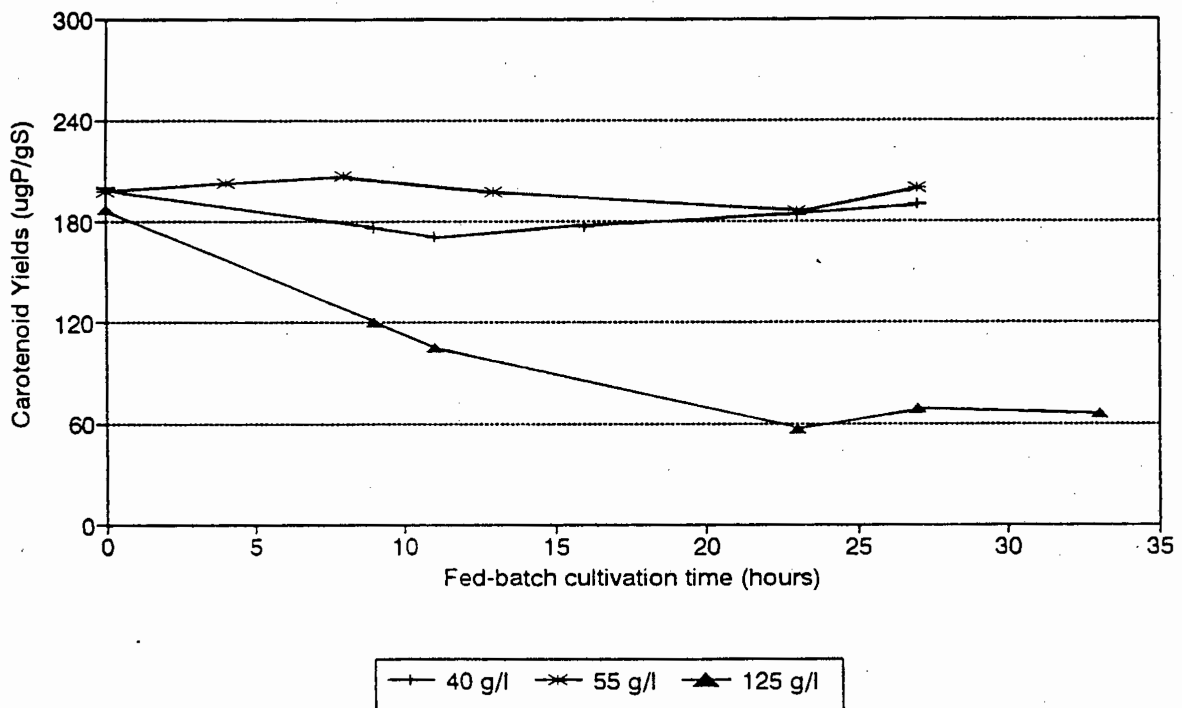


FIGURE 6.12. Total carotenoid yields (μgP/gS) for the fed-batch cultivation of *P. rhodozyma* (UCT-1N-3693) at various feed glucose concentrations.

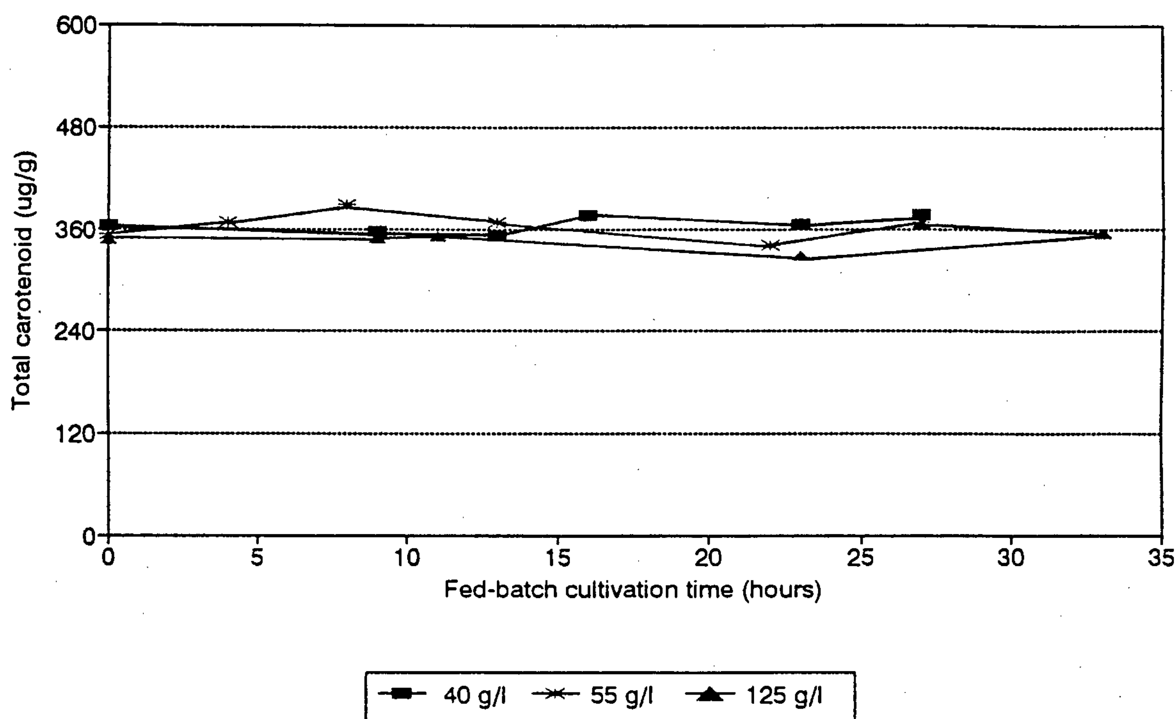


FIGURE 6.13. Total carotenoid content (μgP/gX) of *P. rhodozyma* (UCT-1N-3693) grown in fed-batch cultivation at various feed glucose concentrations.

6.3.3. Confirmation of the Crabtree effect during continuous cultivation of *Phaffia rhodozyma*

During continuous cultivation of *P. rhodozyma* with a feed glucose concentration of 10 g/l and at dilution rates greater than 0.1 h^{-1}, an increase in the residual glucose concentration with a corresponding decrease in the biomass concentration was observed (Figs. 4.7 and 6.14). Aerobic culture conditions (Section 6.3.1) were again confirmed by checking that the rate of oxygen transfer (0.192 mg/l.s) exceeded the maximum rate of oxygen utilization (0.112 mg/l.s). The increase in residual glucose concentration corresponds to detection of ethanol in the supernatant of culture samples (Fig 6.14). This alludes to a transition in the metabolism of *P. rhodozyma* from aerobic respiration to aerobic fermentation, indicating the existence of the Crabtree effect. A similar shift in metabolism was also reflected in the

results for continuous cultivation of the Crabtree positive yeasts *Brettanomyces lambicus* and *Schizosaccharomyces pombe* by Wöhrer *et al.* (1981).

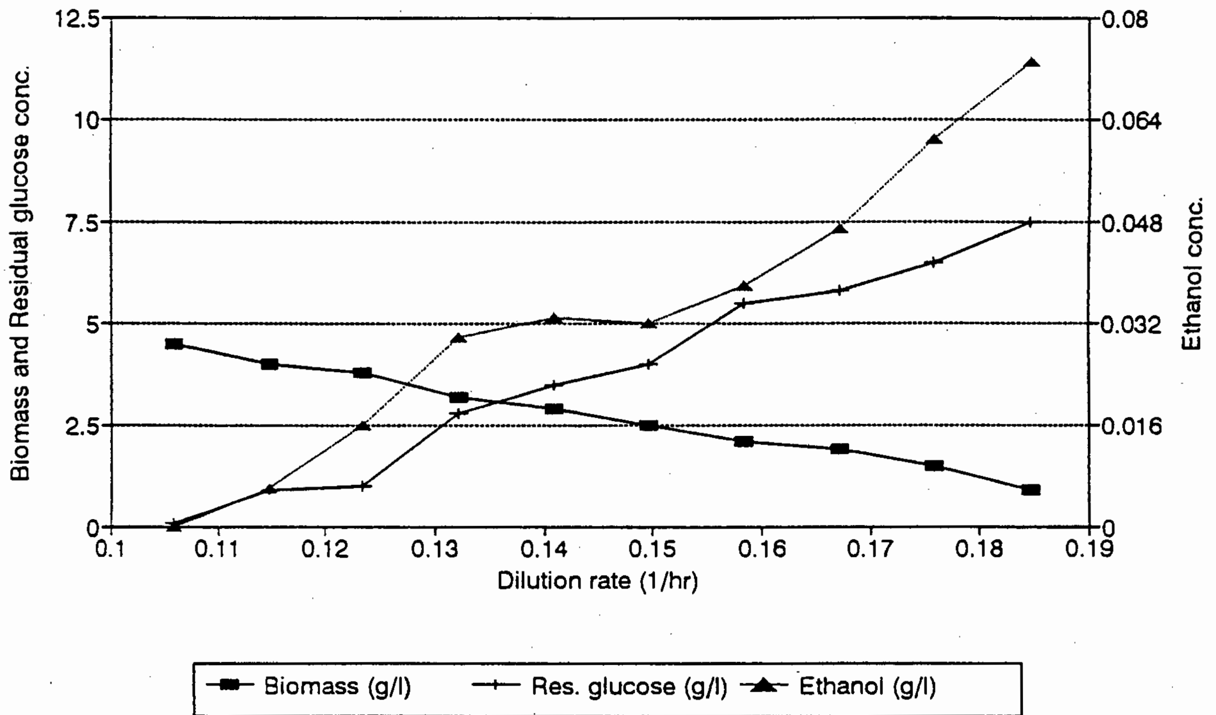


FIGURE 6.14. Continuous cultivation of *P. rhodozyma* (UCT-1N-3693) at 22°C, pH 5.0 and a feed glucose concentration of 10 g/l.

6.4. CONCLUSIONS

Fed-batch and continuous cultivation of *P. rhodozyma* under defined aerobic conditions, at high glucose feed concentrations, and high dilution rates respectively, resulted in the formation of typical yeast fermentation products. Furthermore a decrease in the biomass yield was observed under these conditions. It can therefore be concluded that the phenomena known as the Crabtree effect is exhibited by *P. rhodozyma*.

The Crabtree effect affects the final level of *P. rhodozyma* biomass formation as a result of glucose degradation proceeding partially via aerobic fermentation at high glucose concentrations. Consequently the carotenoid formation yield is also affected significantly. This is as a result of

P. rhodozyma carotenoid production being growth associated. The substrate inhibition of biomass and carotenoid formation in *P. rhodozyma*, as a result of this yeast demonstrating the Crabtree effect, is an important consideration for the industrial production of astaxanthin. This serves to further highlight the need for a fed-batch process where low residual sugar concentrations can be maintained.

CHAPTER 7

CONCLUSIONS

The principle aim of this research project was to study biomass and pigment production by the yeast *Phaffia rhodozyma* with a view to its commercial production by local industry.

Accordingly, emphasis was placed on investigating the following areas: (i) the possibility of obtaining a yeast strain with an increased pigment level and a high growth rate; (ii) assessment of the growth and pigmentation of *P. rhodozyma* in shake flask, batch, continuous and fed-batch cultivation, using both glucose and molasses as a growth substrate; (iii) modelling the growth and pigment formation in *P. rhodozyma*; (iv) evaluation of substrate inhibition effects on *P. rhodozyma* growth and pigmentation

A mutation programme, using NTG as a mutagen and ß-Ionone as a selective means for carotenoid over-producing strains, resulted in the selection of *P. rhodozyma* mutant UCT-1N-3693. The mutant had an improved total carotenoid content that was 1.5 times greater than the wild strain. Shake flask evaluation of the *P. rhodozyma* mutant resulted in a total carotenoid content of 455 μg/g and a maximum specific growth rate of 0.21 h^{-1}. Although the pigment level is low compared to other *P. rhodozyma* strains (Table 2.1), the growth rate of UCT-1N-3693 is high. A compromise between high levels of pigmentation and a high growth rate is clearly required. This compromise is essential due to the fact that both the protein and astaxanthin content of *P. rhodozyma* is important for use of the yeast as an animal feed.

Batch growth of *P. rhodozyma* (UCT-1N-3693) on glucose medium (10 g/l) resulted in a cell dry mass of 6.1 g/l, and a total carotenoid content of 416 μg/g. Furthermore carotenoid formation was growth associated. Batch cultivation of *P. rhodozyma* (UCT-1N-3693) on molasses medium (total sugar concentration of 10 g/l) supplemented with $(NH_4)_2SO_4$ and yeast extract resulted in a cell dry mass of 5.4 g/l and a total carotenoid content of 410 μg/g.

During continuous cultivation of *P. rhodozyma* on glucose medium (10 g/l) at increased dilution rates, a drop in the biomass concentration above dilution rates of 0.09 h^{-1} was observed. A concurrent increase in the

residual glucose concentration was also observed at these high dilution rates. Kinetic constants of $\mu_{max}=0.21\ h^{-1}$ and $K_s=1.9$ g/l were obtained by a Lineweaver-Burk plot of the data. Increasing the initial glucose concentration from 5 to 25 g/l during continuous cultivation at a constant dilution rate of 0.1 h^{-1} led to a decrease in the biomass yield and a coexistent increase in the residual glucose concentration. Again this suggested the presence of the Crabtree effect. Supplementation of the air supplied to the culture with pure oxygen did not induce carotenoid biosynthesis during continuous cultivation of *P. rhodozyma* on glucose medium (10 g/l) at $D=0.1\ h^{-1}$.

A high biomass yield is essential for the production of yeast biomass as a protein source . During shake flask cultivation of *P. rhodozyma* on minimal (YNB) media and varying the initial glucose concentration from 1 to 40 g/l, resulted in a decrease in the biomass yield from 0.52 to 0.29. A similar decrease in the biomass yield from 0.48 to 0.30 was observed with an increase in the sugar concentration of the molasses medium (from 1 to 40 g/l). These results suggested that the Crabtree effect may be exhibited by *P. rhodozyma*.

The presence of the Crabtee effect was confirmed by the detection of typical yeast fermentation products (such as ethanol and acetic acid) in culture samples during both fed-batch cultivation at a glucose concentration of 125 g/l in the feed and continuous cultivation of *P. rhodozyma* at high dilution rates under defined aerobic conditions. In both cases a drop in the biomass yield coincided with detection of residual glucose in the culture samples.

Use was made of an exponential incremental feed scheme during fed-batch cultivation of *P. rhodozyma* in order to overcome the detrimental effects of substrate inhibition during yeast cultivation at high sugar concentrations. Fed-batch cultivation of *P. rhodozyma* at feed glucose concentrations of 27, 40, and 55 g/l resulted in final biomass concentrations of 11.0, 13.7 and 19.0 g/l respectively. A yield coefficient greater than 0.5 g/g and a total carotenoid content in excess of 400 μg/g was observed in each case. In addition fed-batch cultivation of *P. rhodozyma* on molasses medium with a

feed sugar concentration of 40 g/l compared favourably with cultivation on glucose medium (40 g/l). This demonstrated the suitability of molasses as a substrate for large scale production.

A model was developed in order to predict the growth and pigmentation of *P. rhodozyma* during fed-batch cultivation. The model involved a mass balance approach to establish a feed pattern and was based on exponential cell growth, Monod substrate kinetics and growth associated product formation. Biomass and carotenoid formation in *P. rhodozyma* was successfully predicted by mathematical equations during fed-batch cultivation of *P. rhodozyma*. The model was shown to breakdown at high residual glucose concentrations in the culture as a result of substrate inhibition caused by the Crabtree effect.

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APPENDIX A

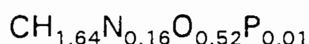
Calculation of the limiting nitrogen and phosphate values in culture medium.

This calculation is based on the elemental composition of *S. cerevisiae* (Bailey and Ollis 1986) and the stoichiometry for its conversion of substrate to biomass.

Elemental composition of yeast (% by weight):

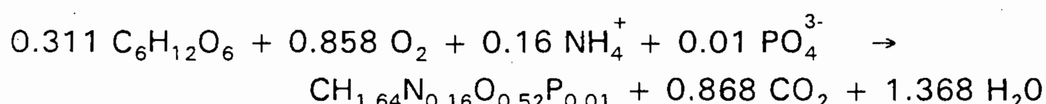
C = 44.7
H = 6.2
N = 8.5
O = 31.2
P = 1.08

Empirical chemical formula:



$$CH_{1.64}N_{0.16}O_{0.52}P_{0.01}$$

Therefore the stoichiometric equation for the conversion of various substrates to biomass (based on a cell yield of 0.50 g/g) is:



$$0.311\ C_6H_{12}O_6 + 0.858\ O_2 + 0.16\ NH_4^+ + 0.01\ PO_4^{3-} \rightarrow CH_{1.64}N_{0.16}O_{0.52}P_{0.01} + 0.868\ CO_2 + 1.368\ H_2O$$

Based on 1 liter of culture containing 6.5 g/l yeast biomass (dry weight) and the above stoichiometry, the nitrogen, phosphate and glucose requirements are calculated:

NH_4^+ required = 1.314 g/l
PO_4^{3-} required = 0.459 g/l
$C_6H_{12}O_6$ required = 27.07 g/l

Thus per 10 g of glucose in the medium at least 1.8 g of $(NH_4)_2SO_4$ and 0.25 g KH_2PO_4 is required.