# OPTIMISING THE GROWTH OF *CRYPTOCOCCUS* SPECIES SS1, A POTENTIAL PROBIOTIC FOR FARMED ABALONE

by

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*Over the course of my studies I have learnt countless lessons that I will keep with me, always. Thank you to my Creator for providing me with the opportunity to walk on this earth and for always opening the next door.*

**"The important thing is not to stop questioning" – Albert Einstein**

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## ABSTRACT

Farmed abalone is a reliable and good quality source of abalone. *Cryptococcus* species SS1 was isolated from the gut of the South African abalone, *Haliotis midae*, and has been identified as a potential probiotic for farmed abalone. The implementation of strain SS1 as a probiotic for aquacultured abalone required the design of a fermentation system to produce high concentrations of the yeast strain in order to supply the probiotic to commercial abalone producers. The aim of this project was to assist in the recommendation of a commercial fermentation process that is economically feasible for the production of strain SS1. This involved evaluation of all the main factors that will contribute to the cost of the fermentation; i.e. cultivation medium, fermentation space and time, and productivity.

The effect of environmental parameters on the growth of strain SS1 was evaluated to determine optimum culture conditions. The parameters evaluated included the medium, temperature, pH and sodium chloride concentration. A laboratory medium consisting of only 8.8 $gl^{-1}$ yeast extract and 20 g glucose $l^{-1}$ was sufficient for achieving high cell yields of strain SS1, with the yeast extract providing essential components for growth. The *Cryptococcus* species was able to grow at 25°C but not at 30°C or above. Growth of strain SS1 was not affected by pH values between 5.0 and 7.5. The yeast was able to tolerate salt concentrations of up to 50 mM but did not exhibit an obligate requirement for sodium chloride in the medium.

The highest cell density obtained during batch cultivation in the stirred tank reactor was approximately 15 $gl^{-1}$. Increasing the size of the inoculum from 0.5% to 10% of the new medium volume decreased the fermentation time from 47 h to 30 h. The result was that productivity increased with increasing inoculum size. The highest productivity (0.50 $gl^{-1}h^{-1}$) was observed in the culture that received the 10% inoculum.

A protein source (in addition to yeast extract) is not needed for growth of SS1 in shake flasks. Also, strain SS1 was unable to utilise the soluble protein in the medium as a nutrient source during batch cultivation both in the shake flasks and in the stirred tank reactor. There was no optimal glucose concentration that resulted in maximal growth of strain SS1 in shake flasks. In contrast, glucose was important for growth of strain SS1 and productivity of the batch cultivation process in the stirred tank reactor. Also, the specific growth rate and specific glucose utilisation rate of strain SS1 was affected by the glucose concentration of the medium. Excluding glucose from the medium decreased the biomass concentration to only 3 $gl^{-1}$. The highest productivity (0.50 $gl^{-1}h^{-1}$) and maximum specific growth rate (0.23 $h^{-1}$) was observed when SS1 was cultivated in the standard medium containing 20 $gl^{-1}$ glucose. Doubling the initial glucose concentration to 40 $gl^{-1}$ decreased the productivity to 0.32 $gl^{-1}h^{-1}$ and the maximum specific growth rate to 0.19 $h^{-1}$. The growth of strain SS1 was characterised by multiple exponential phases during growth in the standard medium indicating that growth of the yeast was dependent on more than one substrate. Since these substrates could not be identified during this study, Monod kinetics was not used to model strain SS1 growth.

Oxygen was a growth-limiting substrate for strain SS1 in standard Erlenmeyer flasks. The introduction of baffles in the flasks improved oxygen transfer and resulted in better growth of strain SS1. The specific growth rate decreased when strain SS1 was cultivated in standard Erlenmeyer flasks containing increasing volumes of medium due to a decrease in oxygen transfer rate from 0.090 $mgO_2l^{-1}$ at a working volume of 20% to 0.058 $mgO_2l^{-1}$ at a working volume of 5%. The agitation rate was increased from 600 rpm to 800 rpm as a means to investigate the effect of increased oxygen transfer on the growth of strain SS1 in the stirred tank reactor. Increasing the agitation rate to 800 rpm inhibited the growth of strain SS1. The maximum specific

growth rate decreased from 0.23 $h^{-1}$ to 0.15 $h^{-1}$, reducing the productivity of the process from 0.50 $gl^{-1}h^{-1}$ to 0.25 $gl^{-1}h^{-1}$.

The highest final cell concentration obtained during fed-batch cultivation was approximately 20 $gl^{-1}$ using a constant feeding strategy. The best time to initiate feeding was during the first 10 h of growth when both the specific growth rate and the specific glucose utilization rate were optimal. The productivity of the fed-batch cultivation process using the constant feeding strategy (0.40 $gl^{-1}h^{-1}$) was lower than for batch cultivation (0.50 $gl^{-1}h^{-1}$). As a result, the fed-batch cultivation of strain SS1 using the strategies employed in this study was regarded as uneconomical.

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# NOMENCLATURE

**Abbreviations:**

atm - atmosphere
DO - Dissolved oxygen
g - gram
h - hour (s)
mg - milligram
min - minute
ml - millilitre
OTR - Oxygen transfer rate
OUR - Oxygen utilization rate
rpm - revolutions per minute
sec - second
STR - Stirred tank reactor
vvm - volume air flow per volume culture per minute
µg - microgram
µl - microlitre

**Symbols:**

a - Gas-liquid interfacial area per unit volume of fluid
C - Dissolved oxygen concentration
$C^*$ - Saturated dissolved oxygen concentration
H - Henry's constant (atm $m^3$ Kmole)
K - Overall mass transfer
$k_L$ - Liquid phase mass transfer coefficient
$K_s$ - Saturation constant for substrate
$OD_{600}$ - Optical density at a specified wavelength
$pO_2$ - Partial pressure of oxygen
S - Concentration of growth limiting substrate
$S_0$ - Initial concentration of growth limiting substrate

| | | |
|---|---|---|
| T | - | Time |
| V | - | Culture volume in culture vessel |
| X | - | Biomass concentration |
| $\mu$ | - | Specific growth rate |
| $\mu_{max}$ | - | Maximum specific growth rate |
| $Y_{x/s}$ | - | Yield of biomass on substrate |

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# CHAPTER 1

## GENERAL INTRODUCTION

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## 1.1. INTRODUCTION

All abalone belong to the genus *Haliotis*. They belong to the Molluscs and are related to oysters, mussels, scallops, as well as the garden snail. Their large muscular foot constitutes 28-46% of the wet weight of the animal and is the primary reason why abalone are considered such a sought after seafood delicacy. The demand for abalone is especially high in the Far East where they are prepared for special or ceremonial occasions. Of the six abalone species endemic to South Africa (*H. midae, H. parvum L., H. spadisea, H. queketti, H. speciosa,* and *H. pustula*) only *Haliotis midae*, commonly known as perlemoen, is of commercial importance (Sales and Britz, 2001).

## 1.2. THE STATUS OF ABALONE AQUACULTURE

Modern aquaculture involves the intensive production of finfish, crustaceans, molluscs, and algal plants under "controllable" conditions (Olafsen, 2001). This industry has shown an annual increase in production of approximately 10% for the period 1984-1995 and is rapidly becoming one of the fastest growing food-producing sectors (Verschuere *et al.*, 2000). In 1998 the total world production was 38 million tons with a net value of US$52 billion (http://www.afma.co.za).

### 1.2.1. Worldwide abalone aquaculture

There are 90 species of abalone worldwide of which 15 are harvested commercially. The major abalone fisheries occur in Japan, China, Korea, Taiwan, Australia, New Zealand and South Africa (http://www.fishtech.com). Over the past two decades the commercial abalone fishery has declined dramatically due to:

- Mortality of juvenile abalone due to disease outbreaks, consumption by predators etc.
- Over harvesting and illegal harvesting of abalone
- Loss of natural habitat due to coastal development, pollution and competition with other species

Over the past decade commercial abalone fishery has declined 30% while abalone aquaculture has increased by more than 600% (http://www.fishtech.com). The decrease in the commercial abalone fishery has resulted in a concurrent increase in the price of abalone meat. Typically a decline in fishery also enhances the economic viability of producing the species commercially (Erasmus, 1996). This has caused a huge demand for abalone in the world market and has promoted the development of abalone aquaculture. Cultured abalone provides a reliable, good quality source of abalone and can demand high prices on the international market.

### 1.2.2. Abalone aquaculture in South Africa

Although abalone fisheries have existed in South Africa since 1949 (Sales and Britz, 2001), South Africans were reluctant to get involved in the practice of abalone aquaculture for two main reasons. Firstly, *H. midae* exhibits a very slow growth rate taking approximately 8-9 years to reach maturity and 12 years to reach the minimum legal harvestable size of 114 mm (Erasmus, 1996). Secondly, abalone on the west coast is primarily fed on the seaweed *Ecklonia maxima*. The supply of the kelp in sufficient amounts for commercial farming was perceived to be a major constraint (Sales and Britz, 2001).

Interest in abalone aquaculture developed when it was determined that *H. midae* spawned successfully in captivity and that cultured abalone exhibited higher growth rates than those in the wild (Erasmus, 1996). Also, the development of artificial feeds to improve the growth of abalone has made this a more cost-effective and manageable industry (Sales and Britz, 2001). Although *H. midae* would require an estimated period of 30 years to reach a shell length of 200 mm, abalone aquaculture is concentrated on the production of 100 mm animals in 4 years (Erasmus, 1996). South African produced abalone is currently sold on the export market at approximately US$32/kg (http://www.afma.co.za). Thus abalone aquaculture is a very profitable industry in South Africa.

## 1.3. PROBIOTICS

The outbreak of diseases is a major constraint affecting the growth and productivity of aquaculture (Olafsen, 2001). Traditional methods such as antibiotics used to treat or prevent disease as well as to promote the growth of the animals are a concern as they promote the emergence of multiple antibiotic resistance in microorganisms, a property that is readily transferable to other microorganisms (Moriarty, 1997; Verschuere *et al.*, 2000). Alternative strategies devised to improve the growth rate and prevent disease in invertebrates include: the development of affordable and efficient vaccines, the use of immunostimulants and nonspecific immune enhancers and the use of probiotics and bioaugmentation (Gatesoupe, 1999; Ringo and Birbeck, 1999; Verschuere *et al.*, 2000).

### 1.3.1. The definition of probiotics

The concept of probiotics is traditionally associated with the use of beneficial microorganisms to restore the microbial balance in the intestine and to treat or prevent diseases or disorders in terrestrial organisms (Gatesoupe, 1999). The use of indigenous microflora to improve human and animal nutrition is well documented (Fuller, 1989; Holzapfel *et al.*, 1998). Accordingly, probiotics are defined as "mono or mixed cultures of live organisms which when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora" (Verschuere *et al.*, 2000). Increasing scientific evidence suggests that microorganisms that colonise the digestive tract of various invertebrates have similar beneficial properties (Gomez-Gil *et al.*, 2000, Olafsen, 2001).

In aquaculture the relationship that exists between the environment and the host is very different and more intense than that for terrestrial organisms (Gatesoupe, 1999; Verschuere *et al.*, 2000). Aquatic organisms can obtain probiotics both from the environment and the feed. The definition of probiotics must be slightly modified when it is applied to aquaculture. Thus, with respect to aquaculture, a probiotic is defined as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-association or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards

disease, or by improving the quality of its ambient environment" (Verschuere *et al.*, 2000). According to this definition, a probiont must thus be able to: prevent proliferation of pathogens; aid in the digestion of feed; improve water quality; or stimulate the host immune response (Verschuere *et al.*, 2000).

### 1.3.2. Possible modes of action of aquatic probiotics

Although a number of studies have been conducted on the use of probiotics to increase growth and prevent disease the exact mode of action has not been elucidated. Various authors have suggested possible modes of action based on empirical evidence. Verschuere *et al.* (2000) reviewed the following mechanisms:

- Production of inhibitory compounds
- Competition for chemicals or available energy
- Competition for adhesion sites
- Enhancement of the immune response
- Improvement of water quality
- Interaction with phytoplankton
- Enzymatic contribution to digestion

### 1.3.3. Selection criteria for aquatic probiotic strains

A probiotic can be introduced into the digestive system of the culture organism in various ways (Ringo and Birbeck, 1999). The beneficial bacteria can be added either via the live feed or artificial diet or to the ambient environment. Alternatively, the artificial diet can be supplemented with naturally occurring compounds that selectively stimulate the growth of beneficial bacteria resident in the gut. It is also important that the culture organism ingests the bacteria or the feed containing the bacteria. When ingested, the selected strain must then be able to reach the required location in which it will exert its effect in a viable and active state (Verschuere *et al.*, 2000).

The initial application of probiotics for aquaculture employed commercial probiotics designed for terrestrial animals (Gatesoupe, 1999). However, the ability of these microorganisms to thrive in aquatic animals was questionable. More recent attempts to select for probiotic strains are aimed at autochothonous strains; i.e. microbes that

are able to colonise the gut of aquatic invertebrates (Ringo and Birbeck, 1999). However, to date no experimental evidence has been reported that these microorganisms have an advantage over isolates originating from other culture species or culture environments (Verschuere *et al.*, 2000).

### 1.3.4. Microorganisms used as probiotics for aquaculture

Although the use of probiotics for abalone aquaculture is still a novel concept various microorganisms are being studied for their potential as probiotics in other shellfish and fish. Bacteria belonging to the genera *Vibrio, Pseudomonas, Bacillus* and *Lactobacillus* have been investigated as probiotics for the culture of shrimp, crab, oyster and fish (Gatesoupe, 1999; Gomez-Gil *et al.*, 2000). The use of yeast and yeast-derived products to promote growth and prevent disease in invertebrates are also receiving attention (Scholz *et al.*, 1999). The focus has been on *Saccharomyces cerevisiae* and especially waste yeast from the brewery and baking industries (Scholz *et al.*, 1999; Suphantharika *et al.,* 2003). Yeast is well-known for their immunostimulating properties (Sakai, 1999; Wasser and Weis, 1999; Williams *et al.*, 1996). This is primarily attributed to the high β-glucan content of the yeast cell wall (Freimund *et al.*, 2003). Attempts to elicit a probiotic effect in invertebrates by administrating pure β-glucan produced mixed results (Scholz *et al.*, 1999). The effectiveness of this method appears to be dependent on the mode of β-glucan preparation and the development of a suitable method has generated much interest (Freidmund *et al.*, 2003). Another yeast receiving attention is *Phaffia rhodozyma.* These yeasts contain carotenoid pigments, including astaxanthin, which is considered to act as a fat-soluble growth factor (Scholtz *et al.*, 1999). The ability of yeast to aid in gut maturation due to the excretion of spermidine and spermine has also been investigated (Tovar, *et al.*, 2002).

## 1.4. PROJECT OBJECTIVES

Research is currently in progress at the University of Cape Town on the use of probiotics for the production of farmed abalone. Two of the selection criteria for probiotic microorganisms are aiding in the enzymatic digestion of the feed and enhancement of the immune response of *H. midae*. Erasmus (1996) found that the gut bacteria of *H. midae* were able to hydrolyse agar, carrageenan, carboxymethylcellulose, laminarin and alginic acid all of which are complex polysaccharides that occur in seaweed. Some 70-90% of the enzymatic activities of these microorganisms was extracellular and would be excreted into the lumen of the abalone digestive tract. Thus, the indigenous gut bacteria of *H. midae* could potentially assist in the digestion of seaweed (Erasmus *et al.*, 1997).

Various bacterial and yeast isolates that produce enzymes that degrade the main components of Abfeed® are currently being investigated as prospective probiotics for farmed abalone (Macey *et al.*, 2003). Abfeed® is an artificial feed which is formulated and produced by Sea Plant Products Limited to increase the feeding efficiency of abalone. The main ingredients in Abfeed® are fishmeal, starch, minerals and vitamins. As a result, Abfeed® has a high protein (34%) and carbohydrate (43.3%) content (Table 1.1).

**Table 1.1**[a]: Proximate analysis of Abfeed®

| Component | Estimated content[b] |
|---|---|
| Protein | 34% |
| Carbohydrate | 43.3% |
| Fat | 5.3% |
| Crude fibre | 1.2% |
| Ash | 5.7% |
| Moisture | 10.0% |
| Total energy | 1490 kJ/100g |

[a] Data obtained from an informational booklet distributed by Sea Plant Products Limited

[b] (% given as w/w)

*Cryptococcus* sp. SS1 was previously isolated from the digestive tract of *H. midae* cultured on Abfeed® (Macey *et al.*, 2003). The yeast excretes an extracellular amylase (Figure 1.1) that could contribute to the enzymatic degradation of the complex carbohydrate component of Abfeed ingested by abalone (Table 1.1). Strain SS1 is one of three microorganisms which are under investigation as a probiotic mixture for farmed abalone. Preliminary investigations have indicated that the microbial mixture improves the growth rate of cultured *H. midae* (Macey *et al.*, 2003). The microorganisms also provide protection from infection by pathogenic strains by stimulating the immune system of *H. midae* (Macey *et al.*, 2003). Thus strain SS1 has been proposed as a potential probiotic for farmed abalone.

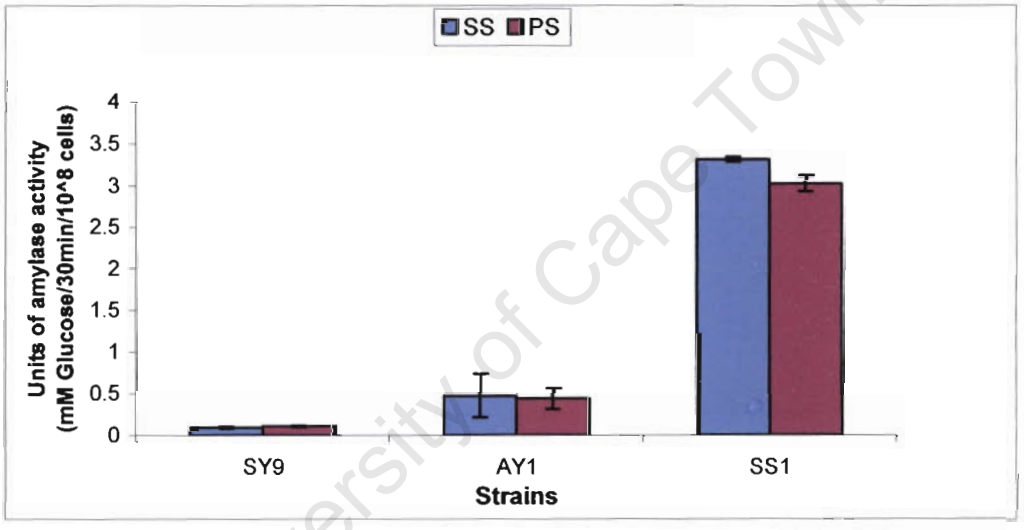


**Figure 1.1:** Amylase activity of the three microbial strains used in the probiotic mixture to supplement Abfeed. SS=soluble starch, PS=potato starch (Macey, 2001).



The ecological origin of the probiotic isolates including strain SS1, cannot be determined with certainty. Erasmus (1996) found that the microbial enzymatic activities in abalone varied according to their diet. The amylase activity of strain SS1 can be correlated to the starch component of Abfeed®. Thus it is likely that strain SS1 was introduced into the abalone via the feed. However, as mentioned previously, due to the nature of the relationship between abalone and the aquatic environment it is also possible that strain SS1 originated from the seawater that is circulated through the raceways in which abalone are cultured. Thus due to the limited available knowledge, strain SS1 cannot be classified as either marine or terrestrial with confidence.

The commercial production of abalone in South Africa is still in the developmental stage. There are twelve abalone farms in South Africa with an estimated investment of US$12 million and a projected production of 500 - 800 tons per annum (Sales and Britz, 2001). The implementation of probiotics for abalone aquaculture would require that large volumes of the appropriate probionts be generated in order to supply probiotics to commercial abalone producers in South Africa. Hence it is necessary to have a fermentation system in place to produce high concentrations of the microbial strains selected as probiotics.

The objective of this project is to study the growth and kinetics of *Cryptococcus* sp. SS1. The integral components include:

- Determination of environmental conditions for optimum growth;
- The optimisation of a suitable cultivation medium;
- Modeling the growth kinetics of the strain;
- Development of a fermentation strategy.

To assist in the recommendation of a commercial fermentation process that is economically feasible, it is important that the main factors that will contribute to the cost of the fermentation i.e. cultivation medium, fermentation space and time, productivity and product recovery, be carefully evaluated. The results obtained are used to recommend protocols in order to devise a fermentation process that will facilitate commercial scale production of the probiotic.

The commercial production of probiotics is likely to generate plenty of interest in both the scientific and business communities. As discussed previously, most studies to date have focussed on the selection and application of microorganisms as probiotics for aquaculture. The present study is one of the first attempts to investigate dense culture production of a probiotic for use in abalone aquaculture. Although no available literature deals with the commercial production of probiotics for use in aquaculture, there are publications on the production of probiotics for human nutrition (Schiraldi *et al.*, 2002). Similar studies investigating commercial scale production processes for probiotics for aquaculture are inevitable.

The purpose of this general introduction was to give a brief yet comprehensive overview of the factors that contributed to the initiation of the present project and to outline the primary objectives that must be reached. Chapter 2 reviews the literature that relates to dense culture production of yeasts. Attempts are made to correlate the literature to the current project. Chapter 3 contains the experimental procedures used during this study. The experimental results are presented, analysed and discussed in chapters 4, 5 and 6. Chapter 4 focuses on medium development. Yeast cultivation media containing glucose as the primary carbon source are investigated. Chapter 5 reports on the growth kinetics of strain SS1. Chapter 6 investigates implementation of the fed-batch cultivation strategy based on the biokinetics of SS1. Chapter 7 summarises all the main conclusions of this study. The appropriate recommendations are made based on the analysis of experimental data generated during the study. In conclusion, suggestions are made for further experiments that need to be conducted to resolve ambiguities and / or discrepancies should any exist or to optimise the current fermentation process.

# CHAPTER 2

## LITERATURE REVIEW

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## CONTENTS

## 2.1. INTRODUCTION

Yeasts have an extensive history of being exploited by humans for biotechnological purposes of which the baking industry and the production of alcoholic beverages are prime examples (Walker, 1998). These processes primarily employed organisms that belong to the genus *Saccharomyces* (Berry, 1983). Today, *S. cerevisiae* is still the most widely exploited yeast and large amounts of biomass are produced, the bulk of which is for Baker's yeast (Walker, 1998).

**Table 2.1:** Non-*Saccharomyces* yeast biomass with biotechnological uses (Walker, 1998).

| **Yeast** | **Uses / potential uses of biomass** |
|---|---|
| *Kluyveromyces marxianus* and *K. lactis* | Animal feed yeast biomass from whey lactose. Sources of lactase |
| *Candida utilis* | Single-cell protein (SCP) from sulphite waste liquor and wood sugars |
| *Phaffia rhodozyma* | Carotene pigment (red food colorant) |
| *Saccharomyces boulardii* | Biotherapeutic agent |
| *Pichia pastoris* and *Hansenula polymorpha* | SCP and recombinant proteins from methanol |
| *Yarrowia lipolytica* and *Candida paraffinica* | SCP from n-alkanes |
| *Schwanniomyces castellii* | SCP from starch |
| *Pichia stipitis* and *Candida shehatae* | SCP from lignocellulosic biomass |
| *Rhodotorula glutinis, Lipomyces lipofer, Cryptococcus curvatus* and *Candida* sp. (e.g. *C. palmioleophila)* | Single cell oil (SCO), as substitutes for edible and non-edible oils from cheap carbon sources (e.g. whey, molasses, n-alkanes) |

Modern biotechnology has evolved to include industrially important yeast from other genera (Table 2.1). Besides the baking industry, yeast biomass and yeast cell extracts are also produced for other industrial applications (Table 2.2).

**Table 2.2:** Industrial uses of yeast biomass (Walker, 1998)

| | Type of yeast product | Examples of uses |
|---|---|---|
| Whole-cell products | Compressed baker's yeast/ active dried yeast | Baking, brewing, winemaking and distilling |
| | Yeast cream | Baking and distilling |
| | Fodder yeast/ single-cell protein | Animal feed |
| | Biotherapeutic/ growth factor yeasts | Human/animal probiotics |
| | 'Reagent' yeasts | Biocatalysts in organic chemistry |
| | Biosorbent yeasts | Heavy metal sequestration |
| | 'Mineral yeasts' | Nutritional trace elements (Cr, Se) source |
| | Cosmetic/pharmaceutical yeasts | Skin respiratory factor |
| | Pigmented yeasts | Feed colorants |
| | Biological control yeasts | Antifungal agents in agriculture |
| | Pollution control yeasts | BOD reductions |
| Extracted-cell products | Yeast extracts | Food use and microbiological growth media |
| | Yeast RNA derivatives | Flavour enhancers and pharmaceutical use |
| | Yeast cell walls | Food and pharmaceutical use |
| | Yeast B-complex vitamins | Capsules/tablets for dietary supplements |
| | Yeast enzymes | Invertase and lactase for food use |
| | Recombinant yeasts | Therapeutic proteins |

Researchers have become increasingly aware of the potential application of marine microorganisms in biotechnology, bioremediation and medicine (Austin, 1988). Table 2.3 presents some of the 177 yeast species isolated from the marine environment, some of which are truly obligate. Knowledge concerning the growth and metabolism of marine yeasts is limited and they are often considered to be metabolically identical to their terrestrial counterparts. However, there are indications that marine yeast isolates are less active than those from terrestrial sources and can degrade and assimilate a broader range of substrates (Litchfield, 1976). This is to be expected since in their natural environment these microorganisms are frequently exposed to dilute concentrations of complex nutrients.

**Table 2.3:** Marine yeasts (Austin, 1998)

| Sub-division | Family | Genus |
|---|---|---|
| Ascomycotina | Metschnikowiaceae | *Metschnikowia*[a] |
| | Saccharomycetaceae | *Debaryomyces, Hanseniaspora, Hansenula, Kluyveromyces*[a]*, Pichia*[a]*, Saccharomyces* |
| Basidiomycotina | Sporobolomycetaceae | *Leucosporidium*[a]*, Rhodosporidium*[a]*, Sporobolomyces* |
| Deuteromycotina | Torulopsidaceae | *Candida*[a]*, Cryptococcus*[a]*, Kloeckera, Rhodotorula*[a]*, Sterigmatomyces*[a]*, Sympodiomyces*[a]*, Torulopsis*[a]*, Trichosporon* |
| | (Yeast-like cells) | *Aureobasidium / Pullularia* |

[a] Includes obligate marine species.

The common feature of most biotechnological processes involving yeast is that dense culture production is often a prerequisite for optimum productivity (Lee, 1996). The successful cultivation of yeast in the laboratory or for fermentation processes requires an understanding of their nutritional requirements (Walker, 1998). However, besides the biological requirements, nutrients are also selected based on cost-effectiveness, availability and consistency (Dahod, 1999). The cost-effectiveness of the medium is especially important in fermentations where biomass is the primary product (Casida, 1968; Reed, 1982). These processes are highly capital intensive

especially with respect to the cost of the raw material and energy (Reed, 1982). Raw materials and energy are estimated to account for 45-75% and 12-37%, respectively, of the total production cost (Reed, 1982). The energy expenditure is primarily due to oxygen transfer and heat removal (Reed, 1982; Spencer and Spencer, 1989). Recently, medium selection has often been integrated into downstream processing so that the medium does not obstruct, and sometimes even facilitates, the recovery of the product (Dahod, 1999).

This chapter gives a general literature review of the various factors involved in the cultivation of yeasts. Since the main objective of this project is to recommend a dense culture strategy to facilitate future commercial production of strain SS1, the focus is especially on the factors that contribute to the production cost of yeast biomass. The general nutrient requirements of yeasts are evaluated. The incorporation of the nutrient requirements into the formulation and selection of suitable yeast cultivation media is also discussed. Since it is possible that strain SS1 originates from the marine environment brief reference is made to the growth requirements of marine yeasts. The dense culture production of yeasts is reviewed focussing on the fed-batch cultivation technique. The different feeding strategies employed for fed-batch cultivation, as well as the effect of limited oxygen transfer capacity on dense culture production, are also discussed. The chapter concludes with a brief discussion of the methods commonly employed commercially for the recovery of yeast cells from the fermentation medium.



## 2.2. YEAST NUTRITION

The yeast cell is composed of the major elemental building blocks (carbon, hydrogen, oxygen, nitrogen, phosphorous and sulphur), macromolecules (proteins, polysaccharides, nucleic acids, and lipids), bulk inorganic ions (potassium, magnesium) and trace elements (Walker, 1998). During growth, yeasts acquire essential elements from their environment and convert these into cellular material (Bailey and Ollis, 1986; Walker, 1998). The medium governs the chemical or nutritional environmental conditions that the organism is exposed to and has a direct

affect on the productivity of the cultivation process (Zhang and Greasham, 1999). Thus the medium is a critical component of any cultivation process both for research and for industry (Casida, 1968; Lee, 1996; Zhang and Greasham, 1999).

### 2.2.1. Medium formulation

There are various methods available for designing a suitable cultivation medium. The stoichiometric approach relates the elemental composition of the microorganism to the expected amount of biomass (Bailey and Ollis, 1986; Fiechter *et al.*, 1981; Zhang and Greasham, 1999). The medium is formulated to incorporate all the elements found in the cell in the correct proportions (Miller and Churchill, 1986; Zhang and Greasham, 1999). In general, the elemental composition of a cell varies according to the type of organism i.e. bacterium, yeast or fungus and the growth conditions (Bailey and Ollis, 1986; Dahod, 1999). The composition of some yeasts is given in Table 2.4 (Kroschwitz and Howe-Grant, 1992). Considering the values in Table 2.4, a medium that is formulated to grow e.g. 100 g dry weight of baker's yeast would require a minimum of 8.5 g nitrogen, 1.0 g phosphorous, 2.0 g potassium etc.

The amount of carbon required for yeast growth demands knowledge of the efficiency with which nutrient carbon is incorporated into cellular carbon (Bailey and Ollis, 1986). For example a facultative organism typically incorporates 10% of nutrient carbon during anaerobic growth and between 50 and 55% during aerobic growth. Thus, during aerobic growth, the amount of carbon in the medium must be double the desired amount of biomass. According to Table 2.4 Baker's yeast contains approximately 47% carbon, and therefore, the carbon required for Baker's yeast growth would be 0.47 times the specified total biomass. The stoichiometric approach is typically applied to the formulation of defined media (Fiechter *et al.*, 1981). Due to the nature of the individual substrates, complex media are usually formulated based on observations from trial-and-error-based experiments (Lee, 1996).

### 2.2.2. Carbon and nitrogen requirements

The carbon and nitrogen sources are very important constituents of the cultivation medium as both the microbial cells and fermentation products are primarily composed of these elements (Casida, 1968). Different yeast species have different

metabolic capabilities regarding the carbon and nitrogen sources they can utilise (Phaff *et al.*, 1978).

**Table 2.4:** Composition of Yeast (Kroschwitz and Howe-Grant, 1992)

| Component | Baker's yeast* | Brewer's yeast* | *Candida* sp. |
|---|---|---|---|
| C, wt% | 47.0 | | 45,9 |
| H,wt% | 6.0 | | 6.7 |
| N,wt% | 8.5 | | 7.3 |
| O,wt% | 32.5 | | 32.1 |
| ash, wt% | 6.0 | 6.4 | 7.8 |
| Ca, wt% | 0.06 | 0.13 | 0.57 |
| Fe, wt% | 0.003 | 0.01 | 0.01 |
| Mg, wt% | 0.13 | 0.23 | 0.13 |
| P, wt% | 1.0 | 1.4 | 1.7 |
| K,wt% | 2.0 | 1.7 | 1.9 |
| Na, wt% | 0.03 | 0.07 | 0.01 |
| Co, mg/kg | | 0.2 | |
| Cu, mg/kg | 8.0 | 33.0 | 12.4 |
| Mn, mg/kg | 5.9 | 5.7 | 38.7 |
| Zn, mg/kg | 197 | 38.7 | 99.2 |
| dry matter, wt% | 94.0 | 93.0 | 93.0 |
| crude fibre, wt% | | 3.0 | 2.0 |
| ether extract, wt% | | 1.1 | 2.5 |
| protein (N x 6.25), wt% | 45 | 44.6 | 48.3 |
| digestible protein, wt% | | 38.4 | 41.5 |
| thiamine, mg/kg | 90 | 91.7 | 6.2 |
| riboflavin, mg/kg | 45 | 35 | 44 |
| nicotinic acid, mg/kg | | 448 | 500 |
| pyridoxine, mg/kg | 40 | 43 | 30 |
| biotin, mg/kg | 1.3 | | 1.1 |
| pantothenate, mg/kg | 65 | 110 | 83 |
| folic acid, mg/kg | 15 | 10 | 23 |
| choline, mg/kg | 4000 | 3885 | 2911 |

* Baker's and Brewer's yeast generally refer to *S. cerevisiae*



2.2.2.1. Carbon

All yeasts can utilise glucose as a carbon and energy source (Rolland *et al.*, 2002). As a result, glucose has been the focus of carbon metabolism in yeast (Käppeli, 1986). The common pathway in sugar metabolism is the conversion of glucose and related sugars to pyruvate (Flores *et al.*, 2000). The pathway by which pyruvate is further metabolised is dependent on both the yeast species and the presence or absence of oxygen (Käppeli, 1986). Under aerobic conditions glucose-insensitive

yeasts oxidize pyruvate to carbon dioxide through the tricarboxylic acid (TCA) cycle resulting in fast growth rates and high biomass yields (Flores *et. al.*, 2000). Glucose-sensitive yeasts such as *S. cerevisiae* are able to metabolise low amounts of glucose to carbon dioxide and water under these conditions (Berry, 1983). However, glucose concentrations in excess of 0.1% to 0.2% results in repression of respiration, production of large concentrations of ethanol and a reduced biomass yield (Kroschwitz and Howe-Grant, 1992).

2.2.2.2. Nitrogen

Yeasts are capable of utilising a variety of organic and inorganic nitrogen sources. Ammonium sulphate is utilised by most yeast and consequently, is commonly used as a nitrogen source in yeast media (Suomalainen and Oura, 1970; Walker, 1998). Urea is also frequently used to satisfy nitrogenous requirements although it is not utilised by all yeasts (Walker, 1998). Although *S. cerevisiae* is unable to utilise either nitrate or extracellular protein such as low molecular peptides as nitrogen sources, other yeasts have this ability (Berry, 1983; Walker, 1998). Other utilisable nitrogen sources include amino acids, purines, pyrimidines, and amines (Berry, 1983; Walker, 1998).

### 2.2.3. Yeast cultivation media

The cultivation medium must contain all the components required for growth and product formation (Lee, 1996). This does not necessarily mean medium components should be supplied in excessive quantities. Some nutrients can inhibit or even poison cell growth if they occur above a certain concentration. Alternatively, the extensive growth resulting from excess nutrient concentrations can cause the accumulation of metabolic by-products that will have a negative effect on growth (Bailey and Ollis, 1986). Also, residual nutrients from excess nutrients create unnecessary waste. Yeast media can be classified as defined or complex depending on the composition.

2.2.3.1. Defined medium

A chemically defined medium is composed of relatively pure compounds that occur in known quantities (Casida, 1968; Prescott *et al.*, 1999). This type of medium is ideal for medium design and can be used to determine the specific requirements for growth and product formation of an organism and is preferred for laboratory studies

(Zhang and Greasham, 1999). Theoretically this is the medium of choice for high cell density cultivation of microorganisms using fed-batch fermentation, as the yield coefficients and growth rates are easily determined and simplify the feeding strategy used (Lee, 1996; Zhang and Greasham, 1999). Defined media promotes reproducibility between different fermentations and eliminates any errors due to medium composition (Casida, 1968).

Simplified and relatively inexpensive defined media have been used successfully for commercial fermentations (Zhang and Greasham, 1999). However they are frequently expensive primarily because of the cost of the pure chemical components (Casida, 1968). When grown in a defined medium a microorganism is required to synthesise all cellular components from the simple chemical elements. Thus this type of medium is impractical for microorganisms that require large amounts of additives. Yeast nitrogen base and yeast carbon base (Table 2.5) are two defined media that are commercially available for growing yeast cells (Phaff *et al.*, 1978). Yeast nitrogen base is deficient in a carbon source and can be used to determine the carbon requirements of yeasts. Conversely, yeast carbon base requires the addition of a nitrogen source (Walker, 1998). Both media also contain the vitamins that are generally required for yeast growth and would most likely be too expensive to consider for industrial fermentations.



2.2.3.2. Complex medium

A complex medium is composed of one or more chemically undefined substrates (Prescott *et al.*, 1999). One of the primary reasons for employing complex media is their relatively low cost (Dahod, 1999). Consequently, complex fermentation substrates frequently consist of waste products from the brewery, bakery and corn-milling industries (Casida, 1968). Although yeasts and multicellular fungi are generally less fastidious than bacteria, their cultivation frequently requires the addition of vitamins and/or growth factors (Walker, 1998). Yeast extract-peptone-glucose (YEPG) is routinely used for laboratory cultivation and maintenance of yeast strains (Walker, 1998). Yeast extract is industrially prepared by the autolysis or hydrolysis of yeast cells and is used to satisfy the vitamin and/or growth factor requirement of yeast cells (Crueger and Crueger, 1989). Although a complex substrate is generally added to the medium to provide a single nutrient, they often

contain multiple nutrients for microbial growth (Dahod, 1999). Yeast extract is composed of a mixture of amino acids and peptides, water-soluble vitamins, and carbohydrates (Table 2.6). The carbohydrates are primarily glycogen and trehalose which are hydrolysed to glucose during the yeast extract preparation process (Bridson and Brecker, 1970; Crueger and Crueger, 1989).

**Table 2.5:** Composition of two chemically defined media for growing yeasts (amounts are given per litre of distilled water) (Phaff *et al.* 1978)

| INGREDIENTS | YEAST NITROGEN BASE | YEAST CARBON BASE |
|---|---|---|
| **Carbon source** | grams | grams |
| D-glucose | none[a] | 10 |
| **Nitrogen source** | | |
| $(NH4)_2S0_4$ | 5 | none[b] |
| **Salts** | | |
| $KH_2PO_4$ | 1.0 | 1.0 |
| $MgSO_4.7H_2O$ | 0.5 | 0.5 |
| NaCl | 0.1 | 0.1 |
| $CaCl_2.2H_2O$ | 0.1 | 0.1 |
| **Amino Acids** | milligrams | milligrams |
| L-histidine.HCl.$H_2O$ | 10 | 1.0[c] |
| DL-methionine | 20 | 2.0[c] |
| DL-tryptophan | 20 | 2.0[c] |
| **Compounds supplying trace elements** | micrograms | micrograms |
| $H_3BO_3$ | 500 | 500 |
| $CUSO_4.5H_2O$ | 40 | 40 |
| KI | 100 | 100 |
| $FeCl_3.6H_2O$ | 200 | 200 |
| $MnSO_4.H_2O$ | 400 | 400 |
| $Na_2MoO_4.2H_2O$ | 200 | 200 |
| $ZnSO_4.7H_2O$ | 400 | 400 |
| **Vitamins** | | |
| Biotin | 2 | 2 |
| Calcium pantothenate | 400 | 400 |
| Folic acid | 2 | 2 |
| Inositol | 2000 | 2000 |
| Niacin | 400 | 400 |
| Para-aminobenzoic acid | 200 | 200 |
| Pyridoxine·HCl | 400 | 400 |
| Riboflavin | 200 | 200 |
| Thiamine·HCl | 400 | 400 |

[a] The desired carbon source must be added.
[b] The desired nitrogen source must be added.
[C] The nitrogen contained in these three amino acids is insufficient to support visible growth.

**Table 2.6:** General composition of yeast extract (Bridson and Brecker, 1970)

*A General Analysis of Yeast Extract Paste*

| | (g/100g) |
|---|---|
| Total nitrogen | 7.5-10.5 |
| Amino nitrogen | 3.4-4.8 |
| Chlorides as NaCl | 0.07-1.3 |
| Moisture | 30 |
| Phosphates as $P_2O_5$ | 3.8 |
| Carbohydrates | 8.2 |
| Purine nitrogen | 0.27 |
| Fat | trace |
| Sodium | 5.6 |
| Potassium | 3.0 |
| Calcium | 0.01 |
| Iron | 0.005 |
| Magnesium | 0.2 |
| Copper | 0.005 |
| Zinc | 0.005 |
| Manganese | 0.0005 |
| Cobalt | 0.0005 |

*Range of Values of Vitamin Content (μg/g) of Yeast Extract*

| | |
|---|---|
| Thiamine | 18-40 |
| Riboflavin | 18-150 |
| Nicotinic acid | 300-1250 |
| Pantothenic acid | 20-100 |
| Pyridoxine | 25-35 |
| Folic acid | 5-10 |
| Inositol | 1000-1700 |
| Choline | 1000-2000 |
| Biotin | 0.5-1.0 |
| *p*-Aminobenzoic acid | 6 |
| Vitamin B12 | 0.01 |

*Amino acid composition (g/100g) of Yeast Extract*

| | |
|---|---|
| Alanine | 3.4 |
| Arginine | 2.0 |
| Aspartic acid | 4.5 |
| Cystine | 0.45 |
| Glutamic acid | 6.7 |
| Glycine | 2.3 |
| Histidine | 1.2 |
| Isoleucine | 2.3 |
| Leucine | 3.0 |
| Lysine | 3.5 |
| Methionine | 0.7 |
| Phenylalanine | 1.7 |
| Proline | 1.7 |
| Serine | 2.3 |
| Threonine | 2.3 |
| Tryptophan | 0.5 |
| Tyrosine | 1.6 |
| Valine | 2.5 |

### 2.2.4. Marine yeast nutrition

The ocean is a source of a wide variety of dissolved organic compounds. These range from simple acids such as formic and acetic acid to more cornplex polymers of carbohydrates and amino acids (Parsons and Strickland, 1962). However these compounds are present in low quantities (a few μg/litre). According to Duursma (1960) an organic carbon content of 1000mg C/$m^3$ is a good estimate for the bulk ocean and is 5-10 times higher in surface waters and near the shore. The distribution of yeast in the marine environment depends primarily on the availability of organic material. The diversity of the yeast species and the total numbers of yeast cells are higher in coastal regions characterised by a higher organic carbon concentration, whereas the numbers are as low as 2-3 colony-forming units per litre of seawater in the open ocean (Ahearn, 1973; Phaff *et al.*, 1978). The digestive tract and possibly other sections of marine organisms are also a rich source of organic material (Azam and Hodson, 1981).

Microorganisms from the marine environment have such different nutrient requirements that it would be impossible to formulate a medium for the growth of all (Carlucci and Pramer, 1957). Attempts to isolate these organisms generally make use of media which are vastly different when compared to the low nutrient concentration present in the natural environment (Jannasch, 1967). Fell *et al.* (1960) isolated marine yeasts in medium containing 2% glucose. They found that the growth rate of the yeasts increased when the medium was supplemented with 0.1% yeast extract and 0.5% peptone. Reportedly some marine yeasts exhibit a nutritional requirement for biotin and thiamine. However the majority of yeasts do not require biotin and thiamine (Litchfield, 1976).



## 2.3. PHYSICAL PARAMETERS THAT AFFECT YEAST GROWTH

Besides the formation of biomass, the energy generated from catabolism is used in other processes called maintenance functions (Verduyn, 1991). Environmental conditions such as temperature, pH, osmotic stress etc., will determine how much energy is used for maintenance (Verduyn, 1991). Maintenance functions could have

a negative effect on the biomass yield if too much energy is expended for this purpose. The growth rate of microorganisms is also temperature and pH dependent (Blanch and Clark, 1996).

### 2.3.1. Temperature

Although most yeasts are able to grow at 20-25°C (Phaff *et al.*, 1978), a number of free-living yeasts are unable to grow at 30°C. The exception being yeasts isolated from warm-blooded animals that prefer higher temperatures and yeasts isolated from arctic regions that prefer lower temperatures. *Saccharomyces* strains mostly exhibit optimum grow rates at 28-34°C (Matthews and Webb, 1991). However the optimum temperature for yeast growth is species-dependent (Phaff *et al.*, 1978). Also, the optimum biomass yield does not necessarily occur at the optimum temperature i.e. the temperature at which the growth rate is optimal (Verduyn, 1991). In a study to determine the effect of temperature on yeast growth, van Uden (1984) found that high temperatures resulted in petite mutants and affected the permeability of the plasma membrane. Temperature also affected both the biomass composition and the biomass yield.

### 2.3.2. pH

Although yeasts are able to grow over a wide pH range (pH 3 to pH 8) most grow well between pH 4.5 and 6.5 (Walker, 1998). Regardless of the external pH, the intracellular pH is strictly regulated between 5.8 and 6.3. Large-scale cultivation processes are generally conducted at low pH values (between 4 and 5) in order to limit bacterial contamination (Matthews and Webb, 1991).

### 2.3.3. Osmotic tolerance

The addition of high salt concentrations causes water to leave the cell resulting in osmotic stress. Under these conditions algae and fungi increase intracellular concentrations of compatible solutes such as sucrose, arabitol, glycerol and mannitol to restore osmotic equilibrium (Prescott *et al.*, 1999). In *S. cerevisiae* osmotic stress induces increased production of glycerol while in other yeasts both glycerol and arabitol accumulates (Verduyn, 1991). A few yeasts, especially yeast from the genus *Debaryomyces,* are highly osmotolerant. Several marine microorganisms have been

isolated that have no dependence on sodium ions. However this observation is frequently made for complex media that contain traces of sodium ions (Litchfield, 1976). Most marine yeast isolates are able to grow equally well in media with no salt and in media containing 3-4% sodium chloride (Phaff *et al.*, 1978).

## 2.4. YEAST CULTIVATION SYSTEMS

### 2.4.1. Batch cultivation

When inoculated into new media, yeast growth proceeds through four typical phases: lag phase, log phase, stationary phase and death phase (Crueger and Crueger, 1989). During the initial lag phase the cells adjust metabolically to the new growth conditions. This is followed by a period of logarithmic or exponential growth. The onset of stationary phase occurs when the nutrients become depleted and when the metabolic by-products become inhibitory to growth. An exponential decline in the viable cell numbers is indicative of the death phase. The productivity of batch cultivation is increased by forcing the process to larger volumes and higher cell densities (Larsson *et al.,* 1997). However, since additional nutrients are not added and the waste products are not removed, dense culture production is restricted due to biological reasons e.g. overflow metabolism that results from high nutrient concentrations (Lee, 1996).

### 2.4.2. Fed-batch cultivation

Industrially, the fed-batch technique is used to overcome problems such as overflow metabolism associated with batch cultivation (Lee, 1996). The basic fed-batch fermentation process consists of at least two phases (Crueger, and Crueger, 1989). During the initial batch phase the cells grow at the maximum specific growth rate and utilise all the nutrients present in the medium. This is followed by a feeding phase during which a defined nutrient is added to the reactor at growth-limiting concentrations such that the cells grow at a reduced specific growth rate. The reduced specific growth rate is necessary to prevent conditions such as overflow metabolism, accumulation of toxic metabolic products, oxygen limitation and other

factors that contribute to inhibition of growth (Lee, 1996, Riesenberg and Guthke, 1999).

### 2.4.3. Continuous cultivation

Continuous cultivation allows microorganisms to be cultured over long periods of time (Shuler and Kargi, 1992). Nutrients are supplied at a constant rate while waste products and some cells are removed at the same rate (Crueger and Crueger, 1989). This allows the environmental conditions in the bioreactor to remain unchanged. Although continuous cultivations are the most productive their long duration makes it difficult to maintain aseptic conditions (Crueger and Crueger, 1989; Stafford, 1986).

## 2.5. FED-BATCH PRODUCTION OF DENSE CULTURES

An important objective of fermentation for both research and industry is to optimise volumetric productivity, i.e. to obtain the maximum amount of product in a given volume within a certain time (Lee, 1996; Riesenberg and Guthke, 1999). Since high culture densities are often a prerequisite for high productivity (Riesenberg and Guthke), it is important to develop a cultivation method that allows for the production of dense cultures (Lee *et al.*, 1999). Fed-batch fermentation is the most common strategy used in industry for the cultivation of dense microbial cultures (Lee *et al.*, 1999; Lee, 1996). This method is particularly useful for the cultivation of microbes where substrate inhibition or catabolite repression is a problem (Lee, 1996). Thus it is very important to implement the appropriate feeding strategy (Lee *et al.*, 1999). The fed-batch process is used industrially for the production of yeast biomass e.g. Baker's yeast (Spencer and Spencer, 1989). Since oxygen transfer is a key parameter in industrial processes for yeast biomass production, it is critical that sufficient oxygen is maintained in the bioreactor for rapid yeast growth (Berry, 1983; Walker, 1998).



### 2.5.1. Fed-batch feeding strategies

During fed-batch cultivation high substrate concentrations must be fed into the reactor in a controlled manner. As a result, many feeding strategies have been

developed to obtain high cell densities (Riesenberg and Guthke, 1999). The implementation of the appropriate feeding strategy is very important for the fed-batch fermentation process. Feeding strategies can be simple or very complex with feedback control. Simple feeding strategies include constant feeding, stepwise increased feeding and exponential feeding (Lee, 1996). During constant feeding substrate is fed into the reactor at a constant rate. However, the cell population and the volume constantly increase. As a result the substrate is no longer able to support the nutritional requirements of the growing population and the maximum specific growth rate decreases. In order to prevent this, the substrate is fed into the reactor with increasing steps. The optimum feed rate is to feed so that exponential growth is maintained. More sophisticated feeding methods couple the feeding strategy with the measurement of physical parameters such as dissolved oxygen, pH, microbial heat, $CO_2$ evolution rate, etc. (Lee *et al.*, 1999; Lee, 1996). However, these methods require sensitive and often expensive sensors and computer control systems. Simple feeding strategies have been used successfully to produce dense cultures of *E. coli* (Lee, 1996), *Cryptococcus curvatus* (Hassan *et. al.,* 1996), *Candida brassicae* (Yano *et. al.*, 1985) and others.

### 2.5.2. Oxygen transfer in the bioreactor

Oxygen transfer is a key parameter in aerobic fermentation processes (Crueger and Crueger, 1989; Noorman, 2001). According to Bailey and Ollis (1986), the oxygen consumption rate of an actively respiring yeast population with an estimated population density of $10^9$ cells per millilitre is ca. 6 g of oxygen per litre per hour. Considering that the oxygen concentration at saturation is 8.10 mg per litre (25°C, 1 atm) it is obvious that the oxygen demand of the actively respiring population per hour is 750 times greater than the oxygen saturation value. Aerobic conditions are achieved and maintained in fermentation vessels by sparging sterile air into the medium (Crueger and Crueger, 1989). However, due to its low solubility in aqueous solutions (Table 2.7), oxygen often becomes limiting during dense culture production (Lee, 1996). The major reason for oxygen depletion is due to microbial respiration (Bailey and Ollis, 1986). However, other factors such as changes in temperature, foaming and surface active compounds can also affect oxygen transfer (Bailey and Ollis, 1986).

**Table 2.7:** Oxygen solubility and Henry's constant for pure water (Blanche and Clarke, 1996)

| Temperature (°C) | Oxygen conc[a] mg $l^{-1}$ | Henry's constant, H atm $mg^{-1}$ |
|---|---|---|
| 25 | 8.10 | 0.0258 |
| 35 | 6.99 | 0.0299 |

[a] In equilibrium with air at 1 atm ($pO_2$ = 0.209 atm)

The transfer of oxygen from the air bubble to the cell involves a chain of mass transfer steps as shown in Figure 2.1 (Noorman, 2001). These mass transfer steps occur both in the liquid and gas phases. However, because oxygen is poorly soluble in liquid, the rate-limiting step for oxygen transfer is diffusion through the liquid layer surrounding the air bubble.

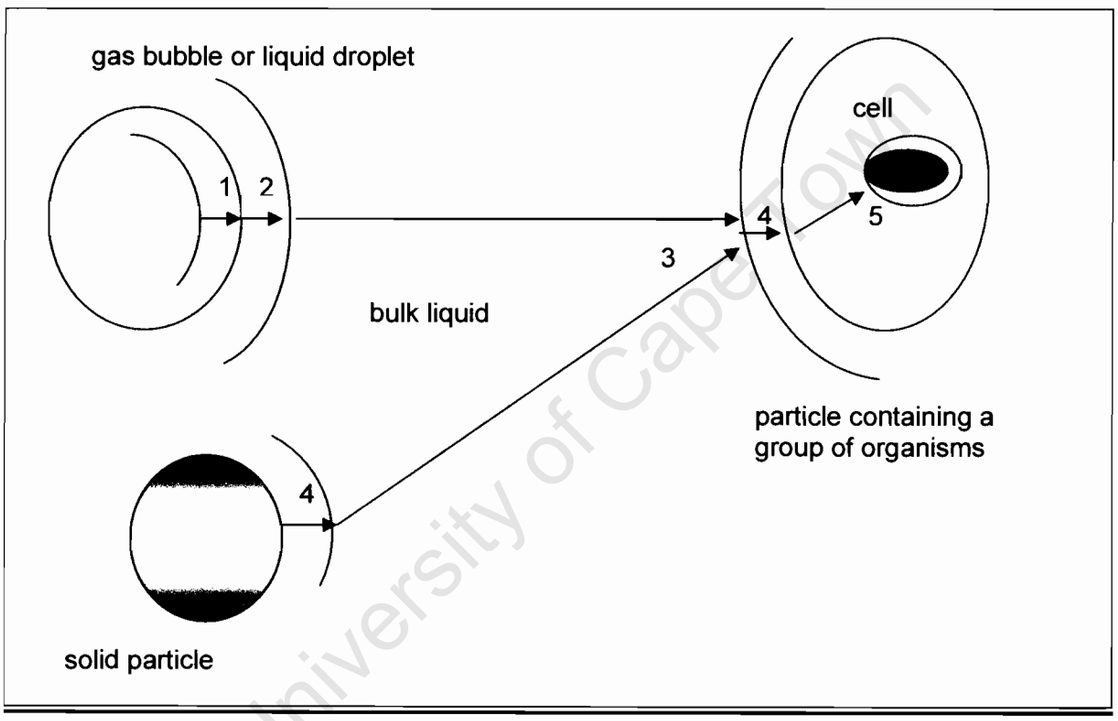




**Figure 2.1:** Chain of mass transfer steps for a substrate or nutrient from a gas bubble, liquid droplet or solid particle towards the site of reaction inside a cell. 1: Transfer (mainly by diffusion) of substrates from gas, liquid or solid phase to the interface with the liquid water phase; 2: Transport (most often by a combination of diffusion and convection) across a thin, rather stagnant layer of water phase that surrounds the gas bubble, liquid droplet or solid particle; 3: Transport (usually by convection or turbulence) through the bulk liquid phase to a thin layer surrounding a single micro-organism; 4: Transport (diffusive) across this layer to the cell surface; 5: Transport (passive by diffusion and/or active with a transport enzyme) over the cell envelope to a site inside the cell where the reaction takes place. NB: Products formed take the reverse route. Reproduced from Noorman (2001).

The overall transfer of oxygen is given by:

$$OTR = k_L a(C^* - C) \qquad \text{Equation 2.1}$$

where,

OTR = Oxygen transfer rate

$(C^* - C)$ = the overall driving force i.e. the difference between the saturated oxygen concentration, $C^*$, and the oxygen concentration in the liquid medium, C.

$k_L$ = liquid phase mass transfer coefficient ($ms^{-1}$)

a = is the gas-liquid interfacial area per unit volume of fluid ($m^{-1}$)

Accordingly, the three parameters involved in oxygen transfer are $k_L$, a, and C*-C. The first two parameters are often treated as one parameter; namely, the volumetric transfer coefficient, $k_La$, which is dependent on the gas flow rate, speed of agitation, oxygen partial pressure, and the addition of an antifoam agent. The oxygen supply in a bioreactor can be increased by increasing the aeration rate or agitation speed or by supplementing the air stream with pure oxygen (Lee, 1996). Alternatively, the oxygen demand of cells can be reduced by decreasing the growth rate.

## 2.6. RECOVERY OF YEAST CELLS

The recovery and purification of the product is a very important component of any industrial fermentation process (Crueger and Crueger, 1989; Shuler and Kargi, 1992). The method selected for the recovery and purification process is dependent on the size and nature of the product (Crueger and Crueger, 1989). The product may be cellular material (biomass), an extracellular component or an intracellular component (Shuler and Kargi, 1992). Regardless of this the primary step in product recovery is usually the separation of solid components such as biomass, insoluble particles and macromolecules from the fermentation broth (Hatti-Kaul and Mattiasson, 2001; Shuler and Kargi, 1992). If biomass is the product this is the major step in the recovery process (Shuler and Kargi, 1992). The methods commonly employed for solid-liquid separation are coagulation and flocculation, filtration and centrifugation.

### 2.6.1. Coagulation and flocculation

Sometimes the fermentation broth is pre-treated prior to filtration and centrifugation to promote the formation of cell aggregates to facilitate the solid-liquid separation process (Crueger and Crueger, 1989; Hatti-Kaul and Mattiasson, 2001; Shuler and Kargi, 1992). Coagulation is the formation of small flocs due to the addition of a coagulating agent, which are usually simple electrolytes (Shuler and Kargi, 1992). A flocculating agent, usually a polyelectrolyte or certain salts, is then used to agglomerate the small flocs into larger particles which will settle easier (Crueger and Crueger, 1989).

### 2.6.2. Centrifugation

Centrifugation is used for the separation of microbial cells and small particles from the culture broth (Hatti-Kaul and Mattiasson, 2001). This method is sometimes used to harvest yeast cells (Bailey and Ollis, 1986).

### 2.6.3. Filtration

Filtration is the simplest and most economical method for the separation of large solid particles and cells from the fermentation medium (Shuler and Kargi, 1992). The fermentation broth is passed through the filter medium. A filter cake is formed on the filter surface due to the retention of solid particles while the liquid passes through the filter. Filtration is used for the separation of 10 to 40% solids by volume and 0.5 to 10 μm by particle size (Hatti-Kaul and Mattiasson, 2001). Continuous rotary filters or rotary drum precoat filters are commonly employed in the fermentation industry for the filtration of filamentous fungi and yeast cells (Hatti-Kaul and Mattiasson, 2001; Shuler and Kargi, 1992). However, since filtration is strictly dependent on the pore size and the particle size, clogging of filters is problematic (Crueger and Crueger, 1989). As a result cross-flow filtration was developed to prevent clogging. Three types of cross-flow filtration processes are recognised based on the pore size: reverse osmosis, ultrafiltration and microfiltration (Crueger and Crueger, 1989). Microporous filters with pore sizes of 0.2 to 2 μm are used for the separation of yeast cells (Crueger and Crueger, 1989; Shuler and Kargi, 1992).

# CHAPTER 3

## EXPERIMENTAL PROCEDURES

## CONTENTS

## 3.1. INTRODUCTION

This chapter describes the techniques employed for the cultivation and evaluation of strain SS1 for efficient biomass production. These include yeast strain and maintenance (section 3.2), medium and cultivation conditions (section 3.3) as well as analytical methods (section 3.4) used for the assessment of growth and metabolism. The chapter concludes with an experimental approach (section 3.5), which describes how the experimental procedures were implemented in order to achieve the aims and objectives of the project.

## 3.2. YEAST STRAIN AND MAINTENANCE

### 3.2.1. Yeast strain

The yeast strain used in this study, commonly referred to as strain SS1, was isolated from cultured *H. midae*. The yeast was identified as belonging to the genus *Cryptococcus* by 16S DNA analysis (Personal communication B. M. Macey)[1]. The strain was thought to be a potential probiotic because it was shown to produce an extracellular amylase. The yeast could also potentially aid in disease prevention by stimulating the immune system of *H. midae* (Personal communication B. M. Macey)[2].

### 3.2.2. Maintenance

Strain SS1 was cultured from glycerol stocks once a month and maintained on YEPG agar plates (Appendix A.2) at ambient (room temperature, ~22°C) for a maximum period of two weeks. The yeast was transferred on a weekly basis to new plates until a month had expired when it was re-streaked from glycerol stocks. For long-term storage glycerol stocks were prepared in cryotubes by mixing an overnight yeast culture 2:1 with 50% glycerol in a total volume of 1 ml. The tubes were vortexed to ensure that the cells were evenly distributed and immediately stored at -70°C.

[1] B. M. Macey, PhD student, University of Cape Town, Unpublished data

[2] *Ibid.*

## 3.3. MEDIUM AND CULTIVATION CONDITIONS

### 3.3.1. Medium

The standard medium for cultivation of strain SS1 was YEPG, which consisted of yeast extract (10 $gl^{-1}$), proteose peptone (20 $gl^{-1}$) and glucose (20 $gl^{-1}$). The proteose peptone was later substituted with either bacteriological peptone or tryptone as indicated. All cultivation vessels were sterilised containing all the medium components except glucose in order to avoid the Maillard reaction. A 10x concentrated glucose solution was prepared in a volume that corresponds to 10% of the total cultivation volume and autoclaved separately.

### 3.3.2. Shake flask cultivation

All shake flask cultivation experiments were conducted as indicated in 50 ml, 100 ml or 5-litre Erlenmeyer flasks containing a medium volume corresponding to 10% of the volumetric capacity of the relevant flask unless otherwise stated. The inoculum was prepared by inoculating 5 ml of medium with a single colony. This 5 ml culture was subsequently used to inoculate experimental flasks. In cases where the 5 ml volume was insufficient, it was inoculated into 50 ml media, which was used as inoculum for further experiments. All cultures were incubated for the time period indicated at room temperature on a rotary shaker set at 100 rpm to ensure adequate aeration.

### 3.3.3. Bioreactor cultivation

3.3.3.1. Equipment

All batch and fed-batch cultivations were conducted in a 7-litre Chemap stirred tank reactor (STR) as shown in Figure 3.1. The reaction vessel was sterilised with all the medium components except the glucose, which was autoclaved separately. The glucose and inoculum were diluted 1/10 into the bioreactor to obtain a glucose concentration of 20 $gl^{-1}$ and the required starting optical density as indicated. Temperature was maintained at 25°C by circulating water through an internal cooling coil.

The dissolved oxygen tension (DOT) in the reactor was measured with a polarographic oxygen probe (Mettler Toledo). Compressed air was sparged into the


**Figure 3.1:** The 7*l* Chemap stirred tank reactor.

reactor at a rate of 1 vvm or alternatively set to maintain the DOT above 20% to prevent oxygen limitation. pH was monitored with an Ingold pH probe and was not controlled unless stated otherwise. In cases where pH control was necessary, 5 M sodium hydroxide or 2 M hydrochloric acid was used. Foaming was prevented by the addition of 0.02% Organic antifoam (Sigma) prior to sterilisation.

3.3.3.2. Inoculum preparation

The culture used to inoculate the fermenter was passed through several pre-inoculum stages in order to optimise the growth rate of the strain. A single colony was inoculated into 5-ml medium in a universal container and incubated at room temperature for 24 h. This 5 ml culture was used to inoculate 50 ml of broth that was in turn used to inoculate 500 ml broth. In initial experiments the reactor was inoculated with the required volume to standardise to an $OD_{600}$ of 0.02 that was the standard optical density used for shake flask experiments. The starting $OD_{600}$ was later increased 10-fold to 0.2 in order to eliminate the lag phase. A starting $OD_{600}$ of 0.4 was used to achieve high cell densities in the STR.

3.3.3.3. Batch procedure

Batch cultivation was performed using a 5- or 6-litre working volume. Batch cultivation was carried out until the cells reached stationary phase. The data from batch cultivation studies was used to determine the kinetic parameters needed for fed-batch studies.

3.3.3.4. Fed-batch procedure

Fed-batch cultivation was initiated as a batch culture. During the batch phase, the glucose concentration was monitored as described in section 3.4.2. The feeding phase was initiated once the glucose levels decreased to a certain concentration or after a required amount of time had expired. The substrate was fed into the reactor from a reservoir with a Masterflex peristaltic pump using a constant or a stepwise increasing feeding strategy as indicated. Feeding proceeded until the culture level reached the appropriate volume. The cells were then cultured as a batch process until the depletion of glucose or the onset of stationary phase. During the feeding phase the volumetric air supply was increased to maintain the DOT above 20%.

## 3.4. ANALYTICAL METHODS

### 3.4.1. Yeast growth

During cultivation in the shake flasks, a 500 µl yeast suspension was sampled and the growth monitored by measuring the optical density at 600 nm ($OD_{600}$). For studies in the STR, a 5 ml yeast suspension was sampled aseptically at regular intervals through a steam sterilised sample port and immediately processed for further analysis. The progression in cell growth was monitored gravimetrically by determining dry cell weights, spectrophotometrically by measuring the $OD_{600}$ and by direct cell counts using a haemocytometer.

#### 3.4.1.1. Dry cell weight

Aliquots (1 ml) of the yeast sample were dispensed into three labelled pre-weighed and pre-dried Eppendorf tubes and pelleted by ultracentrifugation (14000 rpm) for 2 minutes. The supernatants were stored at -20°C for further analysis. The pellets were re-suspended in 1 ml deionised water and re-centrifuged. The water was discarded and the tubes containing the pellets dried at 80°C for 72 h. The tubes were cooled in a desiccator, re-weighed and the yeast dry cell weight determined.

#### 3.4.1.2. Optical density

Growth was monitored spectrophotometrically (Beckman DU530) by measuring the optical density of cultures at 600 nm ($OD_{600}$). At OD values above 0.6, dilutions were made to ensure a linear relationship between cell concentration and optical density.

#### 3.4.1.3. Direct cell counts

The total cell count was performed microscopically under 400x magnification using a standard haemocytometer. The culture was diluted appropriately with distilled water and a minimum of 300 cells were counted to ensure statistical accuracy.

### 3.4.2. Reducing sugar determination

The dinitrosalicylic acid (DNS) reducing sugar method (Appendix C.1; Miller, 1956) was used to determine the amount of glucose in the medium. The amount of glucose

in the medium was determined from a glucose concentration standard curve (Appendix D.1).

### 3.4.3. Soluble protein determination

The amount of soluble protein in the medium was determined by a modified version of the protein quantitation assay described by Lowry *et al.* (1951) (Appendix C.2) using bovine serum albumin as a standard (Appendix D.2).

### 3.4.4. Determination of the volumetric oxygen transfer coefficient

3.4.4.1. Stirred tank reactor (STR)

The volumetric transfer coefficient in the STR was determined using the dynamic method first developed by Taguchi and Humphrey (1966) as stated in Atkinson and Mavituna (1983). The calculation of the volumetric mass transfer coefficient, $k_La$, was based on an unsteady state material balance for dissolved oxygen (DO). The airflow of an actively respiring culture was temporarily halted. DO readings was recorded at 5 or 10 second intervals. The air supply was resumed before the DO dropped below the critical concentration of 20%. Below the critical concentration, the culture would be exposed to oxygen-limited conditions which would negatively affect growth. The change in the DO concentration as a function of time is shown in Figures 3.2 (Noorman, 2001) and E.3 (Appendix E).

The mass balance for oxygen over the course of the experiment is given by Equation 3.1:

$$\frac{dC}{dt} = k_L a\left(C^* - C\right) - q_0 X \qquad \text{Equation 3.1}$$

where: $\left(C^* - C\right)$ = the overall driving force i.e. the difference between the saturated oxygen concentration, $C^*$, and the oxygen concentration in the liquid medium, C.

$k_L$ = liquid phase mass transfer coefficient ($ms^{-1}$)

a = is the gas-liquid interfacial area per unit volume of fluid ($m^{-1}$)

$q_0$ = the specific oxygen utilisation rate

and $q_0X$ = the oxygen utilisation rate

Immediately after the air supply is terminated the DO rapidly decreases. This linear decline (line AB in Figure 3.2) represents the oxygen utilisation rate (OUR) of the microorganisms (Figure E.4, Appendix E). The $k_La$ for oxygen becomes zero and Equation 3.1 becomes:

$$-\frac{dC}{dt} = q_0X \qquad \text{Equation 3.2}$$

When the air supply (line BC in Figure 3.2) is restored Equation 3.1 can be rearranged:

$$C = \frac{1}{k_La}\left(\frac{dC}{dt} + q_0X\right) + C^* \qquad \text{Equation 3.3}$$

A plot of C on the y-axis and $\frac{dC}{dt} + q_0X$ on the x-axis will yield a straight line with slope $-\frac{1}{k_La}$ and intercept $C^*$ (Figure E.5, Appendix E).

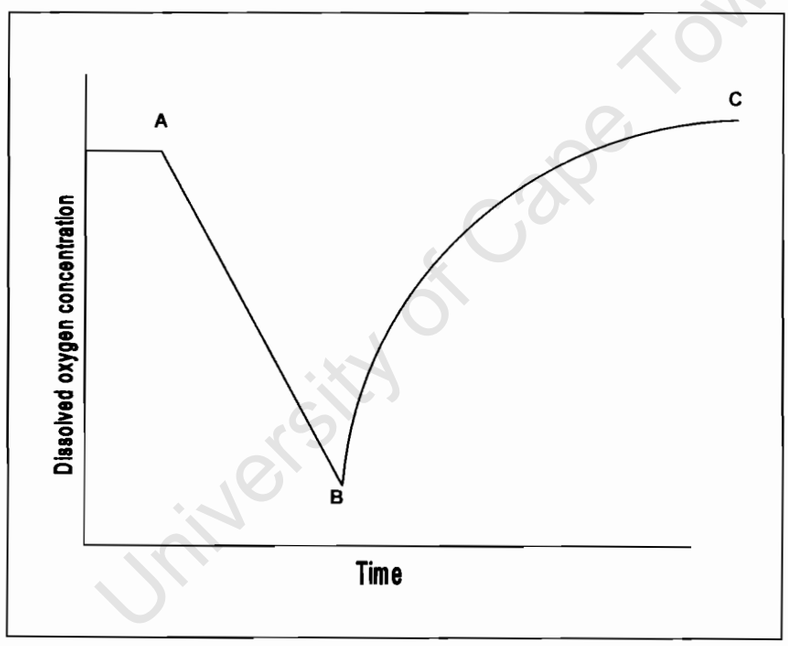


**Figure 3.2:** Relationship between dissolved oxygen concentration and time in dynamic method (Noorman, 2001)

3.4.4.2. Shake flasks

The volumetric oxygen transfer coefficient was determined using the gassing-out technique (van't Riet, 1979). The DO was measured with a polarographic oxygen probe (Mettler Toledo) previously calibrated between zero and saturation with sodium sulphite and compressed air. The shake flask containing the appropriate volume of distilled water was agitated on an orbital shaker at 100 rpm. The oxygen was removed from the flask by sparging the liquid with nitrogen gas until the DO reached zero. Compressed air was then sparged into the liquid at approximately 2.5 to 3.0 litres per minute and the increase in the oxygen concentration was recorded at 10 second intervals. Compressed air was needed because the response time of the DO probe was extremely slow. However, a comparative estimate of $k_La$ for the different liquid volumes would still be achieved if aeration occurred at the same rate. The increase in the DO concentration as a function of time is shown in Figure E.1 (Appendix E).

Since this is a non-fermentative system the OUR, $q_0X$, is zero. Thus Equation 3.1 becomes:

$$\frac{dC}{dt} = k_La\left(C^* - C\right) \qquad \text{Equation 3.4}$$

This can be rearranged and integrated as follows

$$\int_0^C \frac{dC}{\left(C^* - C\right)} = \int_0^t k_La \cdot dt \qquad \text{Equation 3.5}$$

$$-\ln\frac{\left(C^* - C\right)}{C^*} = k_La \cdot t \qquad \text{Equation 3.6}$$

$$\ln\left(1 - \frac{C}{C^*}\right) = -k_La \cdot t \qquad \text{Equation 3.7}$$

Thus a plot of $\ln\left(1 - \frac{C}{C^*}\right)$ on the y-axis and time on the x-axis will yield a straight line with slope $-k_La$ (Figure E.2, Appendix E)

## 3.5. EXPERIMENTAL APPROACH

The design of a dense culture production process requires that three key factors be considered:

a) The effect of environmental parameters on growth must be determined so that the strain is cultured using optimal growth conditions
b) The kinetic parameters are used to model the growth and give a quantitative description of the batch cultivation process
c) A fed-batch cultivation process is developed based on data generated from batch culture experiments

### 3.5.1. Evaluation of environmental conditions on growth

The most important factor that affects biomass formation is the environmental conditions under which the strain is cultivated. This includes medium content as well as physical parameters such as pH, temperature and salt concentration. These studies were used to elucidate which components of the medium are important for growth and the optimum culture conditions for strain SS1.

### 3.5.2. Kinetic modelling of batch growth

Once the environmental parameters that affect growth were optimised for biomass production, batch cultivations was conducted in a stirred tank reactor (STR). The kinetic parameters such as yield coefficients, productivity and specific growth rate were used to give a quantitative description of the batch cultivation process. The major medium components such as soluble protein and glucose were monitored during growth to give an indication of how these components change over the course of the fermentation and to relate substrate consumption with biomass formation.

### 3.5.3. Fed-batch cultivation strategy

The feed substrate and a suitable time to initiate feeding were selected based on batch cultivation experiments. An appropriate fed-batch feeding strategy was then selected and implemented to produce dense cultures of the yeast strain.

# CHAPTER 4

## THE EFFECT OF ENVIRONMENTAL PARAMETERS ON GROWTH

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## CONTENTS

## 4.1. INTRODUCTION

The key element that affects biomass production is the environment in which the cells are grown (Walker, 1998). This includes nutrient availability, pH, temperature, osmotic stress, etc. The medium forms the environment of the microorganism that is being cultivated and thus directly influences cell growth and the productivity of the fermentation process (Casida, 1968). In industrial processes where biomass is the product, the medium also contributes significantly to production cost (Reed, 1982). Thus it is very important to select a medium that contains all the nutrients required for growth and that is cost-effective. Once a suitable medium has been selected it is important to determine the effect of other physicochemical parameters on cell growth. Yeasts are able to tolerate a number of physical and chemical stresses in the natural environment and often certain growth characteristics or requirements are associated with the environment from which the microorganism was isolated (Phaff *et al.*, 1978).

The primary aim of this project was to determine the optimum conditions for growth of strain SS1. The physicochemical parameters evaluated for the purpose of this study were the composition of the medium, pH, temperature and salt concentration. The growth of the yeast was evaluated on different medium substrates to select the most economical medium for dense culture production. Due to the number of experiments that needed to be conducted, most of these studies were performed in shake flasks. However, since the effect of the different medium components is relative (Kennedy, 1994), the medium that generated the greatest biomass in the shake flasks was also expected to be the best medium in the stirred bioreactor. The salt concentration of the medium was investigated because strain SS1 was isolated from a marine invertebrate and hence was potentially a marine yeast which may have an obligate requirement for sodium chloride.

## 4.2. RESULTS

### 4.2.1. Effect of medium composition on growth of strain SS1

Yeasts generally require the addition of vitamins and / or growth factors for dense culture production. As a result they are typically cultivated in media containing complex substrates such as yeast extract and peptone.

#### 4.2.1.1. Effect of protein source on growth

Initially, the standard medium for cultivation of strain SS1 was YEPG (Appendix A.2), which contains yeast extract, proteose peptone and glucose. The proteose peptone in YEPG was very expensive at approximately ZAR2000 per 500 g bottle. The cost of one litre of culture broth was approximately ZAR87, which was primarily due to the high price of proteose peptone. As a result, this medium was impractical even for shake flask studies where relatively small volumes are used. The first task was to reduce the cost of the medium by substituting the proteose peptone with a more economical protein source.

**Table 4.1:** Protein contributed by different substrates

| **MEDIUM** | **YEBD*** | | **YETD*** | | **YEPG*** | |
|---|---|---|---|---|---|---|
| **Protein substrate** | B | YE | T | YE | P | YE |
| **%TN[1]** | 10.0 | 11.3 | 12.7 | 11.3 | 12.0 | 11.3 |
| **Amount added ($gl^{-1}$)** | 20 | 13.4 | 20 | 8.81 | 20 | 10 |
| **Protein contributed[2] ($gl^{-1}$)** | 12.5 | 9.5 | 15.8 | 6.2 | 15.0 | 7.0 |
| **Total [Protein] ($gl^{-1}$)** | **22.0** | | **22.0** | | **22.0** | |

Abbreviations: B=Bacteriological peptone, T=Tryptone, P=Proteose peptone, YE=Yeast extract, G=Glucose

* Appendix A

[1] Percentage total amino nitrogen

[2] Calculated using Equation 4.1

For the purpose of this study bacteriological peptone and tryptone were obvious alternatives due to their low prices and their common use in microbiological media. The proteose peptone in YEPG was substituted with either tryptone or bacteriological peptone. The amount of proteose peptone, tryptone or bacteriological peptone added to the medium remained constant at 20 $gl^{-1}$. However, the amount of protein contributed by each substrate was different as indicated by the percentage total amino nitrogen (Table 4.1). The amount of protein in the medium was kept constant at 22 $gl^{-1}$ by altering the amount of yeast extract. The contribution of each substrate to the protein content of the medium and the amount of yeast extract needed to maintain a constant protein concentration was calculated using Equation 4.1 and is shown in Table 4.1.

$$\mathbf{P = \%TN \times p \times 6.25} \quad \text{(Equation 4.1)}$$

where: P = Protein concentration ($gl^{-1}$)
%TN = Percentage total amino nitrogen
p = mass protein substrate (g)



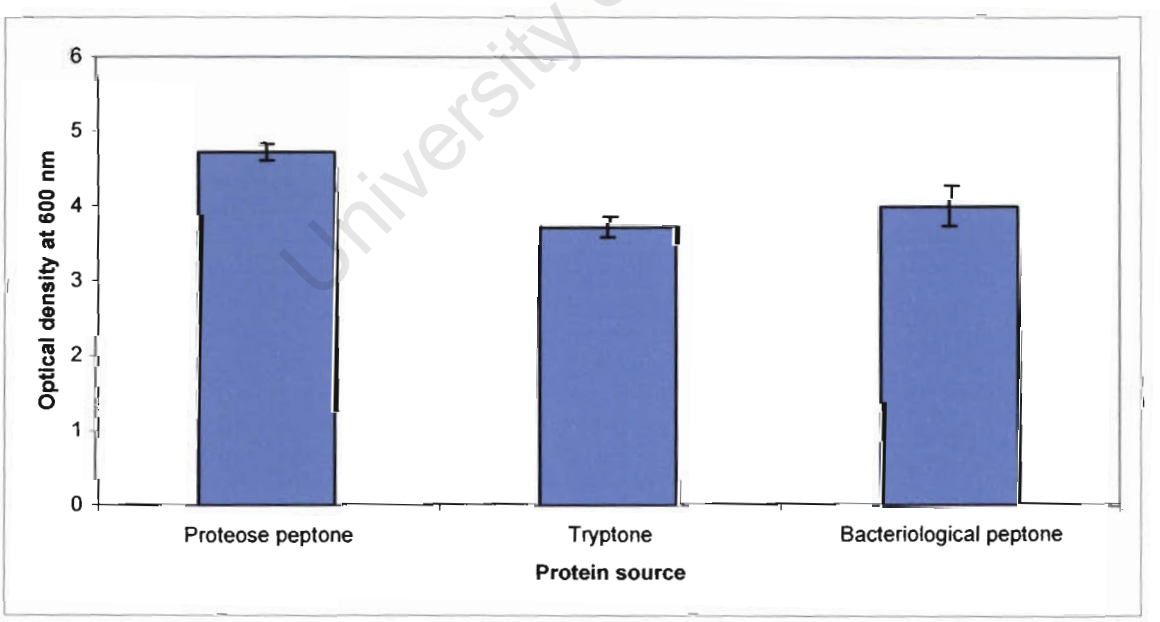



**Figure 4.1:** Effect of protein source on the growth of strain SS1. All cultures were standardised to an initial optical density of 0.02 at 600 nm ($OD_{600}$) and incubated at room temperature for 24 h in Erlenmeyer flasks on a rotary shaker set at 100 rpm to ensure adequate aeration. The error bars indicate the standard error between duplicate experiments.

Both tryptone and bacteriological peptone supported the growth of strain SS1 although growth was better in the medium containing the proteose peptone (Figure 4.1). However, the improved growth in the medium containing proteose peptone was not enough to motivate the use of such an expensive substrate. There was no significant difference between the growth of strain SS1 in the medium containing either tryptone or bacteriological peptone. The prices of the two substrates were also very similar. However, the data for the growth of strain SS1 in medium containing bacteriological peptone was less consistent than growth in medium containing tryptone. Based on these experiments, tryptone was selected as a substitute for proteose peptone in YEPG medium. In keeping with convention this medium was designated YETG (Appendix A.3).

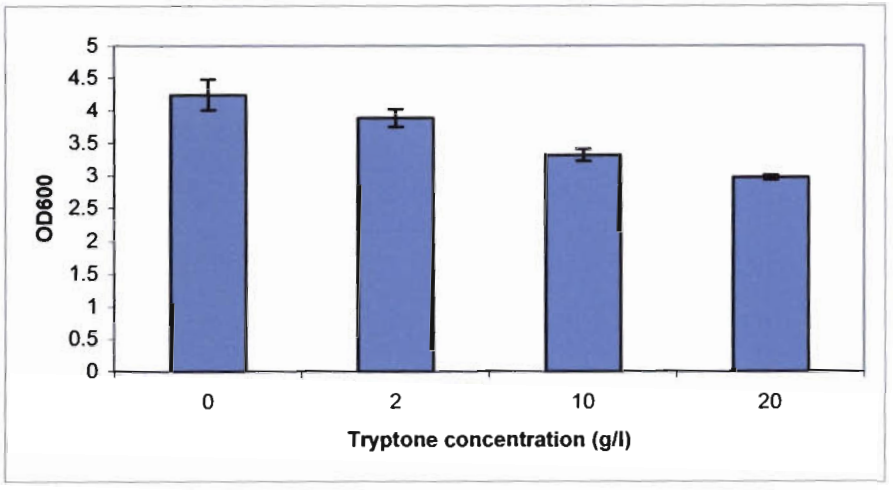
4.2.1.2. Effect of medium component concentration on growth

Earlier studies to find an appropriate substitute for the proteose peptone in YEPG (section 4.2.1.1) were conducted under the premise that the presence of a protein source was important for the growth of strain SS1. However, this assumption was based on literature focussing on general yeast growth rather than on experimental observations. The aim of this study was to determine the effect of the individual components in YETG medium on the growth of strain SS1. This would elucidate whether all the substrates in YETG are required for growth of strain SS1.

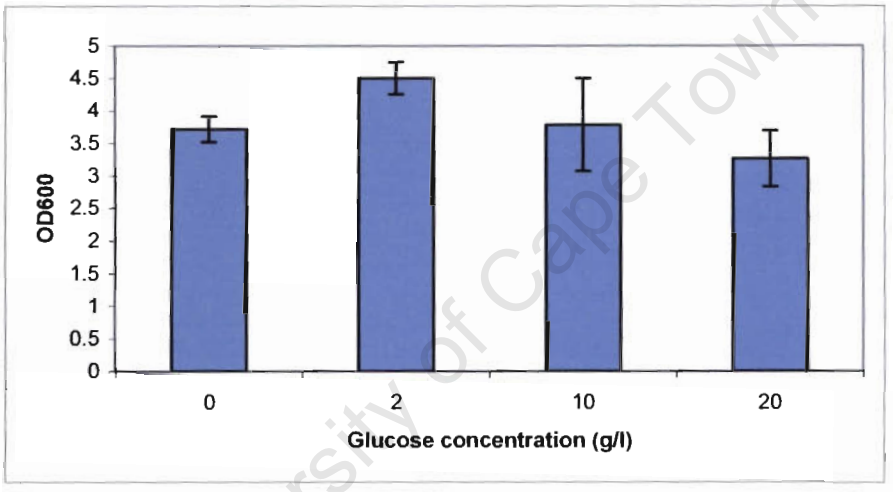
YETG medium consisted of 20 g tryptone $l^{-1}$, 8.81 g yeast extract $l^{-1}$ and 20 g glucose $l^{-1}$. The concentration of each of the substrates in YETG medium was systematically increased from 0 $gl^{-1}$ to the concentration at which it was originally present in YETG while the concentration of the other two substrates were kept constant.

The greatest amount of growth occurred when tryptone was omitted from the medium. Indeed, there was a negative relationship between increasing tryptone concentration and strain SS1 growth (Figure 4.2 a). Therefore it was concluded that tryptone was not required for growth of strain SS1.

**a) Tryptone**




**b) Glucose**




**c) Yeast extract**

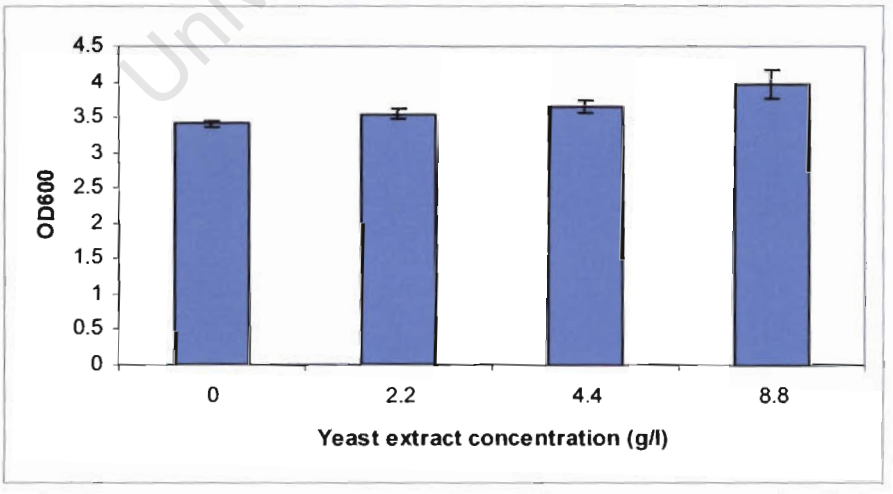





**Figure 4.2:** The effect of medium component concentration on growth of strain SS1. All cultures were standardised to an initial optical density of 0.02 at 600 nm ($OD_{600}$) and incubated at room temperature for 24 h in Erlenmeyer flasks agitated at 100 rpm. The error bars indicate the standard error between duplicate experiments.

The results for growth on different glucose concentrations were inconclusive. No clear relationship between the growth of strain SS1 and glucose concentration was observed (Figure 4.2 b). The growth in the flask containing 2 $gl^{-1}$ appeared slightly better. However, statistical analysis revealed with 95% confidence that there was no significant difference ($P<0.05$) in the growth of strain SS1 in YETG medium containing different glucose concentrations.

The growth of strain SS1 increased with increasing yeast extract concentration (Figure 4.2 c). Statistical analysis revealed with 95% confidence that the improved growth of strain SS1 in YETG medium containing 8.81 g yeast extract $l^{-1}$ was significant ($P<0.05$) compared to the lower yeast extract concentrations.

The results of this study indicated that a medium that consisted of yeast extract and glucose only was required for growth of strain SS1. In keeping with convention this medium was designated YEG (Appendix A.4).

The growth of strain SS1 was compared in YEPG, YETG and YEG media to confirm that YEG medium was indeed the best medium for the growth of strain SS1. The growth of strain SS1 was similar in YEPG and YEG media (Figure 4.3). Statistical analysis showed with 95% confidence that there was no significant difference ($P<0.05$) in the growth of strain SS1 in YEPG and YEG media. However, the growth of strain SS1 was significantly less in YETG medium (Figure 4.3). This confirmed earlier results that the growth of strain SS1 was better when tryptone was omitted from the medium. This study also confirmed that a protein source (in addition to yeast extract) is not needed for the growth of SS1. Also, the overall cost of the medium per litre was effectively reduced from ZAR87 (YEPG) to ZAR14 (YETG) and finally to ZAR7 (YEG).

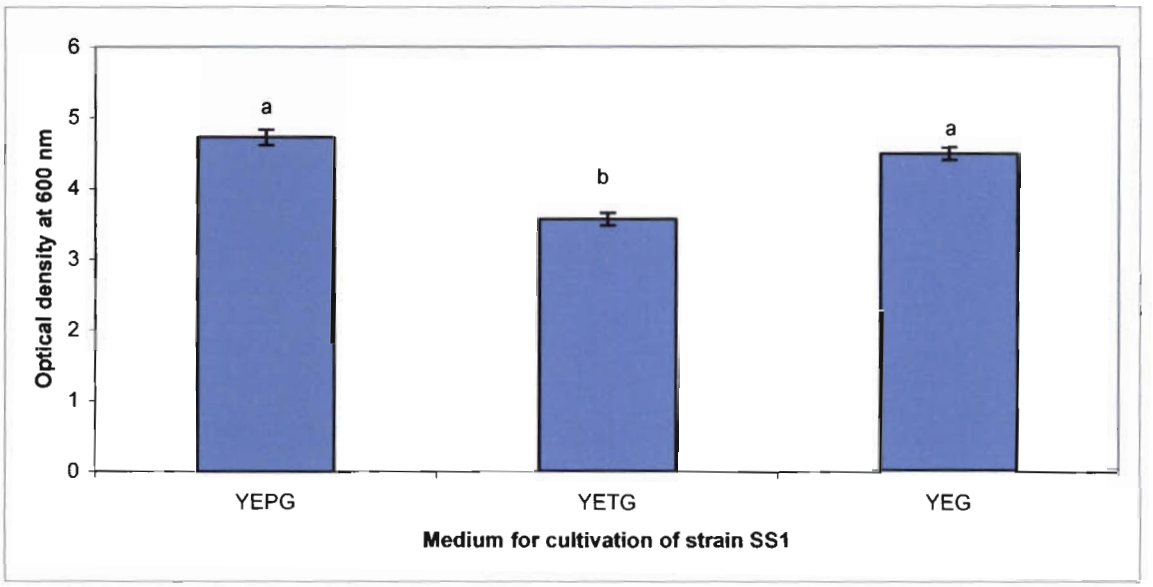



**Figure 4.3:** Comparison of the growth of strain SS1 in the various media used during this study. All cultures were standardised to an initial optical density of 0.02 at 600 nm ($OD_{600}$) and incubated at room temperature for 24 h in Erlenmeyer flasks on a rotary shaker set at 100 rpm to ensure adequate aeration. The error bars indicate the standard error between duplicate experiments. The same letter indicates no significant difference while different letters indicate a significant difference.



### 4.2.2. Effect of temperature on growth of strain SS1

The effect of temperature on growth was studied by incubating strain SS1 at different temperatures. Strain SS1 was cultivated in YEG medium and the extent of growth was determined by measuring the optical density at 600 nm ($OD_{600}$).

**Table 4.2:** Effect of temperature on growth of strain SS1

| Temperature | Growth of strain SS1 ($OD_{600}$) | |
|---|---|---|
| | 24 hours | 48 hours |
| Room temperature* | 2.34 ± 0.057 | 5.26 ± 0.057 |
| 30°C | 0.018 ± 0.005 | 0.015 ± 0.003 |
| 37°C | 0.015 ± 0.002 | 0.012 ± 0.001 |

* Room temperature varied between 22 and 25°C.

All flasks were standardised to an initial optical density ($OD_{600}$) of 0.02 at 600 nm and incubated at the indicated temperature in Erlenmeyer flasks with vigorous aeration.

Data represents the mean $OD_{600}$ ± the standard error between triplicate shake flasks.

Strain SS1 was able to grow at room temperature which was measured as approximately 22°C (Table 4.2). However, no growth was observed when the flasks were incubated at 30°C and 37°C.

### 4.2.3. Effect of medium pH on growth of strain SS1

The effect of pH on the growth of strain SS1 was investigated by cultivating the yeast in YEG medium maintained at different pH values. The pH range evaluated started at pH 5.0 and incrementally increased by 0.5 pH units up to a pH of 7.5. The medium was prepared in a wide range buffer (Appendix B.3) to maintain the pH at the desired value.

Strain SS1 was able to grow relatively well over the entire pH range evaluated (Figure 4.4). Statistical analysis using the t-test at the 95% confidence interval showed no significant difference between growth at pH 7.5, pH 7 pH 6 and pH 5.5. There was a significant difference between growth at pH 7.5 compared to pH 5.0 and pH 6.5. The pH of the supernatant was determined after growth measurements to ensure that the buffer was effective in maintaining the pH at the expected value.



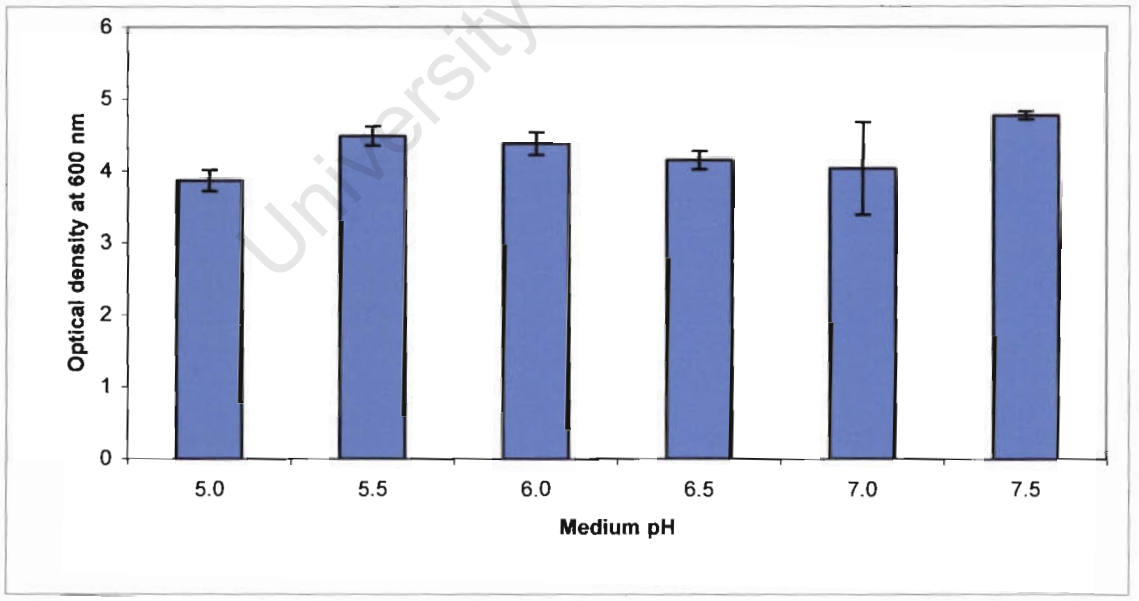



**Figure 4.4:** Growth of strain SS1 at different pH values. Strain SS1 was cultivated in YEG medium at room temperature for 24 h in Erlenmeyer flasks on a rotary shaker set at 100 rpm to ensure adequate aeration. The medium was prepared in a wide range buffer containing sodium phosphate (0.2 M) and citric acid (0.1 M). The error bars indicate the standard error between duplicate experiments.

The pH of the medium was not controlled during standard batch cultivation of strain SS1 in the stirred tank reactor (STR). Changes in medium pH during growth of strain SS1 was monitored with a pH probe. The growth of strain SS1 in relation to the pH profile of the medium is depicted in Figure 4.5. The pH decreased from 6.6 to 5.0 for the first 16 h of growth. At this point a shift occurred and the pH increased for the rest of the cultivation period. The pH of the medium was 6.8 at the end of the cultivation period. Strain SS1 metabolised the glucose in the medium over the entire growth period, even when the medium pH increased.

The effect of controlling the pH of the medium on the growth of strain SS1 during batch cultivation in the STR was investigated. The growth of strain SS1 in YEG medium in which the pH was maintained at pH 5.5 was compared to the growth of strain SS1 when no pH control was employed. The growth of strain SS1 was similar whether the pH was controlled or not (Figure 4.6). Controlling the pH of the medium did not increase the final cell density which remained at ~14 $gl^{-1}$.



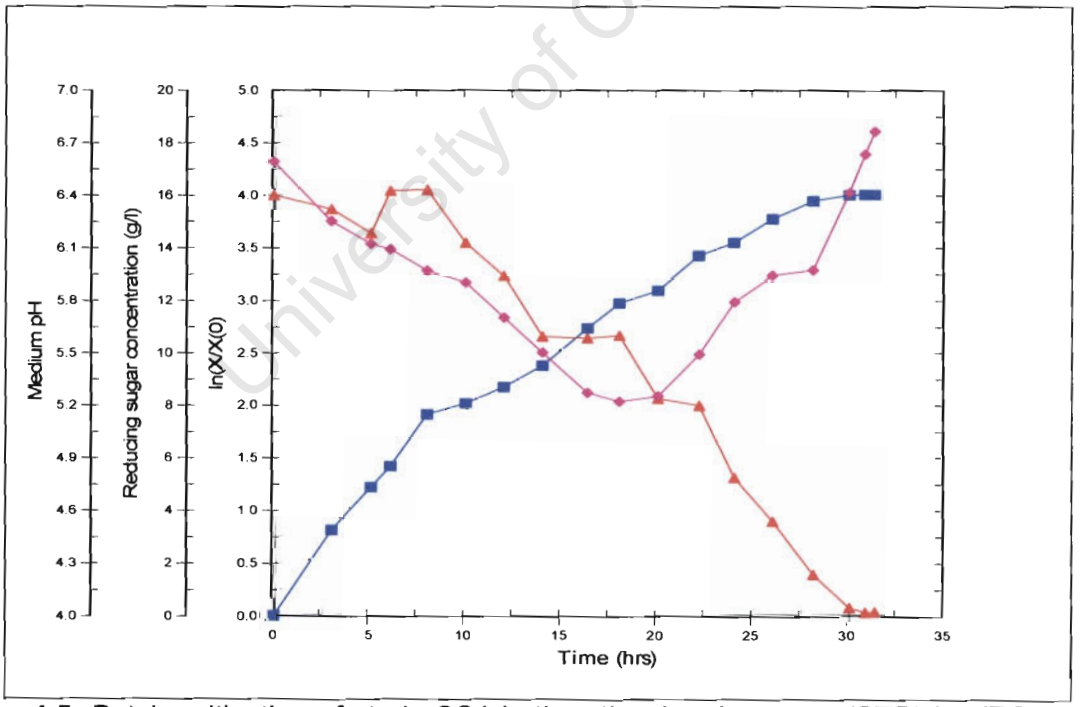



**Figure 4.5:** Batch cultivation of strain SS1 in the stirred tank reactor (STR) in YEG medium illustrating the logarithmic increase in growth (■), change in medium pH (♦) and decrease in reducing sugar concentration (▲). The culture was standardised to an initial optical density of 0.4.

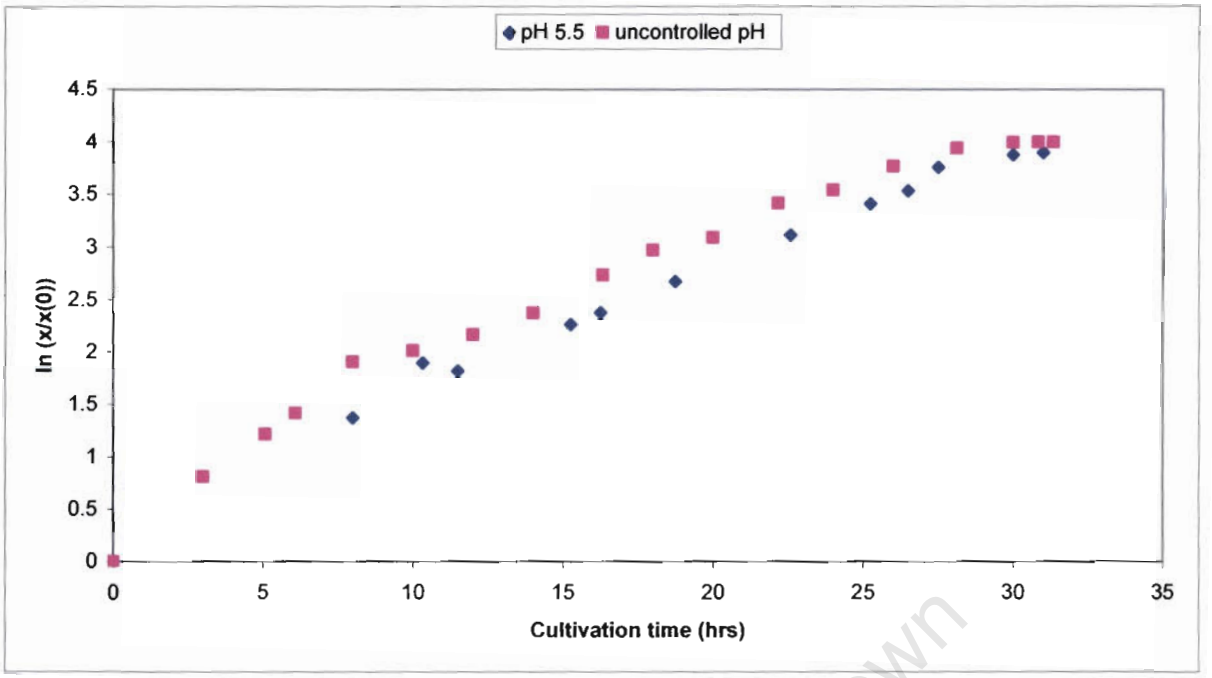



**Figure 4.6:** Semi-logarithmic plot of strain SS1 growth during batch cultivation in the stirred tank reactor (STR). All cultures were standardised to an initial optical density of 0.4 and cultivated in YEG medium. The pH of the medium was maintained at 5.5 (♦) by automatic addition of 5 M sodium hydroxide or 2 M hydrochloric acid. Alternatively the pH was not controlled (■) and consequently a pH profile similar to that in Figure 4.5 was observed (data not shown).

### 4.2.4. Effect of sodium chloride (NaCl) on growth of strain SS1

The effect of increasing salinity on the growth of strain SS1 was studied. Sodium chloride concentrations between 5 mM and 50 mM were investigated. A NaCl concentration of 50 mM (3% w/v) corresponds to the average salt content of seawater.

The yeast strain used in this study was isolated from *H. midae*, which is a marine invertebrate. There was no obvious optimum NaCl concentration where growth of strain SS1 was significantly higher (Figure 4.7). Growth was slightly less in the medium to which no NaCl was added. However, statistical analysis showed with 95% confidence that there was no significant difference between the growth of strain SS1 in the medium that lacked NaCl and the growth in the media that was supplemented with increasing concentrations of NaCl.

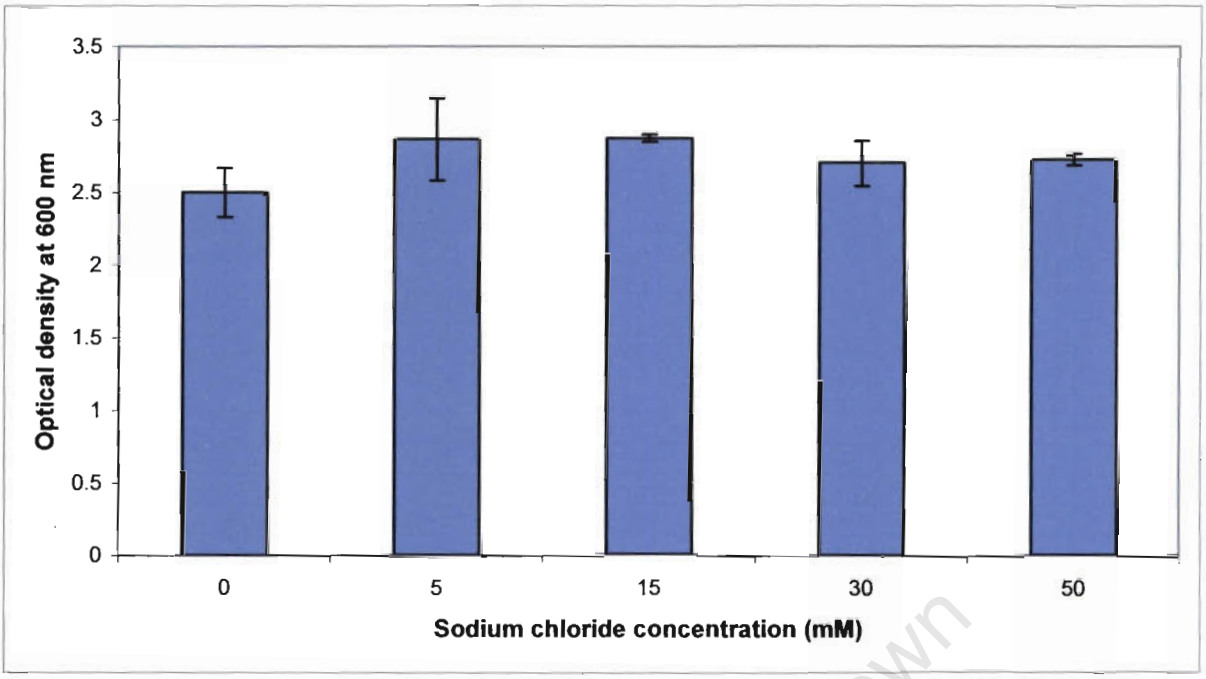



**Figure 4.7:** Effect of increasing sodium chloride concentration on the growth of strain SS1. All cultures were standardised at T=O to an $OD_{600}$ of 0.02. Strain SS1 was cultivated in YEG medium for 24 h in Erlenmeyer flasks with vigorous aeration. The error bars indicate the standard error between duplicate experiments.



## 4.3. DISCUSSION

YEG medium, which consisted of glucose and yeast extract only, was sufficient for the generation of high cell densities of strain SS1. Preliminary studies indicated that tryptone was an adequate substitute for the costly proteose peptone in YEPG medium. The use of tryptone as a protein source reduced the cost of the medium six fold. However, further investigation indicated that the use of tryptone as a protein source was not necessary for the growth of strain SS1. The growth of strain SS1 was better when tryptone was omitted from the medium. In fact, strain SS1 growth decreased as the tryptone concentration in the medium increased (Figure 4.2 a). Also, the growth of strain SS1 in YETG medium was significantly less compared to growth in YEPG and YEG (Figure 4.3). Complex substrates such peptone, tryptone and yeast extract also contain inorganic salts and ions (Bridson and Brecker, 1970). Experimental evidence has shown that high concentrations of complex substrates inhibit microbial growth due to the accumulation of mineral ions in the medium (Park

and Lee, 1999). In addition, high initial concentrations of mineral ions in the medium resulted in a lag phase and decreased both the specific growth rate and the final cell yield of the yeast *Candida brassicae* (Suzuki *et al.*, 1985). Therefore, it is postulated that the decrease in growth of strain SS1 with increasing tryptone concentration was due to the accumulation of mineral ions in the medium. As a result of these experiments tryptone was excluded from the culture medium for strain SS1.

There was no optimal glucose concentration that resulted in maximal growth of strain SS1. Strain SS1 was able to grow equally well in medium containing no glucose and in medium containing 20 g glucose $l^{-1}$ (Figure 4.2 b). However, the presence of a carbon source such as glucose is important since during aerobic yeast growth 50% of the carbon source is converted into biomass. Also, the glucose was respired during growth in the stirred tank reactor (Figure 4.5), possibly because air was constantly supplied into the bioreactor. Increasing the yeast extract concentration resulted in improved growth of strain SS1 (Figure 4.2 c). The increase in growth was statistically significant. Yeast extract is rich in vitamins and growth factors (Table 2.6) that are considered important for the growth of most yeasts (Walker, 1998). The glucose and yeast extract were not excluded from the culture medium since these were the only two components of YEG medium that supported good growth of strain SS1. Also, there was no evidence that either of these substrates inhibited growth of strain SS1.

There was no difference between the growth of strain SS1 in YEG medium compared to YEPG medium (Figure 4.3). It is possible that, like *S. cerevisiae*, strain SS1 does not excrete an extracellular protease and consequently is unable to hydrolyse peptides. Thus the peptides, polypeptides and proteoses in peptone and tryptone are not utilisable nutrient sources for strain SS1. Perhaps the amino acids provided by the yeast extract in the culture medium are an adequate nitrogen source. Thus YEG medium was selected as the most economical medium to support the growth of strain SS1 and was used for further studies both on the effect of other parameters as well as for the batch and fed-batch studies in the following chapters. This medium is 12 times less expensive than the original YEPG medium that was used to culture strain SS1.

Strain SS1 grew well at room temperature which varied between 22°C and 25°C (Table 4.2). The inability of strain SS1 to grow at temperatures of 30°C and 37°C is characteristic of yeasts belonging to the genus *Cryptococcus*. The maximum temperature of growth for most *Cryptococcus* sp. is 28°C (Phaff and Fell, 1970). The only species belonging to this genus able to grow at 37°C is *Cryptococcus neoformans*, which is a human pathogen. Growth of strain SS1 at lower incubation temperatures was not evaluated and therefore room temperature may not necessarily be the optimum temperature for growth of strain SS1.

Strain SS1 was able to grow relatively well over the entire pH range studied although growth was slightly improved at pH 7.5. Generally, yeasts are able to grow over a pH range of 3 to 8 and grow well between pH 4.5 and 6.5 (Walker, 1998). Yeasts also tolerate lower pHs well and have pH optima between pH 4 and 5 (Blanch and Clark, 1996). Rhishipal and Philip (1998) characterised four yeasts isolated from the marine environment and found that three of the isolates had a pH optimum of 5 while one tolerated a pH between 4 and 10 with little difference in growth.

Generally, maintaining the pH at a specified value is important for obtaining high cell densities. Industrial yeast fermentations are normally conducted at a constant pH of 5.5 in the bioreactor since this is close to the pH optimum of most yeasts. Since many bacteria are considered susceptible to low pH values, maintaining a pH of 5.5 in the bioreactor is also used to prevent bacterial contamination (Blanch and Clark, 1996). Since there was no pH value at which the growth of strain SS1 was optimum, the pH of the medium was kept at 5.5. Maintaining the pH of the medium at 5.5 did not improve the growth (Figure 4.6) or the final cell density of strain SS1. It was decided that pH control was not necessary for the cultivation of strain SS1 as the use of the acid and base would only increase production costs while not improving the cultivation process.

A shift in pH occurred during batch cultivation in the STR (Figure 4.5). The decrease in pH over the first 16 h of growth is typically associated with the production of organic acids due to respiration of the carbon source (Berry, 1983). The pH then proceeded to increase back to the initial value. The shift in pH indicated that a

metabolic change had occurred. However, what this change in metabolism entailed is unknown. An increase in pH during yeast growth is usually due to the release of alcoholic compounds; e.g. ethanol by *S. cerevisiae* (Walker, 1998). However, *Cryptococcus* yeasts are obligate aerobes and are unable to ferment sugars (Phaff and Fell, 1970). Amino acids can be used as a carbon and nitrogen source. The use of amino acids as a carbon source involves a de-amination reaction and the release of ammonium ions would result in increased pH (Benslimane *et al.*, 1995). Benslimane *et al.* (1995) have shown that the assimilation of yeast extract amino acids by *Streptomyces ambofaciens* was accompanied by the production of ammonium ions. It could be speculated that the metabolism of the amino acids in yeast extract (Table 2.6) as a carbon source caused the pH of the medium to increase. However, the increase in pH occurred while the glucose in the medium was still being metabolised by strain SS1 (Figure 4.5). It is generally accepted that glucose is the preferential carbon source for most microorganisms and that other carbon sources are only utilised once the glucose becomes depleted (Egli *et al.*, 1983; Fiechter *et al.*, 1981). However, glucose is not necessarily the preferred carbon source for all bacteria (Kompala *et al.*, 1984) and yeasts (Walker, 1998). Also, many microorganisms, including yeasts, are able to metabolise more than one carbon source simultaneously (Egli *et al.*, 1983; Lendenmann *et al.*, 1996). Thus it is possible that the yeast extract amino acids were utilised as a carbon source by strain SS1 and that this occurred while the glucose was being respired. The growth and metabolism of yeast is far too complex to form any conclusions based on the limited data available. Further experiments that investigate the metabolic by-products excreted by strain SS1 need to be conducted.

Strain SS1 was able to grow equally well in YEG medium which either lacked or included NaCl (Figure 4.7). Although strain SS1 did not appear to require sodium chloride in the medium for growth, these experiments were conducted in a medium that was not entirely salt-free due to the inorganic ion content of the yeast extract (Table 2.6). Most marine yeasts are able to grow relatively well with and without the presence of sodium chloride in the medium (Phaff *et al.*, 1978). However, as with the present study, these experiments were frequently conducted in complex media that were not entirely salt-free. Nevertheless, an obligate salt requirement is usually set at 3% NaCl which would be more than the trace amount present in complex

substrates. *Debaromyces hansenii*, one of the most salt-resistant yeasts, was shown to grow equally well in mineral medium containing no NaCl as in mineral medium containing 500 mM salt (Neves *et al.*, 1997). The addition of NaCl to the medium was not considered economically feasible as no significant increase in growth of strain SS1 was observed. The addition of salt to fermentation media is not favoured for industrial fermentation processes as salt may promote corrosion and also negatively affect oxygen transfer in the bioreactors (Marwick *et al.*, 1999). It was concluded that strain SS1 did not have an absolute requirement for sodium ions and therefore may not be an obligate marine yeast. YEG medium without additional NaCl was thus the most economical medium for growth of strain SS1.

# CHAPTER 5

## GROWTH KINETICS OF STRAIN SS1

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## CONTENTS

## 5.1. INTRODUCTION

Mathematical equations are frequently used to describe or model the relationship between different variables in a process (Nielsen, 2001). In order to give a complete description of the growth kinetics of a culture, the structured nature of each cell and the segregation of the culture into individual units (cells) must be considered (Shuler and Kargi, 1992). However, models that include structure and segregation are the most complex description of a system. Although this type of model is the most realistic, it is often difficult to solve due to its complexity (Bailey and Ollis, 1986). In general, the simplest model, i.e. unstructured and nonsegregated, is more commonly used to describe a system or process (Shuler and Kargi, 1992). An unstructured model assumes that the cell composition remains constant. This implies that growth is balanced.

Balanced growth normally occurs during the exponential phase of batch cultivation when nutrients occur in excess and the specific growth rate is independent of the substrate concentration (Blanch and Clark, 1996; Shuler and Kargi, 1992). During exponential growth in a batch bioreactor the specific growth rate can be described by first order kinetics:

$$\frac{dX}{dt} = \mu X \qquad \text{Equation 5.1}$$

Integration of Equation 5.1 results in Equation 5.2:

$$\ln \frac{X}{X_0} = \mu t \qquad \text{Equation 5.2}$$

Thus a plot of $\ln(X/X_0)$ on the y-axis and time on the x-axis yields a straight line where the slope of the curve is the specific growth rate, $\mu$.

One of the most commonly applied unstructured, nonsegregated models of microbial growth developed by Jacques Monod (Shuler and Kargi, 1992) describes a functional

relationship between the specific growth rate, μ, and the substrate concentration, S (Figure 5.1). The Monod equation states that:

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad \text{Equation 5.3}$$

$K_s$ is the concentration of the limiting substrate when the specific growth rate is 0.5 $\mu_{max}$. According to the Monod equation, only the growth-limiting substrate is important in determining the rate of cell growth (Blanch and Clark, 1996). By rearranging Equation 5.1 and substituting $\mu = \frac{1}{X} \cdot \frac{dX}{dt}$ into Equation 5.3, batch growth at constant volume is described as:

$$\frac{dX}{dt} = \frac{\mu_{max} S X}{K_s + S} \qquad \text{Equation 5.4}$$

There are two simplifications to the Monod equation (Shuler and Kargi, 1992):

i. At high substrate concentrations ($S >> K_s$), equation 5.2 is reduced to zero order dependence on substrate concentration i.e. $\mu = \mu_{max}$

ii. At low substrate concentrations ($S << K_s$), the specific growth rate exhibits first order dependence on substrate concentration i.e. $\mu = \frac{\mu_{max}}{K_s} \cdot S$

In general, values of $K_s$ are small implying the specific growth rate is near its maximum value for much of the batch growth period. This occurs during exponential growth when nutrients are in excess and independent of the substrate concentration.

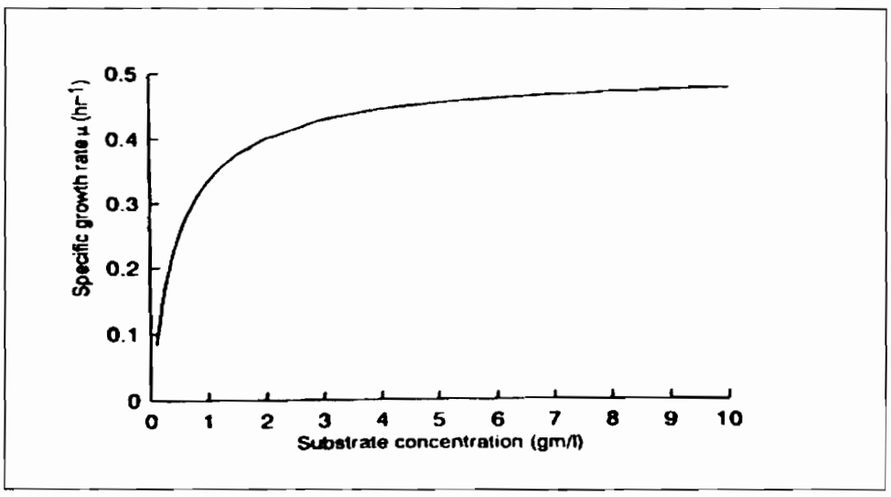



**Figure 5.1:** The specific growth rate as a function of the concentration of the limiting substrate, S, when the Monod model is applied (Blanch and Clark, 1996).

The Monod equation can be modified to describe growth for more than one limiting substrate. If the concentration of the substrates is so low that the growth rate is limited by all then:

$$\mu = \mu_{max} \cdot \left( \frac{k_1 S_1}{K_{s1} + S_1} + \frac{k_2 S_2}{K_{s2} + S_2} + \cdots + \frac{k_i S_i}{K_{si} + S_i} \right) \cdot \left( \frac{1}{\sum_{j=1}^{i} K_{sj}} \right) \qquad \text{Equation 5.5}$$

This expression implies that the growth rate always increases with increasing substrate concentration (Crueger and Crueger, 1989). The Monod equation can also be modified to describe the growth rate at high substrate concentrations i.e.

$$\mu = \mu_{max} \frac{S}{K_s + K_{s0} S_0 + S} \qquad \text{Equation 5.6}$$

The term $K_{s0}S_0$ indicates that the growth rate decreases if the growth-limiting substrate occurs above a certain concentration (Blanch and Clark, 1996). Other modifications of the Monod equation include the introduction of an inhibition term to account for the presence of a growth-inhibiting metabolite. The application of the Monod model or any modification thereof would require knowledge of the identity of the growth-limiting substrate(s).

Since knowledge regarding the growth of strain SS1 was very limited, it was decided to attempt to identify the growth-limiting substrate for strain SS1 and then to employ an unstructured approach based on Monod kinetics to model the growth of the yeast. The aim was to be able to relate the specific growth rate to the rate of substrate utilization and biomass formation. Yield and productivity are important design parameters that provide a quantitative description of cellular processes (Nielsen, 2001). The yield specifies the amount of substrate that is converted into product while the productivity indicates the rate of product formation (Nielsen, 2001). The rate of oxygen transfer to the cells, the production time and the duration of the lag phase are three important criteria that affect the outcome of a fermentation process (Crueger and Crueger, 1989). Consequently, the effects of inoculum size, substrate concentration and oxygen transfer capacity on the specific growth rate, yield and productivity of strain SS1 was investigated during batch cultivation.

Section 5.2.1 investigated batch cultivation of strain SS1 under standard conditions in the shake flasks and in the stirred tank reactor (STR). Duplicate batch fermentations were compared and the reproducibility of experiments was considered.

Section 5.2.2 investigated the effect of inoculum size on the growth of strain SS1. Immediately after cells are inoculated into fresh medium a lag phase occurs during which the cells adapt to the new environment (Shuler and Kargi, 1992). The extent of the lag phase depends on the physiological state or age of the inoculum, the size of the inoculum and the cultivation conditions (Bailey and Ollis, 1986; Blanch and Clark, 1996; Shuler and Kargi, 1992). In order to reduce or eliminate the lag phase, the inoculum should be actively growing exponential phase cells, the inoculum should be large and account for 5-10% of the new medium volume and the cells should be acclimatized to the medium and growth conditions before inoculation (Bailey and Ollis, 1986).

Section 5.2.3 investigated the effect of substrate concentration on the growth of strain SS1. The focus was especially on the glucose concentration of the medium. Glucose is very important in yeast metabolism, functioning both as a nutrient and a signaling molecule (Rolland *et al.*, 2002). Obligate aerobes like strain SS1 are typically classified as glucose insensitive and exhibit high growth rates and high biomass yields under aerobic conditions (Fiechter *et al*, 1981; Käppeli, 1983). Unlike yeasts that are sensitive to the presence of free glucose, such as *S. cerevisiae,* glucose insensitive yeasts are unable to grow in the absence of oxygen and do not excrete ethanol (Fiechter *et al.*, 1981; Käppeli, 1983). The growth and kinetics of strain SS1 was evaluated as a function of the glucose concentration.

Section 5.2.4 investigated the effect of oxygen availability on the growth of strain SS1 in shake flasks and in the STR. *Cryptococcus* species are obligate aerobes that require oxygen for respiration (Phaff and Fell, 1970). As discussed in section 2.5.2 of the literature review, oxygen transfer is a very important factor of an aerobic fermentation process. The oxygen transfer rate (OTR) in a bioreactor is related to the oxygen utilization rate (OUR) and thus to the biomass concentration, X, by Equation 5.7:

$$OTR = OUR = q_{O_2} X \qquad \text{Equation 5.7}$$

where $q_{O_2}$ is the specific oxygen utilization rate.

The volumetric transfer coefficient, $k_L a$, is used as a measure of the oxygen transfer rate (OTR) in a cultivation system.

$$OTR = k_L a \Delta C \qquad \text{Equation 5.8}$$

where $\Delta C$ is the difference between the saturated oxygen concentration, $C^*$, and the oxygen concentration in the liquid medium, C. Since oxygen is sparingly soluble in liquids (Lee, 1996), C is very small and Equation 5.8 becomes:

$$OTR = k_L a C^* \qquad \text{Equation 5.9}$$

Some of the methods used to improve mixing efficiency and thus oxygen transfer in a fermentation system and their effect on the growth of strain SS1 were considered.

## 5.2. RESULTS

### 5.2.1. Batch cultivation of strain SS1

Standard culture conditions were maintained in the shake flasks and the STR (as described in section 3.3.2 and section 3.3.3, respectively) in order to ensure reproducibility between different experiments. The cultures were also standardized to an optical density of 0.02 in the shake flasks and 0.4 in the STR (unless stated otherwise) at the start of each experiment to ensure that comparisons could be drawn between different sets of experiments. Figures 5.2 and 5.3 show duplicate batch growth profiles of strain SS1 in shake flasks and the STR, respectively. Since the logarithm of the biomass concentration was used, the slope of the line indicates the specific growth rate. A maximum specific growth rate ($\mu_{max}$) of 0.23 $h^{-1}$ with a standard error of 0.0037 was obtained during batch cultivation in shake flasks. In the STR, the $\mu_{max}$ was 0.23 $h^{-1}$ with a standard error of 0.0069.



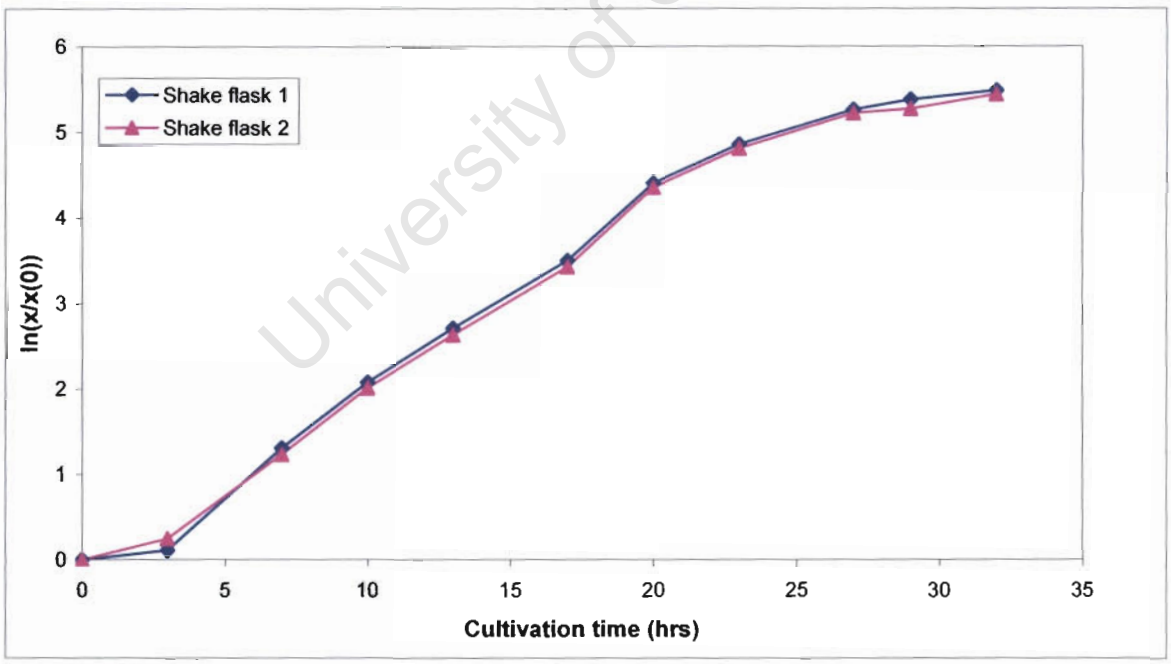



**Figure 5.2:** Duplicate batch cultivations of strain SS1 in shake flasks in YEG medium. All cultures were standardized to an initial optical density of 0.02 at 600 nm.

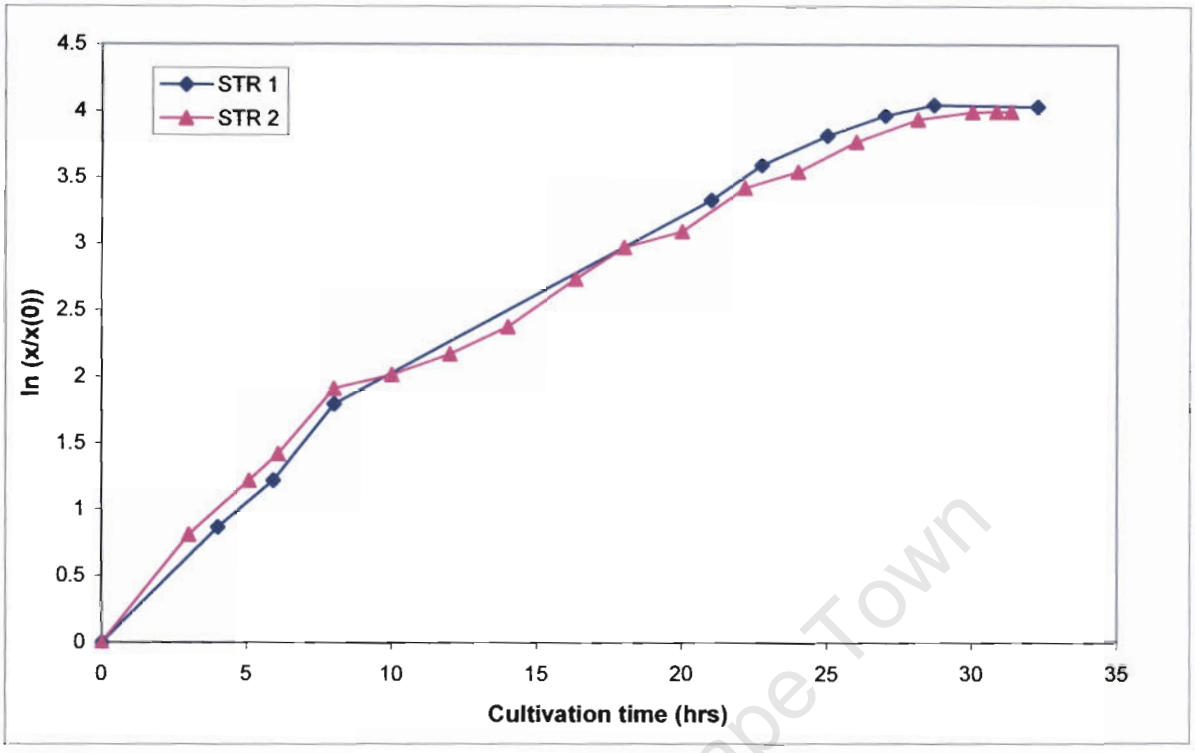



**Figure 5.3:** Duplicate batch cultivations of strain SS1 in the stirred tank reactor (STR) in YEG medium. The culture was standardized to an initial optical density of 0.4 at 600 nm using a 10% inoculum.



### 5.2.2. Inoculum size

The aim of this study was to determine the effect of inoculum size on the growth of strain SS1 and the productivity of the cultivation process. Inoculum sizes of 0.5, 5 and 10% (v/v) were used to standardize the culture in the STR to an OD600 of 0.02, 0.2 and 0.4, respectively, at the beginning of the cultivation process.

**Table 5.1:** Effect of inoculum size on the growth of strain SS1 during bioreactor cultivation

| Initial OD600 | % Inoculum | Lag time (h) | Max. cell density ($gl^{-1}$) | Cultivation time (h) | Productivity ($gl^{-1}h^{-1}$) |
|---|---|---|---|---|---|
| 0.02 | 0.5 | 2-8 | 14.7 | 47 | 0.31 |
| 0.2 | 5 | 0 | 14.5 | 40 | 0.36 |
| 0.4 | 10 | 0 | 14.9 | 30 | 0.50 |

The culture that received an inoculum size of 0.5% of the new medium volume exhibited a lag phase of between 2 to 8 h (Table 5.1). No lag phase was observed for the cultures that received large inocula. The maximum cell densities attained were similar in all cases at approximately 15 $gl^{-1}$. The fermentation time required to achieve the maximum cell density decreased with inoculum size. The total cultivation time is especially important for productivity; i.e. amount of cells produced per unit volume per unit time of a fermentation process. Long fermentation times have a negative affect on productivity. Thus, as the fermentation time decreased from 47 h to 30 h with increasing inoculum size from 0.5% to 10%, the productivity of the fermentation increased. The highest productivity (0.50 $gl^{-1}h^{-1}$) was observed in the culture that received the 10% inoculum (Table 5.1).

### 5.2.3. Substrate concentration

The growth of strain SS1 was studied in YEG medium to determine which substrates were utilized for growth and how efficiently the substrates were converted into biomass. The standard medium contained (per liter) 20 g glucose and 8.81 g yeast extract. An approximate composition of yeast extract is given in Table 2.6. The focus was specifically on utilization of the glucose and soluble protein as these were the principal components of the medium and could easily be measured with standard colorimetric techniques. It was expected that one of these macro nutrients would form the growth limiting substrate whose identification is imperative for the development of a dense culture strategy using fed-batch cultivation as discussed in the following chapter.

Typical growth curves obtained during batch propagation in shake flasks and in the STR are shown in Figures 5.4 and 5.5, respectively.

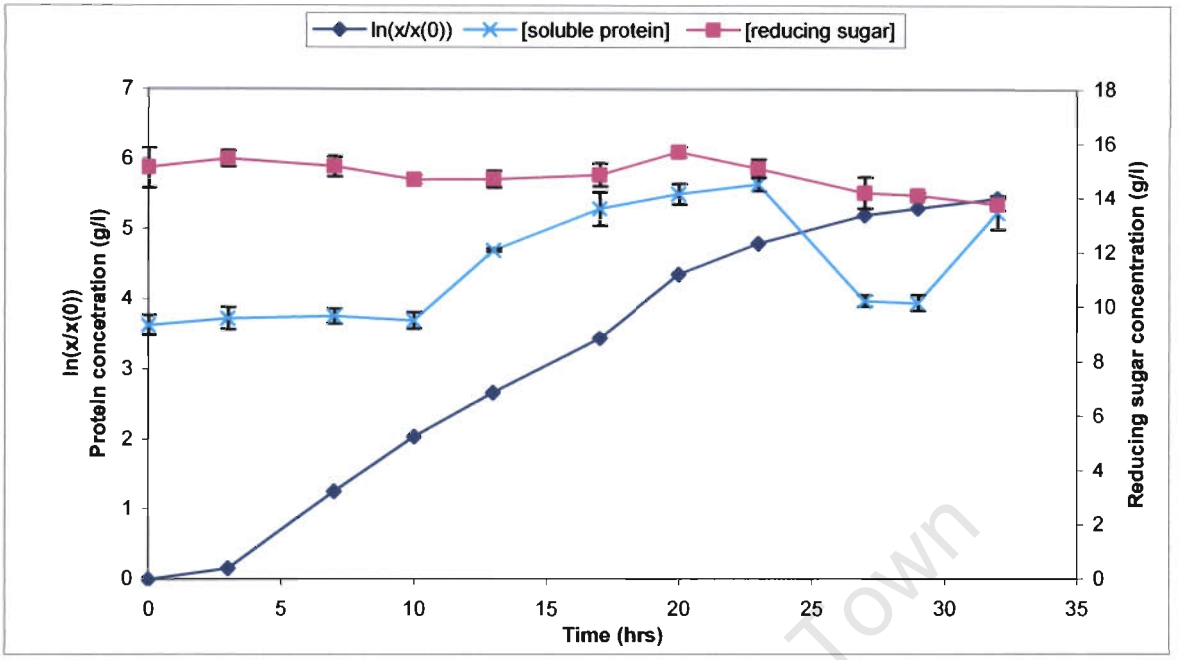



**Figure 5.4:** Shake flask cultivation of strain SS1 in YEG medium containing 20 g glucose $l^{-1}$. Cultures were standardized to an initial optical density of 0.02 at 600 nm. The growth plot was the average of five experiments.

Since the logarithm of the biomass concentration was used, the slope of the line indicates the specific growth rate. The yeast extract was calculated to contain approximately 6 $gl^{-1}$ of protein using Equation 4.1. This calculation was based on a percentage total nitrogen (%TN) of 11.3%. The protein concentration in the medium as measured by the Lowry assay (section 3.4.3) was less than the expected value. The soluble protein concentration fluctuated between 3.5 and 5.5 $gl^{-1}$ during batch growth in the shake flask and between 3 and 4 $gl^{-1}$ in the STR. The metabolism of glucose as carbon source differed depending on the cultivation vessel used. When strain SS1 was cultivated in the shake flask the glucose was not utilized (Figure 5.4) whereas glucose was depleted when strain SS1 was cultured in the STR (Figure 5.5).

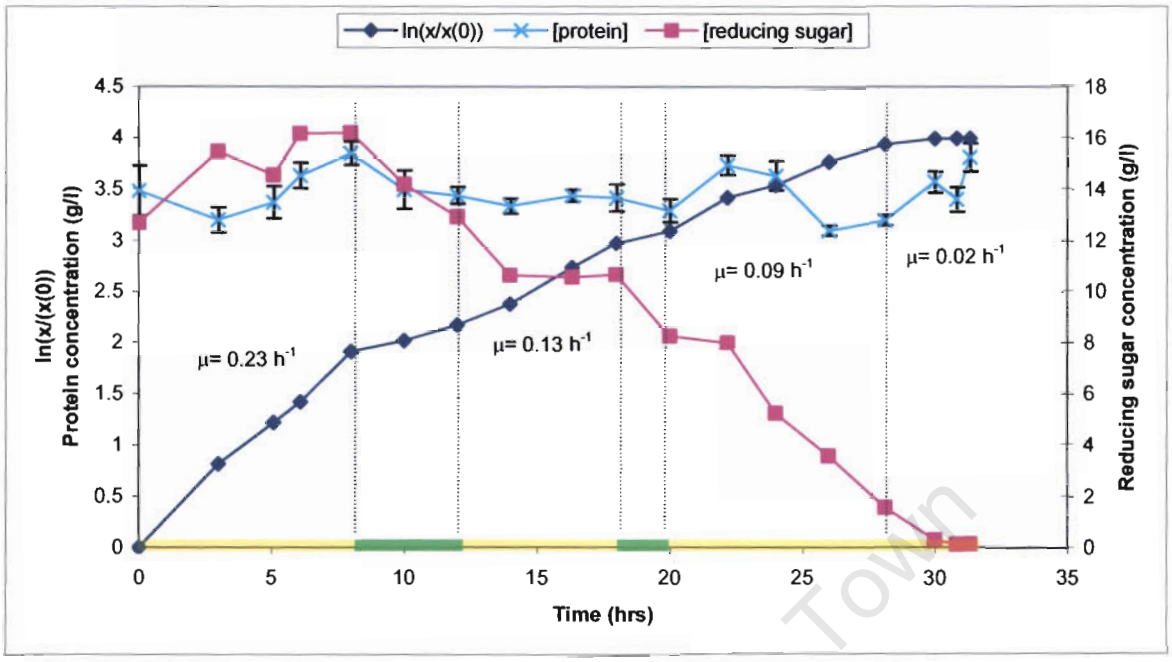



**Figure 5.5:** Batch cultivation of strain SS1 in the stirred tank reactor (STR) in YEG medium containing 20 g glucose $l^{-1}$. The culture was standardized to an initial optical density of 0.4 at 600 nm using a 10% inoculum. (····) indicate zones where changes in the specific growth rate were observed. The change in specific growth rate is marked in yellow and periods of lag in green on the x-axis.

Since the soluble protein was not utilized as a nutrient source by strain SS1, batch cultivations were conducted in the STR to determine the effect of glucose concentration on the growth of the yeast. The growth of strain SS1 in the standard medium was compared to medium without glucose and medium containing 40 g glucose $l^{-1}$.

The absence of glucose in the medium resulted in a final cell density of only 3 $gl^{-1}$ after 30 h (Table 5.2), after which no further increase in cell concentration was observed. The result was a very low productivity (0.10 $gl^{-1}h^{-1}$). The experimental biomass yield was 3.90 based on glucose and 0.43 based on total carbon as substrate (Table 5.2).

**Table 5.2:** Effect of glucose concentration on kinetic parameters of strain SS1

| Initial [glucose] ($gl^{-1}$) | Max. cell density ($gl^{-1}$) | Max. specific growth rate, $\mu^{max}$ ($h^{-1}$) | Cultivation time (h) | Productivity ($gl^{-1}h^{-1}$) | Experimental yield, $Y_{x/s}$ | |
|---|---|---|---|---|---|---|
| | | | | | (g biomass/ g glucose)[1] | (g biomass/ g carbon)[2] |
| 0 | 3.0 | 0.17 | 30 | 0.10 | 3.90 | 0.43 |
| 20 | 14.9 | 0.23 | 30 | 0.50 | 0.92 | 0.65 |
| 40 | 15.3 | 0.19 | 47 | 0.32 | 0.52 | 0.39 |

[1]Calculation for medium without glucose was based on a glucose concentration of 0.14 $gl^{-1}$, as measured by the reducing sugar method

[2]Calculation was based on a carbon content of 39.6% (w/w) for yeast extract (Hassan *et al.*, 1996), 50% (w/w) for yeast biomass (Walker, 1998) and 40% (w/w) for glucose

In the standard medium a maximum cell concentration of 14.9 $gl^{-1}$ was obtained after 30 h (Table 5.2). The $\mu_{max}$ of 0.23 $h^{-1}$ occurred during the first 8 h of growth after which the growth rate decreased to 0.13 $h^{-1}$ between 12 and 18 h (Figure 5.5). The growth rate decreased further to 0.09 $h^{-1}$ between 20 to 28 h. After 28 h the specific growth rate decreased to 0.02 $h^{-1}$ which was maintained until the end of the cultivation period. Sometimes the decrease in the specific growth rate was preceded by a lag period (Figure 5.5). The yield was 0.92 when based on glucose, and 0.65 based on total carbon as substrate (Table 5.2). The highest productivity (0.50 $gl^{-1}h^{-1}$) was obtained with this fermentation process.

Doubling the initial glucose concentration in the medium to 40 $gl^{-1}$ resulted in an maximum cell concentration of 15.3 $gl^{-1}$ after 47 h. The $\mu_{max}$ of 0.19 $h^{-1}$ was observed over the first 6 h of the cultivation process and was slower than that obtained with the standard glucose concentration (Figure 5.6). The specific growth rate was 0.05 $h^{-1}$ between 23 and 33 h. The specific growth rate decreased further after 33 h to 0.01 $h^{-1}$ and was maintained for approximately 15 h. After 47 h no further increase in cell growth was observed. Growth ceased while glucose was still present in the medium (Figure 5.6). The cultivation process resulted in a low productivity (0.32 $gl^{-1}h^{-1}$). The

experimental yield ($Y_{x/s}$) was 0.52 based on glucose and 0.39 based on total carbon as substrate (Table 5.2).

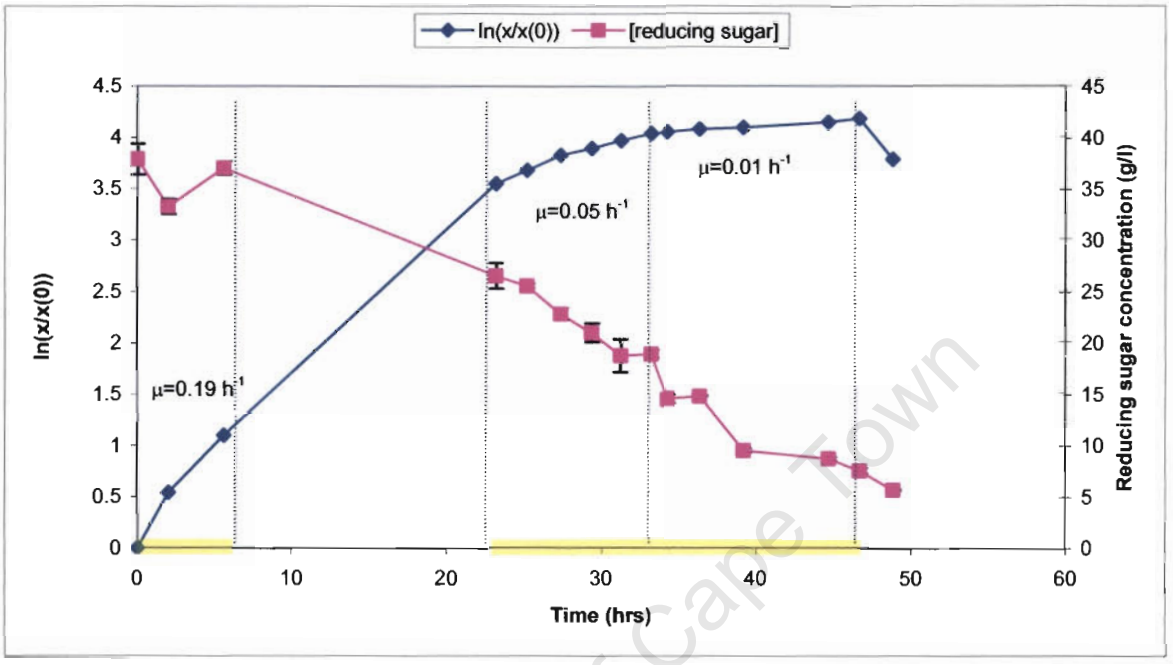


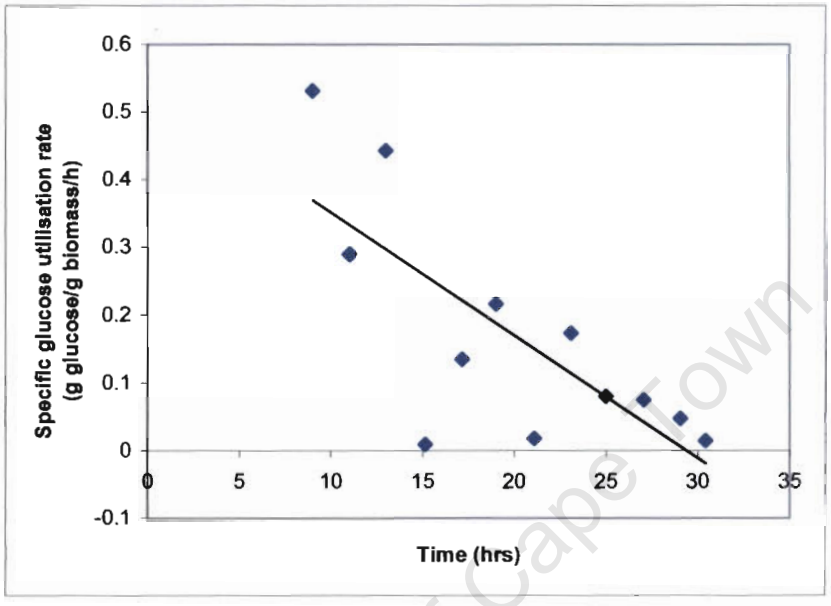

**Figure 5.6:** Batch cultivation of strain SS1 in the STR in YEG medium containing 40 $gl^{-1}$ of glucose. The culture was standardized to an initial optical density of 0.4 at 600 nm using a 10% inoculum. (····) indicate zones where changes in the specific growth rate were observed. The change in specific growth rate is marked in yellow on the x-axis.

Utilization of glucose by strain SS1 was evaluated in YEG medium containing either 20 or 40 g glucose $l^{-1}$. During growth in the standard medium the specific glucose utilization rate exhibited a decreasing trend with time (Figure 5.7 A). In YEG medium containing 40 g glucose $l^{-1}$ the data fluctuated and no clear trends were observed (Figure 5.7 B).

The specific growth rate was evaluated as a function of the glucose concentration in YEG medium containing 20 and 40 g glucose $l^{-1}$. In the standard medium there appeared to be Monod dependence of the specific growth rate on glucose at concentrations of up to ~10 $gl^{-1}$ (Figure 5.8 A ii). However, the specific growth rate did not exhibit Monod dependence for glucose concentrations greater than 10 $gl^{-1}$ (Figure

5.8 A i). Monod dependence was not observed when the medium contained 40 $gl^{-1}$ of glucose (Figure 5.8 B).

A.




B.

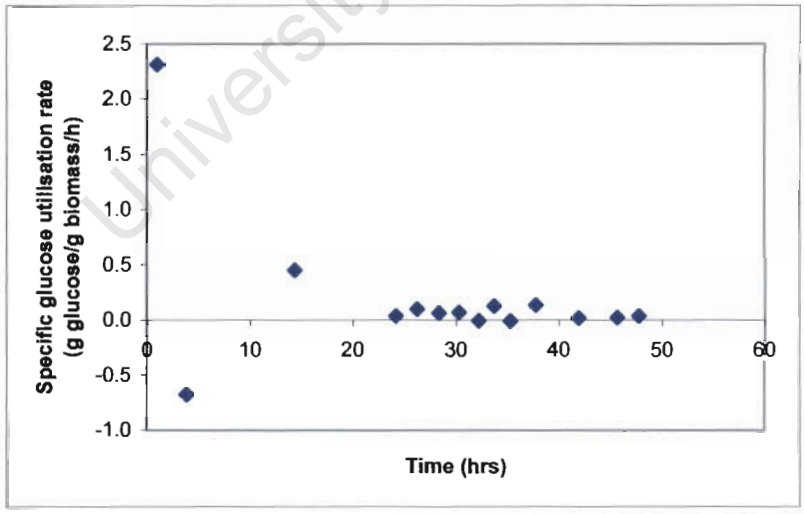
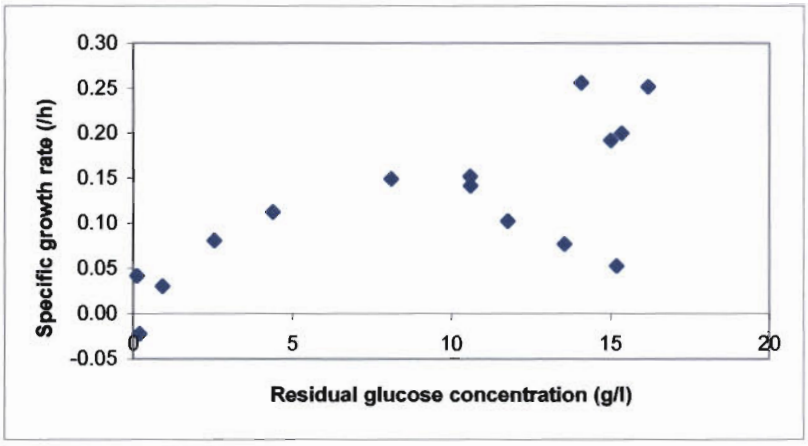


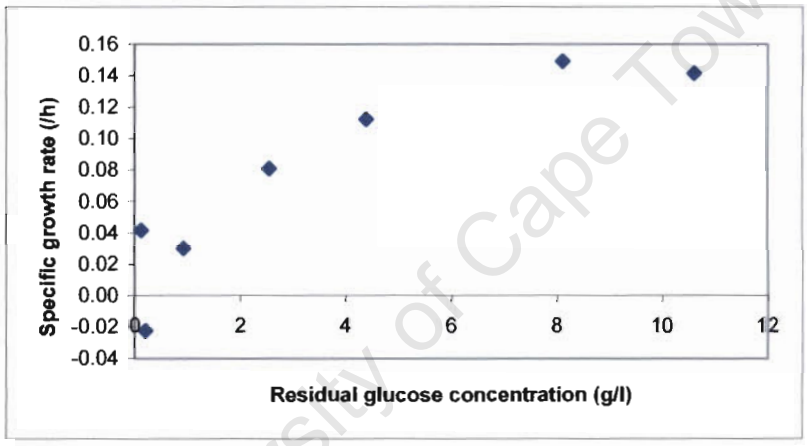



**Figure 5.7:** The specific glucose utilization rate [(dS/dt)/X] of strain SS1 as a function of time during batch cultivation in the STR. The yeast was cultivated in YEG medium containing: (A) 20 $gl^{-1}$ and (B) 40 g glucose $l^{-1}$. All cultures were inoculated with a 10% inoculum and agitated at a rate of 600 rpm. S=glucose concentration ($gl^{-1}$); t=time (h); X=cell concentration ($gl^{-1}$)

A. i




A. ii




B.

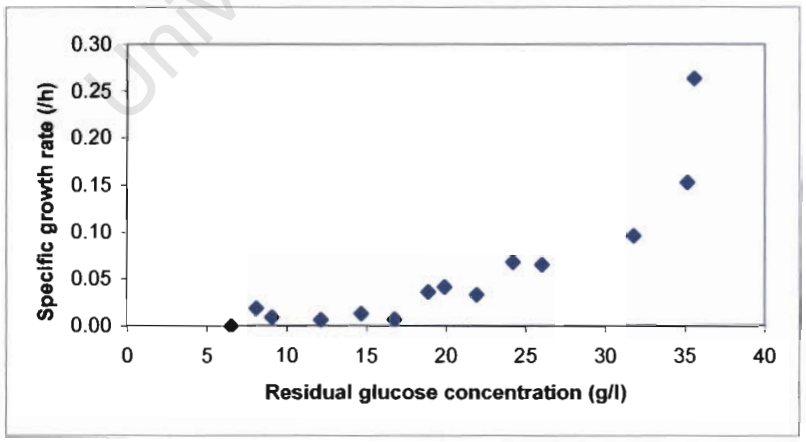





**Figure 5.8:** The specific growth rate of strain SS1 as a function of the residual glucose concentration in YEG medium containing 20 (A) and 40 (B) g glucose $l^{-1}$ during batch cultivation in the STR. Figure Aii is an expansion of the first 10 h of Figure Ai.

### 5.2.4. Oxygen transfer capacity

The aim of this study was to investigate the effect of oxygen availability in shake flasks and in the STR on the growth of strain SS1 through studying the oxygen transfer rate.

#### 5.2.4.1. Volumetric oxygen transfer coefficient in the shake flasks

Oxygen availability in the shake flask was investigated by the introduction of indentations or baffles and by changing the liquid volume in the flask. Firstly, the growth of strain SS1 in baffled Erlenmeyer flasks was compared to standard flasks. The experiments were conducted with and without antifoam present in the medium because the introduction of the baffles promoted excessive foaming which decreased the oxygen transfer rate and could negate the possible positive effect of the baffles. However, the antifoam could also negatively affect the oxygen transfer capacity.



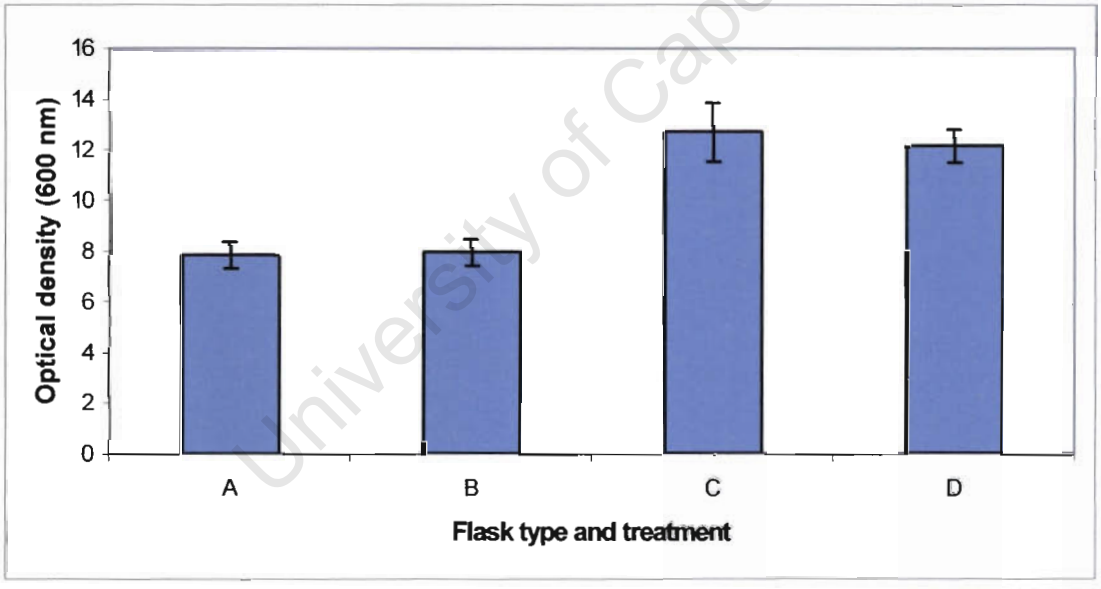



**Figure 5.9:** The effect of baffled vs. unbaffled shake flasks on growth of strain SS1. A=Unbaffled with antifoam; B=Unbaffled without antifoam; C=Baffled with antifoam; D=Baffled without antifoam. All flasks were standardized to an initial optical density of 0.2 and incubated at RT for 24 h on an orbital shaker set at 100 rpm. Experiments were conducted in duplicate in 500 ml capacity flasks containing 50 ml of YEG medium.

The baffled Erlenmeyer flasks improved growth of strain SS1 by more than 60% compared to that observed in the standard flasks (Figure 5.9). The addition of antifoam prevented foaming in the baffled flasks. Comparison of the growth between the two sets of flasks (i.e. comparing flask A with B or flask C with D) showed that growth was reasonably similar. Thus the presence of antifoam did not appear to affect the growth of strain SS1. Secondly, strain SS1 was cultivated in 1-litre Erlenmeyer flasks containing 50, 100 and 200 ml of YEG medium while all other conditions were kept constant. Initially the growth was very similar in all flasks (Figure 5.10). A 2-hr lag phase was observed followed by a period of accelerated growth at the start of the exponential phase at approximately 6 h.

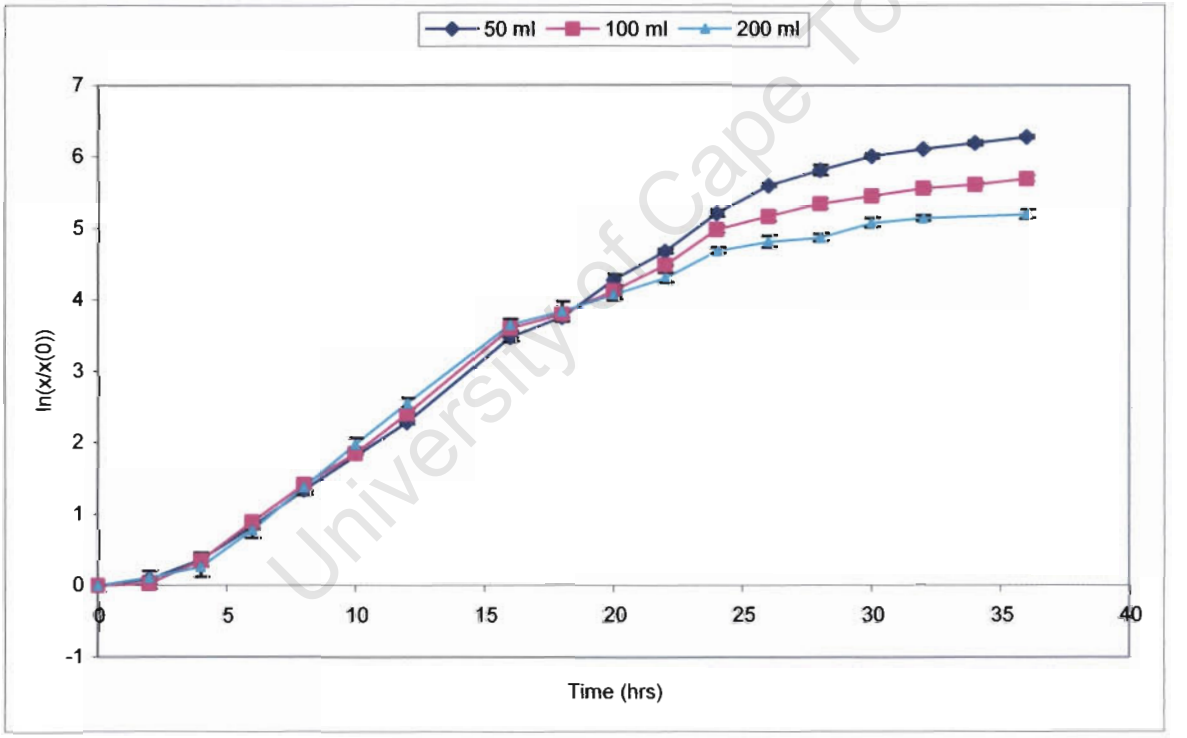





**Figure 5.10:** Effect of oxygen transfer on the growth of strain SS1 during batch cultivation in standard shake flasks. All cultures were initially standardized to an OD600 of 0.02. Each plot was the average of four flasks.

During exponential growth the specific growth rates were relatively similar in all flasks: 0.26 $h^{-1}$ ($R^2$=0.9972), 0.27 $h^{-1}$ ($R^2$=0.9974) and 0.28 $h^{-1}$ ($R^2$=0.9996) for the 50 ml, 100

ml and 200 ml flask, respectively. After approximately 18 h a reduction in the specific growth rate was observed in all flasks; i.e. 0.25 $h^{-1}$ ($R^2$=0.9978), 0.17 $h^{-1}$ ($R^2$=0.9999), 0.12 $h^{-1}$ ($R^2$=1) for the 50 ml, 100 ml and 200 ml flask, respectively. The specific growth rate decreased further in all flasks over the remaining cultivation period. After 36 h the highest growth was observed in the flask that contained 50 ml of broth.

The gassing out method (section 3.4.4.2) was used to determine the volumetric transfer coefficient, $k_La$, in 1-litre Erlenmeyer flasks containing 50, 100 and 200 ml of distilled water. Distilled water was used because the use of YEG medium resulted in excessive foaming, which impeded dissolved oxygen probe measurements. However, since the aim was to establish a comparative estimation of $k_La$ for the different liquid volumes, distilled water was an acceptable alternative.

**Table 5.3:** Determination of volumetric oxygen transfer coefficient in the standard shake flask

| Liquid volume (ml) | $k_La$ ($s^{-1}$) | Regression coefficient |
|---|---|---|
| 50 | 0.0112 | 0.99 |
| 100 | 0.0088 | 0.99 |
| 200 | 0.0073 | 0.99 |

Note: Experiments were performed in duplicate

The increase in volume resulted in a decreased volumetric oxygen transfer coefficient as indicated by the decrease in $k_La$ (Table 5.3). This corresponds to a decrease in OTR from 0.090 $mgO_2l^{-1}$ at a working volume of 5% to 0.058 $mgO_2l^{-1}$ at a working volume of 20% where the saturated dissolved concentration ($C^*$) is 8 $mgl^{-1}$ (22°C, $pO_2$ of 0.21 atm).

5.2.4.2. Volumetric oxygen transfer coefficient in the Chemap STR

Oxygen transfer in the STR was investigated by increasing the rate of agitation from 600 rpm (the standard agitation rate in this study) to 800 rpm.

**Table 5.4:** Effect of agitation rate on growth of strain SS1 during batch cultivation

| Agitation rate (rpm) | Maximum cell density ($gl^{-1}$) | Maximum specific growth rate, $\mu_{max}$ ($h^{-1}$) | Productivity ($gl^{-1}h^{-1}$) |
|---|---|---|---|
| 600 | 14.9 | 0.23 | 0.50 |
| 800 | 11.7 | 0.15 | 0.25 |

The higher agitation rate had a negative effect on the growth of strain SS1. This resulted in a lower maximum cell density (Table 5.4). The $\mu_{max}$ decreased from 0.23 $h^{-1}$ to 0.15 $h^{-1}$ at the higher agitation rate. The cultivation time increased from 30 h to 47 h at the higher agitation rate (Figure 5.11). As a result the productivity markedly decreased from 0.50 $gl^{-1}h^{-1}$ to 0.25 $gl^{-1}h^{-1}$ when the agitation rate was increased from 600 rpm to 800 rpm.



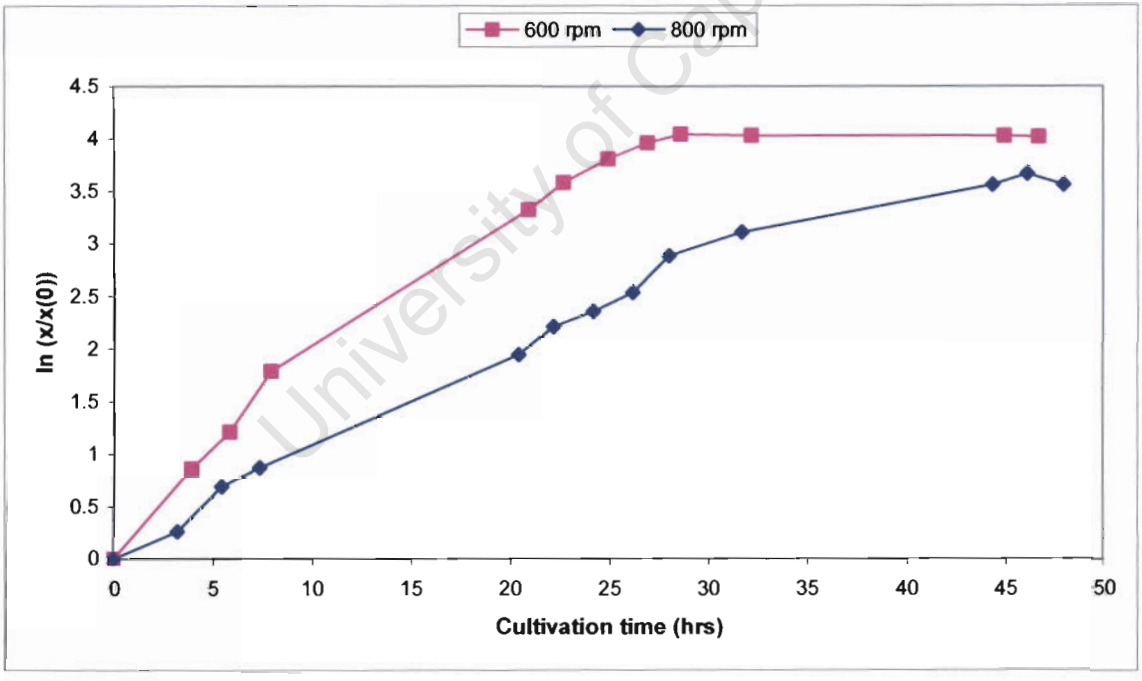



**Figure 5.11:** Effect of agitation rate on the growth of strain SS1 during batch cultivation in the STR. The culture was standardized to an initial optical density of 0.4 at 600 nm using a 10% inoculum. The culture was agitated at a rate of 600 rpm (■) or 800 rpm (♦). Compressed air was sparged into the reactor at a rate of 1 vvm.

The dynamic method (section 3.4.4.1) was used to determine the $k_La$ and the oxygen utilization rate (OUR) of strain SS1 for the two agitation rates. These experiments were conducted 24 h post inoculation when the cell density of the culture agitated at 600 rpm was 3 times greater than that of the culture agitated at 800 rpm (Table 5.5).

**Table 5.5:** Effect of stirrer speed on oxygen transfer parameters in the STR. Compressed air was sparged into the reactor at a rate of 1 vvm

| Stirrer speed, N | Cell density at 24 h, $X_{24}$ ($gl^{-1}$) | $k_La$ ($s^{-1}$) | Oxygen utilization rate, OUR ($mgO_2l^{-1}s^{-1}$) | Specific oxygen utilization rate, $OUR/X_{24}$ ($mgO_2s^{-1}$ g biomass$^{-1}$) |
|---|---|---|---|---|
| 600 rpm | 10 | 0.0201 | 0.034 | 0.0038 |
| 800 rpm | 3 | 0.0212 | 0.0094 | 0.0036 |

Contrary to expectations, increasing the agitation rate to 800 rpm only marginally increased the volumetric oxygen transfer coefficient in the STR (Table 5.5). The OUR of the culture agitated at a rate of 600 rpm was 3 times higher than that of the culture agitated at 800 rpm (Table 5.5). The specific oxygen utilization rate was similar; i.e. 0.0038 $mgO_2s^{-1}$ g biomass$^{-1}$ for the 600 rpm culture and 0.036 $mgO_2s^{-1}$ g biomass$^{-1}$ for the 800 rpm culture.

## 5.3. DISCUSSION

### 5.3.1. Batch cultivation of strain SS1

The reproducibility of batch cultivations of strain SS1 was investigated by considering the growth of the yeast in duplicate experiments under standard batch cultivation conditions. The maximum specific growth rate was determined and standard error between duplicate experiments was calculated. The maximum specific growth rate ($\mu_{max}$) was 0.23 $h^{-1}$ with a standard error of 0.0037 in the shake flask. The low standard error indicated that the $\mu_{max}$ was similar for duplicate batch cultivation experiments in the shake flask. Therefore the growth curves were considered identical. A $\mu_{max}$ of 0.23 $h^{-1}$

with a standard error of 0.0067 was obtained in the stirred tank reactor (STR). Again, the low standard error indicated that the $\mu_{max}$ was similar for duplicate batch cultivation experiments in the STR. Therefore the growth curves were also considered identical.

### 5.3.2. Inoculum size

Inoculum size was an important parameter for the growth of strain SS1. An inoculum size of 10% was concluded to be optimum for dense culture production of strain SS1. An inoculum size of 0.5% resulted in a lag phase which was eliminated with larger inocula. The cultures that received larger inocula (5 and 10% of the new medium volume) exhibited no lag phase. The lag phase for the culture that received the 0.5% inoculum was postulated to be a result of the small inoculum size. The transfer of a small inoculum into a large volume of medium results in the outward diffusion of essential vitamins, cofactors or ions which are required for the activity of intracellular enzymes (Bailey and Ollis, 1986). The extent of the lag phase depends on the time required to synthesize these compounds. The absence of a lag phase in the cultures receiving large inocula was attributed to the fact that large, exponentially growing cultures were inoculated into a relatively rich medium under effectively the same conditions. Thus all the factors that could contribute to a lag phase, including inoculum size, were eliminated.

The size of the inoculum also affected the productivity of the fermentation process. The increase in inoculum size resulted in a reduction of the total fermentation time from 47 h (0.5%) to 40 h (5%) and finally to 30 h (10%). The reduction in the cultivation time ultimately resulted in increased productivity with inoculum size. The highest productivity (0.50 $gl^{-1}h^{-1}$) was observed for the culture that received the 10% inoculum. This value is in agreement with the literature which recommends large inocula (5-10% of the new medium volume) in industrial processes for the production of microbial biomass (Bailey and Ollis, 1986).

### 5.3.3. Substrate concentration

Batch growth studies showed that the soluble protein in the medium was not important for the growth of strain SS1. The soluble protein in YEG medium was not utilized as a protein source when strain SS1 was cultivated both in shake flasks and the STR (Figure 5.4 and Figure 5.5). This confirmed studies aimed at medium development which indicated that a protein source was not needed for optimum growth of strain SS1. It is probable that, like *S. cerevisiae*, strain SS1 does not excrete an extracellular protease and thus is unable to degrade the peptides in the yeast extract. Cultivation of strain SS1 on skim milk agar produced no zones of clearing (data not shown), demonstrating the absence of an extracellular protease in strain SS1. Since strain SS1 was able to grow very well in Yeast nitrogen base without amino acids (data not shown), it was unlikely that the growth limiting substrate was an amino acid.

The glucose in the medium was not utilized by strain SS1 during cultivation in shake flasks (Figure 5.4). This result explained previous experiments which indicated that the growth of strain SS1 was not dependent on the glucose concentration in the medium during batch cultivation in shake flasks (Figure 4.2 b). The glucose was utilized when strain SS1 was grown in the STR (Figure 5.5). Glucose was important for high productivity of the batch cultivation process in the STR. The highest productivity was observed in the standard medium containing 20 g glucose $l^{-1}$ (Table 5.2). When no glucose was present in the medium, biomass formation of strain SS1 was inhibited resulting in the lowest productivity. Doubling the initial glucose concentration in the medium to 40 $gl^{-1}$ resulted in a longer cultivation time, and consequently, in lower productivity.

The initial glucose concentration in the medium affected the growth kinetics of strain SS1. The maximum specific growth rate ($\mu_{max}$) decreased from 0.23 $h^{-1}$ to 0.19 $h^{-1}$ when the glucose concentration was doubled to 40 $gl^{-1}$. This indicated that high glucose concentrations inhibited the growth of strain SS1. In addition, the glucose concentration of the medium influenced the specific glucose utilization rate of strain SS1 (Figure 5.7). During growth in the standard medium, the specific glucose utilization rate decreased

with time (Figure 5.7 A). Thus, although the number of cells increased with time the amount of glucose used by each cell decreased. In the medium containing 40 g glucose $l^{-1}$, the specific glucose utilization rate fluctuated and no clear trends were observed (Figure 5.7 B). The two different profiles observed indicated that glucose utilization was sensitive to the initial glucose concentration present in the medium and that a high glucose concentration inhibited glucose utilization. Not much is known about glucose repression in yeasts such as *Cryptococcus* sp. that are insensitive to the presence of free glucose in the medium. It was shown for *Trichosporon cutaneum*, a glucose insensitive yeast, that during continuous cultivation the maximum specific growth rate decreased from approximately 0.50 $h^{-1}$ to 0.31 $h^{-1}$ at glucose concentrations of 0.5% and 10% (w/v), respectively (Fiechter *et al.*, 1981 as stated in Janshekar, 1979). This illustrated that excess glucose affected the kinetics of the yeast and thus the glucose concentration in the medium is also important for yeast generally considered as glucose insensitive (Fiechter *et al.*, 1981). The upper limit of 10% is much higher than the glucose concentrations investigated in this study. However, the possibility that strain SS1 is a marine isolate may account for its growth being influenced by lower glucose concentrations than other glucose-insensitive yeasts.

The yeast extract in the medium was also utilized as a carbon source by strain SS1. During aerobic yeast growth, 50% of the carbon source is theoretically converted into biomass (Walker, 1998). Thus the expected theoretical yield of biomass from the carbon source is 0.5. For strain SS1, the yield of biomass on glucose only as substrate was 3.90, 0.92 and 0.50 for a glucose concentration of 0 $gl^{-1}$, 20 $gl^{-1}$ and 40 $gl^{-1}$, respectively, in the medium (Table 5.2). With the exception of the medium containing 40 g glucose $l^{-1}$, the experimental yield was considerably higher than the theoretical yield of 0.5. This indicated the presence of more than one carbon source which was attributed to the yeast extract in the medium. Hassan *et al.* (1996) reported that yeast extract contains 39.6% (w/w) carbon. Therefore the yield of biomass from carbon as substrate was calculated taking into account the carbon contributed by both the yeast extract (as reported by Hassan *et al.*) and the glucose. The yield of biomass from total carbon as substrate was 0.43, 0.65 and 0.39 for a glucose concentration of 0 $gl^{-1}$, 20 $gl^{-1}$ and 40 $gl^{-}$

$^{1}$, respectively, in the medium (Table 5.2). The high yield obtained with the standard medium indicated that nutrient carbon was converted efficiently into biomass carbon. The lower yield obtained with the medium containing 40 g glucose $l^{-1}$ was not unexpected as essentially the same biomass concentration was produced from double the amount of glucose. The use of yeast extract as a carbon source explained the 3 $gl^{-1}$ of biomass formed in the medium containing no glucose. Also, the yield for this medium was more acceptable when the carbon contributed by the yeast extract was taken into account. The carbohydrate content of yeast extract is thought to be primarily glucose which results from the hydrolysis of glycogen and trehalose during the production of yeast extract (Crueger and Crueger, 1989). However, any glucose in the medium would be detected by the reducing sugar assay. Perhaps yeast extract also contains other more complex carbohydrates that were not hydrolyzed to completion and that were not detected with the analytical assay used. Yeast extract reportedly contains sucrose (Seydel *et al.*, 2000) and trehalose (Park and Lee, 2000) which are both nonreducing disaccharides that would not be detected with the reducing sugar assay. However, the breakdown products from the hydrolysis of disaccharides would be detected with the assay. That is assuming that sucrose and trehalose are present in the yeast extract used for this study and were hydrolyzed extracellularly by the invertase and trehalase enzymes, respectively. Although it is generally accepted that sucrose is hydrolyzed extracellularly (Stambuk *et al.*, 2000), there is evidence that sucrose is transported into yeast cells (Santos *et al.*, 1982; Stambuk *et al.*, 2000). In addition, Perlman and Halvorson (1981) reported the existence of an intracellular invertase. Nwaka *et al.* (1996) have shown that *S. cerevisiae* can utilize extracellular trehalose as a sole carbon source. Although the mechanism of trehalose transport is unknown, they provided evidence that a trehalase located in the vacuole was necessary for the hydrolysis of trehalose. As mentioned previously, the amino acids in yeast extract can also be used as a carbon source resulting in the release of ammonium ions (Benslimane *et al.*, 1995). The increase in pH during batch cultivation in the STR (Figure 4.5) could reflect the assimilation of yeast extract amino acids as a carbon source. Accordingly, it is not unreasonable to suggest that the yeast extract contained compounds such as sucrose, trehalose and amino acids that were utilized as a carbon source by strain SS1.

Strain SS1 exhibited a complex growth curve that was characterized by the utilization of multiple carbon substrates. The specific growth rate decreased systematically over the course of the cultivation period. Sometimes the decrease in the specific growth rate was preceded by a lag phase (Figure 5.5), which is characteristic of the successive utilization of a carbon source (Kompala *et al.*, 1984, Monod, 1949). This phenomenon was observed by Monod (1949) and usually occurs when a compound partially satisfying an essential nutrient requirement becomes exhausted or when a metabolite accumulates and eventually serves as a secondary nutrient source. This results in multiple exponential phases as the nutrients are exhausted in succession (Figure 5.12). Also the increase in pH during batch cultivation in the STR was proposed to be due to the yeast extract amino acids used as a carbon source. However, this occurred while glucose was still being utilized and could indicate simultaneous utilization of nutrients. In the natural environment, as well as in many industrial fermentation processes, microorganisms are likely to utilize and grow on a mixture of substrates simultaneously (Doshi and Venkatesh, 1998; Lendenmann *et al.*, 1996). Growth on multiple substrates can involve either sequential or simultaneous utilization of nutrients (Venkatesh *et al.*, 1997) or both (Doshi and Venkatesh, 1998). The ability of microorganisms to grow on multiple substrates is likely to be important in the natural environment for fast and efficient growth (Lendenmann *et al.*, 1996). Therefore it is postulated that due to the complex nature of yeast extract, YEG medium contained multiple nutrients that were sequentially and simultaneously utilized by strain SS1. The specific growth rate decreased as nutrients became exhausted and growth only stopped when all the nutrients required for growth were exhausted.

Monod kinetics was not used to model the growth of strain SS1. The specific growth rate was evaluated as a function of the glucose concentration to ascertain the relationship between the two parameters. The purpose was to determine whether the relationship between the specific growth rate of strain SS1 and the glucose concentration of the medium could be described using Monod kinetics and thus whether glucose was a growth-limiting substrate for strain SS1. The relationship between the

specific growth rate and the growth-limiting substrate concentration when Monod kinetics is applicable is shown in Figure 5.1.

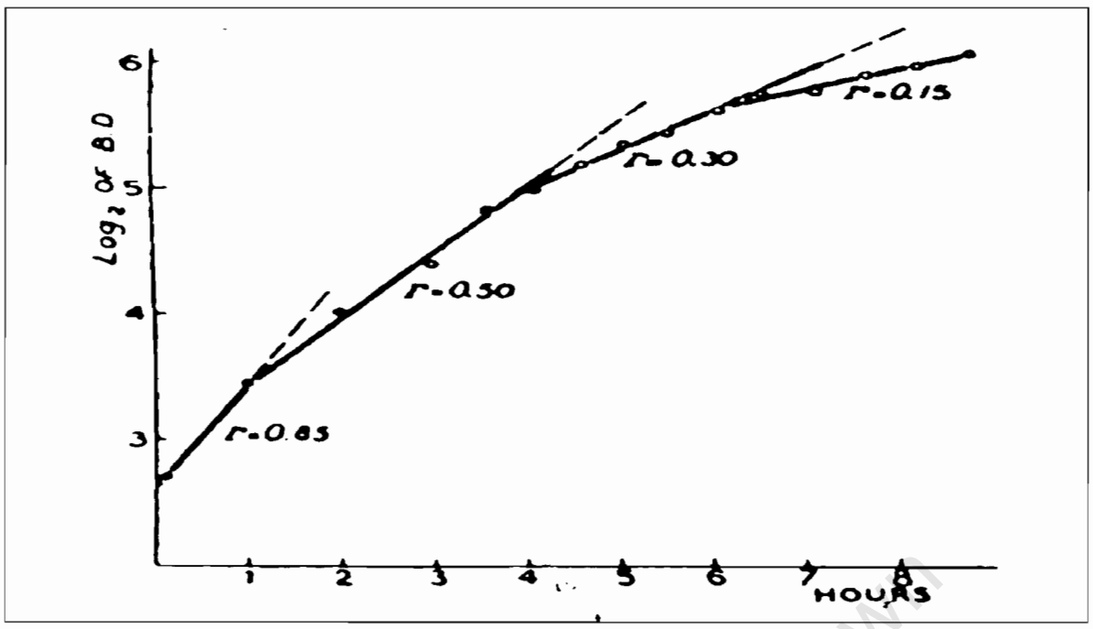



**Figure 5.12:** Multiple exponential phases exhibited by *E. coli* during growth in synthetic medium under suboptimal partial pressure of carbon dioxide. r=growth rate (Monod, 1949)

The relationship between the specific growth rate and the glucose concentration during strain SS1 growth in YEG medium containing 20 $gl^{-1}$ exhibited Monod dependence over a limited glucose concentration (Figure 5.8 A i). This relationship occurred in glucose concentrations of up to 10 $gl^{-1}$ (Figure 5.8 A ii). No such relationship was apparent when the medium contained 40 g glucose $l^{-1}$ (Figure 5.8 B). The data based on the medium containing 20 g glucose $l^{-1}$suggested that it is plausible that glucose was a growth-limiting substrate for strain SS1 over a limited concentration range. However, as mentioned previously, yeast extract was also utilized as a carbon source by strain SS1. Due to the complex culture medium used in this study, the growth of strain SS1 was complex, characterized by multiple exponential phases and possibly multiple growth-limiting substrates. Thus, even if glucose was a growth-limiting substrate, it was not the only growth-limiting substrate for strain SS1. There are a number of models, including modifications of the Monod model, to accommodate the presence of multiple limiting nutrients (Blanch and Clark, 1996; Crueger and Crueger, 1989). Since the identity of all

the limiting nutrients for strain SS1 remains unknown, the Monod equation or any modification thereof could not be applied to model the growth of strain SS1. Modifications of the Monod model are considered inadequate by some researchers to explain the complex processes that occur during growth on multiple substrates (Venkatesh *et al.*, 1997). Although microorganisms are frequently grown on multiple substrates, only a few publications have addressed the kinetics of multi-substrate growth for microorganisms such as *Escherichia coli* (Lendenmann *et al.*, 1996), a marine *Corynebacterium* (Law and Button, 1977) and the yeast *Kloeckera* sp. strain 2201 (Egli *et al.*, 1983). Attempts to model the behavior of microorganisms in multisubstrate environments resulted in structured models such as the optimal (Venkatesh *et al.*, 1997) and cybernetic (Kompala *et al.*, 1984) models. These models are very complicated and to some extent consider the complexity of the cell and the regulation of cellular processes.

### 5.3.4. Oxygen transfer capacity

Oxygen was a growth-limiting nutrient for strain SS1 during batch culture in standard Erlenmeyer flasks. The effect of oxygen availability on the growth of strain SS1 was investigated by the introduction of baffles and by changing the liquid volume in the flasks. The use of baffled flasks improved growth of strain SS1 compared to standard Erlenmeyer flasks (Figure 5.9). Baffles are commonly used to increase oxygen transfer in shaking bioreactors (Büchs, 2001). Therefore it is postulated that the baffled flasks provided better aeration resulting in improved growth of strain SS1, and consequently, that growth in the standard Erlenmeyer flasks was restricted by oxygen limitation. The importance of oxygen transfer for growth of strain SS1 was further demonstrated by cultivating the yeast in different volumes of media while the nominal volume of the flasks remained constant. This is a simple method that is commonly employed to determine whether oxygen limitation limits growth in a shaking culture (Hilton, 1999). The growth rates were very similar in all flasks for the first 16 h of growth (Figure 5.10). However, after approximately 18 h of growth the specific growth rate decreased in the 100 and 200 ml liquid volumes. This was indicative of the probable negative effect of reduced oxygen transfer capacity in the 100 and 200 ml flasks on the growth of strain SS1. The

decrease in oxygen transfer capacity with increasing liquid volume was demonstrated by the decrease in $k_La$ from 0.0112 $s^{-1}$ to 0.0073 $s^{-1}$ (Table 5.3). The resultant decrease in OTR (0.090 $mgO_2l^{-1}$ to 0.058 $mgO_2l^{-1}$) was quantified using Equation 5.9. The rate of transfer of oxygen to the cells governs the rate at which oxygen is utilized by a cell population (Bailey and Ollis, 1986). Furthermore, the oxygen utilization rate (OUR) of a cell population depends on the density of the culture. Therefore, the oxygen transfer rate effectively determines the cell density of a culture (Equation 5.7). Since the increase in cell density is proportional to the specific growth rate of the culture (Equation 5.1), oxygen transfer indirectly determines the specific growth rate of a culture. During the first 16 h the oxygen transfer in all the flasks was sufficient to sustain the increasing growth of the cell population. However, after 18 h of growth oxygen transfer in the 100 and 200 ml flasks was no longer sufficient to sustain the increasing cell population. The specific growth rate of strain SS1 decreased in the flasks where oxygen transfer was insufficient to induce an increase in cell concentration and therefore an increase in the OUR. Thus in the shake flasks oxygen was a growth-limiting nutrient that inhibited strain SS1 growth when oxygen transfer rates were insufficient to sustain the increasing cell population. Odds *et al.* (1995) showed that oxygen was a limiting nutrient for the growth of *Cryptococcus neoformans*. The introduction of baffles or decreasing the medium volume improved mixing efficiency and thus oxygen transfer in the shake flasks, improving the growth of strain SS1.

The agitation rate was increased from 600 rpm to 800 rpm as a means to investigate the effect of increased oxygen transfer on the growth of strain SS1 in the STR. According to the experiments by Dabee (1996) and Clarke (Personal communication) [3], this was still in the range where an improvement in $k_La$ was observed. Dabee (1996) conducted a set of experiments to determine the effect of stirrer speed on the volumetric mass transfer coefficient, $k_La$. These experiments were performed with distilled water in stirred tank Chemap bioreactors identical to the ones used in the current study. The operating conditions are shown in Table 5.6. The study showed that the volumetric oxygen transfer coefficient, $k_La$, increased with increasing agitation rate, N (Figure 5.13).

[3] K.G. Clarke, Senior Lecturer, Dept Chem. Eng., University of Stellenbosch, Unpublished data

However, an upper limit was reached where an increase in the agitation rate did not further improve the $k_La$ value. This is supported by the study of (Personal communication K.G. Clarke) [4].

**Table 5.6:** Dimensions of the 7l Chemap bioreactor and the range of operating conditions examined by Dabee (1996).

| | |
|---|---|
| Tank volume | 7.2 *l* |
| Liquid volume | 5 *l* |
| Tank diameter | 0.186 m |
| Impeller type | 4-bladed Rushton |
| Impeller diameter | 0.090 m |
| Agitation rate | 300 - 1400 rpm |
| Impeller tip speed | 1.4 - 6.60 $ms^{-1}$ |
| Superficial gas velocity | 1.55 – 3.05 x $10^{-3}$ $ms^{-1}$ |
| Gas flow rate | 4.2 – 8.3 x $10^{-5}$ $ms^{-1}$ |
| Temperature | 30°C |

Increasing the agitation rate inhibited the growth of strain SS1 (Figure 5.11). The $\mu_{max}$ decreased from 0.23 $h^{-1}$ to 0.15 $h^{-1}$ when the agitation rate was increased from 600 rpm to 800 rpm (Table 5.4). Consequently, the cultivation time increased from 30 h to 47 h and reduced the productivity of the process (Table 5.4). According to Toma *et al.* (1991), increased agitation may result in increased microbial productivity. However, there is a limit beyond which excess turbulence inhibits microbial growth and productivity. Accordingly, the group showed that the growth of *Brevibacterium flavum* and *S. cerevisiae* was inhibited when the agitation rate was increased beyond 900 rpm and 800 rpm, respectively. The decreased growth was attributed to shear effects which resulted in lower specific growth rates of the microorganisms. Therefore it is proposed that the higher agitation rate (800 rpm) introduced shear stress that negatively affected the specific growth rate of strain SS1 resulting in a less productive cultivation process.

[4] *Ibid.*

The higher agitation rate did not affect the respiration of strain SS1. The OUR was three fold higher when the stirrer speed was 600 rpm compared to a stirrer speed of 800 rpm. However, these measurements were performed when the cell density for the culture agitated at 600 rpm was three fold higher than that of the culture agitated at 800 rpm.

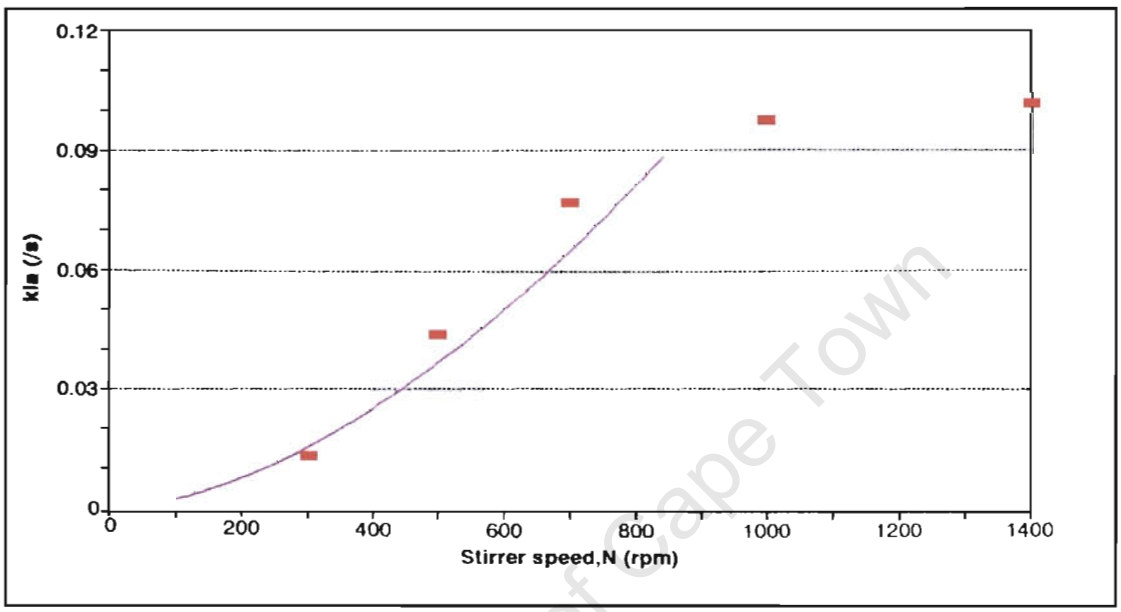



**Figure 5.13:** Effect of stirrer speed N (rpm) on the volumetric oxygen transfer coefficient $k_La$ ($s^{-1}$) with aeration rate of 5 $lmin^{-1}$ (1 vvm) (Dabee, 1996).

The specific OUR, which is indicative of the oxygen utilized per unit of cells, was similar (Table 5.5) indicating that the cultures were respiring at the same rate. Thus the reduction in the specific growth rate at an agitation rate of 800 rpm was not because oxygen uptake, and thus cellular respiration, was impeded.

The higher agitation rate only marginally increased $k_La$ (Table 5.5). The reason for this is not known since, as Figure 5.13 illustrates, a considerable improvement in $k_La$ was expected. The experimentally determined $k_La$ values in Table 5.5 were much lower than those reported by Dabee (Figure 5.13). This can be attributed to the differences in the operating conditions. While Dabee's experiments were conducted in distilled water, the $k_La$ values in this study were determined while the STR contained an actively respiring culture in liquid growth media. The presence of strain SS1, glucose in the media and

antifoam agents are all factors that result in decreased oxygen transfer (Bailey and Ollis, 1986). Furthermore, the culture temperature of 25°C was 5 degrees lower than the temperature used by Dabee (Table 5.6). The oxygen transfer rate increases with increasing temperature between 10°C and 40°C (Doran, 1995). Thus the conditions under which these experiments were performed all contributed to decreased oxygen transfer. However, the difference in $k_La$ in this study and that reported by Dabee (1996) is probably too pronounced for the reasons stated above and needs further investigation. Other methods to increase oxygen transfer include increasing the rate of air supply and supplementing or substituting the air with pure oxygen (Bailey and Ollis, 1986). However, these methods were not considered in this study. The standard rate of air supply of 1 vvm, although relatively high, is in the region of the values used industrially (Spencer and Spencer, 1989). Further increase in aeration rate provides little further increase in $k_La$ (Personal communication K. G. Clarke) [5]. Also, the addition of pure oxygen is not considered economically feasible for biomass production on an industrial scale.



[5] *Ibid.*

# CHAPTER 6

## FED-BATCH CULTIVATION OF STRAIN SS1

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## CONTENTS

## 6.1. INTRODUCTION

Fed-batch cultivation is the preferred industrial method for dense culture production of microorganisms (Riesenberg and Guthke, 1999). This method is primarily used to produce a high product yield under low nutrient or substrate conditions. It is applicable when low substrate concentrations must be maintained in the bioreactor to prevent inhibition of growth or when low substrate concentrations are a prerequisite for high product and biomass yields (Lee, 1996). For example, *S. cerevisiae* is affected by the Crabtree effect and at glucose concentrations greater than 0.1% ferments rather than respires glucose (Fiechter *et al.*, 1981; Käppeli, 1983). During the fed-batch process it is important that the substrates in the medium are fed at a pre-determined rate that does not limit growth (Lee, 1996). This is achieved by controlling the substrate concentration, the feed rate and the feed time of nutrients in fed-batch cultivation. Fed-batch fermentations are usually performed by feeding a specific nutrient that limits growth into the bioreactor. The limiting nutrient is frequently a carbon source such as glucose, glycerol or methanol (Riesenberg and Guthke, 1999). A very important aspect of fed-batch culture is to develop a suitable feeding strategy to ensure the nutrient concentration in the medium is optimal and to prevent both overfeeding and underfeeding (Lee *et al.*, 1999).

The aim of this study was to devise a fed-batch cultivation strategy for the production of dense cultures of strain SS1. This entailed:

- Selection of the appropriate feed substrate
- Application of the appropriate feed strategy
- Selection of the appropriate time to initiate and end the feeding phase



## 6.2. RESULTS

Batch cultivation studies in the STR revealed that although glucose was required for biomass formation, high glucose concentrations inhibited the growth of strain SS1

(section 5.2.3). Consequently, glucose seemed the obvious choice as a feed substrate for the fed-batch cultivation of strain SS1. However, batch cultivation experiments also elucidated that yeast extract was also used as a carbon source and that there was more than one growth limiting substrate. Thus, fed-batch studies were conducted using either glucose or different variations of the medium as feed substrate. The fed-batch cultivation process consisted of a feeding phase which was preceded and followed by a batch phase. Two different fed-batch feeding strategies were investigated as a means of producing dense cultures of strain SS1; namely stepwise increased feeding and constant feeding.

### 6.2.1. Stepwise increased feeding strategy

Batch studies elucidated what appeared to be Monod dependence of the specific growth rate on glucose as substrate at concentrations of up to 10 $gl^{-1}$ (Figure 5.8 Ai). This occurred during the late exponential phase of batch cultivation when a sufficient amount of biomass had accumulated in the bioreactor. The stepwise increased fed-batch strategy was to initiate the feeding phase during this period and to maintain the reducing sugar concentration between 5 and 10 $gl^{-1}$. Since the specific growth rate appeared to be constant and dependent on the reducing sugar concentration over this period, this was expected to result in pseudo steady state conditions.

**Table 6.1:** Operating conditions during fed-batch cultivation using stepwise increased feeding strategy

| | **Fed-batch 1 (SFB1)** | **Fed-batch 2 (SFB2)** |
|---|---|---|
| Starting culture volume (l) | 3 | 3 |
| Final culture volume (l) | 6 | 6 |
| [glucose] in feed ($gl^{-1}$) | 30 | 30 |
| [yeast extract] in feed ($gl^{-1}$) | 8.81 | 0.88 |
| Feed rate 1 (F1) ($mlh^{-1}$) | 100 | 100 |
| Feed rate 2 (F2) ($mlh^{-1}$) | 125 | 125 |
| Feed rate 3 (F3) ($mlh^{-1}$) | 136 | 136 |

The 3 litre batch phase was conducted with YEG medium containing 20 g glucose $l^{-1}$. After approximately 24 h the feeding phase was started and the feed substrate was fed into the bioreactor at an initial rate of 100 $mlh^{-1}$ (Table 6.1). In accordance with previous batch cultivation experiments a cell concentration of between 8 and 9 $gl^{-1}$ was expected in the STR after 24 h. The glucose concentration in the medium was monitored continuously using the reducing sugar assay and the substrate feed rate was manually increased to 125 $mlh^{-1}$ (F2) and later to 137 $mlh^{-1}$ (F3) once a decrease in the residual glucose concentration was observed as indicated in Figures 6.1 and 6.2.

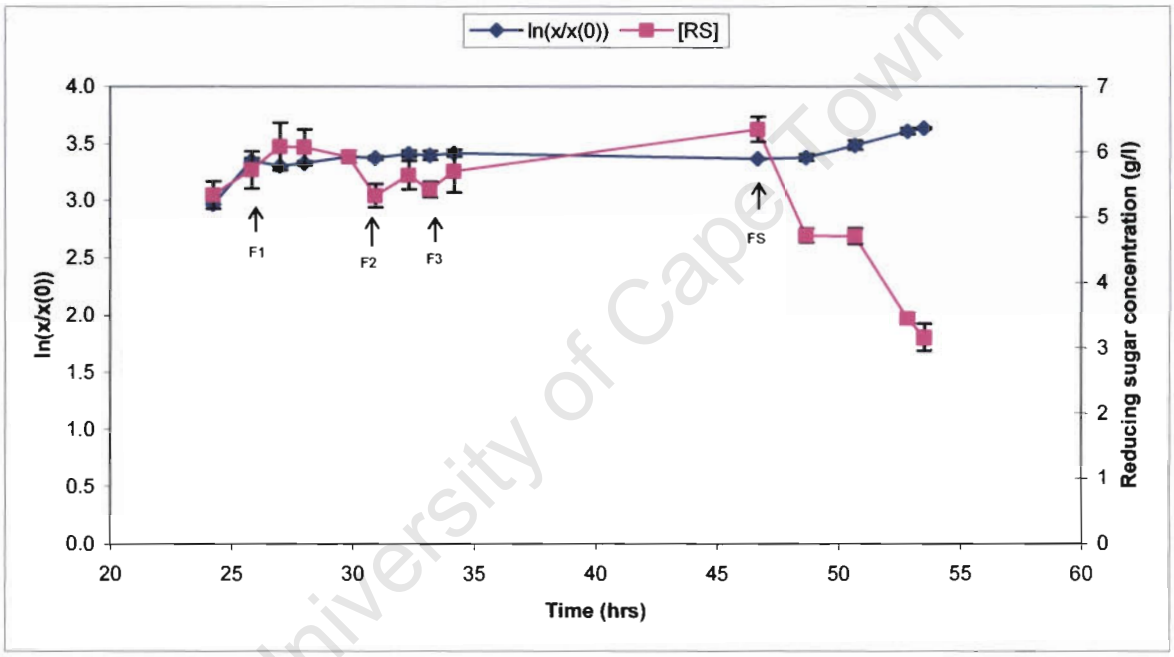




**Figure 6.1:** Fed-batch cultivation of SS1 using stepwise increased feeding strategy SFB1 (Table 6.1). The feeding phase commenced at a rate of 100 $mlh^{-1}$ (F1). The feed rate was manually increased to 125 $mlh^{-1}$ (F2) and 137 $mlh^{-1}$ (F3). The feeding phase was terminated at FS. [RS]=Reducing sugar concentration

For SFB1, YEG medium containing 30 g glucose $l^{-1}$and 8.81 g yeast extract $l^{-1}$ was used as the feed substrate (Table 6.1). The reducing sugar concentration fluctuated between 5 and 6.5 $gl^{-1}$ during the feeding phase (Figure 6.1). Once feeding was terminated the reducing sugar concentration in the medium decreased. The biomass concentration remained relatively constant over the feed period and was determined to be 8.5 $gl^{-1}$ at

the start of the feeding phase and 8.7 $gl^{-1}$ at the end. The biomass increased further during the second batch phase of the fermentation and a final biomass concentration of 11.4 $gl^{-1}$ was obtained after a total cultivation period of 54 h. This resulted in a productivity of 0.21 $gl^{-1}h^{-1}$. The yield was 0.26 based on total carbon as substrate.

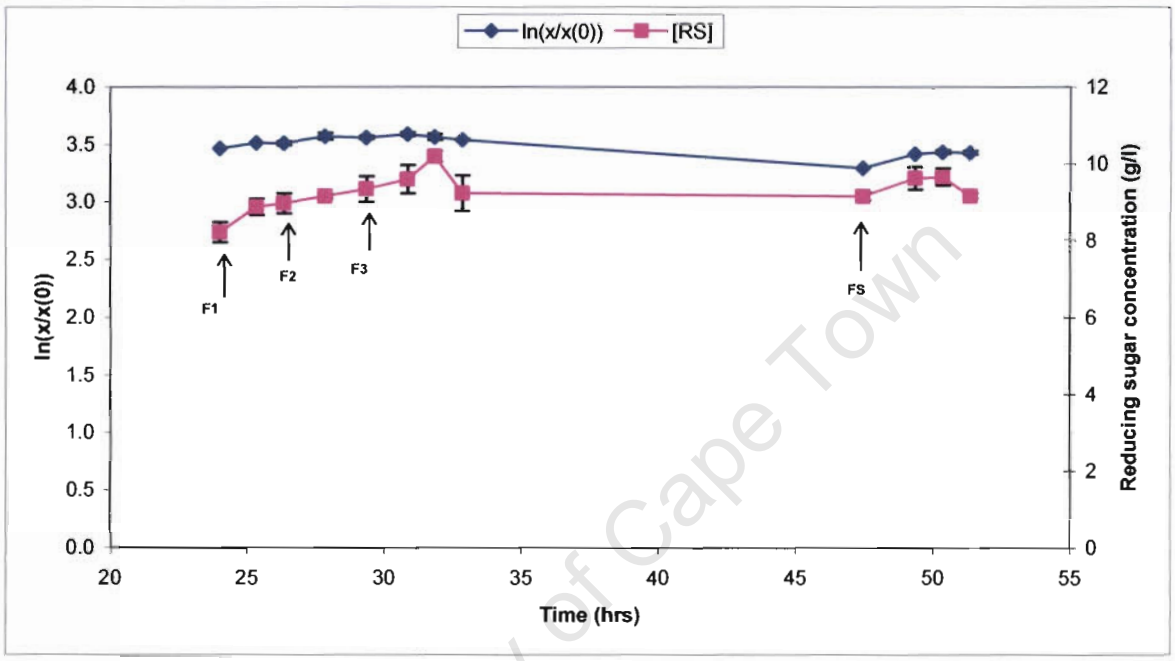


**Figure 6.2:** Fed-batch cultivation of SS1 (SFB2) using stepwise increased feeding strategy SFB2 (Table 6.1). The feeding phase commenced at rate of 100 $mlhr^{-1}$ (F1). The feed rate was manually increased to 125 $mlh^{-1}$ (F2) and 137 $mlh^{-1}$ (F3). The feeding phase was terminated at FS. [RS]=Reducing sugar concentration

For SFB2, YEG medium containing 30 g glucose $l^{-1}$ and 0.88 g yeast extract $l^{-1}$ was used as the feed substrate (Table 6.1). The reducing sugar concentration fluctuated between 8 and 10 $gl^{-1}$ during the feeding phase (Figure 6.2). No reduction in the reducing sugar concentration was observed after feeding was terminated. The biomass concentration was determined to be 8.4 $gl^{-1}$ at the start of the feeding phase and decreased slightly to 7.4 $gl^{-1}$ at the end. The biomass did not increase much further during the second batch phase and a final biomass concentration of 8.2 $gl^{-1}$ was obtained after a total cultivation period of 51 h. This resulted in a productivity of 0.16 $gl^{-1}h^{-1}$. The yield was 0.21 based on total carbon as substrate.

### 6.2.2. Constant feeding strategy

During batch cultivation in the standard medium, the maximum specific growth rate (Figure 5.5) and the highest specific glucose utilization rate of ~0.5 (g glucose) (g biomass)$^{-1}h^{-1}$ (Figure 5.7 A) occurred during the first 10 h of growth. The strategy was to maintain a constant substrate feed rate into the bioreactor based on a specific glucose utilization rate of 1(g glucose) (g biomass)$^{-1}h^{-1}$. Since the fermentation was started as a 5 litre batch culture, the substrate was fed so that 5 g of glucose was introduced into the reactor over a period of 1 hr. Since the glucose concentration in the substrate was 100 $gl^{-1}$, the substrate was fed into the reactor at a rate of 50 $mlh^{-1}$.

**Table 6.2:** Operating conditions during fed-batch cultivation using a constant feeding strategy

| | CFB1 | CFB2 |
|---|---|---|
| Starting culture volume (l) | 5 | 5 |
| Final culture volume (l) | 6 | 6 |
| [glucose] in feed ($gl^{-1}$) | 100 | 100 |
| [yeast extract] in feed ($gl^{-1}$) | 0 | 44 |
| Feed rate ($mlh^{-1}$) | 50 | 50 |

Glucose only (100 $gl^{-1}$) was used as the feed substrate in CFB1 (Table 6.2). The glucose concentration fluctuated between 15 and 20 $gl^{-1}$ during the feeding phase (Figure 6.3). The biomass concentration was determined to be 1.4 $gl^{-1}$ at the start of the feeding phase. The biomass fluctuated during the second batch phase and a final biomass concentration of 5.7 $gl^{-1}$ was obtained after a cultivation period of 40 h. As shown in Figure 6.3 a decrease in the specific growth rate was observed after approximately 24 h. This resulted in a productivity of 0.14 $gl^{-1}h^{-1}$. A yield of 0.12 was determined based on total carbon as substrate.

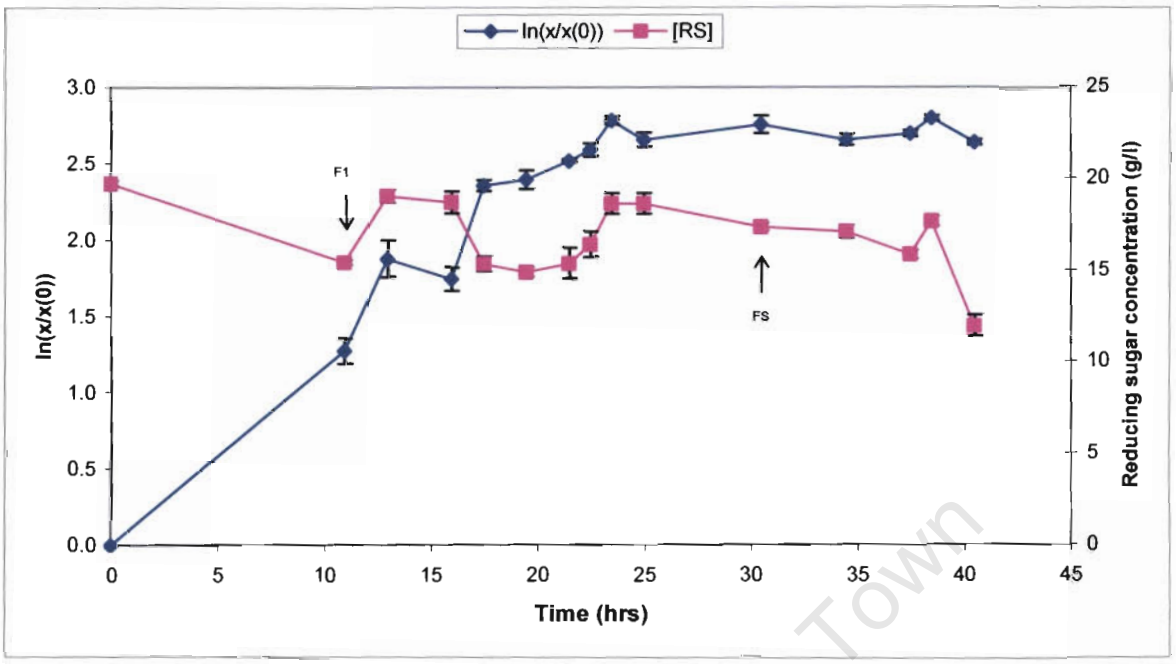


**Figure 6.3:** Fed-batch cultivation of strain SS1 using constant feeding strategy CFB1 (Table 6.2). The feeding phase was commenced at F1 and terminated at FS. [RS]=Reducing sugar concentration

YEG medium containing 100 g glucose $l^{-1}$ and 44 g yeast extract $l^{-1}$ was used as the feed substrate in CFB2 (Table 6.2). The strategy was to maintain a constant feed rate over a 20 hr period. However, feeding was interrupted between 16 and 24 h due to a break in the feed line. This did not result in contamination of the fermentation and did not affect the specific growth rate of strain SS1. The biomass concentration was determined to be 1.7 $gl^{-1}$ at the start of the feeding phase and increased to 9.4 $gl^{-1}$ at the end. The reducing sugar concentration ranged between 10 and 20 $gl^{-1}$ during the feeding phase (Figure 6.4.). The biomass concentration increased further during the second batch phase and a final biomass concentration of 20.6 $gl^{-1}$ was obtained after a total cultivation period of 51 h. This resulted in a productivity of 0.40 $gl^{-1}h^{-1}$. The yield was 0.36 based on total carbon as substrate.

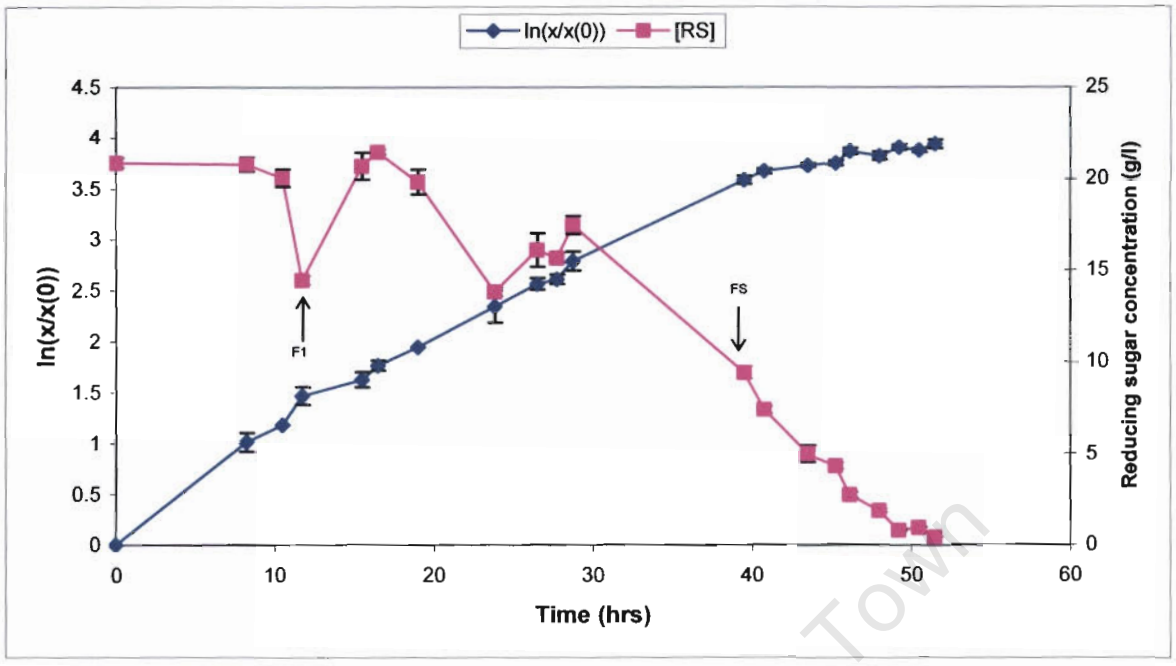


**Figure 6.4:** Fed-batch cultivation of strain SS1 using constant feeding strategy CFB2 (Table 6.2). The feeding phase commenced at F1 and terminated at FS. [RS]=Reducing sugar concentration

## 6.3. DISCUSSION

Modeling the growth of strain SS1 was not as straightforward as anticipated prior to the start of the project. This was primarily because strain SS1 was cultured in a medium containing complex substrates and as a result the identity of the growth-limiting substrates was unknown. Although the fed-batch cultivation of strain SS1 was based on the biokinetic data generated from batch fermentation studies, these were also trial-and-error experiments. The stepwise increased and constant feeding strategies were investigated for the fed-batch cultivation of strain SS1. These are simple feeding strategies that do not require the use of sophisticated and expensive equipment. Each fed-batch strategy was applied to different stages of the batch growth curve where different specific growth rates and specific substrate utilization rates occurred. The optimum productivity obtained using the constant feeding strategy was 0.40 $gl^{-1}h^{-1}$. This was double the productivity achieved with the stepwise increased feeding strategy (0.21

$gl^{-1}h^{-1}$). The difference in productivity is postulated to be due to the time when feeding was initiated as well as the higher yeast extract concentration in the feed. The stepwise increased feeding strategy was initiated near the end of the exponential phase of batch cultivation where the specific growth rate appeared to exhibit Monod dependence on the glucose concentration. The aim was to maintain the glucose concentration between 5 and 10 $gl^{-1}$ by monitoring the glucose in the supernatant with the reducing sugar assay (section 3.4.2). The feed rate was manually increased when a decrease in the glucose concentration was observed. The reducing sugar concentration fluctuated between 5 and 6.5 $gl^{-1}$ (SFB1) and between 8 and 10 $gl^{-1}$ (SFB2). Controlling the concentration of the carbon source at a desired value can be used as a fed-batch strategy for dense culture production (Lee *et al.*, 1999). Kim *et al.* (1994) cultured *Alcaligenes eutrophus* to a final cell concentration of 164 $gl^{-1}$ in 50 h using fed-batch cultivation. The group used a glucose analyzer to maintain the glucose concentration at 15 $gl^{-1}$ when in reality the glucose concentration fluctuated between 10 and 20 $gl^{-1}$. Although for the present study the glucose concentration was kept in the required range without use of expensive equipment, feeding was initiated when strain SS1 was not growing optimally. During batch cultivation in the STR the specific growth rate was optimum during the first 8 h of growth and decreased over the remaining culture period (Figure 5.5). Also, the specific glucose utilization rate decreased during batch cultivation in the standard medium (Figure 5.7 A). Due to selection of an inappropriate time to initiate the feeding phase, a final cell density of 11.4 $gl^{-1}$ (SFB1) and 8.2 $gl^{-1}$ (SFB2) was obtained using the stepwise increased feeding strategy. In contrast, the constant feeding strategy was initiated during the first 10 h of batch cultivation when strain SS1 was growing optimally. Generally in fed-batch cultivation the microorganism is grown as a batch culture and the fed-batch operation is initiated prior to complete exhaustion of the nutrient that limits growth (Reed, 1982). Although feeding nutrients at the beginning of the batch culture of strain SS1 was an unconventional strategy to employ, it is rational to assume that the fed-batch culture for biomass production would be most productive if feeding is initiated when culture growth is optimal. Using concentrated YEG medium as feed substrate, a final cell density of 20.6 $gl^{-1}$ was obtained. This was 4-6 $gl^{-1}$ more than what was obtained during batch cultivation. Therefore it is postulated that for fed-batch culture of

strain SS1 the feeding phase should be initiated during the first 10 h of batch cultivation when the specific growth rate and the specific glucose utilization rate is optimal.

Yeast extract was an important feed substrate for strain SS1. Irrespective of the feeding strategy used the highest maximum cell concentration, yield and productivity was obtained when yeast extract was incorporated in the feed. The use of the stepwise increased feeding strategy and the standard amount of yeast extract in the feed (SFB1) resulted in a final cell density of 11.4 $gl^{-1}$, a yield of 0.26 and a productivity of 0.21 $gl^{-1}h^{-1}$. When the concentration of yeast extract in the feed stream was reduced by a factor of 10 (SFB2), the final cell density decreased to 8 $gl^{-1}$. The yield (0.21) and productivity (0.16 $gl^{-1}h^{-1}$) were also lower. Similarly the use of the constant feeding strategy and concentrated yeast extract (CFB2) in the feed resulted in an increase in the final cell density (5.7 to 20.6 $gl^{-1}$), yield (0.12 to 0.36) and productivity (0.14 to 0.40 $gl^{-1}h^{-1}$) compared to when yeast extract (CFB1) was omitted from the feed. At the end of the feed period the cell density did not increase significantly for SFB2 and CFB1. This was probably because the nutrients provided by the yeast extract were not present in sufficient quantities to sustain further growth of the cell population. Yeast extract is required for the growth of the marine microorganism *Alteromonas putrefaciens*, (Hibino *et al.*, 1993) and stimulated the growth of the microalga *Crypthecodinium cohnii* (de Swaaf *et al.*, 1999) during dense culture production. The utilization of glucose by strain SS1 after feeding was terminated only occurred in the cultures that were fed the standard (SFB1) and concentrated (CFB2) yeast extract concentrations. This indicated that although glucose was important for strain SS1 growth, the yeast extract was even more important.

Fed-batch cultivation of strain SS1 was not considered economically feasible. Ultimately the aim of fed-batch culture is to produce the desired product to a high concentration with a high productivity and yield (Lee *et al.*, 1999). The best result for the fed-batch culture of strain SS1 was when a constant feeding strategy was employed and feeding was initiated during the first 10 h of batch growth (CFB2) using concentrated YEG medium as the feed substrate. Although a final cell density of 20 $gl^{-1}$ was obtained, the

productivity of fed-batch culture (0.40 $gl^{-1}h^{-1}$) was lower than that of the batch process (0.50 $gl^{-1}h^{-1}$). The yield (0.35) was also lower than that obtained for batch cultivation (0.65). This indicated that the nutrients in the medium were not efficiently converted into biomass during fed-batch cultivation. As a result, dense culture production of strain SS1 using fed-batch cultivation is considered uneconomical. However we have identified elements that could lead to an economical dense culture process. It was better to start feeding nutrients during the first 10 h of CFB2 when the culture was still growing well. Also, there was a period between 30 and 40 h post-inoculation in CFB2 when the glucose concentration decreased dramatically even though nutrients were constantly fed into the bioreactor (Figure 6.4) indicating that not enough glucose was fed into the bioreactor. Perhaps a combination of the strategies used in this study i.e. a stepwise increased feeding strategy that is employed while strain SS1 is growing optimally would be more successful for dense culture production.

# CHAPTER 7

## CONCLUSION

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## CONTENTS

## 7.1. CONCLUDING REMARKS

The aim of this study was to optimize the growth of *Cryptococcus* species SS1 for dense culture production. This involved identification of the essential nutrients required for growth and consequently high cell densities. A medium consisting of yeast extract and glucose was sufficient for achieving high cell yields, with the yeast extract providing essential nutrients for growth. Furthermore, the effect of other environmental parameters on growth was studied to determine the optimum conditions for growth. Optimum growth occurred between 22 and 25°C (room ternperature). Growth was not affected by pH values between 5.0 and 7.5. Strain SS1 was able to tolerate salt concentrations of up to 50 mM but did not exhibit an obligate requirement for sodium chloride in the medium.

The growth of strain SS1 was reproducible during batch cultivation both in shake flasks and in the STR. The effect of inoculum size, substrate concentration and agitation rate on the kinetic parameters of strain SS1 was determined during batch cultivation. The highest biomass concentration obtained for strain SS1 during batch cultivation was ~15 $gl^{-1}$. The size of the inoculum affected the productivity of the batch cultivation process in the STR. The productivity of the process increased with inoculum size. An inoculum size of 10% of the new medium volume resulted in the highest productivity (0.50 $h^{-1}$).

The soluble protein in YEG medium was not used as a nutrient source during batch cultivation of strain SS1 in both the shake flask and the STR. The utilization of glucose as a nutrient source was different in the shake flask and the STR. Glucose was not used during batch cultivation of strain SS1 in shake flasks. In contrast, glucose was essential for biomass accumulation during batch cultivation of strain SS1 in the STR. The yeast exhibited sensitivity to the initial glucose concentration in the medium as the specific growth rate was reduced when cultivated in YEG medium containing 40 g glucose $l^{-1}$. In addition, the specific glucose utilization rate profiles of strain SS1 differed when cultured in YEG medium containing either 20 or 40 g glucose $l^{-1}$. Modeling of the growth of strain SS1 using Monod kinetics was not performed as there were multiple

substrates that limited growth and the identity of all the growth limiting substrates could not be determined.

Oxygen was a growth-limiting substrate for strain SS1 when cultured in standard Erlenmeyer flasks. The introduction of baffles in the flasks improved oxygen transfer and resulted in better growth of strain SS1. The importance of oxygen was further demonstrated by cultivating strain SS1 in different volumes of media while the nominal volume of the flasks remained constant. The specific growth rate decreased with increasing medium volume due to decreased oxygen transfer. This was demonstrated by a decrease in $k_La$ from 0.0112 $s^{-1}$ at a working volume of 20% to 0.0073 $s^{-1}$ at a working volume of 5%. The decrease in $k_La$ translated into a decrease in OTR from 0.090 $mgO_2l^{-1}$ to 0.058 $mgO_2l^{-1}$ with increasing medium volume. Increasing the agitation rate of the STR from 600 rpm to 800 rpm was detrimental to the growth of strain SS1, resulting in an inhibition of growth and biomass formation. This was postulated to be due to increased shear effects introduced by the higher agitation speed.

Attempts to produce dense cultures of strain SS1 using fed-batch cultivation were not successful. Application of a stepwise increased feeding strategy initiated during the late exponential growth phase resulted in a fed-batch cultivation process that was much less productive than the batch process. A constant feeding strategy using more concentrated feed substrates that were introduced during the first 10 h of growth when both the specific growth rate and the specific glucose utilization rate was optimal, resulted in a maximum cell concentration of ~20 $gl^{-1}$. This was only 4-6 $gl^{-1}$ higher than the cell concentration obtained during batch cultivation. Furthermore, the cultivation period of the fed-batch process was 20 h longer than the batch process. Thus the productivity of the fed-batch cultivation process using the constant feeding strategy (0.40 $gl^{-1}hr^{-1}$) was lower than that obtained with batch cultivation (0.50 $gl^{-1}hr^{-1}$). As a result, the fed-batch cultivation of strain SS1 using the strategies employed in this study was deemed uneconomical.

## 7.2. FUTURE WORK

The final medium for growth of strain SS1 consisted of relatively expensive laboratory grade substrates. It should be determined whether these substrates can be substituted with inexpensive industrial grade components. It should also be considered whether strain SS1 is able to utilize the cruder substrates, whether the crude substrates do not contain compounds that might inhibit growth and whether the substrates can be converted into biomass with at least the same efficiency as the lab grade substrates. The use of industrial grade substrates such as waste yeast instead of yeast extract would provide the same nutrients but would reduce the cost of the process and make production of strain SS1 economically feasible on an industrial scale.

Dense culture production of strain SS1 necessitates a better understanding of the growth and metabolism of the yeast. Although glucose was important for the growth of strain SS1, there were indications that the yeast was sensitive to high glucose concentrations (40 $gl^{-1}$). Perhaps the kinetics of strain SS1 should be evaluated at initial glucose concentrations lower than 20 $gl^{-1}$. Yeast extract was also important for the growth of strain SS1. However, the complex nature of the substrate complicates studies to determine the reason for its importance. A detailed analysis of the chemical composition of yeast extract would simplify studies to determine the component(s) of the yeast extract important for strain SS1 growth. Alternatively, the use of a defined medium that contains known amounts of specific substrates would make it possible to determine the growth-limiting substrate(s) for strain SS1. Also, an elemental analysis of the yeast would elucidate how the nutrients in the medium are converted into biomass and would give a better understanding of the elements required for biomass formation.

During this study, it was not considered that strain SS1 growth may have stopped due to the accumulation of a metabolic by-product which became inhibitory to growth. During growth, microorganisms excrete metabolic by-products into the culture medium which can accumulate to inhibitory levels. These metabolic by-products differ according to the organism being cultivated. Some well-known examples are acetate for *E. coli*,

propionate for *Bacillis subtilis*, lactate for *Lactococcus lactis* and ethanol for *S. cerevisiae* (Riesenberg and Guthke, 1999). Therefore it is suggested that the spent culture media should be analyzed to determine what metabolites are excreted during growth of strain SS1 and whether these compounds accumulate in the medium.

The dense culture production of strain SS1 using fed-batch cultivation was not considered economically feasible due to the low productivity (0.40 $gl^{-1}h^{-1}$) obtained compared to batch fermentation (0.50 $gl^{-1}h^{-1}$). However, fed-batch cultivation was not completely unsuccessful. The final cell density was increased to 20 $gl^{-1}$. This could be further increased by increasing the yield ($Y_{x/s}$) of biomass from carbon as substrate. During fed-batch cultivation the yield was only 0.36 compared to a yield of 0.65 during batch cultivation. This indicates that nutrients are more efficiently converted into biomass during batch cultivation. Perhaps nutrients will be utilized more efficiently if less nutrients are fed into the bioreactor. Hibino *et al.* (1993) showed that during fed-batch cultivation of the marine microorganism *Alteromonas putrefaciens* using peptone and yeast extract as feeding substrate, the maximum cell concentration increased 1.5 times when the total amounts of the fed nutrients were reduced by half. Alternatively, the same nutrients, or more concentrated nutrients, could be fed very slowly into the bioreactor, thereby extending the feeding period. The ratio of glucose to yeast extract should also be assessed to determine the optimum concentrations of these substrates in the feed. Also, employing a stepwise increased feeding strategy while strain SS1 is growing optimally should be evaluated for dense culture production.

Once a suitable strategy for the dense culture of strain SS1 has been developed, the separation of the yeast from the fermentation broth should be evaluated. The recovery and purification of the product is an integral part of any industrial fermentation process (Crueger and Crueger, 1989; Shuler and Kargi, 1992). When biomass is the product, harvesting of the cells is the primary step in the product recovery process (Shuler and Kargi, 1992). The primary methods used industrially for the separation of yeast cells from the medium that should be considered for the recovery of strain SS1 are discussed in section 2.6 of the literature review.

Finally, it should be determined whether strain SS1 cultivated in the laboratory under conditions for optimum growth is able to survive in the aquaculture environment and whether the yeast retains its probiotic properties. This especially concerns the ability of strain SS1 to produce an extracellular amylase. During this study the objective was to produce dense cultures of strain SS1 in the shortest possible time. Thus the yeast was cultivated in media containing glucose which is an easily utilizable carbon source. Growth of strain SS1 on more complex carbohydrates such as starch is likely to yield slower growth rates and lower cell densities. However, it should be determined whether the yeast acquired from dense culture production retained the ability to degrade complex carbohydrates, especially when strain SS1 is to be used as a probiotic for abalone.

# APPENDICES

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# APPENDIX A

## CULTURE MEDIA

All media were sterilised by autoclaving for 15 minutes at 15 psi and 121°C. Heat labile components of media were sterilised using a 0.22 μm filter. Agar plates contained the same components as the media but with the addition of 15 $gl^{-1}$ of bacteriological agar (Biolab MERCK).

### A.1. Yeast extract-Bacteriological peptone-Glucose (YEBG) medium

| | |
|---|---|
| Yeast Extract (DIFCO Laboratories) | 5.0 g |
| Bacteriological peptone (Biolab MERCK) | 10.0 g |
| Glucose | 20.0 g |
| $dH_2O$ | 1 litre |

### A.2. Yeast extract-Proteose peptone-Glucose (YEPG) medium

| | |
|---|---|
| Proteose peptone (Difco) | 20.0 g |
| Yeast Extract (DIFCO Laboratories) | 10.0 g |
| Glucose (Biolab MERCK) | 20.0 g |
| $dH_2O$ | 1 litre |

### A.3. Yeast extract-Tryptone-Glucose (YETG) medium

| | |
|---|---|
| Yeast Extract (DIFCO Laboratories) | 8.81 g |
| Tryptone (Biolab MERCK) | 10.0 g |
| Glucose (Biolab MERCK) | 20.0 g |
| $dH_2O$ | 1 litre |

### A.4 Yeast extract-Glucose (YEG) medium

| | |
|---|---|
| Yeast Extract (DIFCO Laboratories) | 8.81 g |
| Glucose (Biolab MERCK) | 20.0 g |
| $dH_2O$ | 1 litre |

# APPENDIX B

## BUFFERS AND SOLUTIONS

### B.1. DNS reagent

| | |
|---|---|
| 3, 5-Dinitrosalicylic acid (Sigma) | 10.6 g |
| NaOH (Biolab MERCK) | 19.8 g |
| Sodium potassium tartrate (Biolab MERCK) | 306 g |
| Phenol | 7.6 g |
| Sodium metabisulphite | 8.3 g |
| Distilled water | 1416 ml |

Dissolve the first three reagents completely in water before adding the other constituents and dissolving in turn. The phenol is melted at 50°C.

### B.2. Solutions for Lowry assay

Copper reagent

20 g $Na_2C0_3$ in 260 ml distilled water

0.4 g $CuS0_4.5H_2O$ in 20 ml distilled water

0.2 g Na K Tartrate in 20 ml distilled water

Mix all three solutions for the copper reagent.

1 % SDS

10 g sodium dodecyl sulfate per 100 ml distilled water

1M NaOH

4 g NaOH per 100 ml distilled water

2X Lowry concentrate

3 parts copper reagent

1 part NaOH

1 part SDS

Solution is stable for 2-3 weeks. Warm the solution to 37°C if a white precipitate forms, and discard if there is a black precipitate. Rather keep the 3 stock solutions and mix just before use.

0.2 N Folin reagent

Mix 10 ml 2 N Folin reagent with 90 ml distilled water. Keep in an amber bottle. The dilution is stable for several months.

### B.3. Wide range buffer (pH 5.0-7.5)

Contains x ml 0.2 M $NaH_2PO_4.2H_2O$ and (100-x) ml 0.1 M citric acid

| pH | x |
|---|---|
| 5.0 | 49.0 |
| 5.5 | 43.5 |
| 6.0 | 37.5 |
| 6.5 | 28.3 |
| 7.0 | 17.8 |
| 7.5 | 7.9 |

# APPENDIX C

## STANDARD ASSAYS

### C.1. The dinitrosalicylic acid (DNS) reducing sugar method

Dilute the collected supernatant fraction to within the required range. Add DNS reagent (150 µl) to 50 µl of diluted supernatant sample. Boil the mixture in a water bath for 5 min and immediately place on ice to stop the reaction. Make the volume up to 1 ml with distilled water and determine the absorbency at 510 nm.

### C.2. Lowry protein quantitation assay

i. Dilute samples to an estimated 0.025 - 0.25 mg/ml with buffer. Prepare 400 µl of each dilution. Duplicate or triplicate samples are recommended.

ii. Prepare a reference of 400 µl distilled water. Prepare standards from 0.25 mg/ml BSA by adding 40 - 400 µl to 13 x 1 ml Eppendorf tubes and bring to volume of 400 µl with distilled water.

iii. Add 400 µl of 2x Lowry concentrate, mix thoroughly; incubate at room temperature for 10 min.

iv. Add 200 µl 0.2 N Folin reagent very quickly, and vortex immediately. Complete mixing of the reagent must be accomplished quickly to avoid decomposition of the reagent before it reacts with the protein. Incubate for 30 min at room temperature.

v. Use glass or polystyrene cuvettes to read the optical density at 750 nm. If the absorbencies are too high, they may be read at 500 nm.

# APPENDIX D

## STANDARD CURVES

### D.1. Dinitrosalicylic acid (DNS) assay

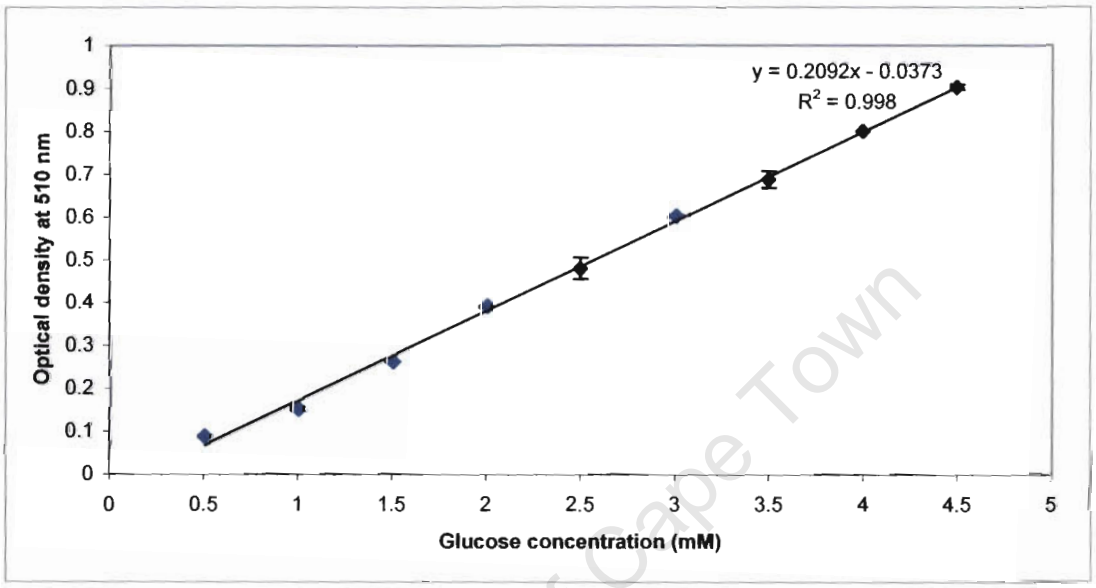


**Figure D.1:** Glucose concentration standard curve for DNS assay

### D.2. Lowry assay

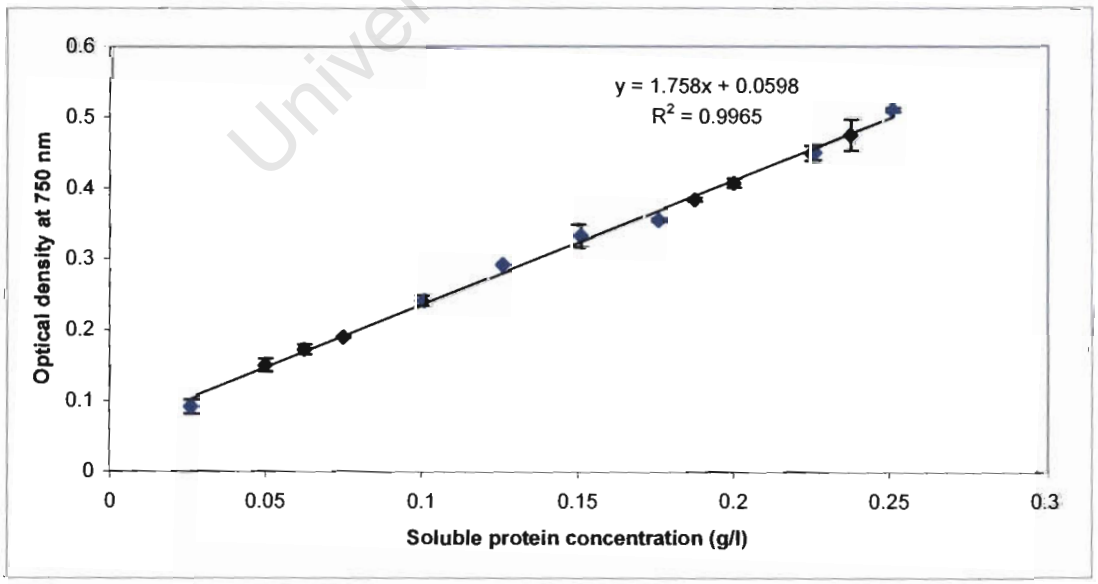


**Figure D.2:** Bovine serum albumin concentration standard curve for Lowry assay

# APPENDIX E

## VOLUMETRIC OXYGEN TRANSFER COEFFICIENT DETERMINATION

### E.1. Shake flasks

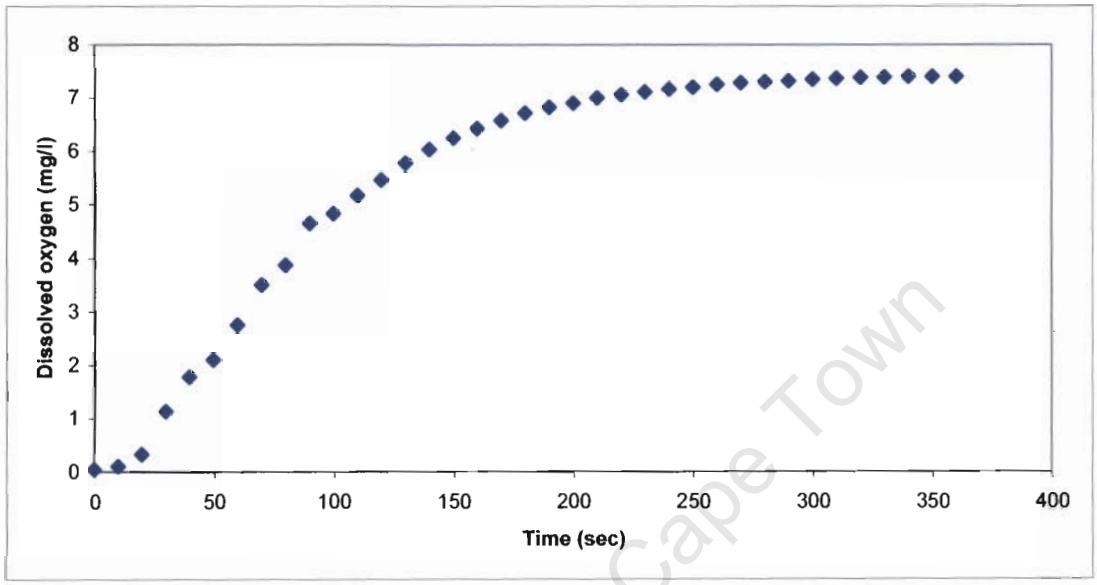


**Figure E.1:** Typical dissolved oxygen profile in shake flasks for volumetric oxygen transfer measurement

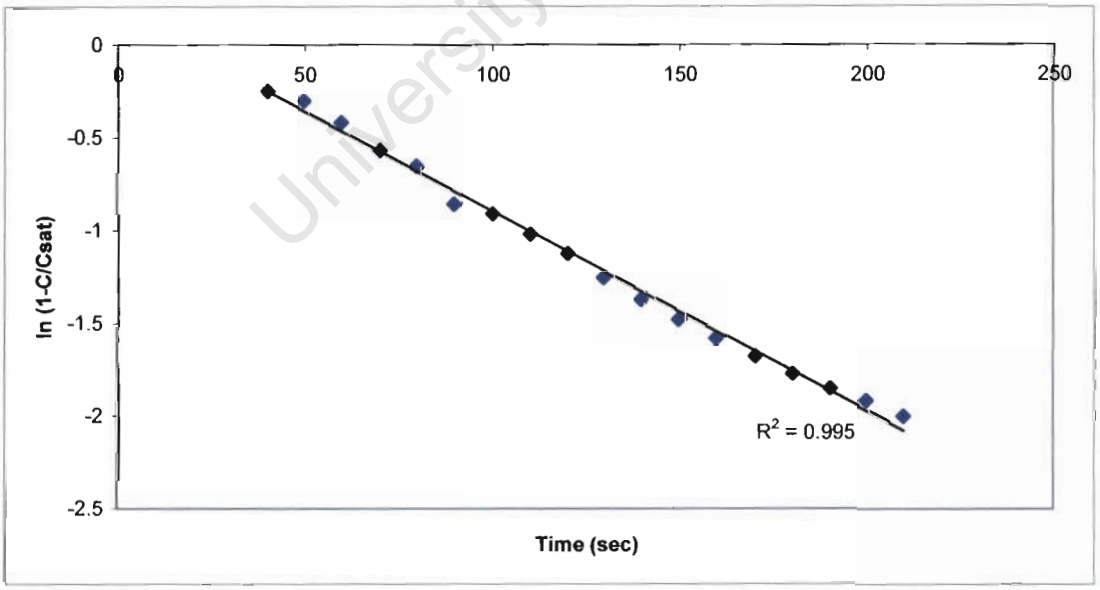


**Figure E.2:** Determination of $k_La$ in the shake flask using the gassing-out technique

## E.2. Stirred tank reactor (STR)

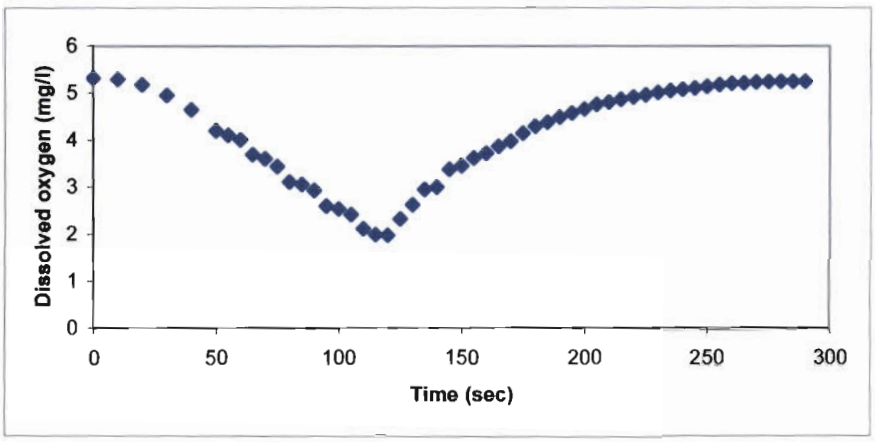


**Figure E.3:** Typical dissolved oxygen profile in the STR for volumetric oxygen transfer measurement

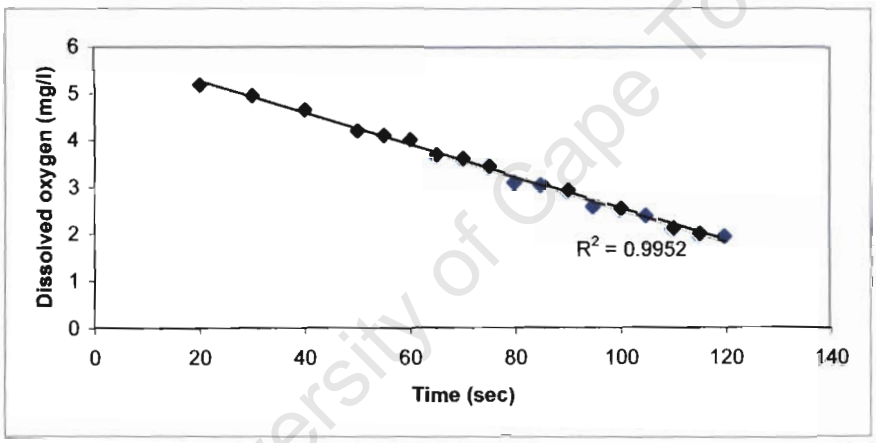


**Figure E.4:** Determination of OTR in the STR using the dynamic method

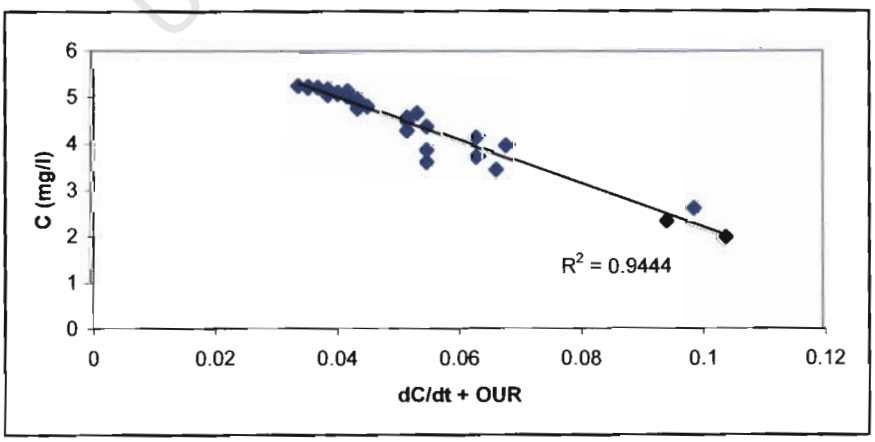


**Figure E.5:** Determination of $k_La$ in the STR using the dynamic method

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