Development of a bioprocess technology for the production of *Vibrio midae*, a probiotic for use in abalone aquaculture

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Submitted in complete fulfilment for Degree of Doctor of Philosophy (Biotechnology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban South Africa

*** SUBMISSION APPROVED FOR EXAMINATION**

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REFERENCE DECLARATION

I, **Ms Ghaneshree Moonsamy** – **20426849** and **Professor Suren Singh** (supervisor) and **Dr Rajesh Lalloo** (co-supervisor) do hereby declare that in respect of the following dissertation:

Title: **Development of a bioprocess technology for the production of *Vibrio midae*, a probiotic for use in abalone aquaculture**

1. As far as we ascertain:
   (a) no other similar dissertation exists;
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Signature of Co-supervisor Date

AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Professor Suren Singh** and **Dr Rajesh Lalloo**.

Signature of Student

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ABSTRACT

The abalone industry of South Africa is under severe pressure due to illegal harvesting and poaching of this seafood delicacy. These abalones are harvested excessively; as a result, these animals do not have a chance to replace themselves in their habitats, ensuing in a drastic decrease in natural stocks of abalone. Abalone, has an extremely slow growth rate, and takes approximately four years to reach a size that is market acceptable, therefore, it was imperative to investigate methods to boost the overall growth rate and immunity of the animal. The University of Cape Town (UCT) began research, which resulted in the isolation of two microorganisms, a yeast isolate *Debaryomyces hansenii* and a bacterial isolate *Vibrio midae*, from the gut of the abalone and characterised them for their probiotic abilities. This work resulted in an internationally competitive concept technology that was patented. The next stage of research was to develop a suitable bioprocess to enable commercial production. Numerous steps were taken to develop an efficient production process for *V. midae*, one of the isolates found by UCT. The initial stages of research resulted in the development of a stable and validated cell bank which allowed the development of a robust inoculum stage. This was followed by optimization of temperature and pH which resulted in improved probiotic production at a temperature of 30^{o}C and a pH of 6.5. Once these critical growth parameters were established further media optimization studies were performed. The two key nutrient supplements investigated were corn steep liquor (CSL) and High Test Molasses (HTM) due to their suitability, availability and affordability. The optimization of CSL (6.4 g.l^{-1}) and HTM (24 g.l^{-1}) concentrations in the growth medium resulted in a 180% increase in cell concentration,

a 5716-fold increase in cell productivity and a 97.2% decrease in the material cost of production when compared to the base case technology. Furthermore, a stable market ready liquid probiotic product, containing viable but not culturable (VBNC) state of *Vibrio midae* cells, was developed during the downstream processing aspect of the study. Finally, the validation of this production technology at full manufacturing scale was demonstrated which further enhances the attractiveness and commercial feasibility of this probiotic production process.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Abalone is one of the most valuable seafood species in the world, whereby demand far exceeds supply, especially in Asia (Gordon and Cook, 2004; Stanford, 2004; Reddy-Lopata *et al*., 2006). The destination of majority of the globally produced abalone is China, were abalone are used primarily as a celebration dish, especially during the Chinese New Year in February. Abalone (family *Haliotidae*) belongs to a class of marine vetigastropod molluscs, which are distributed along rocky shores and reefs of coastal temperate and tropical waters (Degnan *et al*., 2006). The abalone family consists of about 56 species all belonging to the genus *Haliotis* (Geiger, 1999; Geiger and Poppe, 2000). Many members of this family (Table 1.1) have achieved commercial status as fishery and/or aquaculture species, and are of major economic importance.

The best response towards health issues in an aquaculture system has always been the application of antimicrobial drugs. However, the use of such treatments has led to the development of antibiotic resistant bacteria (Schwarz *et al*., 2001), which results in a decreased efficiency of antibiotics. Other than the outbreak of diseases, the abalone industry is also faced with major challenges such as extremely slow growth rate and high mortality rate in culture systems (Naidoo *et al.,* 2006).

Table 1.1 Globally farmed abalone species and their location

SPECIES NAME	COMMON NAME	LOCATION	TYPE OF FISHERY
Haliotis rufescens	Red abalone	N. America	Farmed/ Recreational
Haliotis rufescens	Red abalone	Chile	Farmed
Haliotis cracherodii	Black abalone	N. America	Farmed
Haliotis fulgens	Green abalone	N. America	Wild/Farmed (Mexico)
Haliotis corrugata	Pink abalone	N. America	Wild/Farmed (Mexico)
Haliotis kamtschatkana	Pinto abalone	N. America	Farmed
Haliotis midae	Perlemoen	South Africa	Wild/Farmed
Haliotis laevigata	Green-lip abalone	S. Australia	Wild
Haliotis rubra	Black-lip abalone	S. Australia	Wild/Farmed
Haliotis roei	Roe's abalone	Australia	Wild
Haliotis iris	Black footed paua	New Zealand	Wild
Haliotis diversicolor supertexta	Small abalone	Taiwan	Wild/Farmed
Haliotis discus hannai	Disk abalone	Japan, China	Wild/Farmed

However, aquaculture faces major challenges, which include the outbreak of diseases, waste accumulation and deterioration of environmental conditions (Balcazar *et al.*, 2006; Macey and Coyne, 2006). Disease occurrence is usually associated with primary invasion by pathogenic strains as well as mechanical injury coupled to stressful environmental conditions viz., physiochemical changes and poor water quality (Jeney and Jeney, 1995). This predicament has become one of the main barriers towards the successful development in the aquaculture industry, given that it limits the production of aquaculture products in terms of quality, quantity, and regularity (Macey and Coyne, 2005).

Recent developments surrounding the use of beneficial bacteria such as probiotics, to displace pathogenic bacteria, has been gaining attention. This method provides a more appropriate treatment than the use of antimicrobial drugs (Moriarty, 1999). Probiotics offers the aqua-culturist various advantages, but they need to have the ability to colonize and persist in the gastrointestinal tract of the host for a suitable period, in order to confer these benefits (Conway, 1996). However, in cases where probiotic organisms do not persist for long periods, they should be applied at higher dosages more frequently such that a desired effect is exerted (Verschuere *et al.*, 2000; Macey and Coyne, 2006).

The focus of this research is based on previous studies conducted by Macey and Coyne (2005). Their findings indicated that important microbial strains such as *Vibrio midae* and *Debaryomyces hansenii*, amongst others resided in the gastrointestinal tract of the South African abalone (*Haliotis midae*). These strains when used either singularly or in consortium have the ability to improve the health and growth rate of the South African abalone by enhancing the immune system and increasing digestibility (Macey and Coyne, 2005).

The intention of this technology is to deliver a viable and robust probiotic product that will be incorporated into abalone feed. To ensure that the product offers the desired effect to the intended host, an increased concentration of viable cells must be included into the feed mix, as viability can be severely compromised during the feed production process (Burr and Gatlin, 2005). Increased cell numbers and yield of probiotics can be achieved by the optimization of media and key physiochemical factors such as pH and

temperature during the production process (van Hoek *et al.*, 1998; Riesenberg and Guthke 1999).

In this study, a number of stages were researched in an attempt to develop a suitable and robust production process for *V. midae*. This organism is a gram-negative rod, approximately 0.7µm in diameter and 2µm in length with 1 to 3 polar flagella of approximately 6.6µm in length (Coyne, 2009). It is non-pigmenting, motile, oxidase and catalase positive. It is also non-luminous and has the ability to swarm on marine agar plates (Coyne, 2009). Each stage of research undertaken in this study (Figure 1.1) involved critical assessment and development to result in an efficient bioprocess technology for production of *V. midae*.

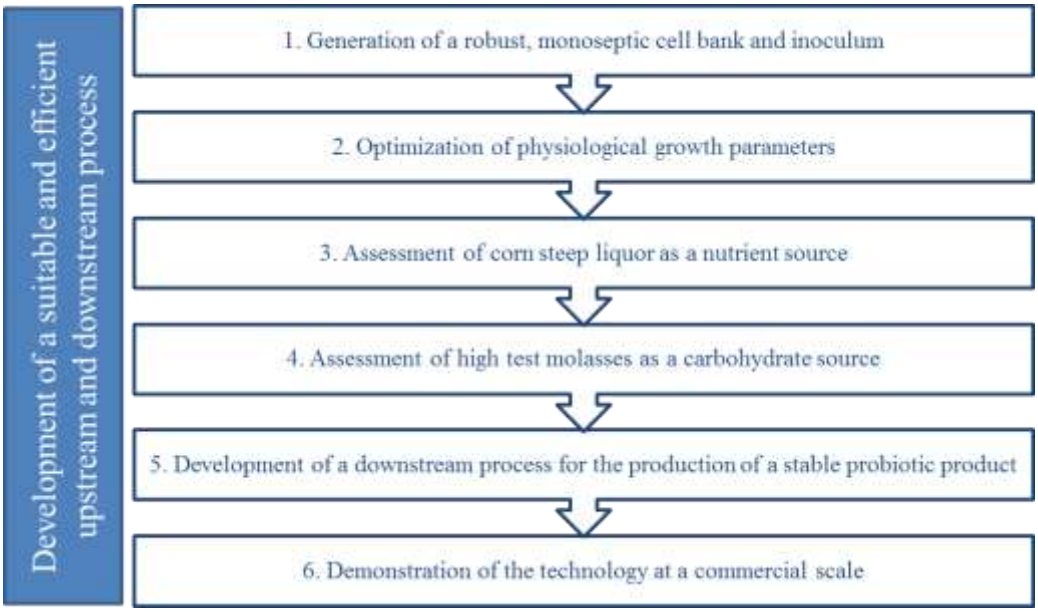

Figure 1.1. Research approach used for the development of a production process for an abalone probiotic (*V. midae).*

In general bioprocess development identifies areas to improve process performance in both upstream (USP) and downstream (DSP) process steps. The first stage of USP development involved the generation of a monoseptic, viable and robust inoculum containing *V. midae,* which is outlined in chapter 2. The inoculum affects the production process in terms of productivity, profitability and process control (Okonkowski *et al.,* 2005). An important step in the preparation of a suitable inoculum was the generation of a viable, monoseptic cell bank. A cell bank should contain a homogenous, pure culture of selected microorganism, which is stored under controlled conditions that ensure viability and genetic stability of the organism (Meza *et al*., 2004). Once a suitable method for cell bank storage was identified, the resultant cell bank was assessed for viability, purity, process costs, amount of culture, and the frequency of use.

Subsequent to the generation of a monoseptic, reproducible inoculum, the next stage in upstream process development involves the optimization of medium components, physiological culture conditions as well as mode of cultivation. This study involved the investigation of the effect of physiological parameters (viz., temperature and pH) on the growth of the *V. midae,* as highlighted in chapter 3. Temperature has a significant effect and is a key variable that influences the growth rate of microorganisms (Ratkowsky *et al.,* 1982; Kurita and Yamazaki, 2002; Bonaïti *et al*., 2004). Furthermore, temperature is the primary factor that determines the metabolic rate of microorganisms (Britz, 1996). In addition to the influence of temperature on growth rate, it also affects the growth yield of a culture because the relative energy requirements for maintenance increase when growth rate of the microorganism slows

down (Knoblauch and Jorgensen, 1999). In this study, the effect of pH on the growth of *V. midae* was also assessed. Microorganisms have a remarkable ability to maintain the intracellular pH at a constant level, even with the occurrence of large variations of pH in the extracellular medium. This occurs at the expense of a significant increase in cell maintenance demand. It has been observed that in some cells, the plasma membrane has the ability to regulate internal pH of a microbial cell, independent of the external medium pH (Pirt, 1975).

Once these key physiological parameters were determined, the next stage of process development involved the formulation of a commercially feasible production medium, which is elaborated on in chapter 4 and 5. The suitability of corn steep liquor (CSL) and high-test molasses (HTM) as more commercially feasible nutrient and carbohydrate substrates were assessed and comparative assessments were made to conventionally used medium components, such as yeast extracts, polypeptones and glucose.

Upon completion of upstream process development, research was focussed on the DSP aspects, which included the separation of the produced biomass from the culture broth and thereafter developing a suitable stabilisation and formulation strategy of the probiotic product, which is captured in chapter 6 and 7.

Subsequent to the completion of the downstream processing aspect of this study, the resultant technology was demonstrated at production scale. The findings of this study are described in chapter 8. The outcome of this study was the development of an

efficient and robust bioprocess technology, with enhanced growth rates, cell productivities, and a stable final product with a reduced cost of production.

1.2 AIM AND OBJECTIVES OF THE STUDY

To address the technology challenges the aim of this study was to develop a suitable and robust fermentation upstream and downstream process for the efficient and cost-effective production of *Vibrio midae,* a probiotic for use in abalone aquaculture.

The following objectives were developed to answer the research questions:

i. To effectively cryopreserve *V. midae* and develop a suitable inoculum
ii. To optimize key growth variables viz., temperature and pH, for the production of *V. midae* using 2 L scale fermentation studies
iii. To investigate the effect of varying nutrient sources on the production of *V. midae* in 10 L fermentation studies
iv. To evaluate different carbohydrate sources and optimize for carbohydrate concentration for production of *V. midae* in 10 L fermentation studies
v. To develop a suitable *V. midae* cell harvesting technology
vi. To formulate *V. midae* into a stable, commercially feasible abalone probiotic product
vii. To demonstrate and validate the upstream and downstream process unit operations at full manufacturing scale

1.3 LITERATURE REVIEW

1.3.1 The impact of abalone mariculture on developing economies

Aquatic animals are nutritionally important for human consumption, as they are an excellent source of proteins, trace elements, and polyunsaturated fatty acids (Marques *et al*., 2006). There has been a significant increase in the demand for an array of both fish and shellfish products as a result of growth in the global population (Subasinghe, 2005). Fisheries itself, cannot provide sufficient amounts of aquatic products to fulfil the demands of the consumer; therefore, aquaculture provides a crucial alternative resource (Marques *et al*., 2006; Britz, 2007). Aquaculture has become more significant and intensive over the last few decades and is presently the fastest growing food production industry. An average industrial growth of more than 6% in the period between 1985 and 2005 has been reported, with an annual increase of approximately 3.2% per annum during the period up to 2009 (Marques *et al*., 2006, FAO, 2012).

Modern aquaculture involves the intensive production of finfish, crustaceans, molluscs, and algal plants under controlled conditions (Olafsen, 2001; Edwards, 2015). Aquaculture yields far exceeds that of natural fishing, and provides an effective means for a constant, year round supply of good quality seafood and seafood products (Gordon and Cook, 2004; Stanford, 2004; Reddy-Lopata *et al*., 2006). The practice of aquaculture not only provides local food security, but also improves the livelihoods of people in many poorly developed coastal regions (Subasinghe, 2005).

Commercial abalone mariculture has become a thriving, global industry. It has a promising future due to the high prices being paid for abalone, coupled to a worldwide decline in fisheries production because of overfishing and poaching (Reddy-Lopata *et al*., 2006; Troell *et al*., 2006). Abalone is one of the most valuable seafood species in the world, whereby demand far exceeds supply, especially in Asian countries such as; Hong Kong, China, Japan, Taiwan and Singapore which are major destination markets (Gordon and Cook, 2004; Stanford, 2004; Reddy-Lopata *et al*., 2006, Aquaculture Association of South Africa, 2013). Abalone is used primarily as a celebratory dish, especially during weddings and other special occasions such as the Chinese New Year (Raemaekers and Britz, 2009). On account of the ever-growing demand of live, dried and canned abalone, current prices (R500 per kg) of this seafood delicacy is expected to escalate (DAFF, 2018). In 2007, it was reported that abalone was supplied to export markets in the following product forms; dried (7%), frozen (24%), live (18%) and canned (51%). Live abalone achieves higher revenues however, it does deem problematic in terms of transportation and related logistics (WCADI, 2012). Due to the demand of this prestigious seafood, supply of abalone is under severe pressure; and has led to the increase in the occurrence of abalone farming facilities around the world.

Cultivation of abalone is widespread in many countries, including USA, Mexico, South Africa, Australia, New Zealand, Japan, Taiwan, China, Ireland, Chile and Iceland (Oakes and Ponte, 1996; Gordon and Cook, 2004; Flores-Aguilar *et al*., 2007; PAUA 2009). In 2006; Troell *et al*., highlighted China as the world's largest abalone producer over 300 farms and a total production of approximately 4500 metric tonnes.

This figure increased to 56 000 metric tons in 2010, and 90 694 metric tons by 2014 (Cook, 2014). Although it is the highest producer of farmed and live product, it is still the major market for abalone produced worldwide (Reddy-Lopata *et al*., 2006). This occurrence is closely related to the economic growth and the increase in personal wealth of the Chinese population in particular the growth of the Chinese middle class (Britz, 2007; WCADI 2012). Global production of abalone via aquaculture is said to reach 103 464 metric tons by 2014. It has been reported that the total abalone from harvesting, illegal poaching and natural supply, does not meet demand for this seafood delicacy (Gordon and Cook, 2004; Troell *et al*., 2006).

Table 1.2. Global production of abalone from aquaculture and illegal harvest by country; (2010 - www.fishtech.com)

COUNTRY	CULTURE	ILLEGAL HARVEST
China	56 000	-
Korea	6228	-
Taiwan	171	-
South Africa	1036	2000
Japan	200	-
USA	250	200
Australia	456	2000
Chile	794	1000
New Zealand	80	
Other	150	600

1.3.2 The South African Abalone Aquaculture Industry

Abalone produced in South Africa continues to establish itself as a premium brand in Asia, and is a good example of mariculture in a developing country. Abalone farming in SA is a relatively new but dynamic industry and has demonstrated a high production capacity (WCADI, 2012). The abalone mariculture industry started developing in South Africa during the 1990's and has been gaining popularity. As a result, an economic environment whereby abalone aquaculture has become increasingly attractive, as a financial investment has been established (Oakes and Ponte 1996). Abalone rearing facilities employs an intensive system in which abalone is reared at high densities in shore-based aquaculture systems (Sales and Britz, 2001). In spite of these efforts, reports suggest that approximately 2000 tonnes are lost due to illegal poaching, damaging natural marine resources (Britz, 2007).

The South African abalone, *Haliotis midae*, locally known as "perlemoen" is the only one, of the six indigenous species, that is of commercial importance (Evans *et al*., 2004; Reddy-Lopata *et al*., 2006). The abalone *H. midae*, takes over 30 years to reach a maximum size of 200mm (shell length) in natural habitats (Sales and Britz, 2001). Even under farmed conditions, abalone growth rate is slow and often varying with size and age (Macey and Coyne, 2005). *H. midae* takes approximately 4 to 5 years to reach a marketable size of 100 mm (shell length) before it can be sold for between US$ 34 to 36 per kg on international markets (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008). Mariculture of abalone is thus important to ensure market supply and economic growth and it is for these reasons that alternate approaches involved in the

promotion of abalone growth and an increased immunity to disease of farmed abalone are needed.

Figure 1.2. Holding tanks containing farm-produced abalone on the West coast of South Africa

Land based aquaculture of abalone has increased over the last decade in South Africa (Figure 1.2), and commercially produced abalone has almost completely replaced the wild harvested product (Britz, 2007). In 2011, the output of all facets of abalone harvest totalled 1036 metric tons (Aquaculture Yearbook, 2012). The status of abalone aquaculture in South Africa between the periods of 2004 to 2006 is outlined in Table 1.3. These farms produced 890 tonnes of abalone, and created direct employment to about 840 people. There was an increase in skilled individuals of approximately 7.6% over the 2-year period. Due to the high demand for this seafood delicacy, revenues of

more than R200 million per annum were achieved (Andersson *et al*., 2006). The industry has demonstrated continued growth. In 2003/4, 19 enterprises secured permits to culture this species and by 2007, this number had increased to 24, further highlighting the growth potential of this particular sector (Shipton and Britz, 2007). It was estimated that by 2020 the production of abalone would amount 2895 tons with a value of R551 million, making abalone mariculture the leading subsector contributor in the aquaculture industry (Britz, 2007). This growth has had a direct impact on the socio-economic growth of the country, whereby more than 1200 people with necessary skills are currently employed in the industry.

Table 1.3. The status of abalone aquaculture and total investment in the South African abalone industry between 2004 - 2006 and 2011(Andersson *et al*., 2006; Aquaculture Yearbook, 2012).

Year	No. of producing farms	Investment (R-million)	Tonnes produced	Annual increase in industry (%)	No. of employees
2004	13	-	576	-	556
2005	13	197	745	27	776
2006	13	182	890	21	840
2011	14	-	1036	16	1219

Global aquaculture initiatives have shown that the success of the technology is largely dependent on government sectors for support to enable the creation of a robust and sustainable industry (WCADI, 2012). The mariculture of abalone and on-going growth of this industry is extremely important, as it addresses a number of challenges faced by the South African nation, which are also common to many developing

countries. This practice will contribute to a number of strategic imperatives including economic and enterprise development, job creation, food security as well as the adoption of sustainable mariculture practices (WCADI, 2012).

1.3.3 Challenges faced in abalone mariculture and conventionally used mitigation strategies

1.3.3.1 Challenges faced by the aquaculture industry

Many aquaculture farmers, including those in the abalone mariculture sub-sector are faced with a myriad of challenges (Moriarty, 1999; Mouton and Gummow, 2011). The challenges are further exacerbated as abalone mariculture activities become more intensified to optimise efficiencies in land usage and productivity. Adversities faced include slow growth rate, the outbreak of diseases, waste accumulation and deterioration of environmental conditions (Balcazar *et al*., 2006; Macey and Coyne, 2006). Disease occurrence is usually associated with primary invasion by pathogenic strains as well as mechanical injury coupled to stressful environmental conditions such as physiochemical changes and poor water quality (Jeney and Jeney, 1995). These factors, in an interactive way, challenge the health and immune response of the abalone and can lead to poor growth, ill health and increased mortality. This predicament has become one of the main barriers towards the successful development in the aquaculture industry, given that it limits the production of aquaculture products in terms of quality, quantity, and regularity (Macey and Coyne, 2005).

Disease control is an inherent part of any animal production system, however, in the aquatic environment, the intimate relationship between bacteria and their host, and the use of open production systems adds to this challenge (Olafsen, 2001). Unpredictable mass mortalities still occur in the early life stages as a result of the proliferation of pathogens and opportunistic microorganisms, which are responsible for major economic losses (Marques *et al*., 2006). Abalone like other aquatic species is susceptible to common marine pathogenic organisms such as *Vibrio parahaemolyticus, Vibrio anguillarum* and *Vibrio carchariae*, as well as prokaryotes and viruses (Nicolas *et al*., 2002; Macey and Coyne, 2005). When pathogenic bacteria or viruses are detected, farmers usually apply antimicrobial compounds to the feed and the rearing water (Gram *et al*., 2001). Broad-spectrum anti-microbials have been extensively used as a means of disease control on many aquaculture facilities and unfortunately remains the method of choice for many farmers (Macey and Coyne, 2005). Some farmers also use antibiotics as prophylactics in large quantities, even when pathogens are not evident. This ill-advised practice has led to an increase in *Vibrios*, and other opportunistic pathogens, which possess multiple antibiotic resistances and as a result; leads to an increase in more virulent pathogens (Moriarty, 1999; Nomoto, 2005). Plasmid-carrying resistance determinants have been transferred in-vitro from aquatic pathogens to human pathogens, such as from *V. cholerae* and *V. parahaemolyticus* to *Escherichia coli* by the horizontal spread of plasmids (Gomez *et al*., 2007). Furthermore, the presence of antibiotic residues in the tissues of animals, an imbalance of microorganisms in the gastrointestinal tract of aquatic species and the release of antibiotics into natural waters, pose further challenges. Therefore, the indiscriminate use of antibiotics confers a negative effect on the health of aquatic host

species, the environment and consumers of food products (Moriarty, 1999). Due to these concerns, the European Union has imposed more stringent regulation of antibiotic use in aquaculture (Ronson and Medina, 2002). Since the application of antibiotics is problematic, a strong demand for alternative methods of disease control is required in abalone mariculture.

1.3.3.2. Abalone diet and lifestyle requirements

Abalones are generally regarded as opportunistic herbivores that readily accept a wide range of diets. In natural ecosystems, abalone feed primarily on seaweed or kelp. This food contains a high degree of alginolytic material that is not readily digestible; as a result, enteric microflora is relied upon to effectively digest this material. If the host intestinal flora lacks the ability to produce beneficial enzymes, a very slow digestion process would result, and consequently hinder the growth of the abalone itself. The proper nutrition and resultant growth of cultured abalone are critical factors that require insight in order to successfully culture this mollusc. Appropriate mechanisms for feeding of abalone are therefore very important and it has been shown that different diets result in different growth rates (Naidoo *et al*., 2006). Growth rates, especially at the early life stages of abalone are affected considerably by the diet and the ability of the individuals to utilize available food with a high resultant feed conversion ratio (Erasmus *et al*., 1997). In abalone production systems abalones are fed either formulated diets or seaweed/kelp, and in some instances, a combination of both (Aquaculture Annual Economic Report, 2011). An optimum formulated diet should enable more efficient digestion consequently resulting in higher feed conversion ratios, and ultimately boost the growth of the abalone, but the reality is that diets are

based on raw material availability and minimum cost formulation models. This presents a challenge in digestibility, feed conversion efficiency, animal health and waste generation into the culture environment. The development of artificial feeds and specialized feeding regimes to improve the growth of abalone has assisted in developing this practice into a more cost-effective and manageable industry (Sales and Britz, 2001). It has been reported that abalone fed an artificial diet, have better canning characteristics than that of wild abalone, and canning yields have shown an increase of up to 15% (WCADI, 2012).

Incorrectly formulated diets, may also lead to the accumulation of waste in the culture system that could cause the deterioration of water quality in the culture environment. The propensity of algal blooms and the proliferation of disease-causing parasites and pathogens increases in the event of waste accumulation due to poor husbandry and poor feed digestibility. The abalone itself then becomes highly susceptible to disease due to these negative conditions in the mariculture water and succumbs to such challenging conditions. Additionally, the digestive systems of these aquatic hosts are in constant contact with the rearing water, making the host more prone to infection.

1.3.3.3 Operational challenges faced by the aquaculturist

In conventional mariculture operations, due to the high stocking densities, the generation of elevated stressful conditions in the culture environment is a frequent occurrence (Wang *et al*., 2008). During the sorting process, abalones are presented with further stresses due to excessive handling and may sustain mechanical damage. Both disease and the deterioration of the environmental conditions are the most

significant contributors to mass mortalities in mariculture operations (Bondad-Reantuso *et al*., 2005). Most operations employ land-based cultivation systems and use pump ashore technology which is energy intensive and costly (WCADI, 2012). The dilution of culture water, to reduce waste concentrations, by increasing flow rates is therefore not a feasible option. Regulatory authorities are also becoming more stringent on the poor quality of farm effluent that is returned to the sea, as a result, preservation of the surrounding environment also becomes a serious challenge to abalone farmers. Bearing in mind that these factors are interactive and ultimately; either as singular occurrences or in combination, may result in decreased production and potential negative impact on the entire aquatic system. Improving digestion, reducing the concentration of waste and disease causing agents in the surrounding water and a heightened immune response are logical mitigation considerations to address the challenges of abalone mariculture. However, classical interventions are costly and mass mortalities continue to occur, resulting in severe setbacks on both economic and social fronts. In more serious instances, some farms have had no other option but to cease operations. The abalone mariculture industry is therefore in dire need of suitable interventions that can address these challenges in an affordable and sustainable manner.

1.3.4 Biological agents as an option to address the challenges in abalone aquaculture

1.3.4.1 The use of biological agents in aquaculture

During the past two decades, the use of biological agents, particularly in feed and as water additives, as an alternative to the use of antibiotics and chemicals has shown to be promising in aquaculture, particularly in fish and shellfish larviculture (Tinh *et al*., 2007). The concept of biological agents has been traditionally associated with the use of beneficial microorganisms to restore the microbial balance in the gastro-intestinal tract of the host and to treat or prevent diseases and/or disorders (Gatesoupe, 1999). Biological agents are emerging as a significant microbial supplement in the field of prophylaxis (Gomez *et al*., 2007). Many studies to date have revealed the potential of these beneficial organisms to combat disease in an aquaculture environment (Rengpipat *et al*., 2000; Robertson *et al*., 2000; Vershuere *et al*., 2000; Olafsen, 2001; Chythanya *et al*., 2002; Bautista-Teruel *et al*., 2003; Lalloo *et al*., 2008; Jiang *et al*., 2013).

In aquatic ecosystems, there is an intimate relationship between microorganisms and other biota in the environment (Vershuere *et al*., 2000). Apart from the aquatic animal being surrounded by water, there is also a constant flow of water through the digestive tract of the aquatic animal. This consequently affects the synergistic balance of indigenous microflora associated with the cultured animal. The classical definition of a probiotic being that of microbes added to food, has become modified with respect to aquaculture. It has been applied as a wider term and is defined as "a live microbial

adjunct which has a beneficial effect on the host by modifying the host-association or ambient microbial community. This can be done 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" (Vershuere *et al*., 2000). Some studies have shown that because of intensification of aquaculture farms, a negative impact has been conferred on the composition of the different protective microbial flora interacting with the host (Olafsen, 2001). This occurrence results in an increase in susceptibility of the host to diseases. It has become evident that augmentation of aquaculture systems with biological agents can lead to growth of beneficial bacteria thus improving overall health of the culture system and the host (Olafsen, 2001).

1.3.4.2. Mode of action of biological agents

The use of biological agents in disease control and improvement of aquaculture is important as demand for environmentally friendly aquaculture practices is on the rise. Biological agents that may be applied in aquaculture comprise of isolates belonging to a wide range of yeast, bacteria and even phytoplankton species (Kesarcodi-Watson *et al*., 2008). In abalone aquaculture, potential probiotics listed to date include, *Vibrio spp*., (Sawabe *et al*., 1998; Tanaka *et al*., 2003; Macey and Coyne, 2005; Sawabe *et al*., 2007), *Debaryomyces sp*., *Cryptococcus sp*., and *Pseudoalteromonas sp*., (Macey and Coyne, 2005; Macey and Coyne, 2006; ten Doeschate and Coyne, 2008), *Lactobacillus* and *Enterococcus* sp., (Iehata *et al*., 2009); *Pediococcus* sp. Strain Ab1(Iehata *et al*., 2010), *Agarivorans albus* F1-UMA (Silva-Aciares *et al*., 2011) and *Shewanella sp* (Jiang *et al*., 2013).,

Biological agents have been found to confer beneficial effects on the host by various modes of action. These may occur as a singular or combined effect, and thus far the following have been reported; (1) the production of antimicrobial products; (2) competitive exclusion; (3) colonisation of the gut and improving microbial balance; (4) enhancement of the host immune response; (5) detoxification of harmful compounds; (6) improved growth rate of the host; (7) antiviral effects, (8) provision of nutrients and enzymatic functions; and (9) improved water quality. Further reports by ten Doeschate and Coyne, (2008) stated that the addition of probiotics to the diet of farmed abalone, could possibly lead to a boost in abalone growth by a number of potential strategies. Some of which include (1) increasing the nutrients accessible to the abalone for absorption in the gut, (2) increasing the pool of secreted digestive enzymes in the gut of abalone, and (3) use of bacterial supplements as an alternate or supplementary nutrient source.

In many instances, pathogen inhibition and/or disease control has been observed as a consequence of the release of chemical substances with bactericidal effects by probiotic bacteria (Vershuere *et al*., 2000). The production of antibiotics, bacteriocins, enzymes, hydrogen peroxide, siderophores and the altering of the pH levels due to the generation of organic acids are all traits displayed by biological agents (Vershuere *et al*., 2000; Pandiyan *et al*., 2003; Hong *et al*., 2005; Lalloo *et al*., 2010). In addition, these biological agents compete with pathogens based on intrinsic growth rate and spatial attachment. Microbial colonisation is characterised by the attachment of the biological agent to the mucosal surface and epithelial cells of the host. This prevents the proliferation of opportunistic pathogens thereby preventing infection (Ouwehand

et al., 1999). It is common knowledge that for a pathogen to be active and replicate in a host system, it requires attachment to these surfaces (Ouwehand *et al*., 1999). When probiotics are administered over a long period, they successfully colonize the gastrointestinal tract, even after cessation of feed supplemented with probiotics. This occurs since the multiplication rate of these probiotics is higher than the rate at which they are removed, thus a build-up in the intestinal mucosa of the host is observed (Ouwehand *et al*., 1999).

Host nutrition is improved as the applied probiotics secrete high levels of hydrolytic enzymes such as amylases, proteases and lipases; as well as the provision of growth factors such as fatty acids, amino acids and vitamins (Irianto and Austin, 2002). Some isolates also have the ability to break down potentially indigestible components of the feed thus reducing toxicity and improving feed conversion efficiency (Irianto and Austin, 2002; Macey and Coyne, 2005). Abalones are in most instances, fed a diet consisting mainly of kelp, which is a complex macroalgal polysaccharide deficient in many essential nutrients (Ghosh *et al*., 2007). It is therefore imperative that enteric bacteria in the abalone gut are present in sufficient amounts, which will adequately facilitate digestion by supplying highly effective polysaccharolytic enzymes (Macey and Coyne, 2005). Many bacteria displaying these properties have been found to exist throughout the digestive tract of *H. midae* (Erasmus *et al*., 1997; Macey and Coyne, 2005). Some findings indicated that enteric bacteria isolated from the gastrointestinal tract of abalone were capable of degrading agar, carrageenan, laminarin, and alginate. It was also shown that 70 - 90% of the enzyme activity was extracellular suggesting

that bacterial enzymes were secreted into the lumen of the gut where they were able to hydrolyse complex algal polysaccharides (Erasmus *et al*., 1997).

Related studies have indicated that *Debaryomyces hansenii* HF1 isolated from larvae of European bass (*Dicentrarchus labrax*) demonstrated high levels of amylase and trypsin; which aided in the digestion of feed (Simpson, 1994). Similar studies on a combination of 3 potential probiotic strains (*Agarivorans albus* F1-UMA, *Vibrio sp*. C21-UMA and *Vibrio sp*. F15-UMA) showed significant increases in growth of abalone over a 210 d period (Silva-Aciares *et al*., 2011). An average monthly improvement in growth of 9.58% of length and 15.94% in weight was observed in relevant test systems. Probiotic organisms persisted in the gut up to a concentration of 10^6 CFU.g^{-1} and also remained present for 16 to 19 d in juvenile and adult abalone after cessation of feeding with a probiotic-supplemented diet. Authors, Erasmus and Cook, (1997) and Tovar *et al*., (2002) reported that when probiotics were applied to a host, a higher growth rate was observed, as isolated gut bacteria produced enzymes that were able to aid in digestion thus improving the health of abalone.

Macey and Coyne, (2005), demonstrated an inaugural application of probiotics in abalone aquaculture. They reported that microbes isolated from the gastrointestinal tract of *H. midae* demonstrated an ability to improve digestion, growth and immunity of abalone. From their study, it was discovered that *D. hansenii*, *Cryptococcus sp., V. midae, and Pseudoalteromonas sp.* reside in the intestinal tract of *H. midae* and have the ability to improve the nutritional status of the abalone feed. Further research demonstrated that these probiotics were able to breakdown complex proteins and

starches, hence making the subsequent assimilation by abalone easier. Studies conducted by Macey and Coyne, (2005) indicated that abalones that had been supplemented with probiotics had a survival rate of 62% compared to 25% of untreated abalones; in challenge trials against bacterium *V. anguillarum*. They later formulated a mixture of probiotics using two yeasts and one bacterial strain (*Cryptococcus* sp. SS1, *Debaryomyces hansenii* AY1 and *Vibrio midae* SY9) respectively for abalone. The probiotic cocktail was added to dry feed to a final concentration of 1×10^7 cells.g^{-1}. The growth rate of small abalone (20 mm) improved by 8% and large abalone (60 mm) increased by 34%. In addition, increases in intestinal proteolytic and amylolytic activity were observed, in probiotic fed abalone when compared to abalone fed the standard feed devoid of probiotics (Macey and Coyne, 2006). The GRAS status of these organisms have not yet been determined and documented in literature, however all virulence and safety testing relevant to the organism has been suitably evaluated by UCT during their initial laboratory investigations.

Authors, Iehata *et al*., (2009); isolated lactic acid bacteria (LAB) from different sources and evaluated potential probiotic effects in abalones *in-vitro*, *Lactobacillus* sp. strain a3 and *Enterococcus* sp. strain s6 inhibited the growth of three abalone pathogens namely (*Listonella anguillarum, V. carchariae and V. harveyi*). Furthermore, these organisms were able to colonize the gut of *Haliotis gigantea* thus enhancing the production of volatile short chain fatty acids (VSCFA) such as acetic acid. They later showed that by supplementing commercially available abalone feed with a potential probiotic organism, *Pediococcus sp*. Ab 1, a change in host intestinal

flora was observed. In addition, higher levels of alginate lyase activity and VSCFAs were recorded. All of these factors led to a combined impact by enhancing the growth of the abalone, *H. gigantea* (Iehata *et al*., 2010).

Studies conducted by Jiang *et al*., (2013) revealed that within a week of supplementing the feed of *Haliotis discus hannai Ino* with two probiotic organisms, *Shewanella colwelliana* WA64 and *Shewanella olleyana* WA65, increases in cellular and humoral immune response, higher haemocytes, respiratory burst activity, serum lysozyme activity and total levels of protein were observed. It was therefore concluded that both strains might be used as a dietary probiotic supplement to improve innate immunity and disease resistance in abalone. These studies have shown the promise of using feed probiotics in aquaculture.

1.3.5 Rationale used for the production of probiotics and biological agents

The use of biological agents in aquaculture has gained momentum over the years. It is thus, imperative that these micro-organisms be commercially produced in order to meet market needs. Comprehensive production processes are lacking and needs to be developed and optimised for each biological agent. This can facilitate the commercial rollout of probiotic products of this nature, but is largely dependent on (1) the efficiency of the production process and (2) the ability to produce large quantities of the probiotic in a suitable form with practical shelf stability (Patel *et al*., 2004). Important criteria influencing the commercial use of biological products are cost, efficacy, shelf life and convenience to the end user (Amer and Utkhede, 2000; Keller

et al., 2001). The cultivation of microorganisms at a large scale is influenced by various factors such as the composition of the media, physical and chemical variables, substrate feed, oxygen availability and many others (Maharajh *et al*., 2008); each of which have to be optimized to ensure a cost effective production process.

1.3.5.1. The development of a suitable cultivation medium

The growth medium that is used to support high productivities in commercial bioprocesses is predominantly formulated with inexpensive nutrient sources (Zhang and Greasham, 1999). The choice of medium to be used in production is an essential aspect of process development as it influences the economic competitiveness of the bioprocess technology (Zhang and Greasham, 1999). Nutrient sources generally play a dominant role in the productivity of the production process since they supply nutrient and growth factors that are directly linked with the formation of biomass and metabolites (Lopez *et al*., 2003). It has been suggested that economical and commercially available medium options be investigated in order to reduce production costs (Lawford and Rousseau, 1997; Kona *et al*., 2001). The growth medium used can be either a defined or an undefined medium. A defined medium has known quantities of all the ingredients that constitute the formulation. An undefined medium contains complex ingredients such corn steep liquor (CSL), which consist of a mixture of chemicals in unknown quantities that vary according to supplier and production batches. The undefined medium option is usually applied in industrial processes based on its ~98% lower cost contribution (Zhang and Greasham, 1999).

1.3.5.2. The use of conventional nutrient sources in the cultivation medium

Yeast extract is a commonly used growth medium component, and has been used extensively in many production processes. It is an important nitrogen and nutrient source as it contains an array of amino acids, vitamins and other growth factors required for microbial growth (Payot *et al*, 1998; Lalloo *et al*., 2009; Salgado *et al*., 2009). Several studies have indicated that high cell yields and productivities have been obtained with the use of yeast extract in various production processes (Nohata and Kurane, 1997; Vuolanto *et al*., 2001; Prabakaran *et al*., 2007). However, the use of this nutrient source is avoided such that it does not inflate the cost of final products due to its high cost contribution (Payot *et al*., 1998). Medium development is a major technical hurdle that should be overcome in order to successfully minimize production costs (Salgado *et al*., 2009). Other nutrient sources that have been used include casamino acids and peptone, which are produced via the enzymatic digestion of meat. The use of these products results in expensive production processes; even though these have been shown to be highly effective nutrient sources. Furthermore, these nutrients sources have negative market acceptance, as they are animal by-products (Lalloo *et al*., 2009). Regulations have also exerted significant pressure on the use of these animal by-products, which have limited their availability. It is therefore imperative that cheaper, safer and readily accessible nutrient sources, capable of supporting production of biological agents, be used in order to ensure that a production process is economically attractive.

1.3.5.3. The use of alternative nutrient sources in the cultivation medium

CSL has been identified as a lower cost nutrient substrate (98% cheaper than YE) that can be used in the production of microbes, in comparison to conventional nutrient substrates such as yeast extract, peptone and casamino acids (Lalloo *et al*., 2008). CSL is produced by immersing corn into dilute sulphur dioxide during the starch-manufacturing processes and is a major by-product of the corn starch processing industry (Niwa *et al*., 2001). It has also been shown to be a supplementary source of vitamins and nitrogen to the culture medium (Amartey and Jeffries, 1994; Silveira *et al*., 2001). The use of CSL has had numerous successes in diverse industrial fermentation processes (Lawford and Rousseau, 1997) with high cell yields and productivities being major benefits (Srivastava and Baruah, 1986). Other than the assessment of a suitable nutrient source, alternative carbohydrate sources also need to be assessed as they play a dominant role in the productivity of a production process. These nutrient sources are directly linked with energy provision for the formation of biomass and metabolites (Lopez *et al*., 2003). Different microbes utilise carbohydrate sources in varying ways. Glucose is a relatively expensive carbohydrate source, and its use in large-scale process is limited as a result of relatively high production costs (Yahiro *et al*., 1997). When developing efficient bioprocesses, attempts are made to obtain economical and commercially available carbohydrate sources such that the production costs are minimised (Lee and Chang, 1995; Lawford and Rousseau, 1997; Zhang and Greasham, 1999; Kona *et al*., 2001; Burkert *et al*., 2004). High-test molasses (HTM) is a valuable carbohydrate used commercially due to its local availability and cost competitiveness. HTM has been applied extensively as an alternative carbohydrate source in various production processes (Lawford and

Rousseau, 1997; Zhang and Greasham, 1999; Kona *et al*., 2001; Burkert *et al*., 2004; Gouda *et al*., 2001; Lalloo *et al*., 2009). HTM, unlike conventional molasses, is a purer product form that enhances mass transfer in a production process due to lower amounts of ash and impurities. HTM has been used as a carbohydrate source because it consists of glucose, fructose and sucrose. Inverted HTM is also readily accessible, which contains mainly glucose and fructose in equal proportions with a small amount of residual sucrose. Other than being a carbohydrate source, HTM also provides abundant vitamins and other growth factors required for microbial growth (Malathi and Chakraborty, 1991; Lalloo *et al*., 2009).

1.3.5.4. Other cultivation medium requirements that need consideration

In some instances, microorganisms may require vitamins to be present in the cultivation medium, which can be found in the supplemented complex nutrient sources, whereas others can be cultivated in a medium devoid of vitamins (Kask *et al*., 1999). Vitamins are growth factors required by most microorganisms for the production of enzyme cofactors (Zhang and Greasham, 1999; Chan, 2003). Other than vitamins, microorganisms also require trace elements for their growth. Trace elements form part of enzymes and co-factors and they aid in the catalysis of reactions and maintenance of protein structures (Burrows, 1936; Zhang and Greasham, 1999). Supplementation of exotic trace elements and vitamins can be costly. The advantage of using cheaper nutrient sources containing these essential additives is that further supplementation is avoided when these cheaper nutrient sources are used, as they are able to satisfy the essential requirements for growth of probiotics.

Other than an influence on growth, the type of cultivation medium used in a production process also influences physical parameters such as mass transfer and the formation of foam. Growth media rich in protein usually result in increased foam formation (Morao *et al*., 1999). In addition, the sparging of gas through the growth medium and agitation at high speeds results in excess foam formation, in oxygen intensive processes. Culture medium and more importantly, biomass, can be lost in the foam phase in the event of vessel overflow. The sensitivity of microorganisms to antifoam toxicity is an important factor that must be considered during the development of production processes; as it can result in a significant decrease in process performance (Holmes *et al*., 2006).

1.3.5.5. The determination of optimum process parameters to enable cultivation

Once a suitable fermentation medium has been developed, optimization of physiological growth conditions such as temperature, pH and oxygen sufficiency are imperative, in order to successfully produce biological agents on a large scale. Temperature and pH have been reported to be amongst the most important environmental parameters which influence the activities and growth rates of many microorganisms as it governs physiological processes.

The impact of temperature has been observed at the cellular level, and can either increase or decrease the catalytic activity of pertinent metabolic and digestive enzymes (Ratkowsky *et al*., 1982; Mayo and Noike, 1995; Betts *et al*., 2000; Kurita and Yamazaki, 2002). It has been reported that the alteration of growth conditions to an unsuitable range can significantly increase the lag phase of a wide range of

microorganisms, which is highly undesirable when designing an efficient bioprocess strategy (Xiang *et al*., 2013). Since temperature affects microbial growth rate, it also affects the growth yield of a culture because the relative energy requirements for cell maintenance increases; when growth rates are reduced (Knoblauch and Jorgensen, 1999).

pH homeostasis is another important factor that needs to be considered during the growth of microorganisms (Hutkins and Nannen, 1992). For most microorganisms, there is an increase in growth rate between the minimum and the optimum pH levels and a corresponding decrease in growth rate between the optimum and the maximum pH value (Todar, 2007). It is well known that pH is important in controlling initiation of growth by microorganisms (Lichstein, 1959). The effect of pH on growth include: (1) affecting the production and activity of enzyme systems controlling growth and division, (2) altering the solubility of essential nutrients, (3) modifying the permeability of cells to substances essential for growth, (4) changing the nature of cell surfaces of envelope materials and cell morphology, and (5) modifying the composition of the cultivation medium (Mitchell 1951; Cochrane, 1958; Lichstein, 1959; Pirt 1975).

Oxygen sufficiency is an additional factor to be considered in the design of an optimum bioprocess strategy. In high-cell density cultivations, oxygen limitation can be very challenging, and prevents attainment of high cell concentrations (Fike, 2010). The method of oxygenation must be given a high degree of consideration, as excessive rates of agitation and sparging will encourage foam formation and cause excessive

shear on the microbial cells. On the contrary, inadequate aeration causes oxygen limitation, and has been reported to be highly detrimental to process productivity, in terms of growth rates and product formation as well as cell viability (Morao *et al*., 1999; Fike, 2010).

These factors have an impact on the improved yield and productivity of a process and as a result the overall cost of the production process. In addition, the physiological parameters also confer information on the functionality of the probiotic once it enters the host environment (Burkert *et al*., 2004; Lalloo *et al*., 2008). Bioprocesses are designed such that the overall process has increased cell yields, productivities and a lowered cost, which ultimately results in a feasible and economically attractive production process. It is essential that these requirements, yields and productivity in particular, are met to ensure that biological agents can be affordably adopted for use in abalone aquaculture (Anesiadis *et al*., 2008). Process yield on sugar, protein and oxygen consumed are typical indicators of and efficient bioprocess (Lalloo *et al*., 2009). The assessment of these criteria also enables the optimization of bioprocesses and permits the comparison of various production strategies.

1.3.6 Processing of probiotics into market acceptable products

Once the respective microorganism has been successfully cultivated at a large scale, the resultant fermentation broth needs to be recovered efficiently to be utilized in subsequent processing and formulations steps (Tsun *et al*., 1999; Rowe and Margaritis, 2004). The downstream process has a major influence on product

commercialization as the major constraint in most processes are embedded in harvesting and formulation costs (Brar *et al*., 2006; Prabakaran *et al*., 2007). This includes key aspects such as maximising recovery and preservation of viability, which are essential, in terms of applying an effective biological agent, especially in aquatic systems (Lacroix and Yildirim, 2007). In addition, it is also vital to ensure that the final probiotic product to be administered to the host aligns with end user requirements such as stability, consistency, easy application, efficacy and affordability (Burges and Jones, 1998; Schisler *et al*., 2004; Brar *et al*., 2006). Consequently, robust cost-effective choices of process steps and ingredients, dictated by the end product characteristics, are necessary to improve the commercial success of newly developed biological agents (Brar *et al*., 2006).

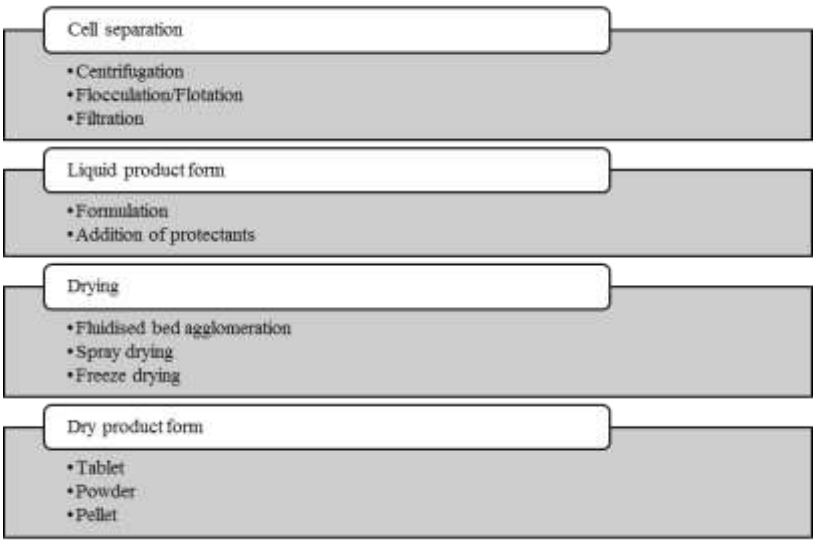

Figure 1.3. Schematic diagram of potential downstream process unit options reproduced with permission (Lalloo *et al*., 2010).

The main objective for downstream processing is to minimise the number of unit operations involved in the process, thus reducing overall capital, validation and

operating costs, while also simplifying process automation (Brar *et al*., 2006). An additional consideration is the final anticipated form of the end product, which has implications on the choice of process options and cost while still meeting customer expectations (Figure 1.3) (Lalloo *et al*., 2010). The downstream process unit operations, completes the process steps; from the upstream fermentation to the end product. It is therefore considered an extremely important prerequisite for commercialization of probiotic technologies. Regrettably, published literature regarding downstream processing and formulation for commercially available products is very limited (Schisler *et al*., 2004; Brar *et al*., 2006).

The harvesting efficacy of the cell separation unit operation governs the marketability of a product, as it affects the potency to cost ratio and influences further processing during formulation and product development. The goal of the recovery process is to produce a product of acceptable quality, in compliance with any regulatory and safety requirements, at an acceptable cost (Lydersen *et al*., 1994). Process options for cell harvesting from fermentation broth include microfiltration, sedimentation, flocculation and ultrafiltration (Figure 1.3) (Luna-Solano *et al*., 2005; Prabakaran *et al*., 2007). Flocculation and flotation using surface action or electrical charge have been reported to be inefficient in the separation of bacterial cells (Lydersen *et al*., 1994). Although there have been some positive reports for harvesting using ultra-filtration the most widely used process remains centrifugation; as it provides a viable alternative for cell harvesting resulting in high recoveries (Puziss *et al*., 1963; Zamola *et al*., 1981; Rojas *et al*., 1996; Brar *et al*., 2006; Prabakaran *et al*., 2007).

Tube centrifugation has been considered to be a useful process that can yield a lower moisture paste thus minimizing the energy required in later stage drying steps if necessary (Torres-Anjel and Hedrik, 1971). Usually, product intermediates are anticipated to be a high cell concentration paste containing the biological agent.

Subsequent to cell separation, product formulation generates a crucial link between the production and application of probiotics. This key step dictates process-ability, economy, shelf life, and efficacy as well as ease of application and provision of a product form that commands customer appeal. The use of a unique combination of the active (probiotic) and nutritional or filler ingredients will improve the administration and efficacy of the product. In the case of probiotics, the end product should seamlessly integrate into standard food production and farming activities (Berovic 1998; Brar *et al*., 2006). Formulation of biological agents can be broadly classified into either dry solids in various forms, or liquid suspensions and emulsions (Rhodes, 1993). The inclusion of additives that enhance process-ability, eco-friendliness and customer acceptance of the final product are also important considerations (Brar *et al*., 2006). Any potential impacts to the host, environment and even the end product consumer, must be thoroughly investigated (Werner *et al*., 1993).

In the case of a probiotic product, the formulation needs to encompass ingredients that aid viability and growth of the probiotic organisms in its intended application. Sugars and proteins are normally the preferred nutrients due to availability and affordability that are included as stabilizers in probiotic products. It also further provides a protective layer for the cells, preventing death and assists in the recovery of injured

cells during processing (Costa *et al*., 2001; Larena *et al*., 2003; Brar *et al*., 2006). The addition of nutrients was also shown to improve storage of a *Pseudomonas fluorescens* F113 strain (Moene-Loccoz *et al*., 1999) and a *Bacillus megaterium* (Wiwattanapatapee *et al*., 2004) for use in biocontrol applications. Appropriate formulations can facilitate easier processing and influence the stability and appeal of an end product in large-scale production (Amer and Utkhede, 2000).

Processing options for abalone biological agents, will include, both dry and liquid product forms (Figure 1.3). Due to the intended use of the selected isolates as a living cell preparation, product options with a high stability are considered most appealing. Refrigerated and frozen cultures occupy large storage volumes and demand higher storage and shipment costs in contrast to dry cultures, which are an economic and practical alternative; however, some microorganisms are highly vulnerable to death when any form of drying is carried out (Klein and Lortal, 1999; Lacroix and Yildirim, 2007; Meng *et al*., 2008).

In addition to production and formulation of user-friendly product formats through a downstream process, the stability and consistency of product intermediates and the end product itself are crucial requirements for successful commercialization (Lewis and Papavizas, 1991). A loss of bioactivity in a product, that is intended to be applied in a viable state, will definitely incur a great deal of process complications and as a result impart a direct increase in usage (Chen and Patel, 2007). In a typical production process, the lag time between process operations can vary due to process integration and scheduling during manufacture. Thus, storage conditions and the addition of

specific stabilizers may be required to prevent vegetative growth or the appearance of contamination in the probiotic product or its relevant intermediates (Soper and Ward, 1981).

The problems of stability during processing, storage and in application have stalled development of biologically based products (Tsuji *et al*., 1997). Accelerated aging studies based on the methodology of death rate plots at different temperatures to generate thermal resistance curves have been shown to be a useful technique for predicting stability (Ramaswamy and Marcotte, 2006) Temperature dependant half-life plots can be generated to predict stability of the probiotic product intermediates as well as the end product, however, the use of this approach has to date not been widely applied (Puziss *et al*., 1963; Wiwattanapatapee *et al*., 2004; Luna-Solano *et al*., 2005).

After addressing the considerations of the actual production process, success of the technology is still not a certainty. It is imperative, that in order to realise the success of using this new technology, the probiotic product must be supplied as a live cell preparation, and must be able to survive not only the feed production process, but also maintain viability in the digestive tract of the host (Gatesoupe, 1999; Vershuere *et al*., 2000). Many probiotics have been successfully applied to land-based animal production practices, however, the aquaculture industry are faced with further limitations as a result of continuous water exposure (Venkat *et al*., 2004). The method of incorporation selected must overcome challenges faced in feed production and the mariculture system itself, such as heat damage and diffusion during feeding, in order to achieve the desired effect of the probiotic technology.

There have been various methods applied to successfully administer viable probiotics to a host in aquaculture environments. These include mixing, soaking, spraying, vacuum infusion, extrusion and bathing (Venkat *et al*., 2004). Incorporation of the probiotic into the feed is usually the method of choice, except when bioremediation agents are added directly to the water. Mixing is the most commonly used method and involves the incorporation of the probiotic into the dry ingredients of the feed during the feed production process. Many researchers including Nikoskelainen *et al*., (2001); Meunpol *et al*., (2003) and Iehata *et al*., (2009), have successfully used this method; however, probiotics that are susceptible to excessive heating and drying during the feed production process do not show high rates of survival (Lacroix and Yildirim, 2007). The soaking method uses preformed feed pellets, which are soaked in a saline broth containing the probiotic organism at a desired concentration (Iehata *et al*., 2010; Keysami *et al*., 2012). Soaked pellets are then dried and stored appropriately for further use. A modified method of soaking, whereby actual fronds of macroalgal species, *Macrocystis integrifolia* were soaked in preconditioned tanks containing bacterial cells were used by Silva-Aciares *et al*., (2011). Upon aeration of these tanks, the bacteria were allowed to colonize the surface of the fronds, and were thereafter fed to test abalone. In other studies, the spraying of feeds with probiotic cells was also carried out (Garcia de la Banda *et al*., 2012). In addition, authors Gildberg and Mekkelsen, (1998), described methods of spray coating feeds with cells that were placed onto plastic trays. Lastly, the bathing option, involved the application of living cells directly into the rearing water (Gullian *et al*., 2004). All potential mechanisms of probiotic inclusion into the feed must be suitably ratified in order to maximize the

potential of the technology. The method selected, should have the ability to integrate easily into existing feed production process, and should in no way negatively impact on the host or the rearing environment. The journey taken to produce a commercially viable probiotic product is by no means forthright. It encompasses innovative process design, effective cell production and formulation technologies, as well as successful maintenance of cell viability and stability. Once all the identified challenges have been effectively overcome, the uptake of this technology and the associated boom in abalone export by means of aquaculture will be inevitable.

1.3.7 Considerations for application of biological agents in abalone aquaculture

Over the past two decades, the applicability of probiotics as solutions to various aquaculture related challenges have been widely reported. However, it is still imperative to consider the safety issues associated with the use of these probiotic products (Balcazar *et al*., 2006; Couvalin 2006; Kesarcodi-Watson, 2008). Safety is the state of being certain that a biological agent used will not have undesirable effects under defined conditions. The production system in which the cell cultivations are conducted must also maintain high levels of sterility to easily facilitate a monoseptic culture, as well as reduce any potential contamination by common food pathogenic bacteria (Lalloo *et al*., 2008).

Once the selected culture has been accurately identified and deposited into a culture repository, extensive literature searches and relevant scientific experimentation must

be carried out in order to obtain information on the biological agent of interest. As the number of isolated probiotic species increase, it is important not to assume biosafety levels and characteristics of each probiotic strain. Furthermore, it is recommended that the exact mode of action of the probiotic organisms be elucidated, in order to achieve the desirable effect when applied to the host system (Lalloo *et al*., 2010). It has been suggested that prior to incorporation of these organisms in abalone aquaculture, it is important to carefully assess the probiotics for pathogenicity, infectivity, toxicity and their resultant metabolite production for quality assurance (Ishibashi and Yamazaki 2001). These critical factors have sometimes been overlooked, and in some instances led to the failure of probiotic technologies (Wang *et al*., 2008). In many case studies, the use of LAB as probiotics have been rendered safe, however, in recent times there have been reports of disease-causing members belonging to *Lactococcus*, *Vagococcus* or *Carnobacteruim* families (Ringo and Gatesoupe, 1998). Additionally, strain testing of potential probiotics should encompass the robustness of the product against process fluctuations under farm conditions and confirmation of non-transmission of drug resistance genes or virulence plasmids (Moubarek *et al*., 2005). Another barrier preventing the worldwide adoption of this technology, relates to the absence of efficacy data, which as a result casts a shadow of doubt over any probiotic related technologies, thereby preventing its uptake by the aqua-culturists.

1.3.8 The impact and benefits of the application of biological agents in abalone aquaculture

Most aquaculture industries are leaning towards the use of probiotic technology as a solution to many of the challenges faced by the industry. The basis for the inclusion of probiotics into the farming environments include higher survival rate of juvenile and adult abalone, improved feed uptake and conversion ratios resulting in faster growth rates, improved resistance to disease and reduced contribution to water pollution (Vershuere *et al*., 2000) Using probiotics is more environmentally friendly because the effluent water is cleaner and there are significant improvements in the gut flora thus enhancing the overall immune response of the host and an increase in food assimilation (Moriarty *et al.,* 2005). However, other factors such as temperature, enzyme levels, water quality and genetic resistance may have an effect on the success of the technology in the farming facility (Balcazar *et al*., 2006).

Thus far, the uptake of the technology is slow-moving. This is due to the fact that farmers expect the probiotic technology to operate using the same basis as antibiotic treatment technology, in that they require and anticipate fast rapid results (Moriarty *et al*., 2005). However, changes in the microbial ecosystems present in the environment is a gradual one; and requires the continuous addition of beneficial microorganisms to compound the desired effect (Ringo and Gatesoupe, 1998). In addition, ineffective and costly probiotic products previously offered in aquaculture has negatively tainted the impact of this technology. Some products include *Clostridium spp*., *Pseudomonas putida* and other potential human pathogens, and others consist of cell densities that are too low to deliver any sort of benefit to the host (Moriarty *et al*., 2005).

The commercial aquaculture sector will make a notable difference in terms creating jobs and economic development in most developing countries embracing this activity. To date, South Africa has validated itself to be a key player in the abalone mariculture arena. With support from government, this industry could experience a further boom, and as a result, assist in reducing the high levels of unemployment that exists (Shipton and Britz, 2007), particularly in coastal areas that can effectively participate in aquaculture practices. Abalone industries not only include direct employment at the farm level, it also indirectly supports interlinked businesses such as the seaweed and abalone processing industries (Troell *et al*., 2006). The challenge is to ensure long-term sustainable growth of the abalone mariculture industry. The use of appropriate and safe biological agents in abalone mariculture has excellent potential to meet the new challenges of this important industry. Commercially feasible production technology for abalone probiotics is however a major gap in the value chain.

CHAPTER 2: CELL BANK AND INOCULUM DEVELOPMENT

2.1 INTRODUCTION

The inoculum for a fermentation process is prepared by cultivating microorganisms from a dormant stock culture to a state that is useful for inoculating a final production fermenter (Hunt and Stieber, 1986). In many microbial fermentation processes, the inoculum can have a substantial impact on process performance in terms of productivity, profitability, and process control (Webb and Kamat, 1993; Okonkowski *et al.,* 2005). It is imperative that the inoculum is in a perfect condition, with a consistent quality, and sufficient volume (Parton and Willis, 1990). A desirable quantity of inoculum should typically be at least 3 to 10% of the medium volume of the next process stage (Stanbury *et al*., 1995).

The purpose of culture preservation and the development of a cell bank is to ensure that organism viability is controlled and maintained in an uncontaminated state without any mutation or variations (Gherna, 1994). The success of the selected cell preservation protocol is dependent on the use of proper growth medium, suitable cryoprotectants, optimal cultivation conditions, and most importantly, the physiological state or age of the culture. Currently, there is a variety of cryopreservation protocols available; however, no universal method has proven successful for all types of microorganisms. Various factors must be considered when selecting an appropriate method of cell preservation. These include: (i) the degree of

viability required subsequent to storage, (ii) the application of the cell population, (iii) the genetic changes that may occur in the organism (particularly important when preserving genetically modified organisms), (iv) the number of samples that require preservation, (v) the frequency of culture withdrawal, and (vi) the unit cost of the particular method chosen (important in commercial operations) (Parton and Willis, 1990).

The use of liquid nitrogen is usually a method of choice for the long-term storage of cultures in a laboratory environment. However, due to the high cost factor associated with the use of liquid nitrogen, ultra-low-temperature freezers with a temperature of approximately -70^{o}C, are being successfully used as an alternative form of long-term storage (Gherna, 1994). Dimethyl sulfoxide (DMSO) and glycerol are typical penetrating cryoprotective agents, which are used to provide cells with both intra and extra cellular protection against freezing (Gherna, 1994, Yu, 2018). Glycerol is known to have anti-freeze properties, and interacts actively with water molecules which enhances the hydrogen bond network of the solvent, and prevents protein unfolding thereby reducing the lethal stress effects of cold temperatures on a bacterial cell (Gherna, 1994; Yu, 2018). Furthermore, it has been shown that DMSO can induce the formation of water pores in the membrane bilayer of cells in order to enhance the penetration of molecules thereby increasing the fluidity the cells hydrophobic core (Yu, 2018). In this study, glycerol was the cryoprotectant of choice due to affordability, availability and successful history of use in our laboratory.

When developing commercially applicable production processes, it is imperative that the isolate of choice, in this case, *V. midae*, be suitably preserved to circumvent the costs of time and effort in the event of re-isolation due to loss of viability (Parton and Willis, 1990). Due to the marine nature of the organism, there is concern surrounding the influence of storage conditions and long-term viability. This requires development of a well-researched and validated protocol for cell bank storage of the organism.

Following a stable, cryopreserved culture, inoculum development is the first phase of the fermentation process. When developing an inoculum, for experimental purposes or manufacturing, it is imperative that the inoculum performs predictably under the selected conditions (Calam, 1976). The choice of cultivation medium of the inoculum should not differ largely from the fermentation growth medium. This will result in a considerable reduction in the culture lag phase, thus substantially reducing process time (Stanbury *et al*., 1995). The growth medium used in this study was a nutrient rich culture medium adapted from a proprietary culture medium. This medium contained yeast extract, peptone and casamino acids as nutrient sources as well as salt to facilitate the growth of the marine *Vibrio*. In some instances, process inadequacies have been attributed to the presence of an unsuitable inoculum culture. These challenges can be avoided or minimized with the use of effective systems for inoculum preparation during fermentation scale up (Kirsop, 1984; Parton and Willis, 1990).

For this particular production process, the growth characteristics, monoseptic status, reproducibility and storage stability of the prepared *V. midae* cell bank were assessed in an attempt to enhance the quality and robustness of the cell bank. This cell bank

provided the starting material used for the development of a suitable, reproducible and efficient inoculum, which forms an integral component of a competitive fermentation process.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of a *V. midae* working cell bank (WCB)

The medium used to cultivate *V. midae* (LMG P-27727), contained the following components ($g.l^{-1}$): 1 $C_6H_{12}O_6$, 3 $(NH_4)_2SO_4$, 0.4 $Ca(NO_3)_2$, 0.04 $MnSO_4.7H_2O$, 0.032 $FeSO_4.7H_2O$, 1 KCl, 30 NaCl, 2.3 $MgCl_2.6H_2O$, 5 casamino acids, 5 yeast extract, 10 peptone (Biolab), 10 glucose and 2.5 $ml.l^{-1}$ H_3PO_4.

The culture medium was prepared to a volume of 700 ml in a 2 L Fernbach flask. The pH was adjusted to 6.5 with 25% $m.v^{-1}$ NH_4OH prior to sterilization (121^oC, 20 min). After sterilization and cooling to 30^oC, a cryovial of *Vibrio midae* culture (2 ml) was aseptically added to the medium. The flask was incubated at 30°C on a rotary shaker at 180 rpm (New Brunswick Scientific, New Jersey, USA). The flask was sampled on an hourly basis, and optical density readings at 660 nm were taken (Genesys 20 spectrophotometer, Spectronic, NY, USA), until an OD of 1 was reached. Thereafter, the broth culture was mixed thoroughly in a ratio of 1:1; with 50% $v.v^{-1}$ glycerol, a procedure adapted from Meza *et al*., (2004). The suspension was then aliquoted (2 ml) under aseptic conditions into sterile cryovials. The cryovials were then stored at -80 °C in Nalgene Mr Frosty ® boxes for controlled cooling for 48 h and thereafter transferred into appropriately labelled cryo-boxes.

2.2.3 Evaluation of cell bank stability

Three cryovials were randomly selected from the cell bank and viable cell counts were performed monthly over a seven-month period. The contents of the cryovials were serially diluted and spread-plated onto MA plates (described in section 2.2.2). The plates were incubated at 30ºC overnight and colony forming units were counted. The shelf life of the cell bank was determined using the procedures outlined by Heldman (2003), using actual data obtained during the course of the investigation. A half-life of a population represents the time (number of days) it would take for a known population to halve, in terms of viable cell number. The half-life was calculated by plotting the log value of viable counts obtained against a function of time. The resultant slope and intercept of this plot, were then used to extrapolate the half-life of the cell bank. The half-life was used to calculate the shelf life of the cell bank to the point where a minimum threshold of 1×10^6 CFU.ml^{-1} was reached.

$$\textbf{Half-life} = ((\text{Log}_{10}(\text{Viability} \times 10^{\text{intercept}})) - \text{Intercept} \div \text{Slope}$$

Equation 2.1

2.2.2 Assessment of monoseptic status, reproducibility and viability of the *V. midae* cell bank

Once the cell bank was prepared, cryovials constituting 5% of the cell bank were randomly selected and assessed for monoseptic status (n = 5). Cultures were viewed at 1000 × magnification using light microscopy (Olympus, Japan) and assessed for the presence of contaminating microorganisms. In addition, a four-way streak of the culture was performed onto marine agar (MA) plates, incubated overnight at 30ºC and thereafter assessed for purity. The *V. midae* culturable cell concentration was also

determined. Samples (100 µL) were serially diluted and spread-plated on marine agar plates (MA) containing ($g.l^{-1}$): 30 NaCl, 2.3 $MgCl_2.6H_2O$, 0.3 KCl, 2 glucose, 5 casamino acid, 1 yeast extract and 12 bacteriological agar. Plates were incubated overnight at 30°C, and resultant colonies were counted and expressed as the number of colony forming units per ml ($CFU.ml^{-1}$). The monoseptic culture was sent to the Department of Molecular and Cell Biology at the University of Cape Town to verify the identity of the cell banked culture using 16S RNA sequencing.

2.2.4 Evaluation of the growth characteristics of the *V. midae* inoculum

Triplicate flasks containing 700 ml of growth medium as described in section 2.2.1 were each inoculated with a cryovial containing 2 ml volume of culture, and were used for the determination of growth characteristics, such as maximum growth rate, doubling time and optimum harvest time. The flasks were incubated at 30°C on a rotary platform shaker set at 180 rpm (New Brunswick Scientific, New Jersey, USA). Flasks were sampled (1 ml) every h until stationary phase was reached. This was determined using OD measurements recorded at 660nm using a spectrophotometer (Genesys^{20}, Spectronic, NY, USA). Growth rates (µ) were calculated by determining the slope of time plots of *ln* OD conforming to linearity ($R^2 > 0.9$). Doubling time, which determines the rate at which the population doubles, was determined by the equation below:

$$\textbf{Doubling time} = ln\,(2) \div \mu$$

Equation 2.2

In addition, the reproducibility of the viable cell concentration of the cell bank was also performed by serially diluting the flask culture in a sterile diluent, and spread-plating 100 µl of sample onto MA plates. Plates were incubated at 30^{o}C overnight and colony forming units were counted and expressed as CFU.ml^{-1}.

2.3. RESULTS AND DISCUSSION

2.3.1. Cell bank production

A working cell bank (WCB) containing 100 cryovials was successfully prepared and stored. A viable cell concentration of $2.56 \times 10^{9} \pm 8.07 \times 10^{7}$ CFU.ml^{-1} was obtained (n = 5; CV = 3.15%) when randomly selected cryovials were enumerated for cell number. The CV of below 10% confirmed the uniformity of the cell bank. This indicates that our procedure, adapted from Meza *et al*. (2004), was successful in producing a working cell bank that was consistent in terms of viable cell number. From our experience in laboratory and industrial scale, process development a random sampling of 5% of the entire cell bank was sufficient to prove that the cell bank was consistent in terms of cell number.

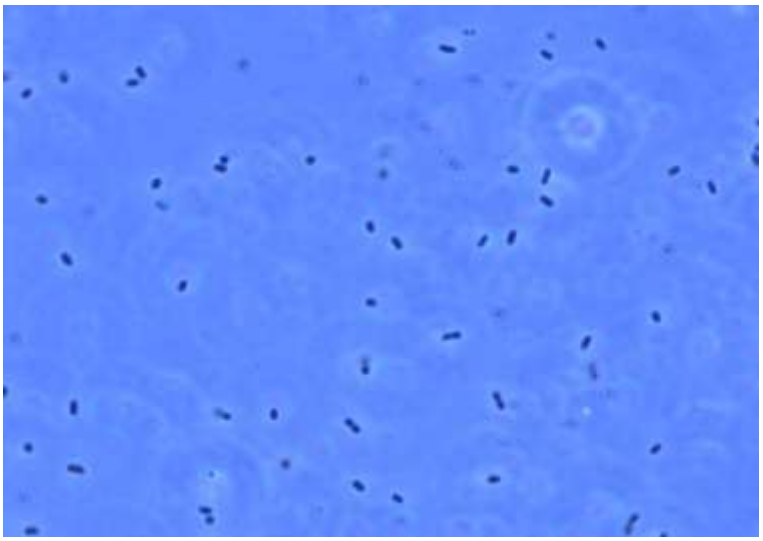

Figure 2.1. Monoseptic culture of *V. midae* viewed at 1000× magnification using light microscopy.

Randomly selected cryovials from the newly prepared cell bank revealed a monoseptic culture of *V. midae*, which was free from contamination, based on visual examination using light microscopy (Figure 2.1). Although not an absolute methodology for monoseptic assessment, the size and nature of the cell morphology was consistent. No atypical morphology was visible, indicating a high probability that the cell bank culture was monoseptic.

Cell culture plates (MA) displayed off-white colonies of identical morphology after overnight incubation at 30^{o}C, thereafter confirmed the monoseptic status of the culture (Figure 2.2). Colonies appeared non-luminous and showed the ability to swarm when grown on MA plates which is typical of the *V. midae* strain of interest (Figure 2.2). No disparity in colony morphology was visible on the test plates.

Figure 2.2. *V. midae* colonies appeared on MA plates after overnight incubation at 30^{o}C.

Furthermore, our research partner at UCT, provided independent confirmation on the identity of our cryovials sent to them as monoseptic cultures of *V. midae* using 16S RNA sequencing. These researchers had previously conducted extensive studies on the isolation, characterisation and identification of the strain using techniques described in Macey and Coyne (2005, 2006). Their results confirmed that our cell bank culture was indeed the strain isolated from the gastrointestinal tract of the South African abalone, *H. midae* during their research.

Cell bank stability was investigated by performing viable cell counts over a nine-month period. Results are shown in Figure 2.3a. During the first 60 days of storage at -80^{o}C, there was a 1.68-fold increase in the *V. midae* viable cell population. Based on our observations, this increase in *V. midae* cell population during the initial storage period was typical of our organism in several shelf life assessments at various

temperatures, and in various product formulations. During this period, the *V. midae* culture possibly increased in number by utilising the residual nutrients from the culture flask for cell replication, prior to the culture reaching the desired storage temperature. After 60 days of storage at -80ºC, a gradual decrease in viability (7.19%) was observed up to 120 days and this was followed by a more drastic decline up to 180 days (31.00%). At 240 days, the viable cell concentration was approximately $2.4 \times 10^9 \pm 2.19 \times 10^8$ CFU.ml^{-1}. Consequently, the resultant data obtained conformed to a linear model with an associated regression coefficient of 0.94.

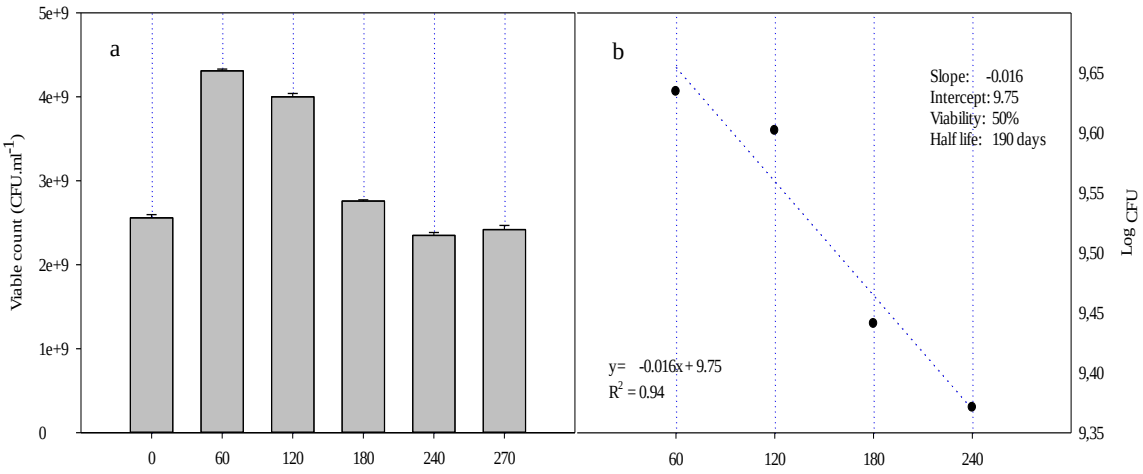

Figure 2.3. Viable cell counts (a) were performed on a monthly basis to assess the storage life of the cell bank. The viable counts obtained from the 60 to 240-day period; were then used to calculate the half-life of the *V. midae* culture (*n*= 3) (b).

The viable cell concentration (CFU.ml^{-1}) was used to calculate the half-life of the cell bank, which was determined to be 190 days (Figure 2.3b) using the curve regression from 60 d to 240 days. It was extrapolated that the resultant cell bank would retain its useable viability for a period of approximately 5.72 years using a minimum threshold viable cell count of 1×10^6 CFU.ml^{-1}. Meza *et al*., (2004) confirmed that if the viable

cell concentration drops below this minimum threshold, an excessive culture lag and poor growth rate would be observed.

Due to the intention of using this *V. midae* organism in an industrial production process, it was vital to produce a monoseptic inoculum containing cells that are suitably preserved to retain viability for as long as possible (Meza *et al*., 2004). The shelf life assessment of the cell bank is an important component of process development because it is imperative that the integrity of the cell bank is known, and that the inherent characteristics and viability maintained. If a deterioration of these factors were apparent, it would negatively influence research quality and the process performance in the commercial production. Similar to our strategy, others have also shown that freezing cultures in combination with a suitable cryoprotectant at -80°C remains the most common method of preserving cultures over a prolonged period of time (Cody *et al*., 2008), as it maintains the culture in its conserved state. The use of glycerol as a cryoprotectant has been widely demonstrated for bacteria, animal and human cell preservation (Proom and Hemmons, 1949; Baker, 1998; Valeri *et al*., 2000), and has been implemented as a cryoprotective agent from as early as 1913 (Cody *et al*., 2008). Glycerol has been found to protect the cells by conferring antifreeze properties, as it lowers the freezing point of the cell culture (Hollander and Nell, 1954). This method of *V. midae* cell storage provided an effective source of starting material for subsequent *V. midae* cultivation studies, as there was minimal variation observed and no contaminants present in the individually prepared cryovials.

A good quality cell bank forms the essential starting material for the production of pure bacterial cultures (Saxelin *et al*., 1999) and it is therefore vital that the inherent characteristics of the organism are preserved. This cell bank with confirmed viability and purity was used for all subsequent research in the development of the production process for *V. midae*. Although the inherent characteristics and viability of the organism were preserved using this protocol, this is not indefinite, and after a certain period, degradation may occur. Upon continuous use and subsequent depletion of the cell bank, a new working cell bank will be produced according to methods outlined in section 2.2.1.

2.3.2. Inoculum stage development

Cultivation of *V. midae* in replicate flasks showed that there was minimal variation in growth rate between the individual flasks ($n = 3$; $p > 0.05$; CV < 5%), thus confirming the reproducibility of the starting material and the growth of the inoculum stage (Figure 2.4). This finding is an important process development consideration, as variations in the inoculum stage often lead to variable research results and batch to batch variation in the production process. Typical examples include production delays due to growth lag in the fermentation process or a complete collapse of the production batch due to a weak inoculum (Stanbury *et al*., 1995; Monaghan *et al*., 1999; Lachhab *et al*., 2001). If the characteristics of the inoculum changes, the impact of the change has knock-on effects on all the subsequent process stages.

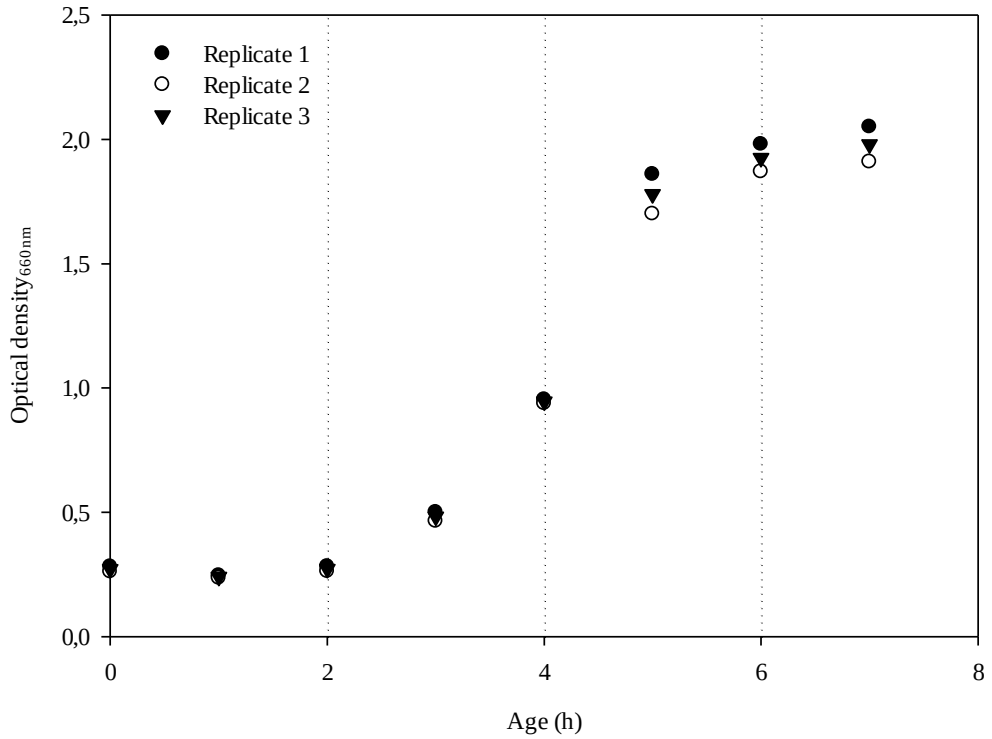

Figure 2.4. Growth of replicate flask cultures of *V. midae* in 2L Fernbach flasks containing 700ml of culture medium, grown at 30°C on an orbital platform shaker at 180 rpm.

Upon assessment of the growth characteristics of the inoculum stage, a maximum growth rate of $0.63h^{-1}$ calculated to the maximum OD_{660nm} point was observed, with a corresponding doubling time of 66 minutes. The time at which the resultant inoculum was ready for transfer to the next process unit operation was determined to be 5 h, with an optical density of 1.98 (Figure 2.4) and a viable cell concentration of $2.12 \times 10^9 \pm 2.19 \times 10^8$ CFU.ml^{-1}. For production process development, it is common practice to transfer the culture from one stage to the next whilst the cells are growing exponentially, preferably in the mid to late logarithmic stage of growth (Speck and Cowman, 1970). For the sake of convenience, this is usually done at a fixed time

after inoculation (Webb and Kamat, 1993). In this study, the inoculum transfer time was determined to be 5 hours. Webb and Kamat, (1993) and Saxelin et al., (1999) reported that if variables such as medium formulation, medium sterilization and culture growth conditions are standardized during inoculum development, there is a reduced possibility of variation in subsequent stages of the process, have documented it. If these factors are kept constant, the reproducibility of the inoculum is ensured, and as a result, a reproducible bioprocess is achieved. Our study has demonstrated that our inoculum stage conforms to these key criteria.

The results of the study demonstrate that a monoseptic and consistent cell bank and inoculum stage was developed. This externally verified *V. midae* cell bank, when used, as a starting material in the production process, was monoseptic, reproducible and stable for up to a period of 5.5 years. This cell bank provided a consistent and reliable starting material to produce an inoculum culture that would ultimately be transferred into a laboratory scale bioreactor. This stage of process development provides the crucial starting material to enable quality research and development of the production process for this abalone probiotic of commercial interest.

CHAPTER 3: TEMPERATURE & pH OPTIMIZATION FOR PRODUCTION OF *V. midae*

3.1 INTRODUCTION

V. midae is a bacterium that was isolated by Macey and Coyne (2005) and was shown to display probiotic characteristics. It was demonstrated that this probiotic enabled the breakdown of complex proteins and starches by producing high levels of relevant digestive enzymes such as alginase, protease and amylase; hence improving the digestibility of abalone feed. Field trial data revealed that with the use of this probiotic, an improved survival and growth rate of abalone was observed, thus improving productivity and yield of farmed abalone (Macey and Coyne, 2005; Macey and Coyne, 2006). This probiotic has significant potential to address the challenges of the abalone mariculture industry, however the technology to produce this probiotic commercially is not available.

Due to the intended application of *V. midae* as an abalone feed probiotic, it is important to produce a culture that has a high cell number and retains high levels of cell viability to ensure commercial feasibility. The viability and cell concentration of probiotics can be affected during the actual application of probiotics into the abalone feed (Burr and Gatlin, 2005). Therefore, to ensure that the maximum activity of the probiotics is maintained during their application, the probiotics must be incorporated at high viable cell numbers into feed.

Literature based on the commercial production technology of *V. midae* is extremely limited. It is therefore important to develop a commercially feasible high cell density cultivation process of this marine probiotic organism. Physical and chemical variables such as pH, temperature, substrate feed, oxygen availability and others, require development in order to optimize productivity and reduce cost. These factors are critical considerations in order to produce this marine microorganism in a commercially viable bio-production process (van Hoek *et al*., 1998; Riesenberg and Guthke, 1999; Maharajh *et al*., 2008). The physiological parameters are primary factors that determine organism metabolic growth rate, which ultimately impacts on the productivity and yield of a bacterial culture (Ratkowsky *et al*., 1982; Knoblauch and Jorgensen, 1999; Kurita and Yamazaki 2002; Bonaiti *et al*., 2004).

During the development of any microbial production process, strong emphasis is placed on increasing the rate of production of cells (cell productivity), as this is a key factor that influences process performance. Both temperature and pH are known to impact productivity of the process. The impact of temperature has been observed at the cellular level, and has been found to either increase or decrease the catalytic activity of metabolic and digestive enzymes (Ratkowsky *et al*., 1982; Mayo and Noike, 1995; Betts *et al*., 2000; Kurita and Yamazaki, 2002). Another important consideration is the pH of the culture environment, as pH homeostasis is also required during the growth of microorganisms (Hutkins and Nannen, 1992). The main objective of assessing these parameters is to maximize volumetric productivity ($g.l^{-1}.h^{-1}$) as this has a major influence on production costs (Riesenberg and Guthke, 1999) and is a key parameter

to ensure affordable use of such a probiotic in mariculture of abalone (Maharajh *et al*., 2008; Riesenberg and Guthke, 1999; Todar, 2007). Therefore, the aim of this research was focussed on evaluating the effect of temperature and pH on the growth of *V. midae*, and determining the optimum temperature and pH for production of this particular isolate.

3.2 MATERIALS AND METHODS

3.2.1 Microorganism and inoculum preparation

A cryopreserved culture of *V. midae* (LMG P-27727), containing approximately 2×10^9 CFU.ml^{-1}, was used as an inoculum source for all bioreactor cultivations conducted in this study. The cryo-preserved culture was prepared as outlined in chapter 2.

A *V. midae* cryovial culture (2 ml) was inoculated into a 1 L Erlenmeyer flask containing 200 ml cultivation medium (chapter 2; section 2.2.1). Culture flasks were incubated on a rotary platform shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 25°C for the temperature study and 30°C for the pH study, with an agitation speed of 150 rpm. Cultures were harvested at mid-exponential phase (OD_{660nm} ~1.50) and used as inocula for all experiments. All materials used in this study were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

3.2.2 Cultivation of *V. midae* in bioreactors

Cultivation of *V. midae* was performed in 2 L Braun Biostat B fermenters (Sartorius BBI Systems, Melsungen, Germany) at a working volume of 1.7 L. The growth media used for the cultivation of *V. midae* was as described in chapter 2; section 2.2.1).

Cultivation of *V. midae* was performed across a range of temperatures (10 ^{o}C, 20 ^{o}C, 25 ^{o}C, 27.5 ^{o}C, 30 ^{o}C, 35 ^{o}C, and 40 ^{o}C) to determine growth response to temperature in the ideal growth range, as well as the extremes. The medium was prepared to a volume of 1500 ml in the fermenter. The pH of the cultivation medium was adjusted to 7.0 with 25% m.v^{-1} NH_4OH and then similarly maintained at this value post sterilization (121^{o}C, 45 min). Inocula (200 ml) were aseptically added into each fermenter. The airflow was maintained at 1 v.v^{-1}.m^{-1} and agitation was ramped manually from 300 rpm upwards to a maximum of 1000 rpm to maintain dissolved oxygen saturation above 30%. Once an optimum cultivation temperature was determined by mathematically modelling the data, actual validation experiments were conducted in triplicate fermentations using the optimum cultivation conditions to test the robustness and reproducibility of the fermentations at the model predicted temperature optimum.

For the pH investigation, cultivation of *V. midae* was performed under the same conditions as the temperature study, but at a constant temperature of 30^{o}C (optimum determined from the temperature study). *V. midae* was cultivated at pH 5, 6, 7, and 8 in 2 L Braun Biostat B fermenters (Sartorius BBI Systems, Melsungen, Germany) to

determine growth response to pH in the ideal growth range as well as the extremes, at a working volume of 1.7 L. Validation assessment of the model predicted pH optimum was also similarly conducted in actual triplicate fermentations operated at the optimum pH.

3.2.3 Sampling, analyses and calculations

All fermenters were sampled (20 ml) on an hourly basis until maximum growth (determined by measuring OD_{660nm}) was reached. As soon as the organism entered the stationary phase, the fermentations were stopped. Data analysis included determination of total cell concentration (cells.ml^{-1}), overall growth rate (h^{-1}), maximum growth rate ($\mu_{max,}$ h^{-1}) and cell productivity (rate of increase in total cell number, cells.ml^{-1}.h^{-1}).

$$Overall\ growth\ rate\ , h^{-1} = \frac{2.303\ (\log OD\ tend - \log OD\ t0\ )}{(tend - t0)}$$

Equation 3.1

$$Maximum\ growth\ rate, h^{-1} = \frac{2.303\ (\log OD\ tmax - \log OD\ t0\ )}{(tmax - t0)}$$

Equation 3.2

$$Cell\ productivity\ , CFU.mL^{-1}.h^{-1} = \frac{CFU_{final}}{Volume_{final}\ \times Process\ age}$$

Equation 3.3

The overall growth rate was calculated using all data points throughout the growth time of *V. midae*. The maximum growth rate (μ_{max}) was calculated using the data points obtained when the organism was in an exponential phase of growth, and the linear slope of the ln OD over time was at its maximum.

All samples were analysed in triplicate. The responses measured focused on the impact of temperature and pH on overall growth rate, cell concentration and cell productivity during the initial exponential phase of growth. Overall growth rate, cell concentration and cell productivity were the responses determined from primary data. The growth rates were determined from regression analysis of the natural logarithm of microscopic cells counts (Thoma® counting chamber, Hawksley and Sons, London, UK) against time. Cell productivity was expressed as the rate change in total cell concentration, determined from the microscopic enumeration of cells over time.

An Arrhenius plot was generated by plotting the *ln* function of growth rate (μ) against the reciprocal of absolute temperature (K) according to methods outlined by Ratkowsky *et al*., (1983). In addition, each of the key responses (overall growth rate, cell concentration and cell productivity) were statistically analysed to determine appropriate fits to quadratic or cubic models. Numerical optima integrating all three responses using their respective mathematical equations were determined by using the optimization function of Design Expert-6 software (Stat-Ease, Minneapolis, USA), excluding responses where R^2 values were less than 0.90 for any of the model fits. This allows the determination of the optimum temperature or pH taking into consideration all of the key responses. Cell productivity was assigned the highest priority (level 5) of the three responses used in the mathematical modelling. Overall growth rate and cell concentration were assigned an equal but lesser priority (level 1). A two-tailed ANOVA test was performed on actual and model predicted data to assess if the resultant data from actual fermentations was statistically similar or

different to the model predicted performance at the temperature and pH optima respectively. Model predicted values and the resultant standard deviations were used to generate upper and lower limits for the model prediction for each respective model.

3.3 RESULTS AND DISCUSSION

3.3.1. The influence of temperature on the growth of *V. midae*

Cell growth as measured by optical density and cell concentration is depicted in Figures 3.1a and 3.1b below. Growth was high in the range of 25 to 30^{o}C and thereafter a decrease was noted at temperatures exceeding 30^{o}C. Below 25^{o}C, the OD and cell concentrations were also lower. The maximum optical density and cell concentration measured were 8.44 and 1.20×10^{10} cells.ml^{-1} respectively at 30^{o}C after approximately 3 h of cultivation. It can be observed that 30^{o}C was seemingly the best temperature for the cultivation of *V. midae* based on this primary data. (Figure 3.1).

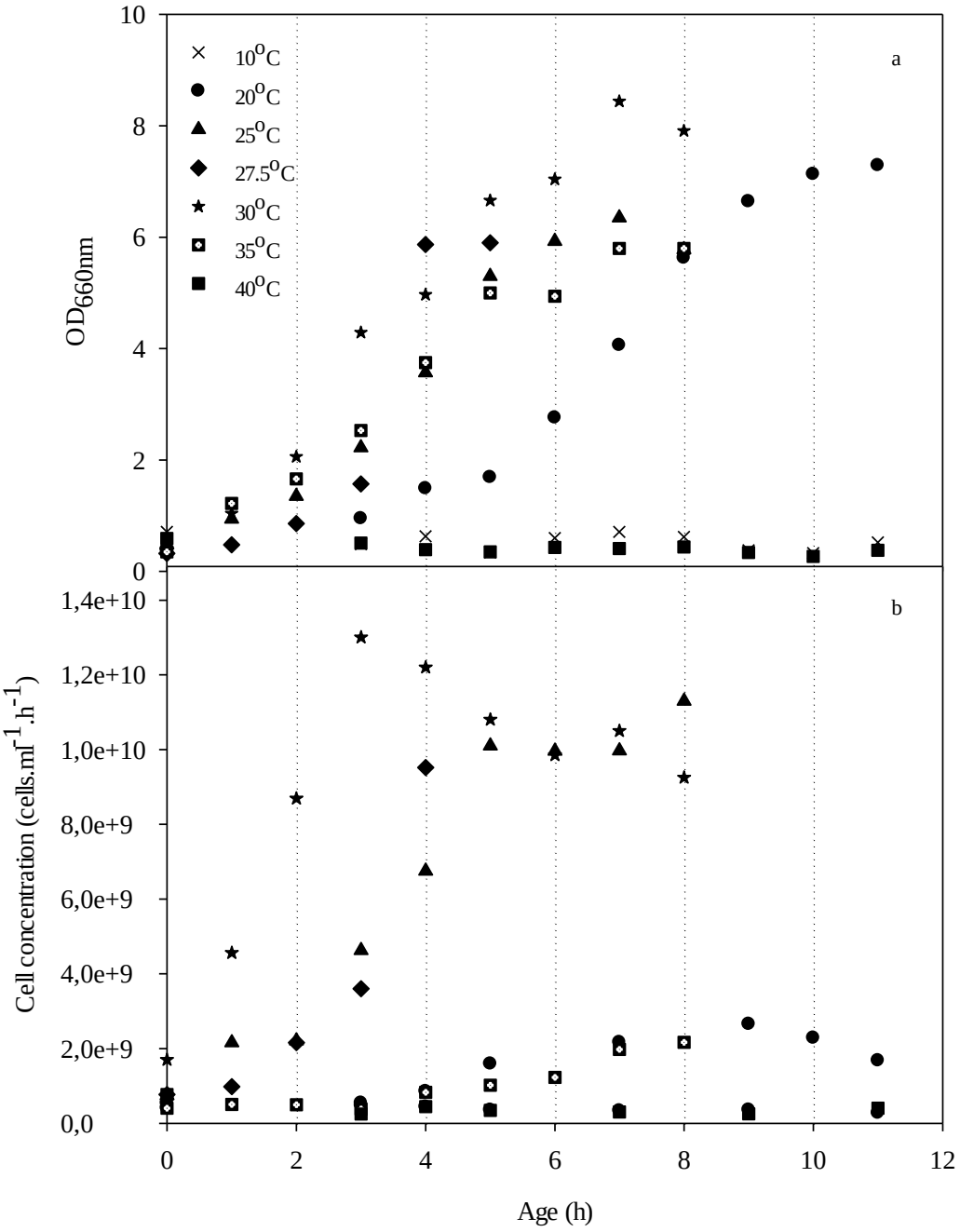

Figure 3.1. Optical densities (a) and cell concentrations (b) observed during the cultivation of *V. midae* at different temperatures 10ºC (×), 20ºC (●), 25ºC (▲), 27.5ºC (♦), 30ºC (*), 35ºC (◘), 40ºC (□) in 2 L bioreactors.

Cultivation temperature influences the intrinsic rate at which a cell grows and replicates, and therefore influences the rate at which microbial production processes occur (Pirt, 1975). In this study, OD and cell concentration were used to monitor *V. midae* growth at different temperatures (Figure 3.1). OD measurements provided at-line, real time process monitoring, but are not sufficiently accurate to calculate the maximum growth rate of the *V. midae* viable population. Therefore, cell concentration was used to determine the effect of temperature on the growth and metabolism of *V. midae*. Maximum specific growth rate (μ_{max}) was used as a measure of the intrinsic growth potential of *V. midae* at different temperatures. Data obtained in the study is depicted below in Figure 3.2.

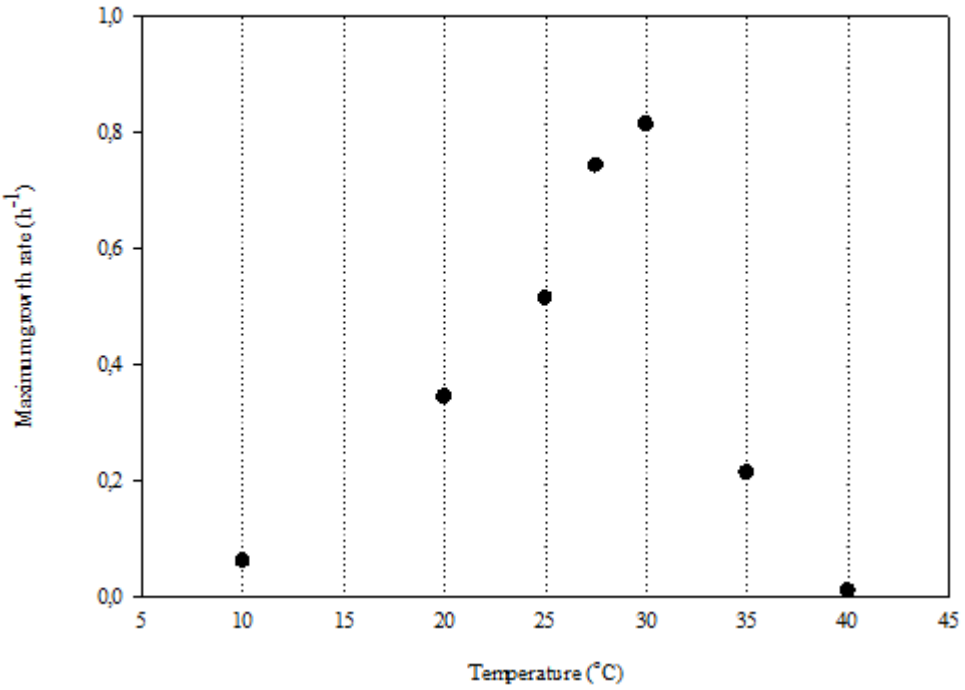

Figure 3.2. Maximum growth rate of *V. midae*, obtained from evaluation of growth in 2 L bioreactors at 10°C to 40°C at pH 7.0.

The maximum specific growth rate (μ_{max}) was highest at 30°C (0.82 h^{-1}), while a visible decrease in growth rate was noted at set points above or below 30°C. The influence of temperature on the rate of microbial processes is always said to be a limiting factor as it affects all chemical and biochemical processes (Pomeroy and Wiebe, 2001), however, other factors such as nutrient sufficiency must be ensured, which was the strategy used in this study. Bacteria are able to regulate their metabolic processes and biosynthesis of their enzymes to achieve maximum efficiency in their environment. This phenomenon generally occurs when the physiological conditions are at an optimum (Lehninger, 1975). The metabolic processes include respiratory rate, substrate utilization, enzyme expression and microbial growth by virtue of cell replication. When these processes are operating under optimal temperature conditions, the maximum specific growth rate of a population can be achieved, as was the case in our study at 30°C. Lee *et al*., (2001) also found that most *Vibrio* strains have an inherently faster growth rate when cultivated at 30 °C and demonstrated reduced growth at temperatures below 22 °C. Although *V. midae* is a marine isolate, it is likely that the mesophilic species of this genus has a near optimum temperature of 30°C due to its intrinsic metabolic make-up. μ_{max} thus increased exponentially between 10°C and 30°C, which follows basic thermodynamic principles. As a consequence, cellular respiration, cell replication, enzyme synthesis and enzyme activity are all expected to be thermodynamically dependant (Russell and Cook, 1995).

Growth was severely hampered at the extremes of temperatures tested in this study (10°C and 40°C), with no apparent increase in cell growth based on optical density or cell concentration (Figure 3.1). The maximum growth rate achieved at 10°C was

negligible (~0.06 h^{-1}), even though nutrients to support microbial growth were present in adequate amounts

in the growth medium. At 10^{o}C cells replicated at a reduced metabolic rate and were unable to utilize available nutrients. Nedwell (1999) stated that at low temperatures, some microorganisms have a lowered affinity to available substrates, as a result of stiffened membrane lipids. This occurrence slows down the functioning of transport proteins used in organism metabolism, which results in reduced growth. Furthermore, Bhakoo and Herbert (1980) showed that *Vibrio* species have the ability to change the composition of fatty acids and phospholipids of the cell membrane to assist in solute transport at low temperatures, thus enabling survival at low temperatures. *Vibrio* cells can also enter an altered physiological state, termed the viable-but-non-culturable state (VBNC) when exposed to low temperatures that enables the cell to survive but not grow. The research done in similar studies, supports our hypothesis that at less than 15^{o}C, our *V. midae* could well have been in this state (Huq and Colwell 1996; Jiang and Chai 1996; Bryan *et al*. 1999, Johnston and Brown, 2002). In our study, observation of the cells incubated at these low temperature using light microscopy demonstrated that cell lysis was not apparent, and therefore the *V. midae* population could have been in a state of VBNC. We confirmed this by re-culturing these cells, using a recovery procedure, to prove their viability (data not shown). Similar studies have also indicated that it is a common occurrence for *Vibrio* populations to remain undetected in marine environments at low temperatures, due to the difficulty in culturing them in a laboratory environment (Bhakoo and Herbert, 1980; Johnston and Brown, 2002; Oliver, 2005).

The maximum grow rate (μ_{max}) was only 0.01 h^{-1} at 40^{o}C (Figure 3.2), confirming that this temperature was not favourable for the growth and proliferation of *V. midae*. We also observed cell lysis at 40^{o}C, indicating that there was physical damage to the cells at high temperature in contrast to a preservation of the morphology at low temperature. When cells are exposed to cultivation conditions above or below their normal range, they display an inability to maximize their growth rate (Guillou and Guespin-Michel, 1996), and therefore perform sub-optimally. This occurrence was clearly evident as shown in both Figures 3.1 and 3.2. Temperature governs the rate at which microbial processes occur, and it has been observed that an increase in cultivation temperature results in a concomitant increase in maximum growth rate; however, further increases above the optimum temperature of a particular organism eventually result in the denaturation of proteins, causing a loss in cell viability (Pirt 1975). Interestingly the decline in μ_{max} between 30^{o}C and 40^{o}C was also exponential indicating a negative thermodynamic influence on cell growth, possibly due to similar thermodynamic effects as relevant in cold domains.

An Arrhenius plot (Figure 3.3) further substantiated our observations that the exponential increase in μ_{max} between 10^{o}C and 30^{o}C was thermodynamically influenced, as was the decline in μ_{max} above 30^{o}C. With an increase in growth temperature from 10 to 30^{o}C, the exponential increase in μ_{max} of *V. midae* corresponds to the *ln* μ_{max} plot of the normal Arrhenius portion of the curve. It could also be observed, that at temperatures exceeding the optimum of 30^{o}C, an exponential decline in μ_{max} was apparent, which correlated with the decrease in growth observed in our study (Figure 3.2). The plot of *ln* μ_{max} against temperature resulted in two linear

regression curves (R^2 > 0.96) with a drastic change in slope at the optimum temperature. This point is known as the critical temperature, which correlates to the point of maximum growth rate of the organism (Figure 3.3). Apart from providing classical thermodynamic information, the presence of low and high temperature domains illustrated by the Arrhenius plot can furthermore be used to infer functionality or survival of the probiotic organisms in product processing and the gut of abalone in response to temperature.

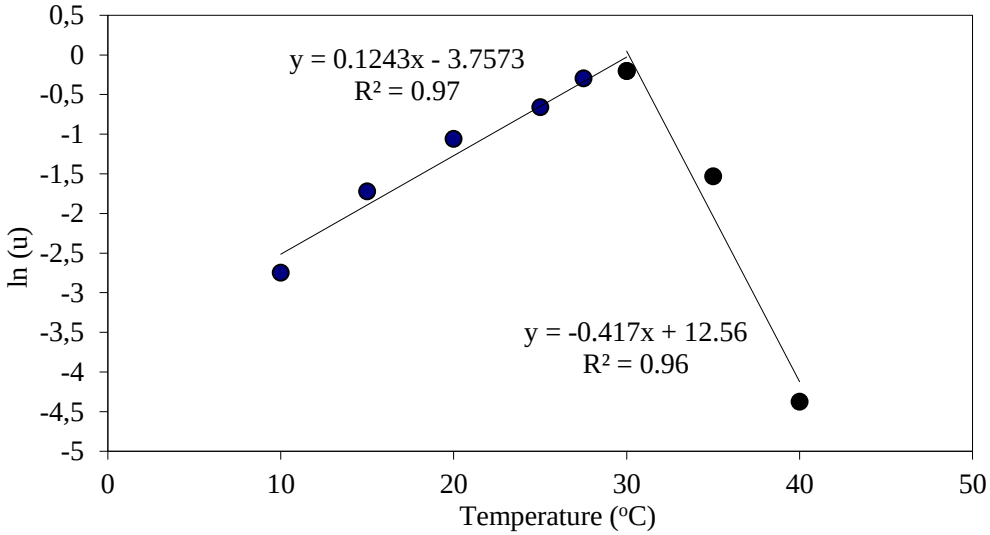

Figure 3.3. Arrhenius plot (*ln* μ_{max} versus temperature) of *V. midae*, indicating the presence of hot and cold temperature domains.

3.3.2 The effect of temperature on overall growth and productivity of *V. midae*

Overall growth rate, cell productivity and cell concentration were selected as marker responses to determine the optimum temperature at which *V. midae* should be cultivated. Overall growth is an indicator of the replication rate of cells throughout the growth period, and differs to the μ_{max} presented in section 3.3.1.1, which in contrast is

an indicator of the maximum metabolic rate. The growth rate data at different temperatures conformed to a cubic model ($R^2 = 0.96$) and a maximum overall growth rate (0.65 h^{-1}) was observed at 30^{o}C. Overall growth rate decreased at all set points above and below 30^{o}C as metabolic processes were sub-optimal at these temperatures, resulting in a longer growth time (Figure 3.4 a). The shape of the curve of overall growth rate (Figure 3.4a) mirrored that of maximum growth rate (Figure 3.2), inferring that the process growth was thermodynamically influenced. For bacterial production processes, temperature must be maintained at a set point at which the growth rate of the organism is at its maximum. If the process is operated at a temperature beyond this optimum, cell energy demands are increased, and as a result, the amount of available energy for metabolic processes related to cell replication is reduced (Guillou and Guespin-Michel 1996; Bonaiti *et al.* 2004).

Productivity of a bioprocess (speed at which a product is formed) is one of the key indicators of process efficiency. Production processes must be designed to produce the maximum amount of viable product in the shortest period of time. In the case of secondary metabolite production, productivity does not necessarily correlate with growth (Luli and Strohl, 1990; Viniegra-Gonzalez *et al*., 2003). In our case; interest was in the actual bacterial cells as a probiotic product and therefore productivity and growth rate data followed a similar trend (Figure 3.4 a and b) as they are both measures of cell replication rate. Cultivation temperature has a key influence on the production process and has a direct impact on the efficiency of a production process. Cell productivity data conformed to a cubic model ($R^2 = 0.95$) with a maximum of 3.43 × 10^9 cells.ml^{-1}.h^{-1} observed at 30^{o}C. Similar to overall growth rate, cell productivity

decreased at temperatures beyond this point (Figure 3.4 b). Under extreme temperatures, beyond the operating range of the organism's tolerable minimum and maximum temperature, productivity of the process is reduced, and in some instances population death occurs, resulting in negative productivity. The latter was clearly observable at the extreme cold and hot temperatures (10 and 40^{o}C respectively), with an exponential increase in productivity observable as temperatures approached the optimum from either the cold or hot domains (Figure 3.4).

Cell concentration is also an important consideration in developing a bioprocess, as the aim of the research is to produce *V. midae* cells for use as an abalone probiotic. Obtaining a high cell concentration positively affects the cost of production, which encourages the adoption of biological agents into current practices (Lalloo *et al*., 2009). In our case, a target cell concentration of 1×10^{10} cells.ml^{-1} was defined up front based on the commercial requirements of the product (high concentration, stable liquid intermediate product for inclusion into the feed components prior to extrusion and drying). During these downstream process operations of centrifugation, formulation, extrusion and drying, some losses in cells and cell viability are experienced. For these reasons, cell concentration targets were stipulated, and this indicator was included as a measure of process success in our optimization study. Cell concentration data against temperature also conformed to a cubic model ($R^2 = 0.98$). The cell concentration target was only achieved at 30^{o}C (1.20×10^{10} cells.ml^{-1}). As noted in Figure 3.4 a and b, cell concentration was also reduced at the extreme temperatures tested, and increased exponentially to a maximum at 30^{o}C for similar reasons to those impacting on growth rate and productivity as previously discussed in this section.

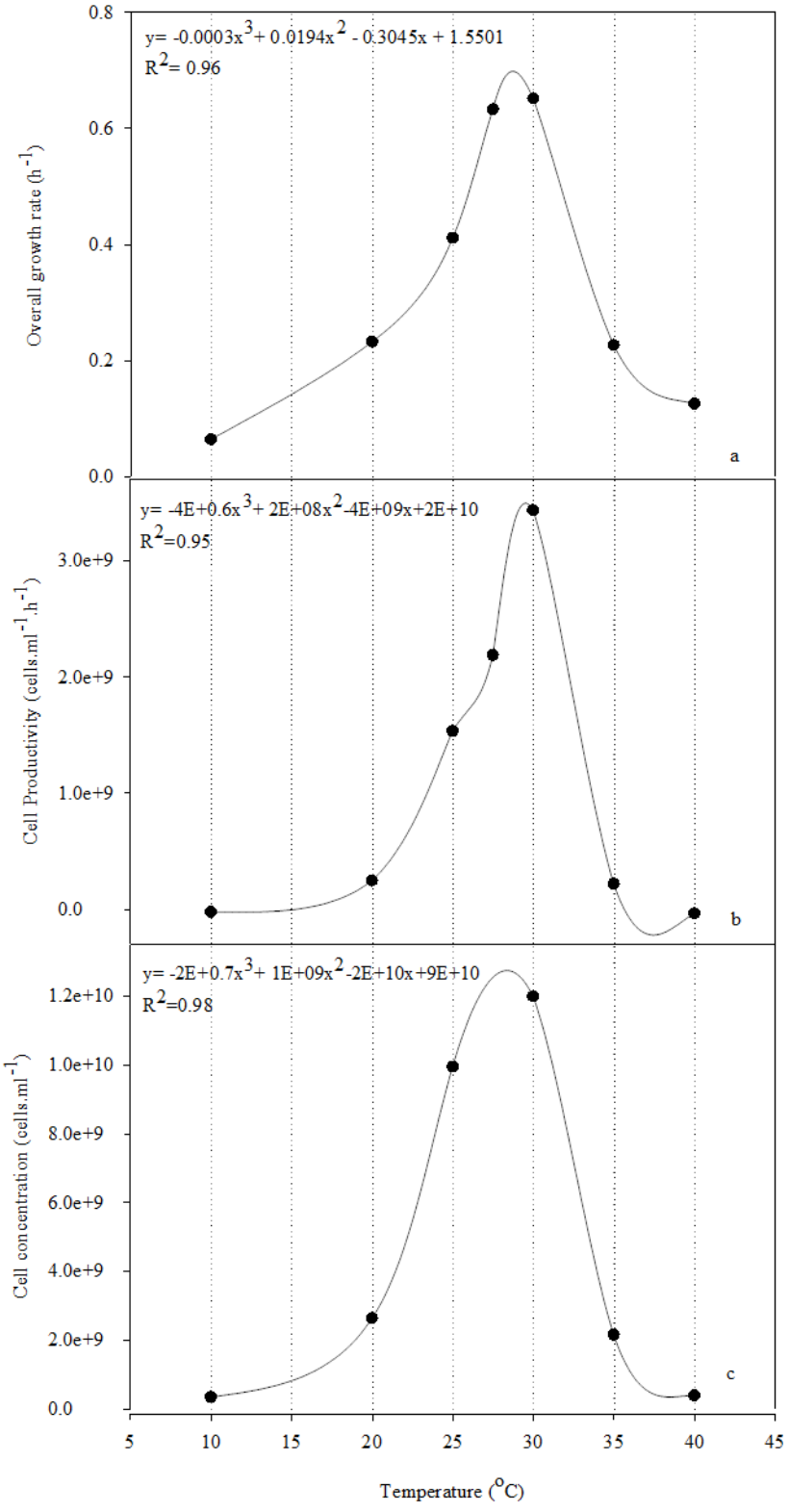

Figure 3.4. Overall growth rate (a), maximum cell concentration (b) and cell productivity (c) assessment of *V. midae* cultivations obtained after evaluation of growth in 2 L bioreactor cultivations at 10°C to 40°C at pH 7.0.

3.3.3. Mathematical optimization of temperature for the cultivation of *V. midae*

Each response (overall growth rate, cell productivity and cell concentration), and their resultant mathematical equations, were simultaneously solved using Design Expert ® in order to predict the optimum temperature conditions for the cultivation of *V. midae.* This technique allows the weighting of responses and the prediction of an optimum using all of the responses in an interactive mathematical model.

Cell productivity was given the highest weighting (level 5), because this response is the commercial indicator of production potential and it directly influences the commercial feasibility of the process. Cell concentration and overall growth rate were weighted with an equal but lesser importance (level 1). All data obtained within the temperature range of 20 to 35^{o}C was statistically analysed for each response. The data from the extreme temperatures did not result in any useful pattern of growth amenable to trend analysis and was excluded. The derived optimum using all three of the responses resulted in a cubic model, with a 90% confidence (desirability co-efficient) in the mathematically predicted optimum temperature of 30^{o}C. (Figure 3.5). The regression co-efficient of individual responses against temperature and the combined model all exceeded 0.95, which provided the statistical basis for acceptance of the model predicted optimum temperature.

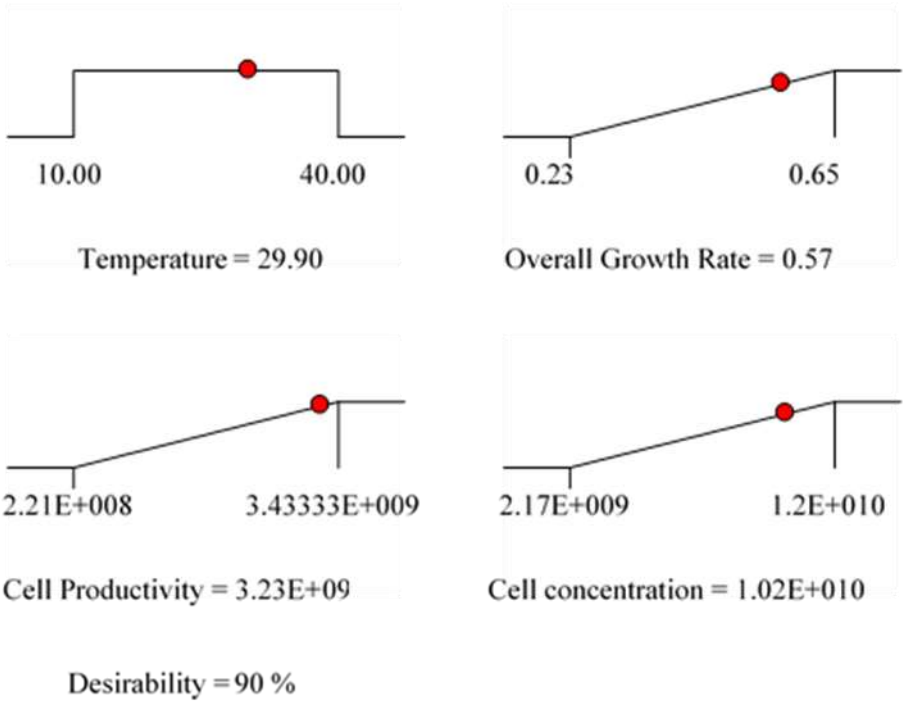

Figure 3.5. Design expert® illustration depicting ramps of key responses used to determine the optimum growth temperature of *V. midae*

The integrated responses against temperature can be visualized using a surface response plot (Figure 3.6). The plot also indicated that when cultivated at 30ºC, *V. midae* growth, productivity and cell concentration were at their maximum.

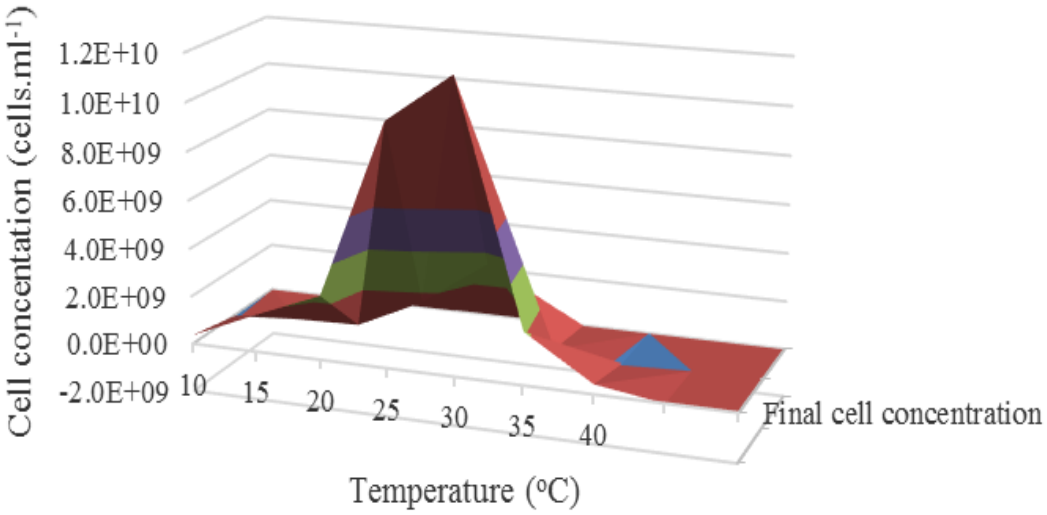

Figure 3.6. Surface response representation of key factors considered during the optimization of temperature for the cultivation of *V. midae* in bioreactors.

3.3.4 Validation of the mathematically predicted optimum

Due to the theoretical nature of mathematical predictions, a more rigorous approach to validate this finding was performed in actual batch cultivations (n = 4) at the model predicted temperature optimum of 30°C. The validation study resulted in an excellent correlation of > 94% for all responses obtained from actual replicate cultivation studies in comparison to their respective mathematically predicted responses (Table 3.1). An overall growth rate of 0.61 (± 0.03) h^{-1}, cell productivity of 3.24×10^9 ($\pm 6.82 \times 10^6$) cells.ml^{-1}.h^{-1} and cell concentration of 9.38×10^9 ($\pm 5.78 \times 10^8$) cells.ml^{-1} was achieved in cultivations performed at the optimum temperature (Table 3.1). Although the comparative data produced low standard deviations between the mathematically predicted optimum and the actual fermentation data, standardized Student T-Test's

(two tailed; equal variance) further confirmed a lack of significance in difference between the two data sets for each of the responses of interest (overall growth rate p= 0.28; cell productivity p= 0.97; cell concentration p= 0.52).

Table 3.1. Validation of modelled optima for overall growth rate, cell concentration and cell productivity of *V. midae* in experiments conducted at the optimum temperature (*n=4; CV < 6%)

Factor	Unit	Modelled response	Actual Data	CV (%)
Growth rate	h^{-1}	0.57	0.61 ± 0.03	4.54
Cell concentration	$cells.ml^{-1}$	1.02×10^{10}	$9.38\times10^{9} \pm 5.78\times10^{8}$	5.90
Cell productivity	$cells.ml^{-1}.h^{-1}$	3.23×10^{9}	$3.24\times10^{9} \pm 6.82\times10^{6}$	0.21

The data obtained in this study revealed that optimization of the growth temperature for *V. midae* had a positive impact on improving the process, and further substantiated our research approach to optimize cultivation temperature when developing a bacterial production process. A 35% increase in overall productivity was achieved using this process improvement strategy, when compared to our base case temperature condition ($22^{o}C$). In addition, it was noted that hot and cold domains were proven to exist for this organism, which provides critical information on the functioning of these organisms in response to different environmental temperatures, such as during production and product processing, in the abalone gut and in the rearing environment. The validity of the mathematically modelled optimum temperature was proven in

actual fermentations which is a critical milestone in confirming the process temperature set-point for future research work and the commercial production process.

All assessments based on primary data, numerical optimization using mathematical modelling as well as validation studies confirmed an optimum temperature of 30ºC. Therefore, the results obtained from the temperature optimization study could be used with confidence in subsequent process development research for production of *V. midae*.

3.3.5 The influence of pH on the growth of *V. midae*

The pH of the cultivation environment is regarded as an important parameter that governs the activity and growth rate of *V. midae* as well as many other microorganisms. Growth studies were thus conducted at varying pH set points while maintaining a constant temperature at 30ºC (based on temperature optimization studies), to evaluate the impact of pH on the growth of *V. midae* (Figure 3.7 a and b). A maximum cell concentration of 3.6×10^{10} cells.ml^{-1} was obtained when *V. midae* was cultivated at pH 6.0 (Figure 3.7 b). *V. midae* was able to grow at a pH of 7.0, however, the maximum growth rate was not optimal. From the primary data, it was noted that *V. midae* exhibited low tolerance to the extremes in pH (5.0 and 8.0, Figure 3.7), based on visible loss in cell viability of the starting population for both pH extremes tested (microscopic observations, data not shown). Bacteria are capable of growing over a wide range of pH conditions (Todar, 2007); however, there are limits to their tolerance. Prescott *et al*., (2002), reported that drastic variations in cytoplasmic

pH can be lethal to sensitive organisms, as it causes the disruption of the plasma membrane or inhibits the activity of enzymes and membrane transport proteins. Some of these mechanisms could explain the death of *V. midae* observed in our studies at the extremes of temperatures tested (5.0 and 8.0).

When pH conditions are optimal for cell growth and plasma membrane function, the maximum specific growth rate (μ_{max}) is achieved. Maximum specific growth rate (μ_{max}) calculated from the cell concentration data, revealed the maximum rate at which the organism is capable of growing. It was highest when *V. midae* was cultivated at a pH of 6 and lowest at a pH of 5.0 (Figure 3.8). *V. midae* performed better at a pH of 6.0 and 7.0, resulting in maximum specific growth rates (μ_{max}) of 1.71 and 1.21 h^{-1} respectively (Figure 3.8). A maximum specific growth rate (μ_{max}) of 0.61 h^{-1} was noted at a pH of 8.0, showing that the organism could tolerate this pH; however, growth was poor under these conditions. Similar studies conducted by Beuchat, (1973), Donovan and van Netten, (1995) and Todar, (2007), which demonstrated that *Vibrio* organisms were able to grow within the range of pH 6.0 to 9.0, correlated to the findings obtained in this study (Figure 3.7). It has been noted that when test organisms are placed at unfavourable pH conditions, the growth rate of the organism slows down at lower or beyond the optimum (Todar, 2007). This was clearly observed during the cultivation of *V. midae* at the sub-optimum pH conditions tested in this study. During typical cell cultivations, microorganisms attempt to establish pH homeostasis between the intracellular and extracellular environments to ensure survival and if for some reason, there is a drastic variation in cytoplasmic pH, this occurrence can be lethal to the organism (Todar, 2007).

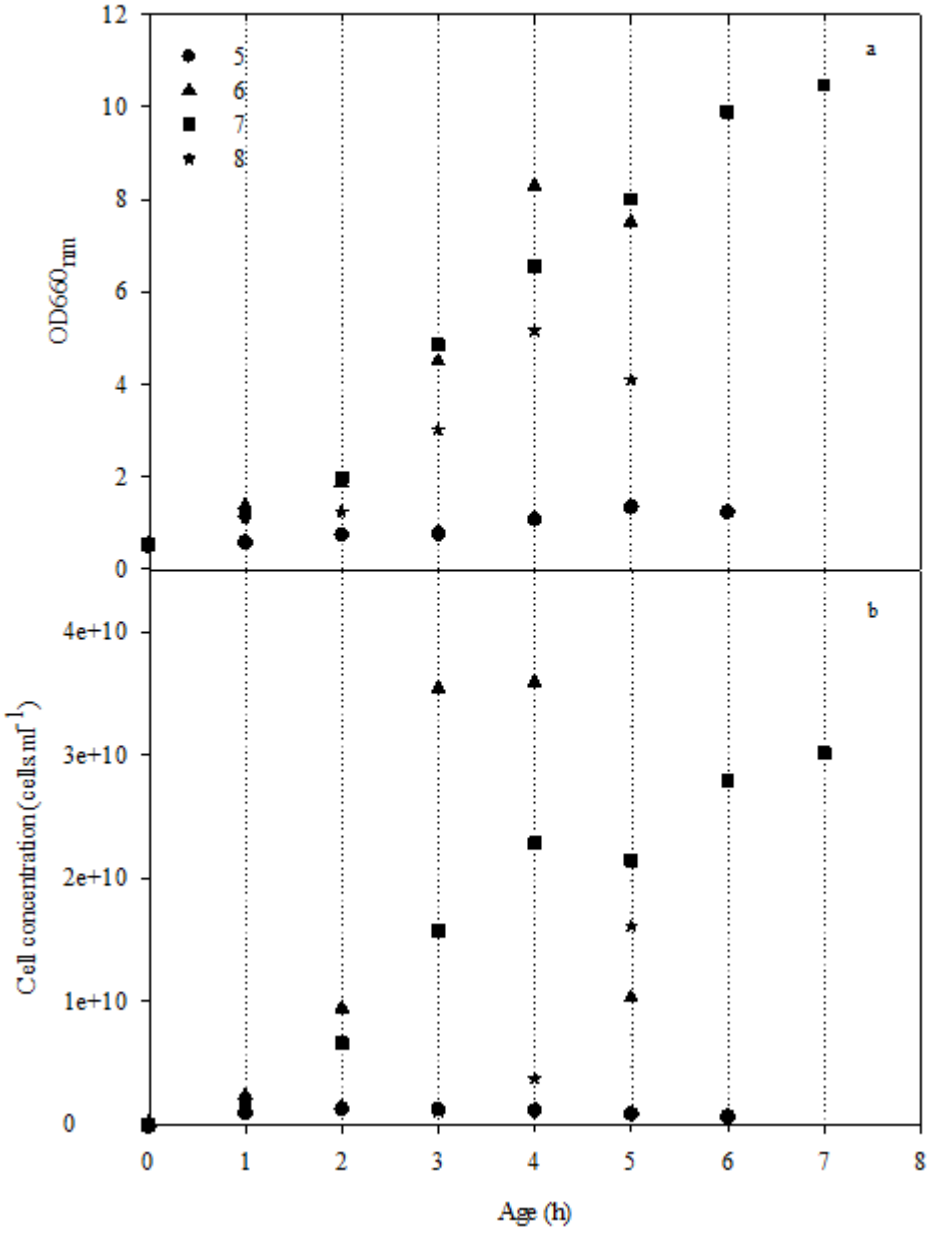

Figure 3.7. Optical densities (a) and cell concentrations (b) observed during the cultivation of *V. midae* in 2 L bioreactors at 30°C and across a pH range of 5 (●), 6 (▲), 7 (■) and 8 (⁎).

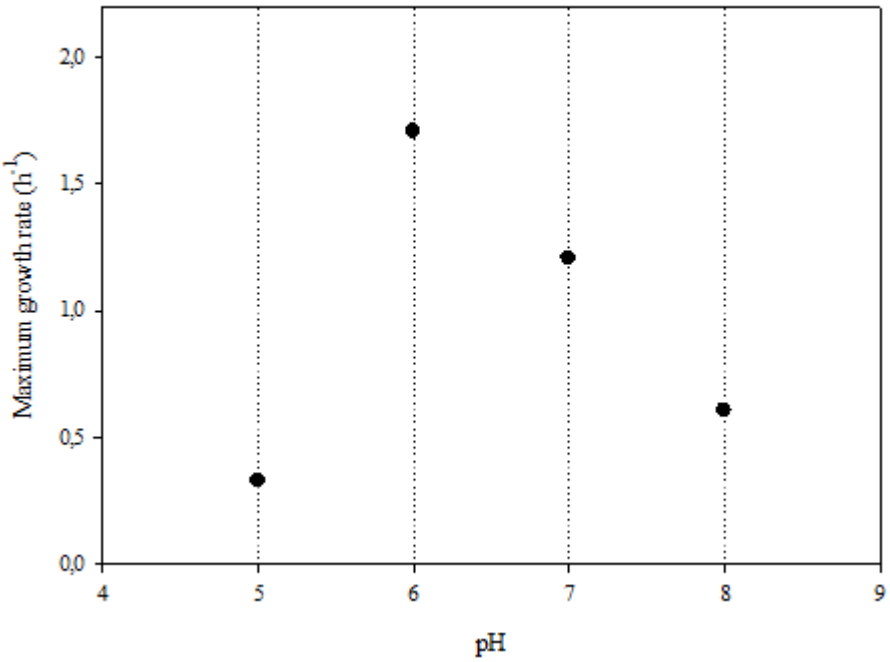

Figure 3.8 Maximum growth rate of *V. midae*, obtained after evaluation of growth in 2 L bioreactors at pH 5 to 8 at 30ºC.

3.3.6 The impact of pH on overall growth and productivity of *V. midae*

In accordance with the temperature study, overall growth rate, cell productivity and cell concentration were selected as marker responses to determine the optimum pH at which *V. midae* should be cultivated. Four cultivations at the respective pH set points were sufficient to generate a mathematical model with an acceptable fit. Overall growth rate data conformed to a quadratic model (R^2 = 0.97), with an optimum at pH 6.0 (1.31 h^{-1}) (Figure 3.9 a). Overall growth rates were lower at the three other pH values tested. *V. midae* showed better growth performance at pH 6 followed by more neutral and alkaline pH conditions (pH 7.0 and 8.0), in comparison to the more acidic pH 5.0. There was a decline in cell number after 4 hours at pH 6 in contrast to pH 7

because growth was faster at a pH of 6, potentially resulting in the earlier depletion of nutrients compared to pH 7 (Figure 3.7). Studies conducted by Huq *et al*., (1984), also indicated that *V. cholerae* was more amenable to growth at a more alkaline pH up to 8.5, but in their studies at a pH value of 6.5, growth was less favourable, in contrast to the optimum found in our study (pH 6). The optimum pH condition will facilitate higher cell productivity, thereby improving the techno-economics of the production process. Similar to overall growth rate, cell productivity data also conformed to a quadratic model (R^2 = 0.93) with a maximum response of 1.19×10^{10} cells.ml^{-1}.h^{-1} observed at a pH of 6.0. At set points beyond this point, a decrease in cell productivity was noted (Figure 3.9 b).

At the onset of this study, the cell concentration target was stated as 1.00×10^{10} cells.ml$^{-1.}$ This target was achieved when *V. midae* was cultivated at pH 6.0, 7.0 and 8.0, but the target cell concentration was not achieved at pH 5.0 (Figure 3.9). Cell concentration data conformed to a quadratic model (R^2 = 0.93). Maximum cell concentration (3.60×10^{10} cells.ml^{-1}) was observed at pH 6.0 (Figure 3.9). At a pH of 5, a drop in cell concentration was observed during the course of the cultivation. Viable culture checks (data not shown), confirmed this, thus indicating that this pH was lethal to *V. midae*. Prescott *et al*., (2002), reported that a drop in the internal bacterial cell pH below 5.5, results in the death of marine cells, and this occurrence was clearly observed during these *V. midae* investigations, whereby a decrease in cell concentration was observed at this pH (Figure 3.7). Furthermore, these findings bear comparison to studies conducted by Wang and Gu, (2004); wherein *Vibrio vulnificus* MP -2, showed no growth when an external pH of 5.2 was maintained in the culture environment.

Reliable information pertaining to the internal pH of the abalone gut was limited and no reference could be made to support the findings in this study.

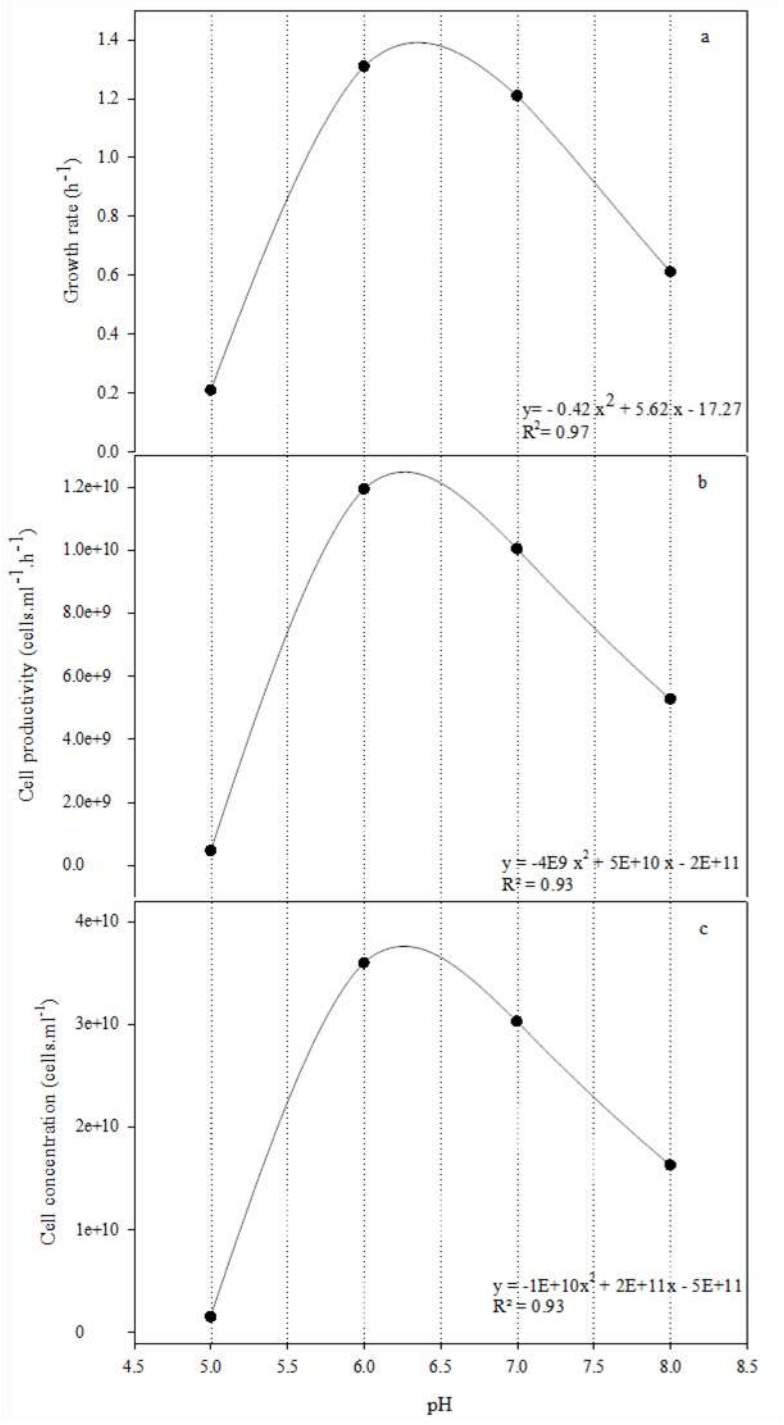

Figure 3.9. Overall growth rate (a), cell concentration (b) and cell productivity (c) data for *V. midae* over a pH range of 5 to 8 at 30ºC.

3.3.7 Mathematical optimization of pH for the cultivation of *V. midae*

The data assessment of the three responses and weighting criteria (overall growth rate, cell productivity and cell concentration) were conducted as described in the temperature optimization study. The selected responses of overall growth rate, cell productivity and cell concentration all conformed to quadratic model fits, with regression coefficients larger than 0.93. These individual mathematical models provided valid inputs into generating an interactive combined mathematical model with the three responses.

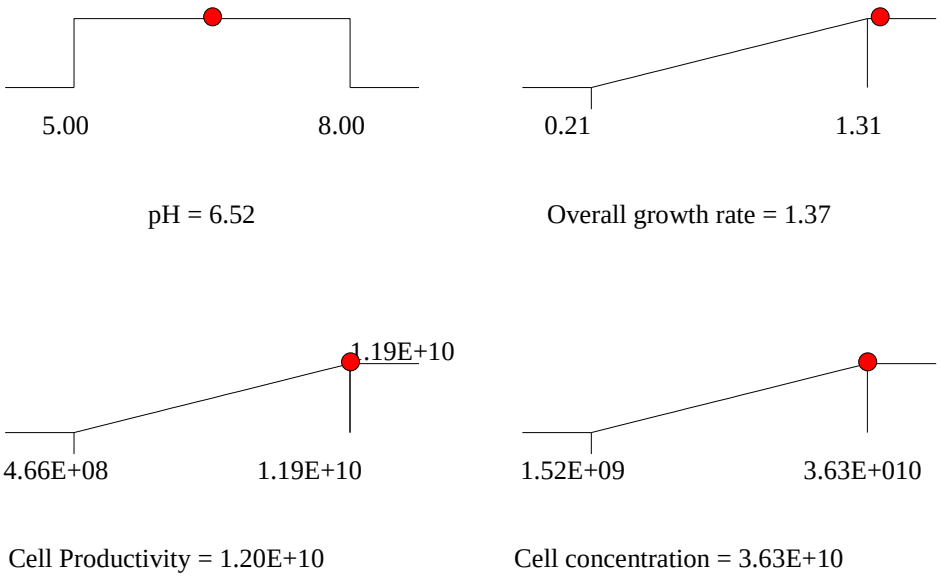

Figure 3.10. Design expert® illustration of ramps depicting growth rate and cell productivity used to determine the optimum cultivation pH of *V. midae*

A quadratic model, with a 100% confidence (desirability co-efficient = 1.00) predicted an optimum pH of 6.5. (Figure 3.10). The regression co-efficient of individual

responses against pH and the combined model all exceeded 0.93, which provided the statistical basis for acceptance of the model predicted optimum pH.

3.3.8 Validation of the mathematically predicted optimum

Although a model desirability of 100% was achieved in the mathematical modelling of data, actual batch cultivations (n = 4) at the model predicted pH optimum of 6.5 still needed to be conducted in order to test the mathematical prediction. The validation study resulted in a correlation of > 88.1% between all responses obtained from actual replicate cultivation studies (Table 3.2). An overall growth rate of 1.61 (± 0.03) h^{-1}, cell productivity of 1.12×10^{10} ($\pm 6.33 \times 10^{8}$) cells.ml^{-1}.h^{-1} and cell concentration of 3.45×10^{10} ($\pm 1.30 \times 10^{9}$) cells.ml^{-1} was achieved in cultivations performed at the optimum pH (Table 3.2). A standardized Student T-Test's (two tailed; equal variance) was performed using the actual data and the model predicted optimum and there was no significance in difference between the two data sets for each of the responses of interest (overall growth rate p= 0.06; cell productivity p= 0.58; cell concentration p= 0.74).

The pH optimization study resulted in a 366% improvement in the productivity of the process in comparison to the baseline technology. Similar to the temperature optimization aspects covered in section 3.3.1; the validity of the mathematically modelled optimum pH was proven in actual fermentations which was a critical milestone in confirming the process pH set-point for future research work and the commercial production process. All assessments based on primary data, numerical

optimization using mathematical modelling, as well as validation studies, confirmed an optimum pH of 6.5. Therefore, the results obtained from the pH optimization study could be used with confidence in the subsequent experiments.

Table 3.2. Validation of modelled predicted optima by conducting actual bioreactor cultivations of *V. midae*

Factor	Unit	Model desirability	Modelled response	Actual data	CV (%)
Growth rate	h^{-1}		1.36	1.61	11.90
Cell concentration	$cells.ml^{-1}$	100%	3.60×10^{10}	3.45×10^{10}	3.01
Cell productivity	$cells.ml^{-1}.h^{-1}$		1.20×10^{10}	1.12×10^{10}	4.82

Research conducted by Riesenberg and Guthke (1999), has shown that one of the main objectives in the development of a bioprocessing strategy is to obtain the maximum amount of product, within a given volume in the shortest period of time. Improvement in process yield, and cost implications of the process were not considered in this study due to scale considerations, however, these are dealt with in later chapters focusing on bioprocess development. This study served to confirm our hypothesis that optimization of temperature and pH would result in substantial improvement in the production of *V. midae.* The study optimizing the temperature and pH resulted in a significant improvement in *V. midae* cell productivity of 136% over the un-optimised base technology. The next step in process development, was to optimize the concentration of cheaper nutrient sources as growth medium components favorable for the cultivation of *V. midae.*

CHAPTER 4: EVALUATION OF CORN STEEP LIQUOR (CSL) AS A NUTRIENT SOURCE FOR THE CULTIVATION OF *V. midae*

4.1 INTRODUCTION

Apart from the physiological growth conditions, cultivation of microorganisms on a large scale is strongly influenced by the composition of the growth medium as it has a substantial impact on productivity, yield and the cost of the production process (Qureshi and Blaschek, 2000; Patel *et al.*, 2004; Preetha *et al.*, 2006). The cultivation medium generally used in fermentation processes provides the organism with basic requirements such as a source of energy, carbon, nitrogen, trace elements and vitamins (Stanbury *et al*., 1995). On a small scale, formulation of growth medium is easier, however, for industrial processes, various factors need to be considered to ensure that the production process is economically feasible and scalable. These include (i) the yield of product needs to be optimized per gram of substrate used, (ii) the production of maximum biomass is required (iii) the maximum rate of product formation is required to optimize capital utilization, (iv) there must be a minimum yield of undesired products, (v) the components used in the medium must be of consistent quality, and readily available throughout the year, (vi) the growth medium must not contain any traces of animal protein due to consumer resistance, (vii) no negative changes must be experienced during medium sterilization, and (viii) the performance of the production process in terms of agitation, aeration, extraction,

purification and waste disposal must not be hampered by the medium composition (Stanbury *et al*., 1995).

Complex media formulations presently dominate the fermentation industry in contrast to defined media due to a substantially lower cost. Complex media can also contribute to a more robust cell growth due to the presence of a matrix of vitamins, minerals and macromolecular precursors (Zhang and Greasham, 1999). Yeast extract, hydrolysates, and polypeptones coupled to carbohydrate sources such as glucose and sucrose have been extensively used in complex medium formulations as nitrogen and sugar rich sources (Nohata and Kurane 1997; Vuolanto *et al.,* 2001; Prabakaran *et al.,* 2007; Lalloo *et al*., 2009). These additives possess an abundance of vitamins and various cofactors required for growth, however, they are expensive and sometimes have negative market acceptance as some have animal origins (Lalloo *et al.,* 2009). As a result, cheaper medium substrates that will result in maximized cell concentration and a corresponding increase in process productivity are constantly sought as alternatives (Burkert *et al*., 2004; Patel *et al.,* 2004).

Probiotics that are usually used in aquaculture belong to the *Lactobacillus*, *Vibrio, Bacillus, Pediococcus* and *Shewanella* genera (Sawabe *et al*., 1998; Tanaka *et al*., 2003; Macey and Coyne, 2005; Sawabe *et al*., 2007, Silva-Aciares *et al*., 2011, Jiang *et al*., 2013). During cultivation of these organisms, standard laboratory based media such as Rogosa's Medium (MRS), Luria broth (LB) and marine broth (MB) (De Man et al.,1960), Macey and Coyne, 2005) are used. However, in the instance of the abalone probiotic, *V. midae,* no information pertaining to suitable culture medium is reported other than marine broth (Macey and Coyne 2005). Therefore, a novel shift in

approach was required in order to obtain a suitable medium to enable the cultivation of this marine probiotic organism. As a result, nutrient substrates such as CSL and HTM were evaluated, and resulted in the generation of a patented bioprocess cultivation medium.

In lieu of the above, corn steep liquor (CSL) was identified as a low cost growth medium additive to be used as a nitrogen rich nutrient source for the production of *V. midae*. Maize is the largest agricultural product produced in the Republic of South Africa (RSA) and production reports revealed that 16.4 million tonnes of maize were cultivated per annum (DAFF, 2012; FAO, 2017). Corn steep liquor is one of the waste products generated from this agro-industry during the production of dextrose and high fructose corn syrup. As this material is readily available and competitively priced; it offers an attractive alternative to conventionally used nutrient substrates in local commercial scale bioprocess technologies.

During the initial suitability assessment, a modified *Vibrio* medium containing CSL was tested in a basic study to assess growth. Once microbial growth in medium containing CSL was demonstrated, the optimum supplementation concentration of this nutrient source was determined to improve the efficiency and economic attractiveness of the *V. midae* production technology. It was hypothesized that the optimization of CSL concentration would further increase cell yield and productivity of the production process while reducing cost of production.

4.2 MATERIALS AND METHOD

4.2.1 Inoculum preparation

A cryopreserved culture of *V. midae* (LMG P-27727), prepared as described in chapter 2, was used to inoculate a 1.8 L Fernbach flask containing 700 ml of modified *Vibrio* medium (MVM). The MVM growth medium in each flask was prepared according to methods outlined in chapter 2; section 2.2.1). The pH of the growth medium was adjusted to 6.5 (optimum from the pH study) using 25% $v.v^{-1}$ NH_4OH, prior to sterilization at 121°C for 15 min. A separate glucose solution (10 $g.l^{-1}$, 55% $m.m^{-1}$ TSAI) was prepared, sterilized and then aseptically added into the culture flask containing the sterile growth medium. Following aseptic inoculation using 1 cryovial, flasks were incubated at 30°C (optimum from the temperature study) and 180 rpm on an orbital shaker (Innova 2300, New Brunswick Scientific, Edison, NJ, USA) for 5 h. A single flask was used to inoculate each fermenter.

4.2.2 Preliminary assessment of the suitability of CSL as a nutrient source for cultivation of *V. midae* at 10 L scale

All fermentation studies were conducted in 15 L Biostat C fermenters (Sartorius BBI systems, Melsungen, Germany) at a working volume of 10 L. The test medium (MVM_{+CSL}) contained an identical composition of salts and antifoam as the MVM described in chapter 2; section 2.2.1; with the exception of the nutrient source (yeast extract, peptone and casamino acids were removed). The MVM_{+CSL} contained CSL supplemented to a total protein concentration of 11.65 $g.l^{-1}$ (total protein

concentration equivalent to the original MVM). The salts, antifoam (1 $ml.l^{-1}$, Pluriol P2000, BASF, Ludwigshafen, Germany) and CSL of the MVM_{+CSL} media were added to the initial charge and made up to a volume of 9.3 L. Subsequent to the *in-situ* sterilization of the initial charge (121ºC, 45 minutes) a separately sterilized glucose solution (10 $g.l^{-1}$, 55% $m.m^{-1}$ TSAI) was added by sterile transfer into the fermenter. The fermentation temperature was maintained at 30 °C. The stirrer speed was set at 500 rpm and ramped to 1000 rpm over 3 h, to maintain the dissolved oxygen above 30% saturation. The pH was maintained at 6.5 using 25% $v.v^{-1}$ NH_4OH and aeration was set at 1 $v.v^{-1}.m^{-1}$. Ammonium hydroxide was used as the preferred base for pH control, as it also serves as a source of inorganic nitrogen improving cell production. For this reason, this base is also widely used in large scale commercial fermentation. All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

The study was conducted in triplicate. Glucose feed commenced when the residual glucose concentration dropped to just below 5 $g.l^{-1}$ (monitored half hourly as described in section 4.2.5). Fermentations were fed with a 55% $m.m^{-1}$ glucose solution at varying feed rates using a gravimetric feed controller to maintain a glucose concentration of 2.0 to 5.0 $g.l^{-1}$. The stop signal for these fermentations was indicated by a decline in the oxygen utilization rate measured on-line and then confirmed by cell number. Both of these parameters were measured as described in sections 4.2.5 and 4.2.6.

4.2.3 Determination of optimum CSL concentration for production of *V. midae*

Once it was confirmed that CSL supported the growth of *V. midae*, growth of the microorganism at a range of CSL concentrations (5 $g.l^{-1}$, 10 $g.l^{-1}$, 15 $g.l^{-1}$, 20 $g.l^{-1}$, 25 $g.l^{-1}$, 30 $g.l^{-1}$, and 40 $g.l^{-1}$) was evaluated in 15 L Biostat C fermenters in fed-batch mode, as described in section 4.2.2. The sugar feed rates for this study, were adjusted such that an oxygen transfer rate of 200 $mM.l^{-1}.h^{-1}$; as described in section 4.2.6; was not exceeded (Rivière 1977; Ozturk 1996). Declines in the oxygen utilization rate and cell number were used as stop signals for these fermentations. A study was conducted to assess the growth of *V. midae* in a medium devoid of CSL however, no growth was observed (data not shown).

4.2.4 Data processing and validation of model predicted optimum

Each of the key responses (cell concentration, cell productivity, cost of production and yields on protein, oxygen and carbohydrate), in response to change in CSL concentration, were statistically analysed to determine appropriate fits to quadratic or cubic models. Numerical optima integrating all three responses using their respective mathematical equations were determined by using the optimization function of Design Expert-6 software (Stat-Ease, Minneapolis, USA), excluding responses where R^2 values were less than 0.90 for any of the model fits. This allows the determination of the optimum CSL concentration taking into consideration all of the key responses. Cell productivity was assigned the highest priority (level 5) of the three responses used in the mathematical modelling. Cost of production (material cost plus cost of capital) and cell concentration were assigned an equal but lesser

priority (level 1). Product yields on protein, substrate and oxygen were not maximized for this study, however, were designated to be in range. Once the model predicted optimum CSL concentration was determined, triplicate fermentations batches were run as described in section 4.2.2 and 4.2.3 to validate the key responses at a CSL concentration of 6.4 $g.l^{-1}$ (mathematically predicted optimum CSL concentration). A two-tailed ANOVA test was performed on actual and model predicted data to assess if the resultant data from actual fermentations was statistically similar to the model predicted performance at the optimum CSL concentration. Model predicted mean values and the resultant standard deviations were used to generate upper and lower limits for each of the modelled responses to enable the statistical comparison.

4.2.5 Sampling and analysis

Bioreactors used in each study were sampled every 30 min and analysed for cell density (660 nm) using a Genesys® 20 spectrophotometer (Spectronic, NY,USA), cell counts were performed using a Thoma® counting chamber (Hawksley and Sons, London), and glucose concentrations were determined using a Accutrend® alpha glucometer (Boehringer Mannheim, Germany). Optical density was only used as a measure to monitor *V. midae* growth in the studies.

4.2.6 Data analysis and calculations

Cell productivity was expressed as the rate change in total cell concentration, determined from the microscopic enumeration of cells over time ($R^2 > 0.9$) (Nori *et al.* 1983). Cost of production was determined by calculating the cost of medium components listed in chapter 2, section 2.2.1(used for the production of one litre of 1

$\times 10^7$ cells as this is the supplementation level per gram of abalone feed according to Macey and Coyne (2005) as well as capital utilization per annum based on an 80% capacity utilization per annum and ten-year straight line depreciation of a production scale system (200L, ~ R10 million). Cost of production was not determined for the 40 $g.l^{-1}$ CSL cultivation, as no increase in growth was observed, and this caused the artificial skewing of model fit for the resultant data set. Exhaust gas analysis was carried out using an Uras 10E gas analyser (Sartorius BBI Systems, Melsungen, Germany). Oxygen utilization (OUR) and carbon dioxide evolution (CER) rates were calculated online using MFCS software, from carbon dioxide and oxygen concentration measurements of the exhaust gases. Oxygen transfer rate was maintained to not exceed 200 $mM.l^{-1}.h^{-1}$ as this is around the safe maximum for stirred tank bioreactors (Rivière 1977; Ozturk 1996). This was calculated by determining the volume corrected OUR at a specific time point in the cultivation based on the actual volume of the reactor and amount of air introduced into the cultivation reactor. Yield co-efficients were also calculated based on data points conforming to high linearity ($R^2 > 0.9$) of plots of total cell number against either total protein (YPP), carbohydrate (YPS) or oxygen (YPO) consumed (Papanikolaou and Aggelis, 2002).

4.3 RESULTS AND DISCUSSION

4.3.1 Assessment of the suitability of CSL as a nutrient source for the production of *V. midae*.

The cost of a microbial production process is one of many key drivers that influence the uptake of the technology in a commercial environment. In order to reduce the cost

of production and increase yields and production efficiencies, cheaper, nutritionally rich nutrient sources have been evaluated in order to improve production processes (Burkert *et al*., 2004; Patel *et al.*, 2004). For biological products such as probiotics, a defined cultivation medium is not required, and therefore readily available agro-industrial by-products such as CSL may be used as suitable nutrient sources.

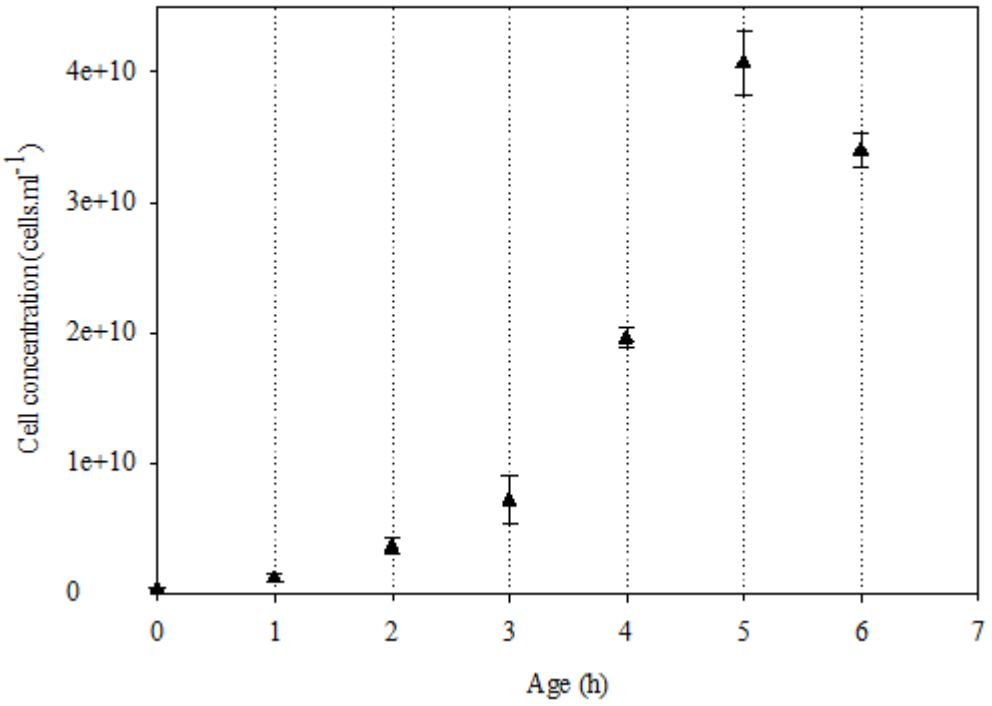

Figure 4.1. Cell concentration profile obtained during the cultivation of *V. midae* in 10 L laboratory-scale bioreactors at 30ºC and pH 6.5 in a modified *Vibrio* cultivation medium supplemented with 11.65 g.L^{-1}CSL (▲). (n= 3)

The ability of *V. midae* to grow in a medium containing CSL was tested in replicate batch fermentations (Figure 4.1). A maximum cell concentration of $3.74 \times 10^{10} \pm 6.93 \times 10^{9}$ cells.ml^{-1} was achieved after 4.5 h of cultivation (Figure 4.1). CSL is commonly used as a nutrient source in several bioprocesses for a variety of purposes as it is cheap

and readily available. This low-cost medium additive serves as a good source of an entire range of essential amino acids, polypeptides and B-complex vitamins (He *et al*., 2004; Schilling *et al*., 2007; Yu *et al*., 2008) which have been shown to facilitate microbial growth. Similar to this study, research conducted by Kuppusamy and Balaraman, (1991), Nancib *et al*., (2005) and Prabakaran *et al*., (2007) has demonstrated higher cell concentrations in various processes when CSL was used to replace conventional nutrient sources. This could be attributed to the specific protein, vitamin and amino acid profiles contained in this complex nutrient source that successfully supports microbial growth. Amino acids are an important component of the CSL, as they are known to be assimilated by various microorganisms and used as sole source of nitrogen during microbial growth (Bapat *et al*., 2006). The specific CSL used in this study contained an abundance of amino acids, with glutamic acid, alanine and leucine known to positively influence microbial growth present in the concentration range of 1 to 2% $m.v^{-1}$, and provided an added advantage of use of a cheaper nutrient source in the cultivation medium (Macko and Estep, 1984). These amino acids serve as both carbon and nitrogen sources to *Vibrio* organisms and serve vital functions in cellular metabolism and protein synthesis (Macko and Estep, 1984). Alanine, glutamic acid and leucine are 3, 5 and 6 carbon compounds respectively. During microbial metabolism leucine and glutamic acid provide more energy than lower carbon containing amino acids such as alanine; and therefore an abundance of the higher carbon containing compounds in the culture medium enables *Vibrio* sp., and other bacterial cells, to obtain high energy levels (Macko and Estep, 1984). Furthermore, CSL is a good source of inorganic nitrogen in the form of ammonium sulphate. Marine organisms prefer inorganic nitrogen in the form of ammonium ion to

nitrates in the culture environment, as it supports high cell growth when it is available (Wheeler and Kokkinakis, 1990, Carpenter and Capone, 2016). However, it can also have cytotoxic effects and reduce cell growth rate due to osmotic pressure when supplemented at high concentrations (Muller *et al*., 2005).

The supplementation of the culture medium with CSL was undertaken in an attempt to boost commercial attractiveness by reducing the overall cost of production of *V. midae.* This study confirmed our hypothesis that *V. midae* could utilize CSL as a nutrient source. The cell concentration achieved in this study was adequate to meet the commercial targets of our technology. Although CSL was supplemented to an equivalent total protein basis of the nutrient sources used in the original MVM, the true value of CSL supplementation could only be confirmed by optimization of the CSL supplementation concentration.

4.3.2 Determination of optimum CSL concentration to increase cell concentration

Once the suitability of CSL as a nutrient source was established, a range of concentrations were assessed in order to determine an optimum CSL supplementation level in the test medium (MVM_{+CSL}). Results revealed that *V. midae* grew well in medium containing CSL at concentrations of 5 $g.l^{-1}$, 10 $g.l^{-1}$ and 15 $g.l^{-1}$ (Figure 4.2). At concentrations exceeding 15 $g.l^{-1}$, longer growth times were observed (Figure 4.2 a and b).

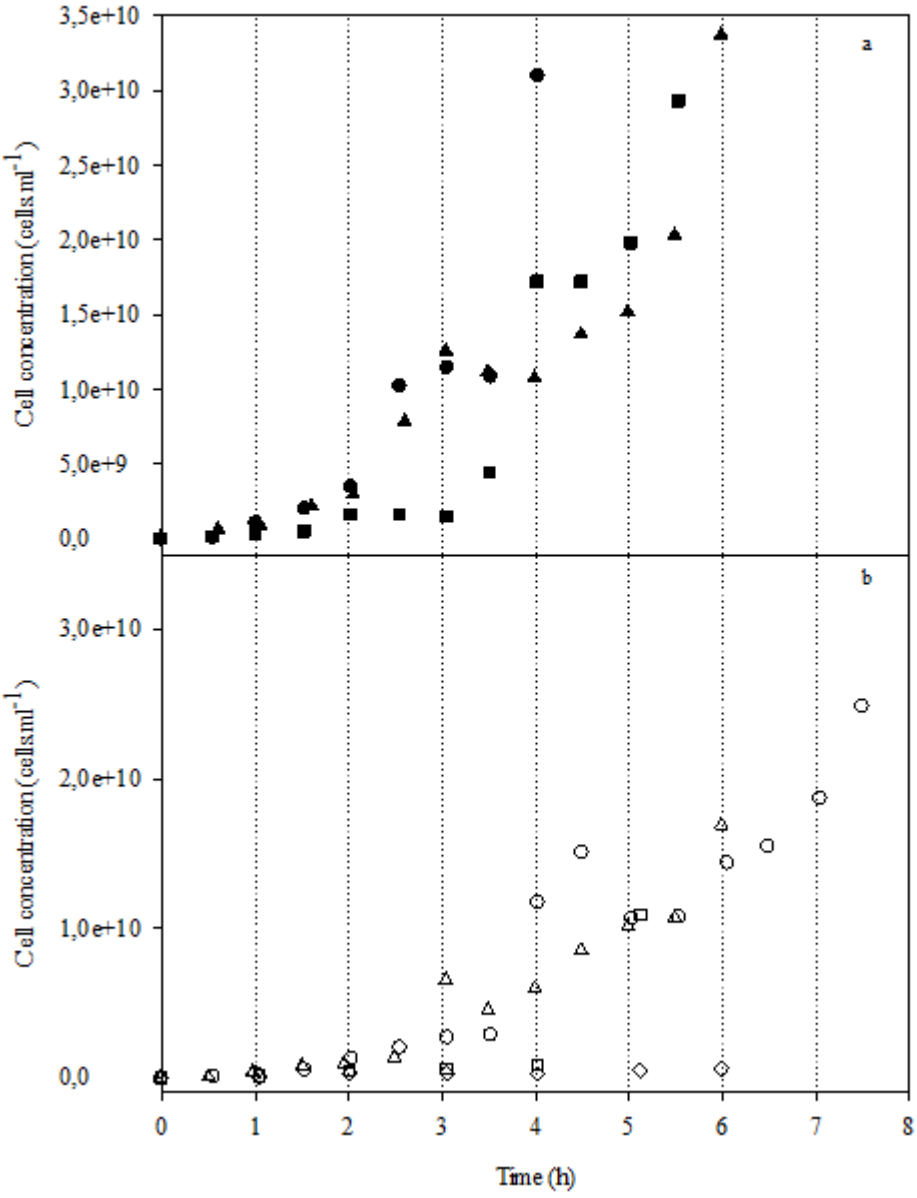

Figure 4.2. Cell concentration profiles obtained from the cultivation of *V. midae* in (a) growth medium containing 5 $g.l^{-1}$ (●), 10 $g.l^{-1}$ (▲) and 15 $g.l^{-1}$ (■) CSL; and (b) growth medium containing 20 $g.l^{-1}$ (○), 25 $g.l^{-1}$ (Δ), 30 $g.l^{-1}$ (□) and 40 $g.l^{-1}$ (◊) CSL in 10 L laboratory-scale bioreactors at 30°C and pH 6.5.

It was observed that when the organism was cultivated in a medium containing a low CSL concentration (5 $g.l^{-1}$), a maximum cell concentration of 2.50×10^{10} $cells.ml^{-1}$

was obtained after a relatively short process time of 4 h (Figure 4.2). This short process time was due to higher growth rate (most likely attributable to lower osmotic load) coupled to quicker consumption of nutrients after which time no further increase in cell concentration was observed. On the contrary; when *V. midae* was grown in a medium containing 40 $g.l^{-1}$ CSL, there was no visible increase in cell concentration (Figure 4.2 b), also verified by optical density data (data not shown). This absence of cell growth could be attributed to the high concentration of ammonium sulphate (50 $g.l^{-1}$) that was added to the medium upon CSL supplementation. At this concentration salts also precipitated out of the fermentation medium. Ammonium sulphate is known to be a preferred nitrogen source for most bacteria; however, it is also notorious for its cytotoxic effects (Muller *et al*., 2005). These unfavourable conditions present at the highest CSL concentration tested (40 $g.l^{-1}$), resulted in an OUR of only 26.73 $mMol.l^{-1}.h^{-1}$. This was approximately 75% less than the oxygen consumption rate by *V. midae* under the same aeration and agitation conditions when CSL was supplemented at 5 $g.l^{-1}$ (max OUR 153.14 $mMol.l^{-1}.h^{-1}$).

It has been reported in similar studies that increased precipitation usually occurs in fermentation media that contain high concentrations of CSL (Champagne *et al*., 1990). A chelating agent, citric acid, was included in the fermentation medium components; however, the concentration of citric acid was not optimized to reduce precipitation as it was outside the scope of this study. When certain compounds precipitate out of solution, they become unavailable for use by the microorganism.

This may cause a metabolic deficiency depending on the type of compound, and as a result, growth of the organism is hindered.

The cell concentration profile conformed to a second order polynomial function (R^2 > 0.90) when plotted against the range of CSL concentrations tested (Figure 4.3 a). A visible increase in cell concentration was observed with an increase in CSL concentration up to 10 $g.l^{-1}$ (3.37×10^{10} $cells.ml^{-1}$) (Figure 4.3 a). Beyond 20 $g.l^{-1}$, a prominent decrease in the resultant cell concentration of *V. midae* was observed with increasing CSL concentration (Figure 4.3 a). This has been noted in several studies on both whole cell production, as well as production of secondary metabolites such as enzymes, where an increase in nutrient supplementation does not result in a linear increase in product formation (Champagne *et al*., 1999; Kona *et al*., 2001). It is known that CSL supplementation at high concentrations has an inhibitory effect on organisms as it contains high concentrations of lactic, butyric and phytic acid that affect substrate metabolism (Sreenath and Jeffries, 1997; Triechel *et al*., 2009), over and above the attenuated growth due to osmotic inhibition from ammonium sulphate at high concentration.

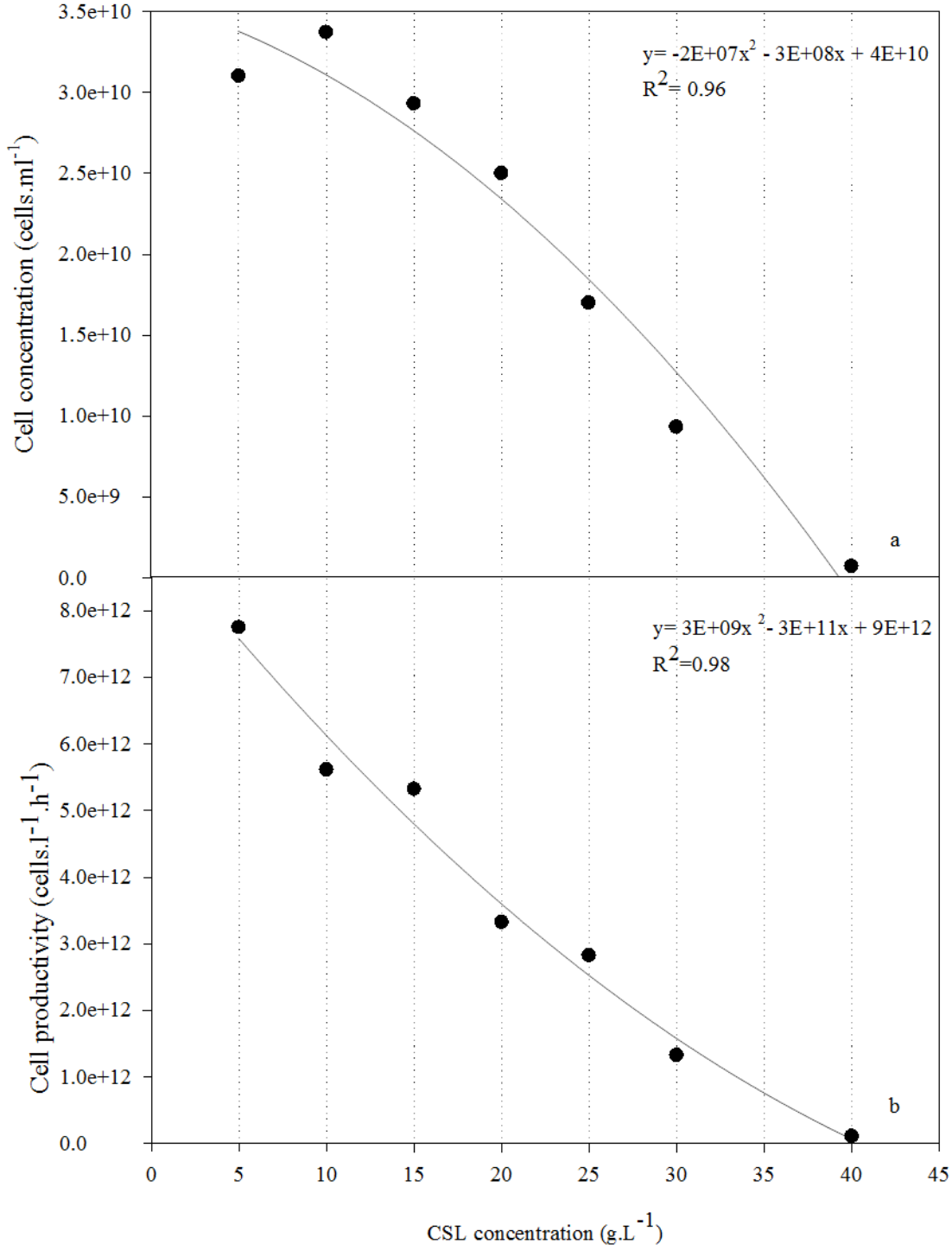

Figure 4.3. Key responses for cell concentration (a) and cell productivity (b) measured during the cultivation of *V. midae* in medium containing various concentrations of CSL in 10 L laboratory-scale bioreactors at 30°C and pH 6.5.

Cell productivity (speed at which a product is formed) was maximum (6.25×10^{12} cells.ml^{-1}.h^{-1}); when *V. midae* was cultivated in a medium containing 5 g.l^{-1} CSL (Figure 4.3 b). Hereafter, a linear decline in overall cell productivity was noted ($R^2 > 0.97$). Higher cell productivity is said to be of higher importance than higher cell concentration, such that the throughput of industrial processes is increased (Tellez-Luis *et al*., 2003). This is in many instances, inextricably linked to economic productivity, which is calculated using process costs of labour, raw materials and capital, as calculated in this study (Figure 4.3 c). Due to the limited growth of *V. midae* at 40 g.l^{-1}, poor process productivity was noted. Although cultivations containing CSL in the range of 10 to 20 g.l^{-1} resulted in high cell concentrations, cell productivity was reduced due to an increase in process time of approximately 27 – 46%. Investigations conducted by other researchers also noted that an increase in CSL concentration did boost product formation, up to a maximum, however after a certain threshold was exceeded, a decrease in product concentration and productivity was noted, which resulted in an associated increase in cost of production (Kona *et al*., 2001). Due to the importance of maximizing productivity of a bioprocess, it is imperative that constituents of the cultivation medium do not hamper process efficiency.

Cost of production, which is also a key driver in bioprocess optimization, is directly influenced by process time, mainly due to capital cost. Longer process times were noted when CSL concentrations exceeded 15 g.l^{-1}. The extended process times had a direct impact on the labour and capital costs, as well as additional material costs (Figure 4.4). It can be observed that there was a gradual increase in production costs between media containing a CSL range of 5 g.l^{-1} to 30 g.l^{-1}. Due to the minimal growth

observed at 40 g.l^{-1}, a substantially higher cost of production was noted, which caused an artificial skewing of the resultant second order polynomial trend ($R^2 > 0.95$), and therefore was not included in the data presentation (Figure 4.4). The use of this particular medium with an associated higher cost of production was not feasible for use, as the techno-economics of the process were adversely affected.

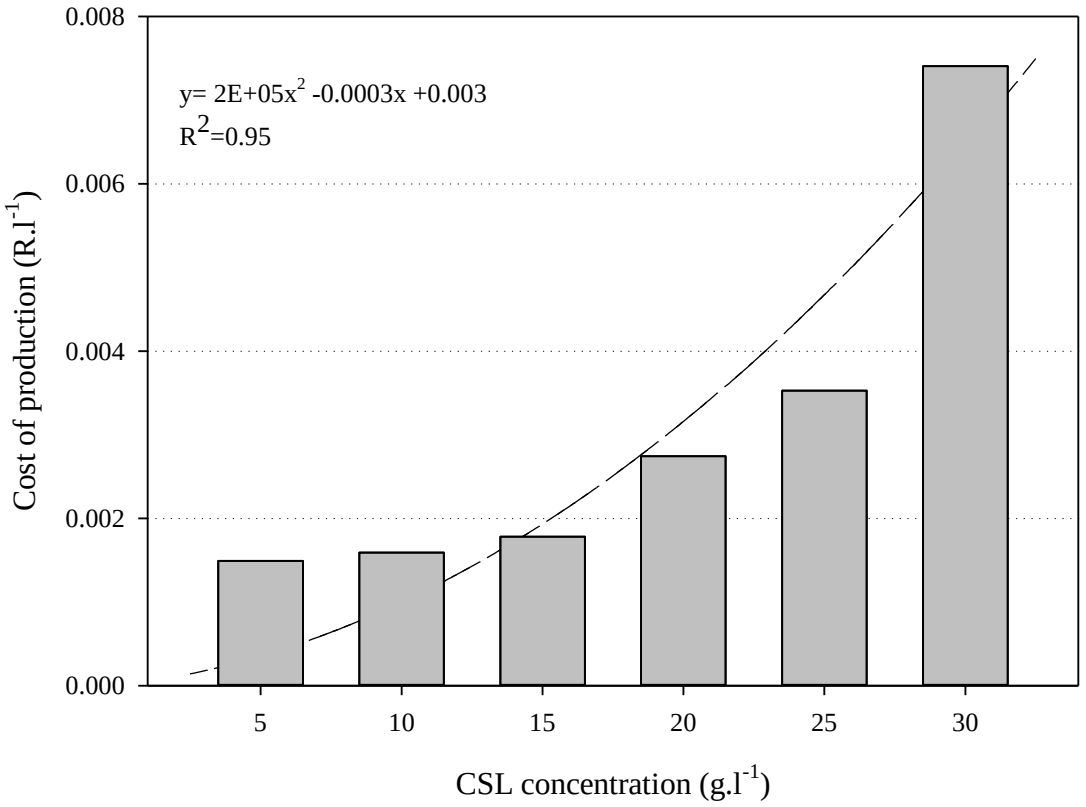

Figure 4.4. Cost of production calculated during the cultivation of *V. midae* in medium containing various concentrations of CSL in 10 L laboratory-scale bioreactors at 30ºC and pH 6.5.

V. midae cell yield on protein conformed to a second order polynomial quadratic function ($R^2 > 0.99$) when plotted against the range of CSL concentrations tested (Figure 4.5 a). It was also observed that cell yield based on protein decreased with an

increase in CSL concentration with a maximum yield noted at 5 $g.l^{-1}$ protein (Figure 4.5 a). In general, higher excess levels of nutrient substrate can decrease process yields, as the metabolic utilization becomes more extravagant often resulting in over-flow metabolism (nutrients not efficiently directed to critical cellular functions for cell synthesis). This was observable for all yield co-efficients calculated in our study.

During the interrogation of YPP, YPO and YPS data, it was noted that an increase in CSL supplementation resulted in a decrease in yield (Figure 4.5 a, b and c) with maximum yields noted at 5 $g.l^{-1}$ (Figure 4.5 b and c). Similar to the results obtained in this *V. midae* investigation, Champagne *et al*. (1990) also demonstrated the inhibitory effect of CSL concentration on yields of lactic acid bacteria when CSL was supplemented at high concentrations. Yield of *V. midae* cells based on oxygen consumption, conformed to a second order polynomial quadratic function ($R^2 > 0.94$) when plotted against the range of CSL concentrations tested (Figure 4.5 c). At the opposite extreme, medium containing CSL at a concentration of 40 $g.l^{-1}$, resulted in extremely poor yields on carbohydrate, protein and oxygen. (Figure 4.5 a, b, c).

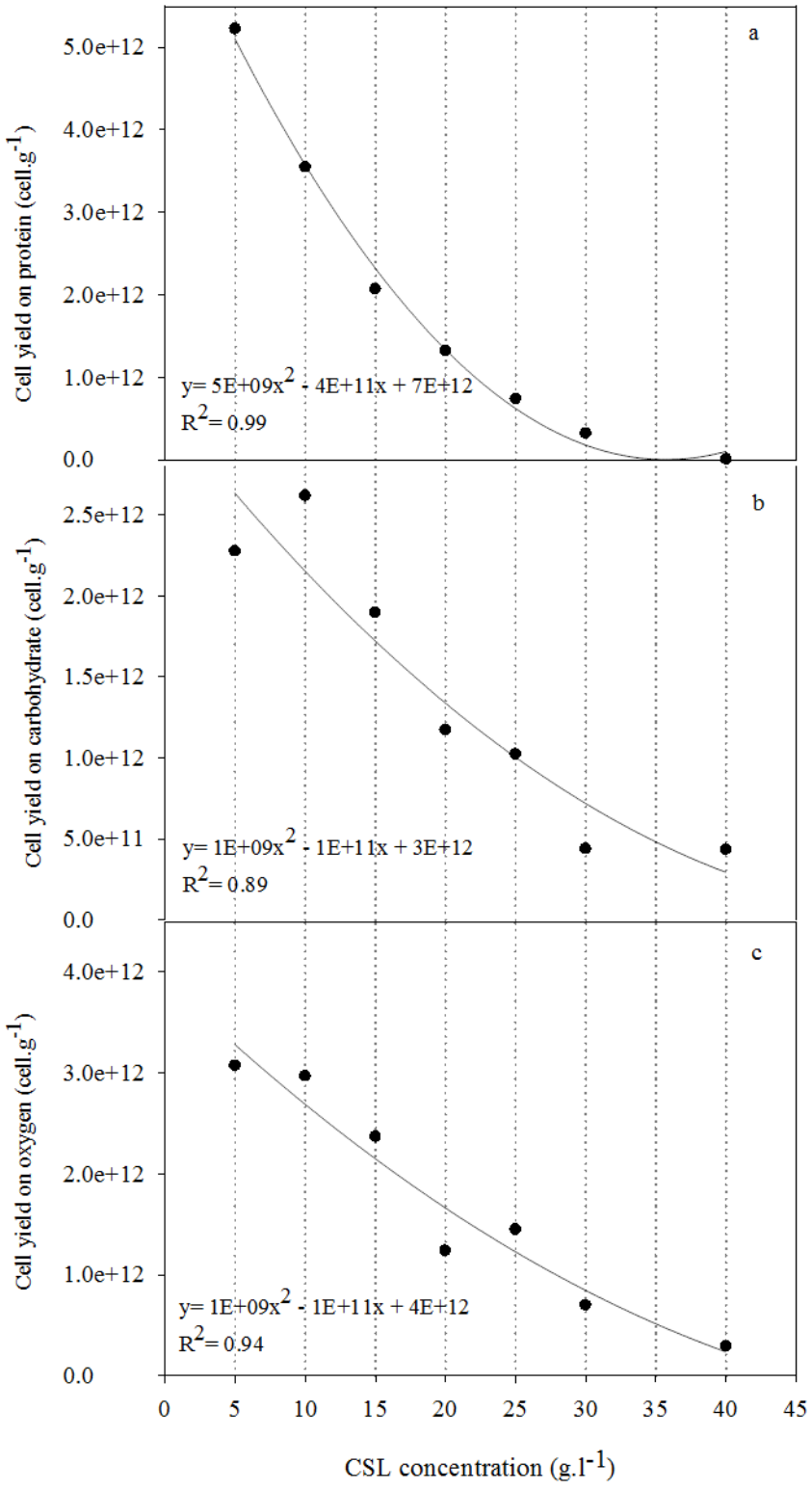

Figure 4.5. *V. midae* cell yield on (a) protein, (b) carbohydrate and (c) oxygen, when cultivated in media containing varying CSL concentrations in 10 L laboratory scale bioreactors at 30ºC and pH 6.5.

4.3.3 Mathematical optimization of CSL concentration for the cultivation of *V. midae*

The modelling of data from the six responses obtained during the cultivation of *V. midae* in medium containing CSL provided valuable input into determining the optimum CSL concentration for the growth of *V. midae*. Each response (cell productivity, cell concentration and cost of production), and their resultant mathematical equations, were simultaneously solved using Design Expert ® in order to predict the optimum CSL concentration for the cultivation of *V. midae.* This technique allows the weighting of responses and the prediction of an optimum using all of the responses in an interactive mathematical model. Cell yields on protein, oxygen and carbohydrate from the resultant data were also examined, as these are key cost and performance drivers of the production process (Figure 4.6). These process indicators were not optimized for this study and were designated to be in range. This optimization rationale was also used by Tellez-Luis *et al*., (2003), who selected concentration, productivity and economic productivity (cost of production) as key variables to optimize CSL concentration in growth medium for the production of lactic acid by *Lactobacillus delbrueckii*. The authors stated that when designing suitable production processes, it is not always necessary to maximize product concentration (*V. midae* cells in our study), but to confer higher importance to technological and economic variables, such as rate of production (cell productivity) and cost of production.

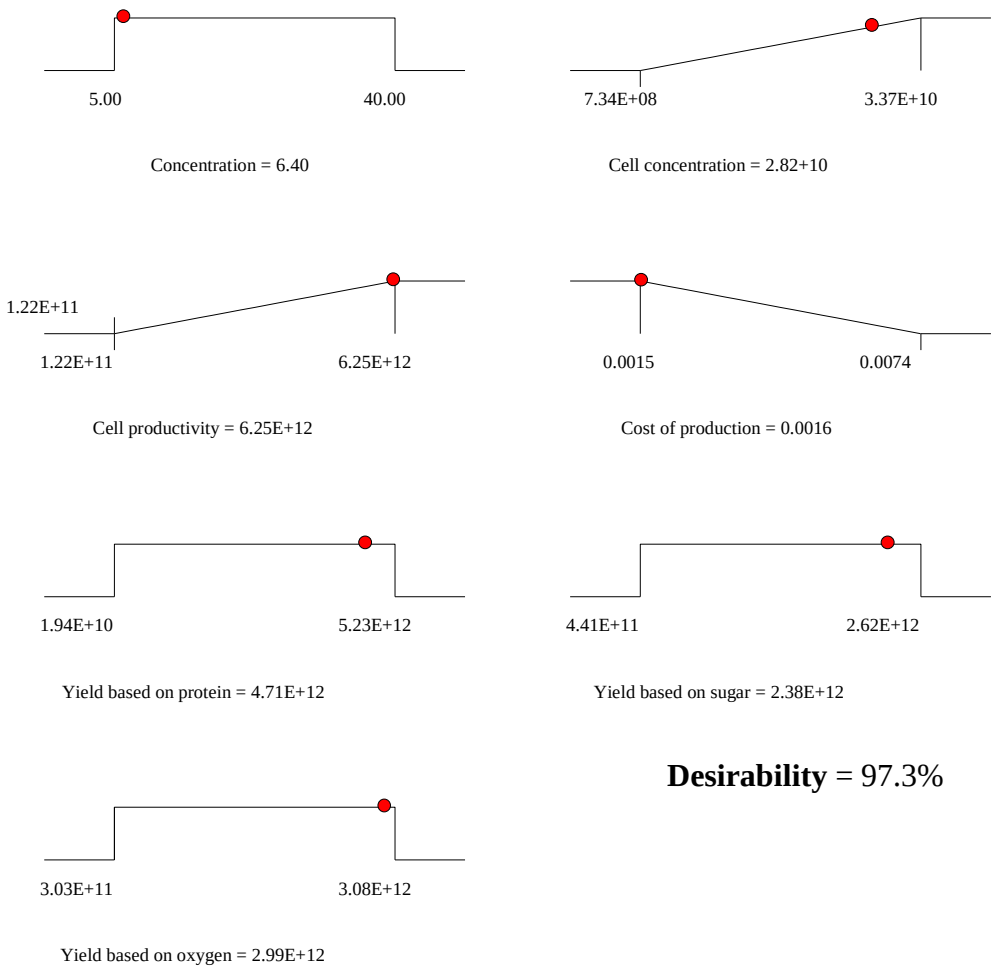

Figure 4.6. Design expert® illustration depicting ramps of key responses used to determine the optimum CSL concentration for growth of *V. midae.*

The derived optimum using all six of the responses resulted in a cubic model, with a 97% confidence level (desirability co-efficient). The mathematically predicted optimum CSL concentration was 6.4 $g.l^{-1}$ (Figure 4.6). It was noted that all regression co-efficients of the six individual responses against CSL concentration and the combined model all exceeded 0.89, which provided the statistical basis for acceptance of the model predicted optimum CSL concentration. As our trends were asymptotic towards zero CSL supplementation concentration, we tested growth in a separate study

to verify if growth would be substantial without CSL supplementation. No significant growth was observed (p= 0.44, n= 4), when comparing initial and final cell concentrations in our study without CSL supplementation, thus indicating that our model predicted optimum was credible.

4.3.4. Validation of the mathematically predicted optimum CSL concentration

Due to the theoretical nature of mathematical predictions, a more rigorous approach to validate this finding was performed in actual batch cultivations (n = 3) at the model predicted optimum CSL concentration of 6.4 $g.l^{-1}$. This study resulted in a correlation of > ~86% between all responses obtained from actual replicate cultivation studies (Table 4.1).

A maximum cell concentration of $3.54 \times 10^{10} \pm 2.53 \times 10^{9}$ $cells.ml^{-1}$ was observed when *V. midae* was cultivated in a medium containing 6.4 $g.l^{-1}$ CSL after 4.5 h (Table 4.1). The comparative data produced relatively low standard deviations between the mathematically predicted optimum and the actual fermentation data (Table 4.1). The findings of this study were statistically evaluated at the 95% confidence level. A standardized Student T-Test's (two tailed; equal variance) further confirmed a lack of significance in difference between the two data sets for two of the responses of interest at the 95% confidence level (cell concentration p= 0.20, cell productivity p= 0.09 and cost of production p= 0.29).

Table 4.1. Validation model predicted key responses measured during the cultivation of *V. midae* in medium containing 6.4 $g.l^{-1}$ CSL (*n* =3)

Factor	Unit	Modelled response	Actual data	CV (%)
Cell concentration	$cells.l^{-1}$	3.31×10^{10}	3.54×10^{10}	4.81
Cell productivity	$cells.l^{-1}.h^{-1}$	7.16×10^{12}	7.86×10^{12}	6.56
Cost of production	$R.l^{-1}$	0.0014	0.0012	8.56
Yield on protein	$cells.g^{-1}$	5.40×10^{12}	5.87×10^{12}	5.88
Yield on sugar	$cells.g^{-1}$	2.83×10^{12}	2.45×10^{12}	10.22
Yield on oxygen	$cells.g^{-1}$	3.57×10^{12}	4.32×10^{12}	13.45

When using agro-industrial residues such as CSL, it is imperative to determine the optimal nutrient substrate concentration in order to achieve best process performance and productivity (Treichel *et al*., 2009). Upon completion of this study, it was observed that overall increases in cell yield on protein (74.3%), cell yield on sugar (54.0%) and cell yield on oxygen (66.0%) were achieved in comparison to the initially used modified *Vibrio* medium (MVM), which contained laboratory based nutrient sources. The determination of optimum CSL concentration resulted in an approximately 10% decrease in overall process time as well as a decrease in cost of production. At an optimum concentration of 6.4 $g.l^{-1}$, the cost of producing a litre of 1×10^7 cells was determined to be R 0.0012, which was 78.0% lower than the MVM medium.

Limited information is available on the development of production processes for *Vibrio* organisms, as well as the use of agro-industry waste residues, as these organisms are not widely produced commercially. As a result, direct comparative assessment of our production process could not be made within the genus of interest. However, extensive literature assessments revealed that CSL has been considered as a suitable fermentation feedstock due to its availability and reasonable cost in several other fermentation processes (Formanek *et al*., 1998; Parekh *et al*., 1999; Kona *et al.*, 2001). Many other researchers optimised CSL concentration and also concluded that the utilization of media components with low costs, such as CSL, at optimum concentration for the growth of a particular organism, leads to a reduction in the overall cost of production due to increases in product yields and higher productivity (Formanek *et al*., 1998; Parekh *et al*., 1999; Kona *et al*., 2001; Kim *et al*., 2002). This contributed to the economic attractiveness of their industrial production processes of interest. Similarly, our study has shown the positive impact of using CSL for the cultivation of *V. midae* and the value of optimizing CSL concentration towards a commercial production process for this probiotic.

CHAPTER 5: EVALUATION OF HIGH TEST MOLASSES (HTM) AS A SUITABLE SUBSTRATE FOR THE CULTIVATION OF V. MIDAE

5.1. INTRODUCTION

The production of industrially relevant microorganisms such as *V. midae*, an abalone probiotic of commercial interest, involves the development of various aspects of the production process. Thus far, research has been performed to determine optimum physiological parameters such as pH and temperature (chapter 3); and the evaluation of using of corn steep liquor (CSL) as a nutrient source in the cultivation medium (chapter 4). These investigations have greatly increased the cell concentration and productivity while minimizing the overall cost of production of this *V. midae* probiotic. The final stage of the upstream process development was focused on evaluating the use of High Test Molasses (HTM) as a lower cost carbohydrate source.

HTM is a locally available, cost competitive substrate, and has been used in a wide array of industrial production processes (Lawford and Rousseau, 1997, Gouda *et al*., 2001, Kona *et al*., 2001, Burkert *et al*., 2004, Srivastava et a., 2015). Maize and sugar cane are the two foremost crops of production in the Republic of South Africa (RSA). Current production reports revealed that 19.9 million tonnes of maize and 11.5 million tonnes of sugar cane are cultivated per annum (DAFF, 2012). Sugar cane molasses and corn steep liquor (CSL) are the waste products generated from the sugar and corn agro-industries respectively. We have already demonstrated the suitability of the maize

based CSL, as a nutrient source and optimized the concentration for the production of *V. midae* (chapter 4). Our study showed that it offered an attractive alternative to conventionally used nutrient substrates due to its availability, lower cost and associated performance improvements in the fermentation process.

Similarly, various sugar mills in South Africa produce molasses as a by-product during sucrose production. Tongaat Hulletts sugar mills in particular, generate high test molasses (HTM) as a process by-product, and trade the substrate as Treacle No 1, to offset sugar production costs. HTM is a by-product that is left over after the sugar is removed from the mother syrup, and is usually quite viscous. It is however, a purer, more refined version of blackstrap molasses because the impurity level is lower, therefore offering better mass transfer in fermentation processes than blackstrap molasses. The substrate is known to contain a mixture of glucose, sucrose and fructose, and an abundance of vitamins and other growth factors which are required for microbial growth (Malathi and Chakraborty, 1991, Farooq *et al*., 2012, Panesar *et al*., 2015, Srivastava *et al*., 2015).

The previously used MVM medium for the production of *V. midae* contained CSL as a nutrient source and glucose as a sole carbohydrate source (as developed in chapter 4). The type of carbohydrate source is known to influence the productivity of a production process as it provides energy for the formation of biomass and metabolites, with different organisms demonstrating different preferences to carbohydrate type (Lopez *et al*., 2003). When designing commercially applicable bioprocesses for the production of bacterial cells, other relatively inexpensive carbohydrate sources need

to be investigated with a view to improve process performance and reduce the material cost of production. This chapter focused on the comparative assessment of HTM against selected commercially relevant pure sugars (glucose, fructose and sucrose), optimization of the HTM supplementation level and validation of the fermentation performance at the optimum level.

5.2 MATERIALS AND METHODS

5.2.1 Assessment of the suitability of HTM as an alternate carbohydrate source for the production of *V. midae*.

5.2.1.1 Inoculum preparation

A *V. midae* (LMG P – 27727) cryovial (2ml) containing approximately 2×10^9 $CFU.ml^{-1}$ viable cells was used to inoculate each 1L Erlenmeyer flask containing 200 ml growth medium (chapter 2, section 2.2.1). Following inoculation, flasks were incubated at 30°C and 180 rpm with shaking (Innova 2300, New Brunswick Scientific, Edison, NJ, USA) for 5 h. Cultures were harvested at mid-exponential phase (OD_{660nm} ~1.50) and used as inocula for all experiments. All materials used in this study were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

5.2.1.2 Comparative assessment of *V. midae* growth in medium containing HTM to medium containing glucose, fructose or sucrose as a carbohydrate source

Cultivation of *V. midae* was performed in 2 L Braun Biostat B fermenters (Sartorius BBI Systems, Melsungen, Germany) at a working volume of 1.7 L. The MVM medium

containing CSL (6.4 $g.l^{-1}$) developed in chapter 4 was prepared to a volume of 1500 ml in the fermenter. Glucose, sucrose, fructose or HTM were prepared to result in a final fermentation concentration of 10 $g.l^{-1}$ Total Sugar As Invert (TSAI). The sugar substrates to be tested were sterilized separately (121ºC, 20 min) and subsequently added to the respective test fermenter for each carbohydrate substrate. The pH of the medium was adjusted to 6.5 with 25% $m.v^{-1}$ NH_4OH and then similarly maintained post sterilization (121ºC, 45 min). The cultivation temperature was set at 30ºC, airflow was maintained at 1 $v.v^{-1}.m^{-1}$ and agitation was ramped from 300 rpm upwards to a maximum of 1000 rpm to maintain dissolved oxygen saturation at approximately 30%. The inoculum (200 ml) was aseptically added into each bioreactor. The stop signal for these cultivations was a decline in total cell number observed during the at-line microscopic counting of cells.

5.2.1.3 Sampling, analysis and calculations

Each bioreactor was sampled on an hourly basis and samples analyzed for cell concentration, determined by the microscopic counting of bacterial cells using a Thoma® counting chamber (Hawksley and Sons, London), as described previously. Cell productivity was expressed as the rate change in total cell concentration, determined from the microscopic enumeration of cells over time. Glucose, fructose and sucrose concentrations were determined using an HPIC system. The system consisted of a Dionex GP40 pump fitted with a TSP AS Autosampler and a Dionex ED40 electrochemical detector. The compounds were separated using a CarboPac™ PA1 column, (Dionex, MA, USA) at ambient temperature. NaOH (150 mM) was used as the mobile phase at a flow rate of 1.20 $ml^3.min^{-1}$. Quantification was performed

using standard curves and internal standards for glucose, fructose and sucrose (Lalloo *et al*., 2009).

5.2.2. Determination of optimum HTM concentration to increase cell concentration, cell productivity and reduce process costs

5.2.2.1 Inoculum preparation

A cryopreserved culture (2ml) of *V. midae* (LMG P -27727) was prepared according to methods outlined in chapter 2, and inoculated into 700 ml of inoculum medium (chapter 2; section 2.2.1) contained in a 2 L Fernbach flask. The pH of the medium was adjusted to 6.5 using 25% NH_4OH, prior to sterilization at 121^{o}C for 20 minutes. Flasks were allowed to cool to ambient temperature and were then inoculated. Culture flasks were incubated at 30^{o}C with shaking at 180 rpm on a rotary platform shaker (Innova 2300, New Brunswick Scientific, USA) for 5 h. Once a transfer OD of 3.5 was reached, flasks were checked for monoseptic status, microscopically under 1000 x magnification using an Olympus BX40 microscope (Olympus, Japan), and aseptically transferred into the vessel.

5.2.2.2 Bioreactor studies

A range of HTM concentrations (1g.l^{-1}, 5 g.l^{-1}, 15 g.l^{-1}, 25 g.l^{-1} and 40 g.l^{-1}) were evaluated in 15 L Biostat Braun C fermenters (Sartorius BBI systems, Melsungen, Germany) with a working volume of 10 L. The growth media in each fermenter consisted of the medium resulting from the CSL optimization studies in chapter 4 (MVM, containing 6.4 g.l^{-1} of CSL).

All fermentation studies were conducted in 15 L Biostat Braun C fermenters (Sartorius BBI systems, Melsungen, Germany) with a working volume of 10 L. The salts, antifoam (1 $ml.l^{-1}$, Pluriol P2000, BASF, Ludwigshafen, Germany) and nutrient sources were added to the initial charge and made up to a volume of 9.3 L. Subsequent to the *in-situ* sterilization of the initial charge, a separately sterilized HTM solution (prepared to the desired concentration at a sugar feed purity of 55% $m.m^{-1}$ TSAI based on product certificate of analysis (CoA)) was added. The fermentation temperature was maintained at 30°C and the stirrer speed was set at 500 rpm and ramped to 1000 rpm, to maintain the dissolved oxygen above 30% saturation. The pH was maintained at 6.5 using 25% $v.v^{-1}$ NH_4OH and aeration was set at 1 $v.v^{-1}.m^{-1}$. All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise stated. The stop signal for these fermentations was indicated by a decline in the oxygen utilization rate measured on-line and then confirmed by decline in cell growth based on microscopic cell counts as described in chapter 4, section 4.2.6.

5.2.3. Data processing, mathematical optimization and validation of model predicted optimum

Each of the key responses (cell concentration, cell productivity, cost of production and yield co-efficients on protein, oxygen and carbohydrate) in response to change in HTM concentration, were statistically analysed to determine appropriate fits to quadratic or cubic models. Numerical optima integrating all three responses using their respective mathematical equations were determined by using the optimization

function of Design Expert-6 software (Stat-Ease, Minneapolis, USA), excluding responses where R^2 values were less than 0.90 for any of the model fits. This allows the determination of the optimum HTM concentration taking into consideration all of the key responses. Cell productivity was assigned the highest priority (level 5) of the three responses used in the mathematical modelling. Cost of production (material cost plus cost of capital) and cell concentration were assigned an equal but lesser priority (level 1). Product yields on protein, substrate and oxygen were not maximized for this study, however, were designated to be in range. Once the model predicted optimum HTM concentration was determined, triplicate fermentations batches were run as described in section 5.2.3 to validate the key responses at a HTM concentration of 24 $g.l^{-1}$ (mathematically predicted optimum HTM concentration). A two tailed ANOVA test was performed on actual and model predicted data to assess if the resultant data from actual fermentations were statistically similar to the model predicted performance at the optimum HTM concentration. Model predicted mean values and the resultant standard deviations were used to generate upper and lower limits for each of the modelled responses to enable the statistical comparison.

5.2.4. Sampling and analysis

The sampling of reactors was performed according to methods outlined in chapter 4, section 4.2.5

5.2.5. Data analysis and calculations

Data handling was performed according to methods described in chapter 4, section 4.2.6

5.3. RESULTS AND DISCUSSION

5.3.1 Assessment of the suitability of HTM as a replacement carbohydrate source for the production of *V. midae*.

Previous work conducted by Coyne (2009), showed that *V. midae* could utilize a wide range of carbohydrate sources including D-glucose, sucrose, D-fructose, D-galactose, xylose, D-mannose, maltose and cellobiose. In this study, a maximum cell concentration of 1.75×10^{10} cells.ml^{-1} at a productivity of 4.69×10^{9} cells.ml^{-1}.h^{-1} was achieved when *V. midae* was cultivated in the MVM medium containing glucose as a carbohydrate source (Figure 5.1). This cell productivity was 18% higher than the cell productivities achieved when *V. midae* was cultivated in a medium containing fructose and 33% higher than medium containing sucrose as sole carbohydrate sources respectively.

When organisms are cultivated in a medium containing various carbohydrate types, such as a mixture of hexose and pentose sugars, they demonstrate a hierarchy of sugar utilization based on their available cellular mechanisms (Bruckner and Titgemeyer, 2002). In the instance of *V. midae,* it can be determined that from the specific sugar utilization rates (data not shown), that the hierarchy of sugar catabolism, was in the order of glucose, fructose and lastly sucrose. Some organisms are not able to break down and utilize sucrose or more complex polysaccharides as they lack the required metabolic machinery. In the instance of Vibrio species, Scholle *et al*., (1987), stated that these organisms are able to utilize sucrose, however, it first needs to be transported into the cell through a Na^{+} electrochemical potential mechanism. Thereafter, it can be

hydrolysed intra-cellularly to form glucose and fructose prior to it being metabolized by the organism, thereby resulting in a lower sugar utilization rate compared to simple monosaccharides. Their explanation is coherent with our observation that the utilization rate of the di-saccharide, sucrose, was slowest compared to the monosaccharides glucose or fructose.

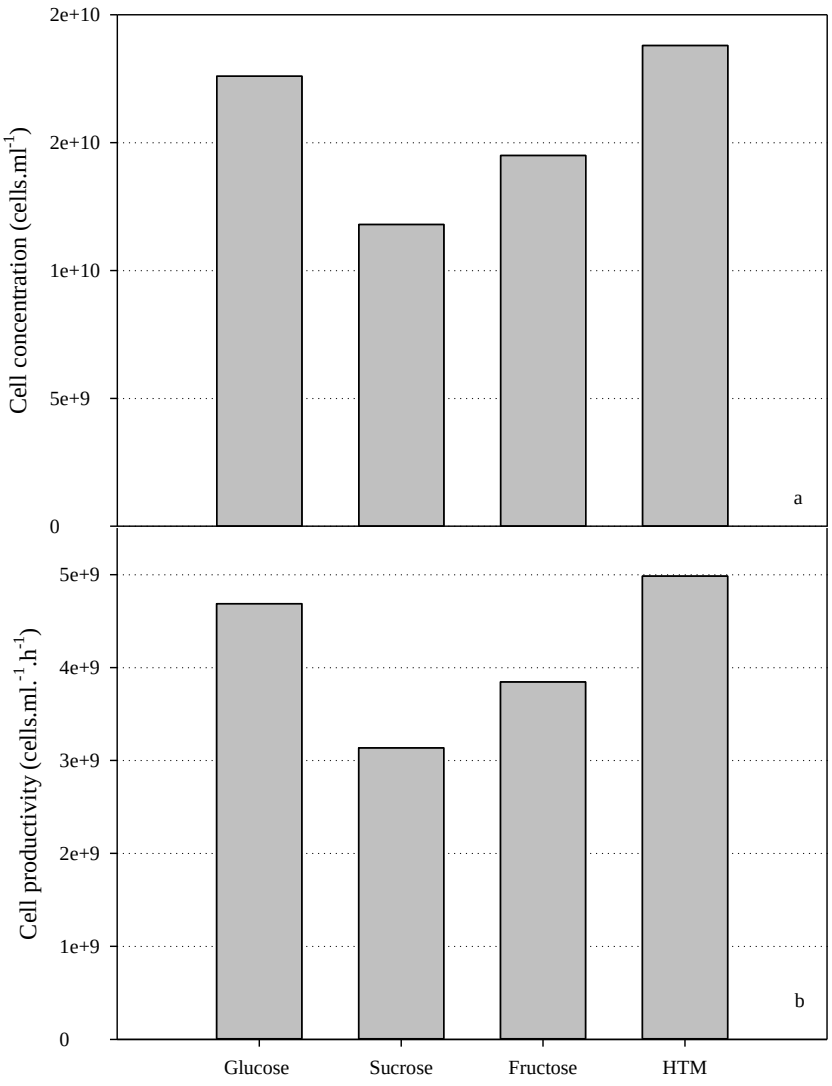

Figure 5.1. Key responses of cell concentration (a) and cell productivity (b) measured during the cultivation of *V. midae* in medium containing various carbohydrate

substrates supplemented to 10 $g.l^{-1}$ TSAI in 2 L laboratory-scale bioreactors at 30ºC and pH 6.5.

When *V. midae* was cultivated in a medium containing HTM, the highest cell productivity of 4.98×10^9 $cells.ml^{-1}.h^{-1}$ was achieved (Figure 5.1 b). The HTM used in this study was supplied as inverted, which contained mainly glucose and fructose in equal proportions with a lower proportion of sucrose (roughly in the ratio of 1:1:0.5 as per the supplier COA and verified by HPIC analysis). In addition, HTM is known be a rich source of trace elements and vitamins, which can boost process performance in comparison to pure carbohydrate sources such as glucose, sucrose or fructose tested in this study (Beaulieu *et al*., 1995). All test substrates used in the study were normalised to a final effective TSAI concentration of 10 $g.l^{-1}$. As a result, the superior performance observed in the HTM study can be attributed to the unique ratio of the sugars or the presence of nutrients found in HTM, such as vitamins, amino acids and trace elements.

V. midae showed the poorest performance when it was cultivated in a medium containing sucrose. A maximum cell concentration of 1.18×10^{10} $cells.ml^{-1}$ was achieved after 4 h of cultivation (Figure 5.1 a). It is likely that a similar performance would be observed if non-inverted HTM was tested, as the sugar form would be predominantly sucrose. Reports in literature does confirm that members of the Vibrio species have the ability to produce intra and extracellular invertase (Scholle *et al*., 1987), explaining their capability to use sucrose. However, this mechanism may cause the organism to grow at a slower rate due to the requirement of an extra metabolic step in using the disaccharide as a carbohydrate source in comparison to using monosaccharides such as glucose and fructose. It is expected that the metabolic energy

for sucrose utilization would be higher than that of the simpler monosaccharides thus resulting in a lower cell yield on sugar, ultimately reflected by a lower cell concentration. This was also observed in our pure sugar study, where *V. midae* cell productivity was 18.5 and 49.5% higher in fructose and glucose containing growth medium, respectively, in comparison to the cultivation medium containing sucrose.

5.3.2 Optimization of HTM concentration to maximise *V. midae* cell growth

Once the growth of *V. midae* was concluded to be favorable using HTM as a carbohydrate substrate in preliminary sugar selection tests, various concentrations of HTM (on TSAI basis) were tested in batch cultivations at 10 L scale to optimize the HTM supplementation concentration in MVM media. Literature regarding carbohydrate substrates used in the production of Vibrio is scarce, nevertheless high cell density cultivations (HCDC) of Vibrio species are commonly linked to similar characteristics observed during the HCDC of *Escherichia coli* (de Mare *et al*., 2003). Once such observation is the formation of acetate and other Krebs cycle overflow metabolites, as a result of being cultivated in media containing excess carbohydrate, resulting in certain metabolic bottlenecks, which can affect metabolic flux to cell replication (de Mare *et al*., 2003). For this reason, optimizing the sugar concentration in growth media becomes an important consideration in maximizing cell production of our probiotic.

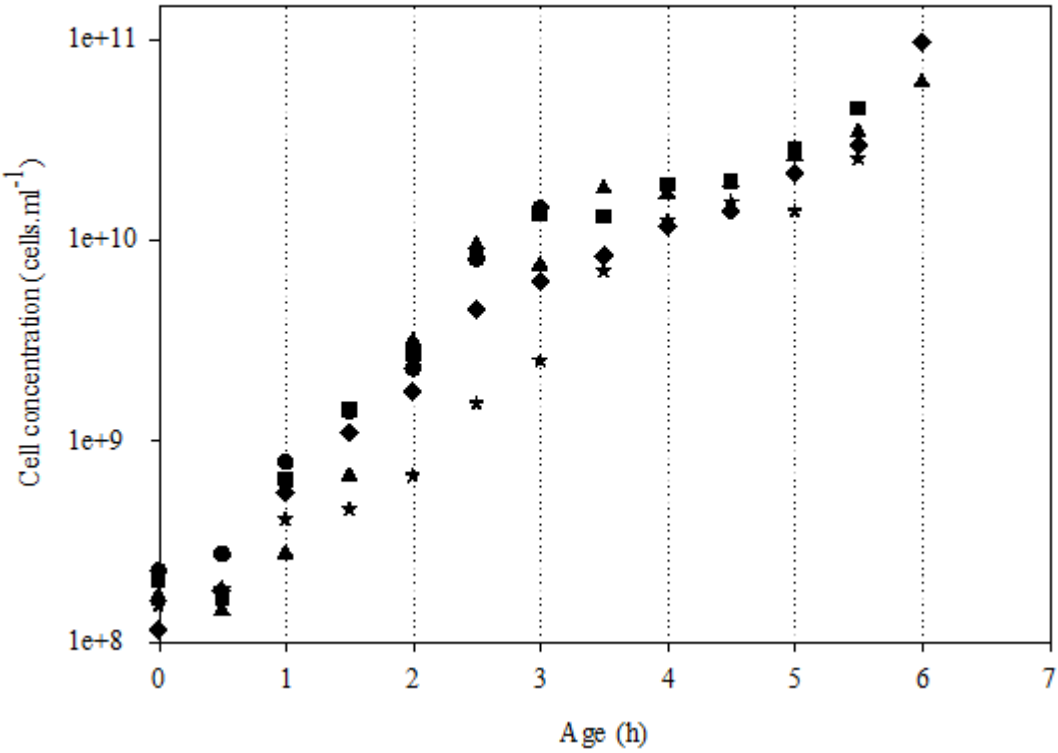

Figure 5.2. Total microscopic cell counts obtained during the cultivation of *V. midae* in medium containing HTM at varying concentrations ($g.l^{-1}$): 1 (●), 5 (■), 15 (▲), 25 (♦) and 40 (⁎).

When an initial charge concentration of 1 $g.l^{-1}$ TSAI was tested, a fermentation production time of 3 h was observed. This short growth time was attributed to the organism rapidly depleting the carbohydrate substrate in the culture medium. Interestingly, initial growth rate was highest, yet the cell concentration achieved was only 1.46×10^{10} $cells.ml^{-1}$ (Figure 5.2). The higher initial growth rate can be explained by reduced osmotic load in the initial charge media (due to low HTM concentration), whilst the lower cell concentration is purely due to carbohydrate substrate depletion. At HTM concentrations exceeding 1 $g.l^{-1}$, there was an increase in cell concentrations of *V. midae* up to a maximum HTM concentration of 25 $g.l^{-1}$. At this HTM

concentration, the maximum cell concentration was 9.72×10^{10} cells.ml^{-1} after 6 h of cultivation (Figure 5.2 and 5.3). At an HTM concentration of 40 g.l^{-1}, a drastic decline in cell concentration was noted (Figure 5.2 and 5.3). This decline in growth rate could be attributed to osmotic load from high sugar concentration and the low cell concentration achieved attributable to the formation of overflow metabolites such as acetate, as a result of maximum carbon flux being directed away from biomass production to waste metabolites. Although we did not measure acetate production, other studies have described this mechanism as an explanation for poor cell yield under sugar excess conditions (Kleman and Strohl, 1992; de Mare *et al*., 2003; Navratil, *et al*., 2005).

Key responses of cell concentration, cell productivity and cost of production were further analysed when plotted against the range of HTM concentrations tested (Figure 5.3). It was observed that with an increase in HTM concentration, there was a visible increase in *V. midae* cell concentration up to a maximum cell concentration of 9.72×10^{10} cells.ml^{-1} at an HTM concentration of 25 g.l^{-1}. At HTM concentrations above this point, a decline in cell concentration was noted. This trend conformed to cubic model with an $R^2 > 0.93$. (Figure 5.3 a). Similarly, cell productivity also increased up to an HTM concentration of 25 g.l^{-1}, thereafter, a decrease in productivity was noted (cubic model, $R^2 > 0.93$). A maximum cell productivity of 1.62×10^{13} cells.l^{-1}.h^{-1} was observed at this concentration (Figure 5.3b).

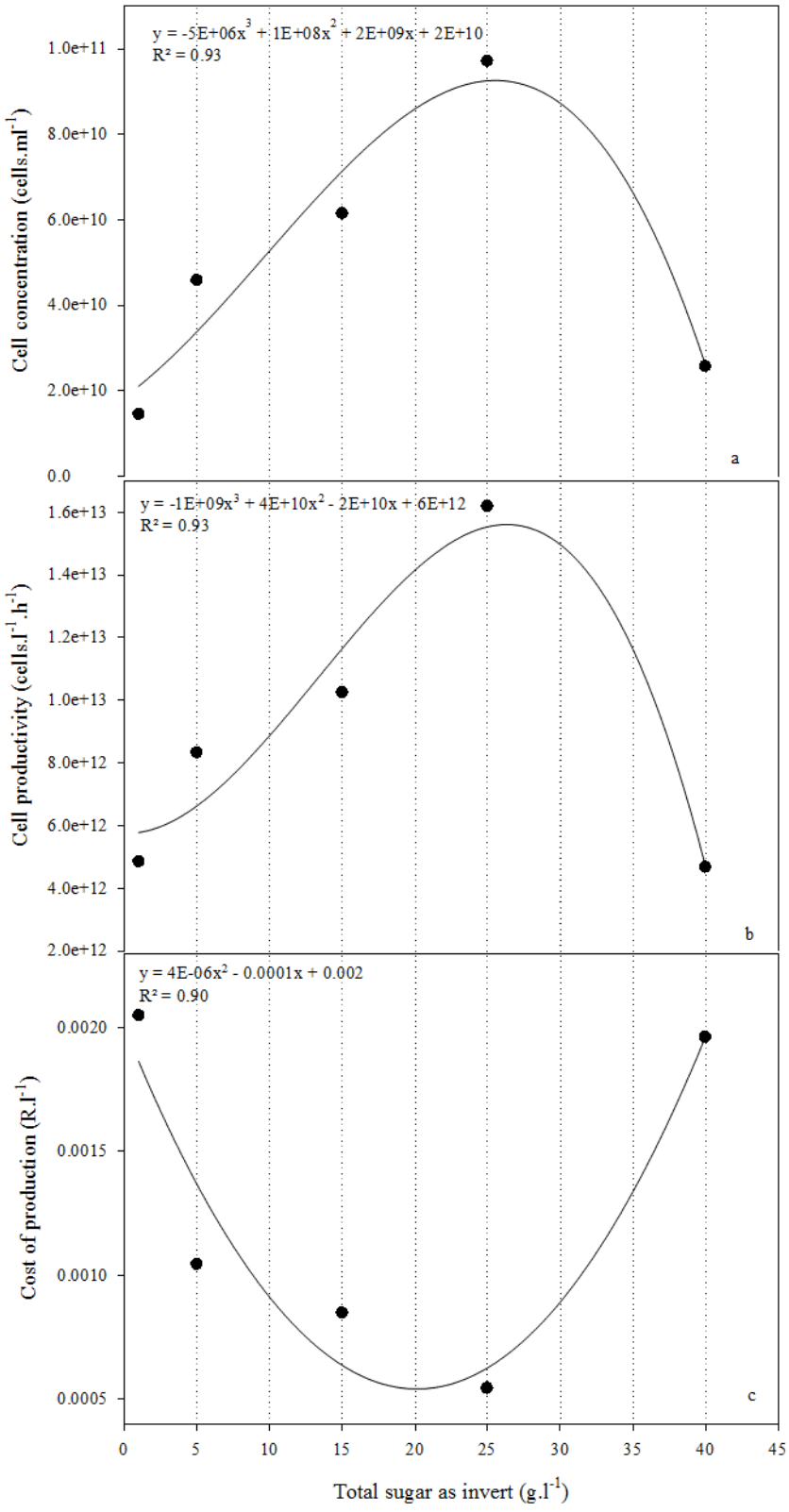

Figure 5.3. Key responses of cell concentration (a), cell productivity (b) and cost of production (c) measured during the cultivation of *V. midae* in medium containing

various concentrations of HTM in 10 L laboratory-scale bioreactors at 30^{o}C and pH 6.5.

When the cost of production was evaluated, an increased cost of production at the minimum and maximum HTM test concentrations (1 and 40 g.l^{-1}) was noted (Figure 5.3 c). This is expected as both cell concentration and productivity were low at these extremes, thus influencing the cost negatively. Reduced costs were noted at HTM concentrations of 5 – 25 g.l^{-1}, with the lowest cost of R 0.0005 per litre of 1×10^{7} cells.ml^{-1} obtained at a HTM concentration of 25 g.l^{-1} (Figure 5.3 c).

The supplementation of HTM to the cultivation medium, in place of glucose, more than halved the cost of production (2.3 fold) in comparison to the MVM medium, and a 15- fold cost reduction in comparison to the original marine broth (MB) medium used at the onset of the research project.

Cell yields based on protein and oxygen conformed to cubic model fits ($R^2 > 0.93$ and $R^2 > 0.97$ respectively) (Figure 5.4 a), and mimicked similar patterns for cell concentration and cell productivity trends in response to HTM concentration. At the low and high extremes of HTM concentration, cell yield on protein or oxygen were negatively impacted due to the lower cell concentration achieved for the same amount of protein supplemented or oxygen provided in the fermentation batch. Both yield on protein and oxygen increased as HTM concentration increase from 5 g.l^{-1} to 25 g.l^{-1} (Figure 5.4).

When cultivated in an optimum medium, the metabolic machinery of the organism performs at an optimal rate using the available substrate to generate biomass. Therefore, the optima for the production indicators correlate well with the optima for the yield on protein and oxygen (~25 $g.l^{-1}$ TSAI concentration). It was also observed that both YPO and YPP data was not optimal at the minimum and maximum TSAI concentrations tested as cellular respiration and protein flux to cell production was not taking place at the optimal rate. As HTM (TSAI) concentration was increased above 25 $g.l^{-1}$, neither the yield on oxygen or protein increased, as cell production was seriously hampered by the low growth rate and final cell concentration.

Cell yield on carbohydrate resulted in a second order polynomial function, with a maximum cell yield of 1.55×10^{10} $cells.ml^{-1}$ during *V. midae* growth in medium containing 1 $g.l^{-1}$ of HTM. (Figure 5.4 b). The data indicates that cell production efficiency on carbohydrate is at its maximum under near starvation conditions and decreases with increasing concentrations of carbohydrate in the media. This can be expected and is quite typical for yield of carbohydrate in several fermentation processes due to overflow metabolism, which directs excessive carbon flux into cellular respiration, saturates the tricarboxylic acid (TCA) cycle or electron transport chain, ultimately resulting in reduced growth rates and productivity of both biomass and product yields (Lee *et al*., 1995, Navrátil *et al*., 2005). Although the yield of cells is highest, when the sugar concentration is lowest, there is a playoff between sugar conversion efficiency and more important parameters such as productivity and cell concentration. Therefore, fermentation process optimization must explore various responses in an integrated way to derive commercially relevant optima.

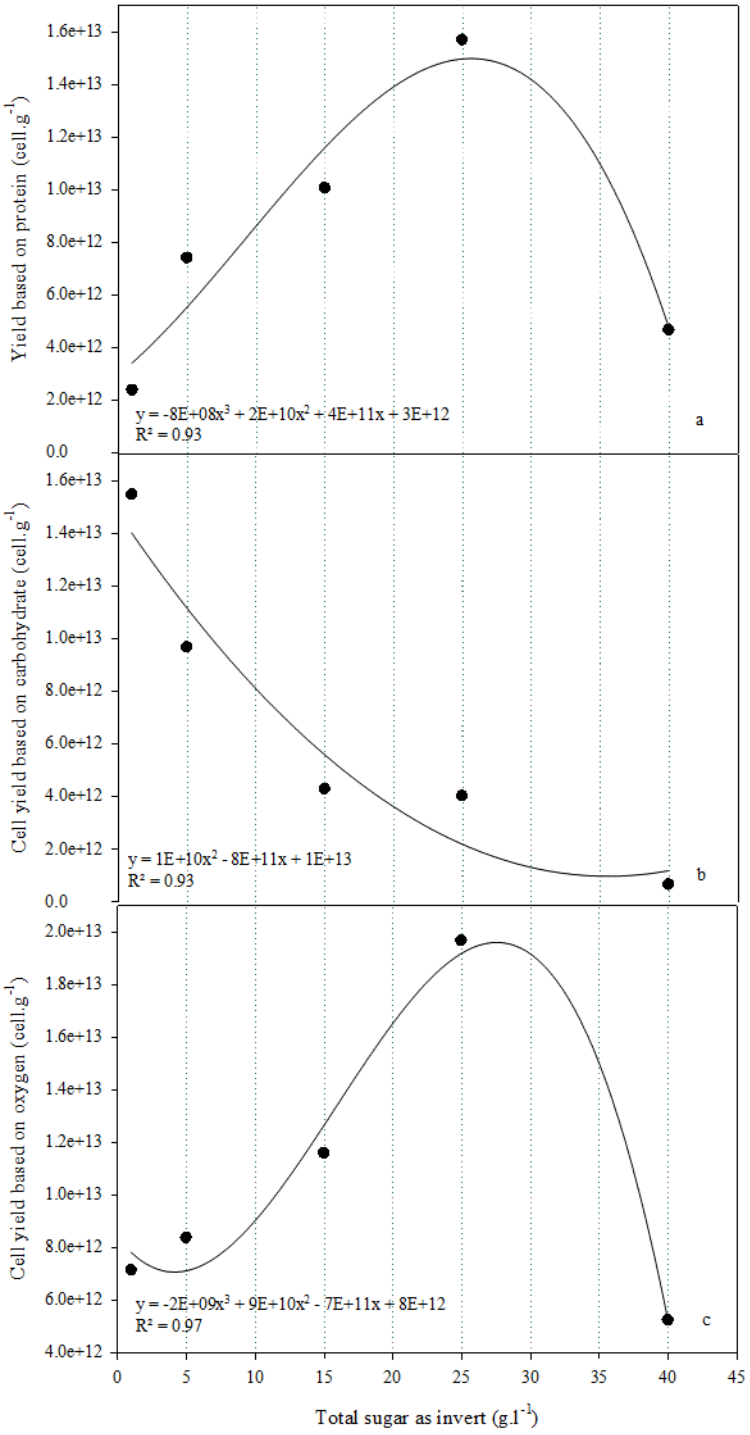

Figure 5.4. *V. midae* cell yield on (a) protein; (b) carbohydrate and (c) oxygen, when cultivated in media containing varying HTM concentrations in 10 L laboratory scale bioreactors at 30°C and pH 6.5

5.3.3 Data processing, mathematical optimization and validation of model predicted optimum

The data generated from this study served to generate a model used to optimize the concentration and productivity of *V. midae* cells, and subsequently minimize the cost to commercially produce this probiotic culture. Each response (cell productivity, cell concentration and cost of production), and their resultant mathematical equations, were simultaneously solved using Design Expert ® software in order to predict the optimum HTM concentration for the cultivation of *V. midae.* This technique allows the weighting of responses and the prediction of an optimum using all of the responses in an interactive mathematical model. Cell yields on protein, oxygen and carbohydrate from the resultant data were also examined as these are key cost and performance contributors to the production process (Figure 5.5). All data obtained within the HTM concentration range of 1 to 40 $g.l^{-1}$ was statistically analysed for each response. Cell productivity was given the highest weighting (level 5), because this response is the commercial indicator of production potential and it directly influences the commercial feasibility of the process, whereas cell concentration and cost of production were weighted equally, but at a lower level. Nancib *et al*., 2005 stated that in order to make biological production processes economically viable, it is imperative to simultaneously optimize process yields and minimize cost. It was noted that the derived optimum using all six of the responses using the optimization function of Design Expert-6 software (Stat-Ease, Minneapolis, USA) resulted in a cubic model, with a 94% confidence level (desirability co-efficient) (Figure 5.5). The mathematically predicted optimum HTM concentration was 24 $g.l^{-1}$. It was noted that all regression co-efficients of the six individual responses against CSL concentration

and the combined model all exceeded 0.90, which provided the statistical basis for acceptance of the model predicted optimum HTM concentration.

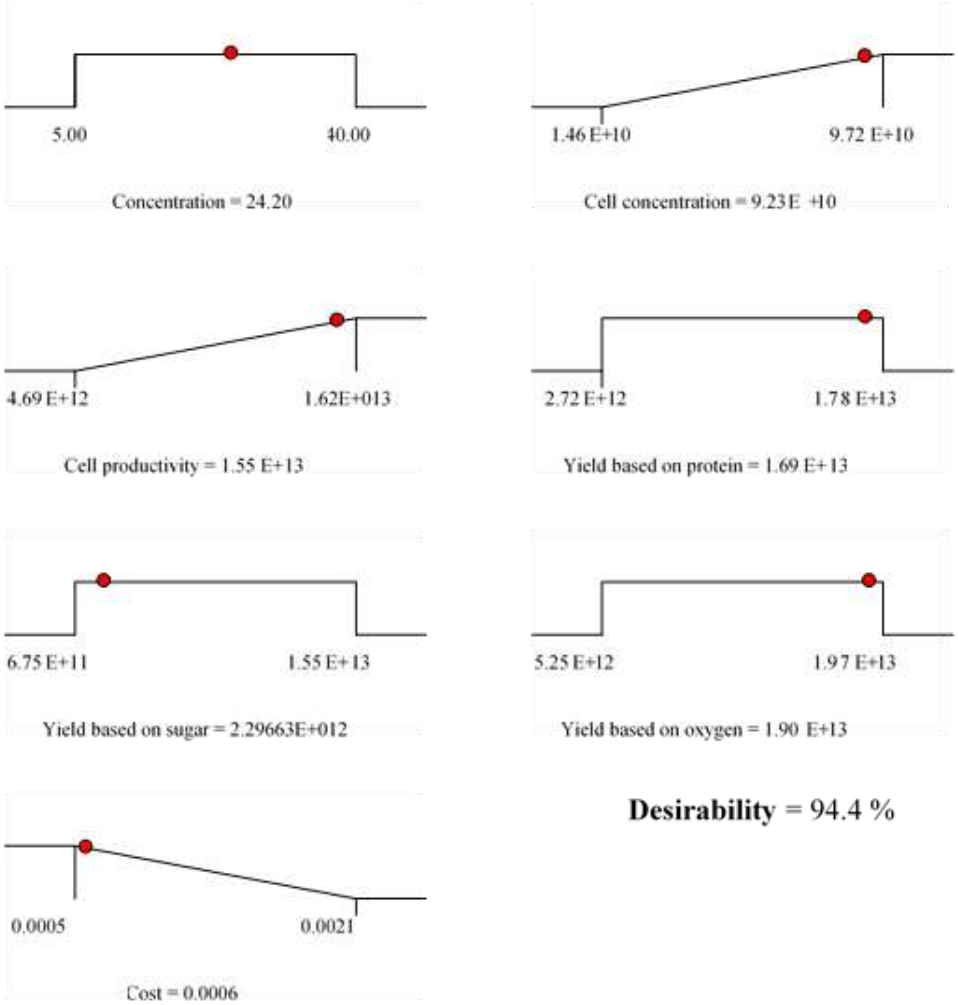

Figure 5.5. Design expert® illustration depicting ramps of key responses used to determine the optimum HTM concentration for growth of *V. midae*

5.3.4 Validation of the mathematically predicted optimum HTM concentration

The modelling of the six key responses obtained during the cultivation of *V. midae* in a MVM medium predicted an optimum HTM concentration of 24.0 $g.l^{-1}$. It was important to verify the model predicted optimum in actual bioreactor studies, to conclude the improvements achieved in process performance to this point. A

maximum optical density of 24.7 ± 1.85 and cell concentration of $7.52 \times 10^{10} \pm 7.88 \times 10^{9}$ cells.ml^{-1} was observed when *V. midae* was produced in a medium containing 24 g.l^{-1} HTM after 6 h of cultivation (data not shown).

Table 5.1. Validation model predicted key responses measured during the cultivation of *V. midae* in medium containing 24.0 g.l^{-1} HTM (*n* =3)

Factor	Unit	Modelled response	Actual data	CV (%)
Cell concentration	cells.l^{-1}	9.21×10^{10}	7.25×10^{10}	14.29
Cell productivity	cells.l^{-1}.h^{-1}	1.54×10^{13}	1.37×10^{13}	8.40
Cost of production	R.l^{-1}	0.00060	0.00064	4.43
Yield on protein	cells.g^{-1}	1.69×10^{13}	1.22×10^{13}	5.88
Yield on sugar	cells.g^{-1}	2.40×10^{12}	3.28×10^{12}	21.85
Yield on oxygen	cells.g^{-1}	1.89×10^{13}	1.88×10^{13}	7.96

The comparison of model predicted data and actual data resulted in a correlation of > 78% between all responses and produced relatively low standard deviations (Table 5.1). The findings of this study were statistically evaluated at the 95% confidence level. A standardized Student T-Test's (two tailed; equal variance) further confirmed a lack of significance in difference between the two data sets for two of the responses of interest at the 95% confidence level (cell concentration p= 0.10, cell productivity p = 0.18 and cost of production p = 0.42). This stage of work confirmed the use of HTM

as a cheap, locally available alternative to glucose, which increased productivities and *V. midae* cell concentration.

It is known that the cost of production of probiotics, for use in aquaculture and other animal rearing systems, must be minimal in order to increase the chances of commercial adoption. In order to achieve a cost effective bioprocess, it is imperative that cell production efficiency must be maximized (Jankovic *et al*., 2010).

Most importantly, the attractiveness of this microbial production process was enhanced because of the lowered process costs (86% lower) achieved through our research. In addition to lowering process cost, the use of the agricultural waste residues such as HTM and CSL provide a more effective means of waste utilization. These studies have demonstrated the efficacy and versatility of using both CSL and molasses as a bioprocess feedstock for the cultivation of *V. midae*. The supplementation of MVM medium with CSL (6.4 $g.l^{-1}$) and HTM (24 $g.l^{-1}$) further lowered the cost of production by 48% in comparison to the MVM with CSL alone. Aside from the economic implications of using lower cost nutrient substrates, and the optimization thereof, a maximum cell productivity of 1.37×10^{13} $cells.l^{-1}.h^{-1}$ was achieved. At this stage of the cultivation, the *V. midae* cells were able to successfully utilize HTM as a nutrient source, and convert the carbohydrates into biomass. Due to the drastically reduced process time, more fermentation cycles could be performed, thereby maximizing capital utilization as the maximum amount of cells were produced in the shortest period of time. This efficient utilization will also positively influence scheduling of other downstream process considerations such as cell separation,

formulation and packaging to occur concurrently, maximizing the efficiency of the overall production process.

Comparative assessments on process performance were difficult to carry out due to the absence of published data on production processes for *V. midae*. Although a comprehensive literature assessment was conducted in an attempt to perform a comparative assessment of our production process to other related studies, no related information was found directly relevant to Vibrio species production. Some examples of the use of agricultural waste residues such as CSL and HTM for the production of lipases (Potumarthi *et al*., 2008); mannitol (Saha, 2006); bacterial nanocellulose (Jung *et al*., 2010); biopesticide (Bt) (Jouzani *et al*., 2015) amongst others, showed that process performance was superior when CSL and HTM were added to the culture medium in place of more commonly used nutrient sources like peptones, yeast extracts and glucose. Due to the novelty of our process, a patent (PA155804/PCT) encompassing the production process was filed by our research group in January 2014 (Lalloo *et al*., 2014).

CHAPTER 6: DEVELOPMENT OF A PROCESS FOR THE SEPARATION OF *V. midae* CELLS FROM THE CULTURE BROTH

6.1 INTRODUCTION

Downstream processing (DSP) is an important aspect of developing biological production processes as it influences the product design, costs and therefore impacts the successful commercialization of products (Tsun, 1999; Rowe and Margaritis, 2004; Prabakaran and Hoti, 2007). The recovery and purification of fermentation products are essential to any commercial process, and the process steps depend on the nature of the desired end product (Shuler and Kargi, 1992).

Depending on the need, various DSP options are available, but selection of appropriate processing unit operations is informed by many considerations such as capital costs, processing costs, throughput requirements, yield potential, product quality, conformance to regulatory requirements, waste treatment needs, continuous or batch processing, automation, the complexity of operations, technical expertise available and personnel health and safety (Stanbury *et al*., 1995). Where the objective is to recover whole cells, as in the case of our abalone probiotic; there are various options that could be used for the separation of cells from the culture medium. However, the separation and purification of cells can be difficult and costly if the most suitable process is not selected (Stanbury *et al*., 1995). An ideal processing step generally will occur at a fast rate, have high recovery efficiencies, will require minimal capital investment and is

able to be operated at minimal life cycle cost (Stanbury *et al*., 1995). The main objective of this aspect of the downstream process technology is to maximise the overall process recovery, and also concurrently minimize overall product processing costs (Lalloo *et al*., 2010). In many instances, the costs associated with recovering a microbial product from a broth medium can be in the region of 15 to 70% of the total manufacturing costs (Atkinson and Sainter, 1982; Shuler and Kargi, 1992). Therefore, it is imperative to make astute decisions on the selection of the most suitable DSP process unit operation, in terms of the desired biological product being produced.

The most commonly used options for probiotic separation are either filtration or centrifugation (Stanbury *et al*., 1995). Due to the small size of microbial cells, filtration aids need to be used in order to improve filtration rates such that losses can be minimised. In many instances, if the correct filtration unit is not selected for use, it results in reduced separation efficiencies, and quicker fouling of membranes, thus resulting in a costly separation process, as several filters are required. This process has a higher operating consumable cost that other units. In order to reduce the frequency of filter fouling, filtration aids can be implemented, however, this may also impose a further challenge especially in terms of cost contribution. Sedimentation, flocculation, electrophoresis and di-electrophoresis, could have also been considered as process options, however, scale up of these processes can be challenging in terms of recovery and operational robustness (Stanbury *et al*., 1995). These alternate process options were not interrogated in this study due to the disadvantages at commercial scale.

Centrifugation provides a suitable tried and tested method of cell separation and is able to operate on a batch, continuous or semi-continuous basis, and was selected on this basis due to the scale of the process. The choice of centrifuge to be used in a process is dependent on the maximum capacity of the centrifuge as well as other characteristics such as speed of operation. These factors are important in order to perform at the planned production rate, with minimal manpower (Stanbury *et al*., 1995).

Due to the intended use of the product as a probiotic for aquaculture (relatively low value application), it is necessary to reduce the number of process unit operations. When recovering microbial cells as a product, the development of a suitable DSP stream must ensure that it not only addresses the aspects of ease of production and its associated costs, but also maintains viability of the whole cell preparation. It is important to mention, that although the development of an efficient bioprocess technology is largely dependent on the selection of the downstream process operations; published data on these developments are not readily accessible (Schisler *et al*., 2004; Brar *et al*., 2006). Probiotics for both human and animal use, are usually freeze dried and packaged accordingly. Although this DSP option of choice is an effective method of maintaining cell viability and product efficacy, freeze dried products are often expensive, as they have a high associated cost of production and require unique lyoprotectant agents to aid in their viability (Castex and Panes, 2012).

In view of the foregoing, this chapter focused on a cell separation process unit operation (PUO) used to remove *V. midae* cells from fermentation broth. This was achieved by evaluating the impact of flow rate on the efficiency of viable cell recovery;

determining the correct de-sludge interval to maximise operational efficiency; and conducting a mass balance closure across the cell separation sub-unit operations.

6.2 MATERIALS AND METHODS

Centrifuges with differing operating modes were available for use in this study. These included a disk stack continuous operation centrifuge (Westfalia SA1, GEA; Germany) and a vertical tube centrifuge (Sharples-Stoke, Pennwalt, USA). The disk stack centrifuge was selected for use in this study, as it has enables continuous processing in contrast to the vertical tube type, which is mainly restricted to batch operation of streams with low solids loading. In our assessment and based on our commercial operating experience, the disc stack centrifuge offered better commercial scaling opportunities in contrast to the vertical tube type.

6.2.1 Determination of centrifuge feed rate to enable efficient *V. midae* cell separation

6.2.1.1 Broth preparation

A *V. midae* cell culture (whole broth) with a concentration of ~ 8.5×10^{10} cells.ml^{-1} was harvested from the fermenter directly into a pre-disinfected storage tank with constant mixing at ambient conditions using an overhead mixer (Heidolph RZR 2102, Kelheim Germany). This culture was used to develop a cell separation protocol in order to maximize cell recovery. Storage tanks were cleaned-in-place (CIP) using a

4% sodium hydroxide solution and a 10% sulphuric acid solution, and thereafter washed thoroughly with water prior to use, until a neutral pH of 7.0 was obtained.

6.2.1.2 Assessment of recovery efficiency of the cell separation process unit operation

Cell separation was performed continuously using a disk stack centrifuge (Westfalia, SA1, GEA, Germany) operated according to the equipment manual. The cell separation process was performed using a two pass process (double centrifugation steps in sequence). The centrifuge was operated at a constant speed of 9000 rpm and the bowl pressure was maintained at 100 kPa by adjusting the back pressure valve. Various flow rates in the range of 10.2 to 52.8 $l.h^{-1}$ were assessed in order to select operating conditions which minimized *V. midae* cell losses. Cost of production was determined by calculating the material cost (used for the production of one litre of 1×10^7 $cells.ml^{-1}$ solution informed by the basis that the supplementation level is 1 ml per gram of abalone feed of this solution, as envisaged by Macey and Coyne, (2005) as well as capital utilization based on an 80% capacity utilization per annum and ten-year straight line depreciation of a production scale system (disk stack centrifuge, ~ R 2.1 million). The cost of harvesting of the cells from the fermentation broth was calculated such that suitable operating conditions could be determined based on cell recovery and process efficiency for the cell separation unit operation.

$$Cost\ of\ recovery = \frac{Total\ capital\ cost + Material\ cost}{Total\ cells\ recovered} \times (1 \times 10^7 cells)$$

Equation 6.1

Each of the key responses (process time, process recovery, total cells recovered and cost of cell removal) in response to flow rate, were statistically analysed to determine appropriate fits to quadratic or cubic models. Numerical optima integrating all responses using their respective mathematical equations were determined by using the optimization function of Design Expert-6 software (Stat-Ease, Minneapolis, USA), excluding responses where R^2 values were less than 0.90 for any of the model fits. This allows the determination of the optimum conditions taking into consideration all of the key responses. Cost of cell removal was assigned the highest priority (level 5) of the four responses used in the mathematical modelling, as this response is the key commercial indicator of the production process potential and it furthermore directly influences the commercial feasibility. In many instances, the DSP costs account for the majority of the total cost in biological processes and therefore must be kept to a minimum. Total cells recovered and process recovery were an equal but lesser priority (level 1). Process time was not optimized in this study, being an independent variable, but was however to be in range.

Once an optimum flow rate was determined, a second pass of *V. midae* cell material was processed through the centrifuge. The first pass is generally performed to remove gross medium components from the culture broth, and the second pass to remove any remaining traces of nutrients to result in a medium-free suspension of cells in a buffer. This practise reduces the likelihood of product fouling.

6.2.2 Determination of de-sludge intervals to maximize process efficiency

During continuous cell separation using the disk stack centrifuge, the cells remain in the bowl of the centrifuge and need to be de-sludged using process water. These de-sludging intervals also need to be determined in order to obtain proper cell separation while ensuring process efficiency. In order to determine purging intervals, the process was repeated as described in section 6.2.1.2, but the flow rate was set at 26.4 $l.h^{-1}$ (based on the results of the flow rate study section 6.2.1.2). Samples of the resultant supernatant were collected, and the OD and cell concentration were determined for each of these fractions in order to ascertain the cell break-through point. The centrifuge bowl was de-sludged at selected intervals (1, 2, 3 and 4 minutes) to determine the impact of the desludge interval on process efficiency. The biomass paste was thereafter reconstituted in a saline phosphate buffer (PB) (KH_2PO_4 0.11, K_2HPO_4 0.71, NaCl 2.91 $g.l^{-1}$), per litre of de-ionized water.

The resultant biomass pellet was reconstituted into 150 L of saline phosphate buffer (PBS) and re-centrifuged using the flow rate and de-sludge intervals determined in section 6.2.1 and 6.2.2. All biomass fractions were combined and reconstituted into a homogenous solution using PB and analyzed as described in section 6.2.4. The biomass slurry was stored in sealable 20 L buckets at 4^oC for further use. The supernatant fractions were collected and analyzed as described in section 6.2.4, as these represented the process losses.

6.2.3 Assessment of process repeatability of the cell separation process

In order to verify the repeatability of the process once the flow rate and de-sludge interval were determined, the full cell harvesting process unit operation was repeated in triplicate. A full "mass balance" (cell number) closure was done, whereby all biomass and supernatant fractions that were obtained during the cell separation process were accounted for. In addition, cell recovery analysis was also conducted according to the following equations:

$$\%\ Cell\ Recovery = \frac{Total\ cells\ Out}{Total\ cells\ In} \times 100$$

Equation 6.2

$$\%\ Closure\ cells\ = \frac{No.of\ cells\ In\ Biomass\ Slurry + No.of\ cells\ In\ Supernatant}{Initial\ No.of\ cells\ Fed} \times 100$$

Equation 6.3

6.2.4 Sampling and analysis

Samples were analyzed for total cell concentration and optical density were appropriate. Optical densities were measured at 660 nm using a DU800 spectrophotometer (Beckman Coulter, Germany). Cell concentrations were determined by microscopic cell counting as previously outlined in chapter 4, section 4.2.5. Random samples were selected and checked for culturable cell number ($CFU.ml^{-1}$) using the plate count method as described in chapter 2, section 2.2.2), to verify that the cells harvested were actually viable.

6.3 RESULTS AND DISCUSSION

6.3.1 Selection of a suitable cell separation process option for *V. midae* cells

Centrifugation was selected as a suitable process unit operation for the separation of *V. midae* cells from the culture broth. Although costs involved with acquiring and operation of a centrifuge are higher than that of other cell separation processing options such as filtration, the advantages of its use in comparison to filtration are apparent (Stanbury *et al*., 1995). Some of the main advantages informing the selection for our process included the availability of cell separation equipment, the ease of operation as well the process efficiency of the unit operation.

It was also essential to develop a fully integrated, functional DSP flow-sheet by selecting the correct cell separation equipment that enabled correct material processing. Non-continuous (batch) centrifuges, such as bench top, basket-type, or vertical tube Sharples-Stokes centrifuges were excluded from use; based on their reduced capacity and impact on process efficiency (Stanbury *et al*., 1995). As our fermentation optimization research resulted in high cell density culture broths, the preferred mode of operation was continuous cell separation with automated de-sludge harvesting. The disk stack centrifuge, which was selected for use in this study is able to operate in a continuous mode and relies on the disks in the rotor or bowl for separation efficiency. Its design is based on a central inlet pipe that is surrounded by a stack of stainless steel conical disks. The broth is fed into the centrifuge through the central feed pipe, and then is directed upwards and inwards between the disks at a 45^{o} angle, which enables rapid sedimentation of the cell slurry. The cell solids are then

removed from the centrifuge using automated purges of rinse water without contributing to major process interruption (Stanbury *et al.*, 1995). The schematic process flow diagram for cell separation is indicated in Figure 6.1.

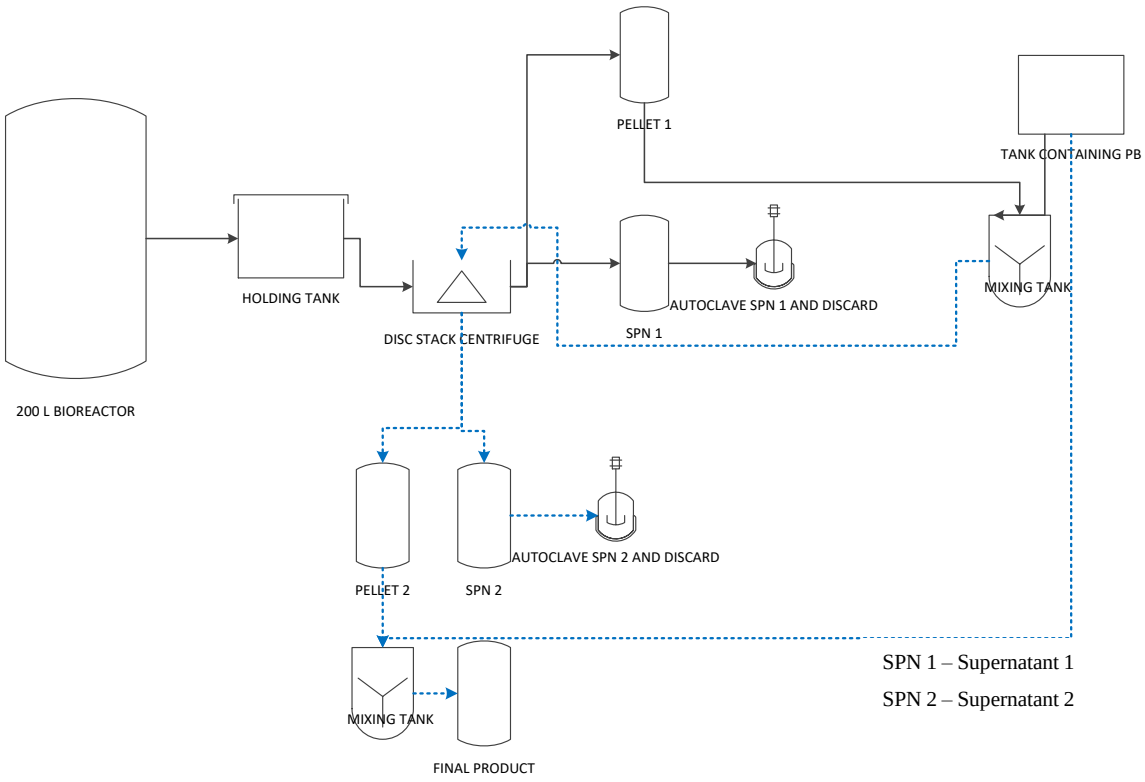

Figure 6.1. Process schematic of cell separation process unit operation (PUO 3) used to separate *V. midae* cells from the culture broth. The supernatants generated post cell separation contain spent media and extracellular products. Black solid lines denote process for pass1 and blue dotted lines denote process for pass 2.

6.3.2 Assessment of various flow rates on cell separation efficiency

A preliminary test was done which assessed eleven different flow rates (between 10.2 and 52.8 $l.h^{-1}$ in order to determine the operating flow rate for the first round of centrifugation using the disk stack centrifuge. During the investigation it was found

that at flow rates lower than 26.40 $l.h^{-1}$, the retention time was too long and as a result, inadequate cell separation occurred, due to irreversible clogging of the cells on the discs. The lower flow rates were furthermore too low to sufficiently fill the centrifuge bowl with culture to create the necessary operating back pressure required, thus hampering process efficiency. Relevant data for five of the twelve flow rates (26.40 to 52.80 $l.h^{-1}$) are represented in Figure 6.3. Flow rates in the range of 26.4 to 52.8 $l.h^{-1}$; resulted in cell losses in the range of 8.82 to 55.73% (Figure 6.3c). OD values were used as an indicator of process recovery to assess breakthrough point, and ranged in the region of 0.80 to 7.73 (Figure 6.2 a). OD values of 0.80 (flow rate of 26.4 $l.h^{-1}$) and 3.63 (flow rate of 31.0 $l.h^{-1}$) correlated to cell concentrations of 7.50×10^{9} $cells.ml^{-1}$ and 2.55×10^{10} $cells.ml^{-1}$ respectively (Figure 6.2 b). Higher flow rates resulted in higher OD values and higher cell concentrations of the supernatant (Figure 6.2 b and c) due to inadequate cell separation because of the reduced retention time within the centrifuge bowl. The high flow rate condition also results in a higher loading of solids per unit time and thus a quicker breakthrough point. The flow rate is thus an essential operating parameter which plays off throughput (process efficiency) against recovery (process yield). Upon the assessment of the viability of the cells post centrifugation, it was noted that 97% of the population was still viable, which is an important finding, as the cell separation is intended to separate cells from the culture broth at a high efficiency rate without negatively affecting the viability of the cells.

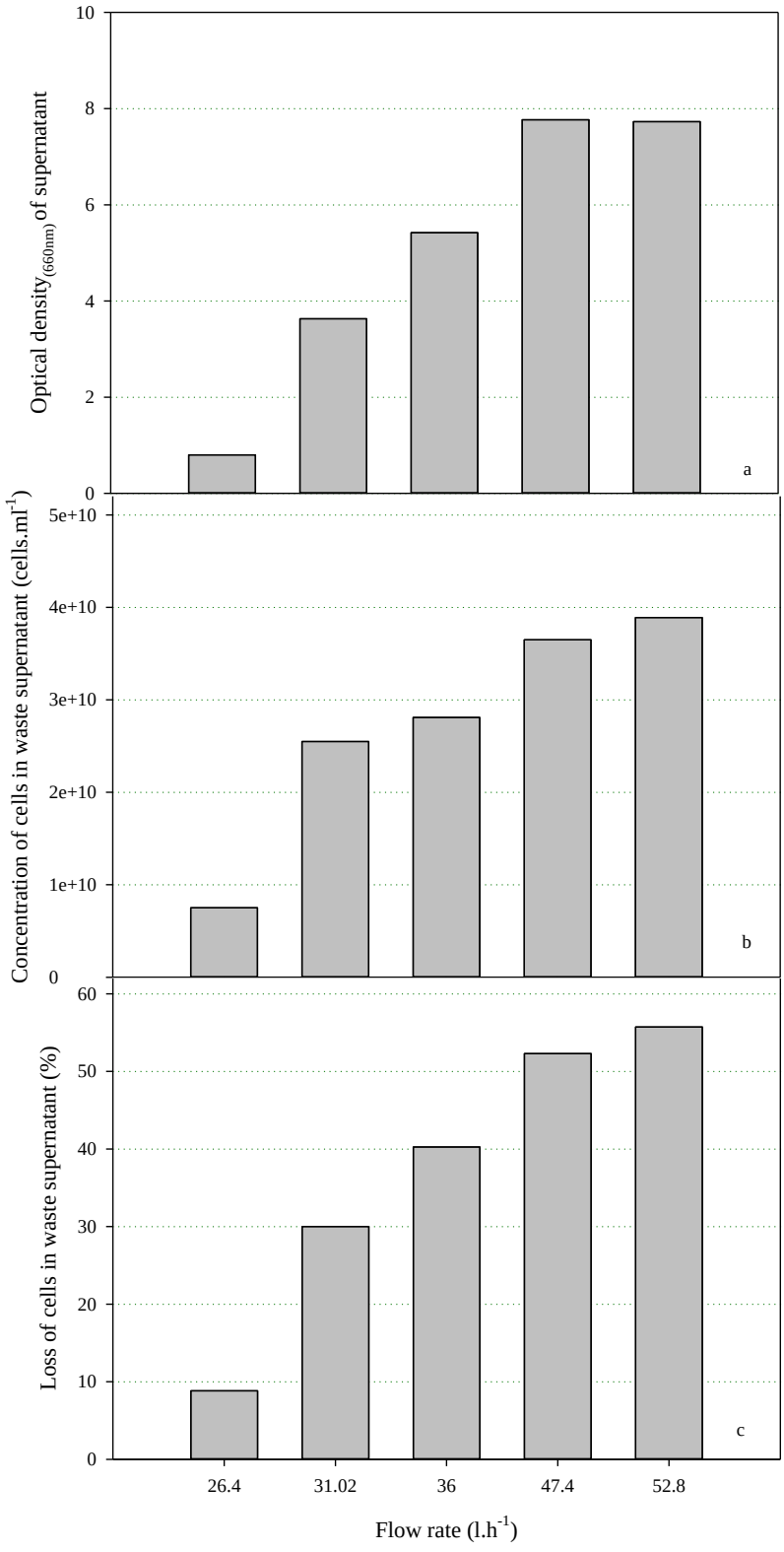

Figure 6.2. Optical densities (a), cell concentration (b) and cell losses (c) of supernatant fractions obtained using different flow rates illustrating the effect on *V. midae* cell recovery.

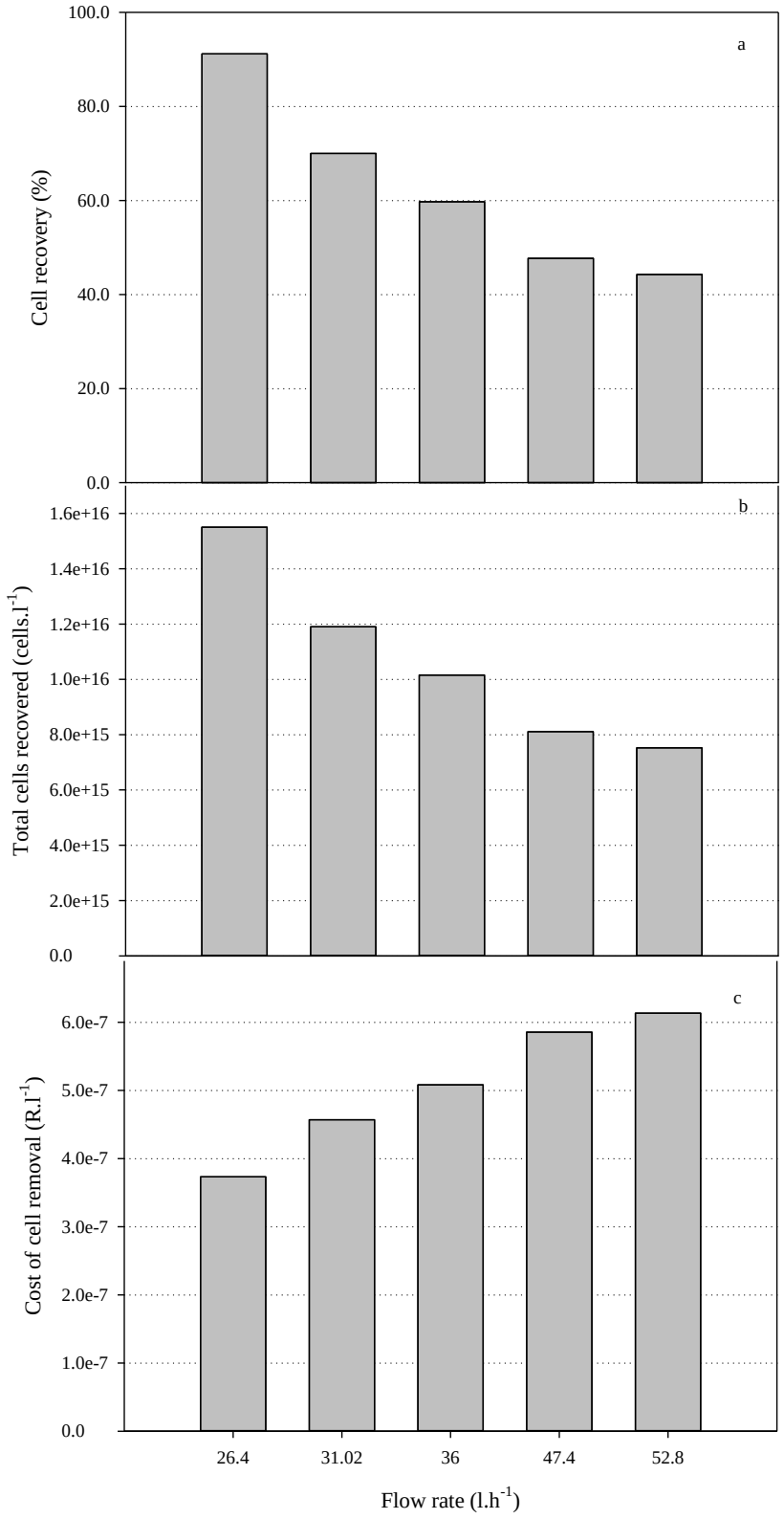

Figure 6.3. Process efficiency of centrifugation at different flow rates assessing (a) cell recovery, (b) total cells recovered and (c) the cost of cell removal (product equivalence).

The cost (expressed per litre of 1×10^7 cells) for harvesting of cells from the fermentation broth was calculated in order to select the operating conditions of the centrifuge (Figure 6.3). It was observed that higher flow rates resulted in faster throughput and hence a reduced process time (reduced cost of labour and improved capital utilization) to process a batch of *V. midae* cells (Figure 6.4). However, these faster flow rates resulted in lowered cell recoveries (Figure 6.3 b). The playoff between these two major cost-contributing factors was modelled to determine the unitised cost of recovery of cells (Figure 6.3 c). It was observed that a flow rate of 26.4 $l.h^{-1}$, the lowest cost of cell harvesting (R 3.73×10^{-7} per cell) was observed.

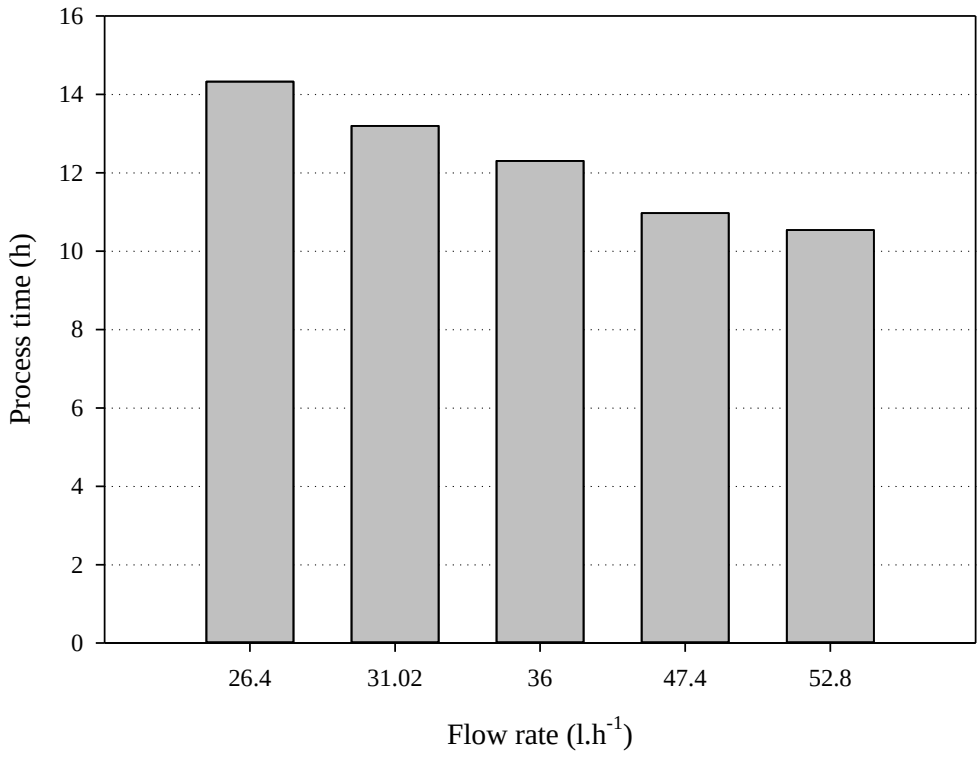

Figure 6.4. Assessment of process time required to perform *V. midae* cell separation using a disk stack centrifuge operated at five different flow rates.

The data generated from this study served as inputs to generate a model used to optimize the flow rate for the separation of *V. midae* cells from the culture broth, and subsequently minimize the cost to produce this probiotic culture. Each response (process time, process recovery, total cells recovered and cost of cell removal), using their resultant mathematical equations, were simultaneously solved using Design Expert ® in order to predict the optimum flow rate. This technique allows the weighting of responses and the prediction of an optimum using all of the responses in an interactive mathematical model. All data obtained within the flow rate range of 26.40 to 52.8 $l.h^{-1}$ was statistically analysed for each response.

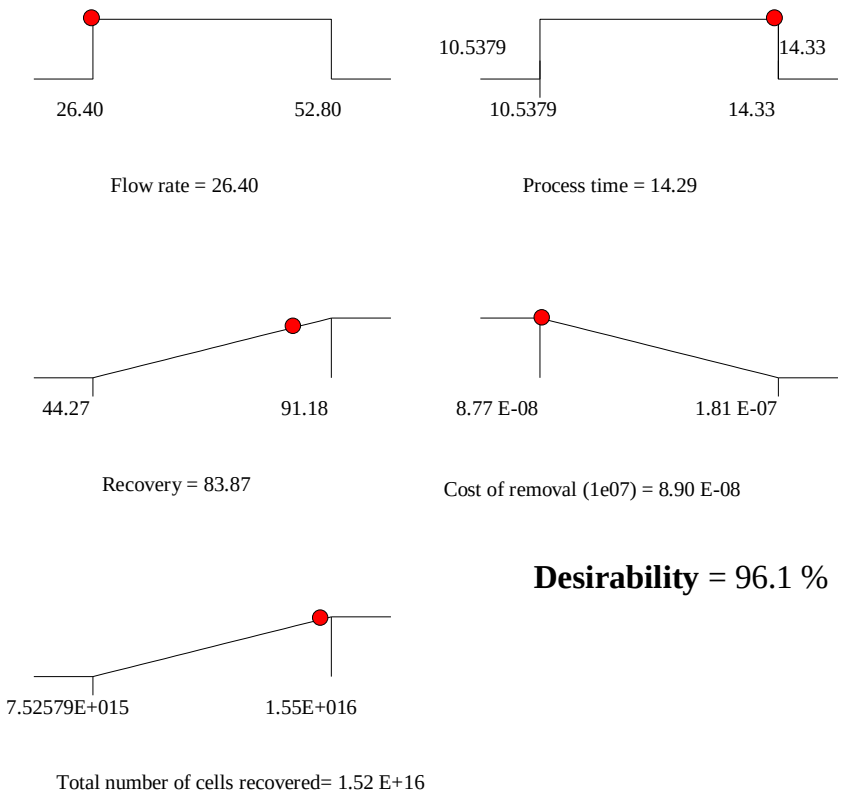

Figure 6.5. Design expert® illustration depicting ramps of key responses used to determine the optimum flow rate for the separation of *V. midae* cells from the culture broth

The derived optimum flow rate of 26.40 $l.h^{-1}$ using all four of the responses, resulted in a quadratic model, with a 96% confidence level (desirability co-efficient) (Figure 6.5). It was noted that all regression co-efficients of the four individual responses against flow rates and the combined model exceeded 0.90, which provided the statistical basis for acceptance of the model predicted value. This flow rate was therefore selected for further assessment in order to determine the de-sludge interval. Disk stack centrifuges are operated at varying speeds depending on the separation process. Manufacturers specify operating speed parameters but do not specify the input feed rates in order to optimize separation efficiency. The operating manual for this specific Westfalia disk stack centrifuge did not stipulate conditions for a minimum or maximum input feed rate, as a result a range of feed rates needed to be tested to determine the feed rate at which process efficiency was maximized.

6.3.3 Determination of de-sludging intervals to maximize process efficiency

In order to harvest the cells collected in the centrifuge bowl on a semi-continuous basis and to limit the loss of cells to the supernatant fraction, purging of the centrifuge bowl with process water, had to be performed at specific time intervals. It is important to consider the downtime experienced during the purging of the centrifuge bowl, during the de-sludging step as an increase in de-sludge interval results in slower throughput because the feed to the centrifuge has to be stopped in order to clear the contents of the bowl. If the bowl purging interval is too high, separation time is increased which impacts on the cost efficiency of the cell separation process by increasing batch process time and reducing capital utilization efficiency.

Supernatant samples were collected at 1 minute intervals at the specified flow rate and the OD was measured to assess the breakthrough point. Process recovery and corresponding cost of individual cell removal are indicated in Figure 6.6. Lower de-sludge frequencies resulted in supernatants containing higher OD values. This was an indication that the centrifuge required more frequent de-sludging in order to reduce the loss of cells to the supernatant fraction.

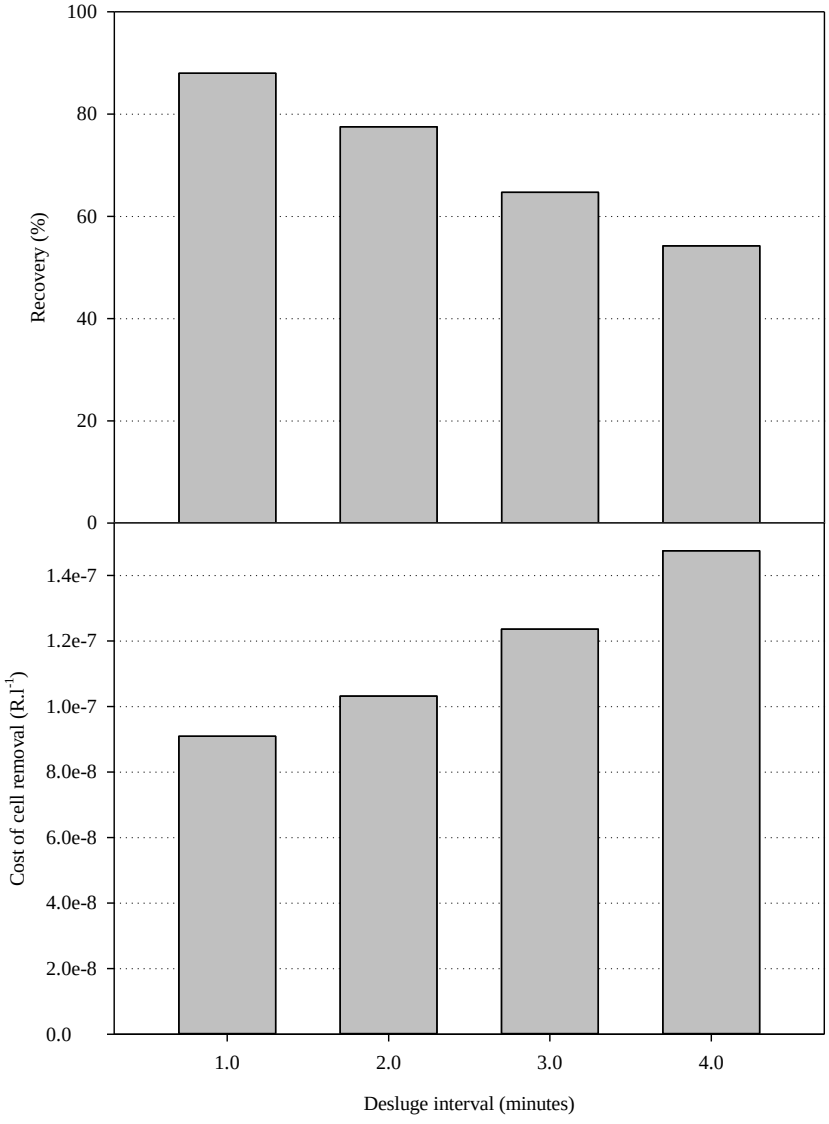

Figure 6.6. Process efficiency assessing (a) cell recovery and (b) the cost of cell removal using a disk stack centrifuge operated at varying de-sludge intervals.

It was observed that at a broth feed rate of 26.4 $l.h^{-1}$, a de-sludge interval of two minutes resulted in the lowest cost of removal of R 1.03×10^{-7} per litre of 1×10^{7} cells, and was therefore selected as the preferred operating conditions. Process recovery and total cells recovered were maximum at the one minute intervals, however, this process parameter could not be selected, as it increased the cost of removal by 30%. Once the first centrifugation pass using a flow rate of 26.40 $l.h^{-1}$ and a purging interval of 2 minutes was completed, the broth was reconstituted to 150 L in a saline phosphate buffer and the centrifugation process repeated. It was hypothesized that this reduced volume (150 L) would further reduce the process time of the downstream process unit operation (based on the hypothesis that the biomass is cleaner and would separate easier from the buffer). This process change was adopted on all subsequent batches. The reconstituted pellet could possibly be suspended in a lesser volume of buffer to reduce the process time of the second centrifugation pass, however, this aspect of research was outside the scope of this study. The OD and cell concentration of the supernatant, as well as *V. midae* cell losses were evaluated using the same process conditions determined in section 6.3.2 and 6.3.3. At the specified flow rate of 26.40 $l.h^{-1}$ and de-sludge interval of 2 minutes, a cell recovery of 99.07% was observed.

6.3.4 Assessment of the repeatability and robustness of the cell separation unit operation

A mass balance recovery was conducted on the cell separation PUO using the conditions determined in section 6.3.2 and 6.3.3. A key aspect of this study was to check repeatability of the process and to ensure that maximum recovery of the

produced biomass through the cell harvesting process steps was obtained. Centrifugation flow-rates, de-sludge time, and cell recovery from the centrifuge itself are important factors to prevent major losses from occurring, which ultimately affects the cost of production of this abalone probiotic.

Table 6.1. Mass balance and cell recoveries (%) obtained for all sub unit operations across replicate batches (*n*=3).

Process step	Indicator	Min	Max	Ave	SD	CV
First pass	Cell recovery	68.71	69.27	68.71	0.50	0.72
	Mass balance closure	74.48	74.00	72.48	0.98	1.35
	Loss to supernatant	3.23	4.24	3.78	0.51	13.50
Second pass	Cell recovery	98.69	108.28	103.60	4.80	4.63
	Mass balance closure	99.82	110.98	105.65	5.60	4.43
	Loss to supernatant	1.13	2.70	2.05	0.82	39.97
Overall	Cell recovery	68.36	74.21	71.17	2.93	4.12
	Mass balance closure	73.38	78.06	75.21	2.51	3.33
	Loss to supernatant	4.81	5.71	5.18	0.47	9.12

The amount of cells recovered during both centrifugation steps are reported in Table 6.2. The first pass demonstrated a lower mass balance recovery with a corresponding lower cell recovery than the second spin. This indicates that the lower recovery was not due to separation efficiency but rather a loss of material mass, further confirmed by low losses to the supernatant fraction. This can be attributed to the cells contained in the solids that remained in the centrifuge bowl and disks which could not be recovered in this step, possibly due to stickiness of the whole fermentation broth.

Another possible explanation is the lower difference in specific gravity of the cells to the whole broth, which contains media components such as nutrients and salts, thus increasing the dissolved solids. In contrast, the difference in specific gravity of the cells to the buffer is higher after the reconstitution step, explaining the improvement in efficiency of the second centrifugation step.

The overall cell recovery across the cell separation unit operation was found to be 71.2 ± 2.9% with a supernatant loss of 5.2%. The mass balance closure indicated that 24.8% of the losses were not accounted for in the various streams (Table 6.2). This was predominantly the solids material remaining within the centrifuge bowl during the first separation step. The cell recoveries can be improved by continuous operation in a commercial process with frequent purging of the spore paste, which would minimize the error in mass balance closure due to unrecoverable (unaccounted for) solids hold up in the centrifuge bowl, as was the case in this experiment (Lalloo *et al,* 2010). Other studies conducted by Torres-Anjel and Hedrick (1971) have indicated that the accumulation of solids in the machine during cell separation results in product losses and it has been reported that recovery can also be compromised by high initial cell loads. If required, further investigations can be performed to optimize the cell concentration that is fed into the centrifuge to reduce the losses experiences during the first pass. The cells of *Vibrio spp.* are small, and as a consequence could prove difficult to recover by merely purging the bowl at more frequent intervals. Another mechanism to recover these cells would be to dismantle the centrifuge after every pass, however this would contradict the selection of a continuous operation centrifuge. If the recovery

of these cells was that significant, it would occur at the expense of reduced process efficiency as process time and cost of recovery would increase.

The conditions specified in this study will be applied to cell separation process which will be conducted on manufacturing scale to effectively recover cells produced during the *V. midae* production process.

CHAPTER 7: FORMULATION OF THE *V. midae* PROBIOTIC INTO A MARKET ACCEPTABLE PRODUCT

7.1 INTRODUCTION

Once the cell separation process unit operation was developed, the final stage of the downstream process development of the *V. midae* abalone probiotic required the formulation of the organism into a stable and consistent final product. Based on the inputs obtained from abalone industry experts, which included a panel of researchers, abalone feed producers and abalone aquaculture farm owners and managers; the preferred end product form was a liquid product containing 1×10^7 cells per gram of feed. Kosin and Rakshit (2006), and Wirupan *et al*., (2016) stated that in order for the probiotic to confer its intended benefit it should be applied to the feed at a concentration typically between $10^6 – 10^7$ $CFU.g^{-1}$. The product also needed to be integrated seamlessly into the current abalone feed production process, and prior activation or pre-formulation of the product at farm stage was undesirable.

The Vibrio production technology did not contain a stabilization step of any sort, in contrast to the likes of a Bacillus spore stabilization process for example. The latter usually maintains the cell preparation in a spore form, which is an advantage in that stable product preparations are obtained. If a biological product contains actively

growing cells, as was the case with our Vibrio probiotic, it may result in a less favourable product preparation due to variability, and possible product fouling.

Vibrios are not spore-forming organisms, and therefore an alternate "stabilization" strategy was required. An extensive literature survey was conducted to understand the fundamentals of Vibrio product processing. It has been revealed that *Vibrio* organisms enter a viable but non-culturable (VBNC) state (Roszak and Colwell, 1987; Wong and Wang, 2004). The existence of this state remains relatively unclear as cells that are in this VBNC state appear dead, as they lose their ability to grow typically on a conventional solid media that once supported growth within a known period of time (Oliver, 2000a). Microorganisms enter a VBNC state as a result of exposure to a form of natural stress (Oliver, 2000b). These stresses include starvation of the population, incubation of the organism outside its range of growth temperatures, elevated osmotic concentration or exposure to white-light (Oliver 2000a). Many researchers have applied these methods to successfully induce various *Vibrio* populations into the VBNC state to obtain stable products (Oliver, 2000a, Oliver 2000b, Oliver 2005).

We explored this strategy of inducing actively growing *V. midae* into the VBNC state, and obtained a stable, viable and robust product formulation, according to Wong *et al*., (2004). Enumeration of the VBNC population was conducted using a commercial viability stain and the products generated were assessed accordingly to show applicability. Furthermore, the VBNC material was used to produce liquid products and these were then assessed for suitability to be included into the feed production

process. Due to the nature of the probiotic product, it was essential to maintain viability of this marine organism throughout the final product processing stream.

The shelf - life of each product prototype that we produced was thereafter determined to enable proper long term storage of the probiotic product and further enhance commercialization potential of this abalone probiotic. The suitable formulation of the *V. midae* probiotic offers several technological benefits to abalone feed producers, such as the stability of the microbes during feed production, distribution and storage. It also confirms proper shelf - life data for on farm storage under typically harsh ware-housing conditions. Therefore, the successful development of a product formulation containing the abalone probiotic strain is a necessary step for the development of a commercial product that meets the requirements outlined by the end user and can be mass produced for use in abalone aquaculture facilities.

7.2 MATERIALS AND METHODS

7.2.1 Measurement of *V. midae* cells in a VBNC state using the LIVE/DEAD BacLight™ assay stain

7.2.1.1 Culture preparation

A *V. midae* fermentation culture was induced into the VBNC state via nutrient limitation (Oliver 2000 a, Oliver 2000 b). The population of VBNC, culturable and dead cells were then differentiated using the LIVE/DEAD BacLight™ stain and conventional analytical methods. Once the different populations in the sample were determined, the VBNC portion of the culture was resuscitated in order to show viability and culturability of the population.

7.2.1.2 Stain preparation

The commercial stain kit comprised of two components, a SYTO 9 nucleic acid stain (component A), and propidium iodide (component B). Equal volumes of component A and B were added into a micro-centrifuge tube as stated by the supplier, mixed thoroughly and stored in the dark until required (Molecular Probes, Kit L7007 technical manual).

7.2.1.3 Test sample preparation, data analysis and calculations

The prepared stain (3 µL) was added to 1 mL of culture. Samples were mixed thoroughly and incubated for 15 minutes in the dark. Stained samples of each cell preparation (5 µL) were placed under a coverslip on a microscope slide. Samples were viewed using fluorescent microscopy and filter set (Olympus, Japan). Five different fields were viewed and three replicates were obtained for each sample. The concentration of viable cells (stained green) was expressed as a percentage of the total population.

$$Viability\ (\%) = \frac{no\ of\ viable\ cells}{total\ cells} \times 100$$

Equation 7.1

Culturable cell counts were determined (in triplicate) for the *V. midae* cell preparation by serially diluting and plating on MA plates (as described in chapter 2; section 2.2.4). Plates were sealed and incubated for 5 d at 30 ^{o}C, and resulting colonies were counted and expressed as the number of colony forming units per ml (CFU.ml^{-1}). The total concentration of cells within each sample was determined in triplicate by the

microscopic counting of cells using a Thoma® counting chamber (Hawksley and Sons, London, UK). The concentration of cells was represented as cells.ml^{-1}.

The following equations were used to distinguish the various *V. midae* cells states in the test cultures.

$$\boldsymbol{Viability_{BacLight}}\,(\%) = \frac{no\ of\ viable\ cells}{total\ cells} \times 100$$

Equation 7.2

$$\boldsymbol{Number\ of\ viable\ cells} = \frac{Viability_{(BacLight)}}{100} \times Total\ cells$$

Equation 7.3

$$\boldsymbol{Culturable\ cells}\,(\%) = \frac{no\ of\ culturable\ cells_{(CFU)}}{Total\ cells} \times 100$$

Equation 7.4

$$\boldsymbol{Number\ of\ culturable\ cells} = \frac{Culturable\ cells\ (\%)}{100} \times Total\ cells$$

Equation 7.5

$$\boldsymbol{Culturable\ cells}\,(\%) = \frac{no\ of\ culturable\ cells_{(CFU)}}{Total\ cells} \times 100$$

Equation 7.6

$$\boldsymbol{Dead\ cells}\,(\%) = \ 100\ - \ Viability_{BacLight}\,(\%)$$

Equation 7.7

$$\boldsymbol{Number\ of\ dead\ cells} = \frac{Dead\ cells\ (\%)}{100} \times Total\ cells$$

Equation 7.8

$$\textbf{\textit{Number of VBNC cells}} = no\ of\ viable\ cells_{(BacLight)} - no\ of\ culturable\ cells - no\ of\ dead\ cells$$

Equation 7.9

7.2.2. Production of liquid product prototypes of the *V. midae* probiotic

7.2.2.1 Prototype formulation

Once the viability and culturability of the VBNC culture was assessed, this material was used to formulate liquid product prototypes. *V. midae,* cells were produced and harvested as described in chapter 6.

The resultant cell pellet was used to prepare four liquid prototypes by combining *V. midae* cells with: (a) saline phosphate buffer (PBS) containing KH_2PO_4 0.11, K_2HPO_4 0.71 and NaCl 29.1 $g.l^{-1;}$ (b) glycerol (50% $^{v}/_{v}$) mixed with PBS, (c) artificial seawater (ASW) containing 16.78 $g.l^{-1}$ NaCl, 0.39 $g.l^{-1}$ KCl, 0.30 $g.l^{-1}$ $NaHCO_3$, 0.006 $g.l^{-1}$ H_3BO_3, 2.6 $g.l^{-1}$ $MgCl_2.6H_2O$, 0.77 $g.l^{-1}$ $CaCl_2$ and 3.56 $g.l^{-1}$ $MgSO_4.7H_2O$ and (d) glycerol (50% $^{v}/_{v}$) mixed with ASW. These buffers were selected on the basis of creating a marine based medium to enable long term storage of the product prototypes. Glycerol was added in order to assess if the addition of this lyoprotectant would positively influence the longevity of the product.

In preparation for the shelf-life assessment, samples of each of the four different product prototypes were aliquoted into pre-sterilised 2 ml Eppendorf® tubes (Plastpro Scientific, SA) and stored in a static environment at 4 ^{o}C, 25 ^{o}C and 30 ^{o}C. These temperatures were selected to represent storage under refrigeration (4^{o}C), at ambient temperature (25^{o}C) and at the optimum growth temperature of the organism (30^{o}C).

7.2.2.2 Sampling and data analysis

Each sample (1.5 mL) was processed such that the following cell titres were determined; (a) culturable cell counts, (b) total microscopic counts and (c) total viable counts. The culturable cell counts were measured according to methods described in section 7.2.1.3. The total microscopic counts and viable counts were determined in triplicate by microscopic counting of either stained or unstained cells respectively, using a Thoma® counting chamber (Hawksley and Sons, London, UK). The concentration of cells was represented as cells.ml^{-1}.

7.2.3. Assessment of the stability of the final liquid product prototype using accelerated stability kinetics

Samples (2 ml) of *V. midae* cells in the selected storage buffer (section 7.2.2.1) were aseptically dispensed into sterile Eppendorf® tubes. Tubes were placed in a static environment at 40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C and 60 ^{o}C for the duration of the trial. Samples (1.5 mL) were harvested at regular time intervals and total microscopic cell concentration, cell culturability and cell viability were determined on all samples according to methods outlined in section 7.2.1.3. The stability kinetics of the prepared samples was performed according to the procedures outlined by Heldman (2003).

For each sample stored at a specific temperature, the logarithmic value of the viable cell count was plotted against time for the duration of the trial. The D value was calculated as the negative inverse of the resultant slope and was temperature specific for any given sample. To relate the shelf life of a product to any temperature, the z value was determined. To obtain the z value, the logarithmic value of the D values at each temperature were plotted against the temperatures tested. The negative inverse of this resultant slope represents the z value.

To calculate the shelf life at any temperature, the following equation was used:

$$\mathbf{F_{temp} = \log (N_o \div N_s) \times D_{temp}}$$

where, N_o = initial number of organisms

N_s = final number of organisms

D_{temp} = D value at the stored temperature

Equation 7.10

To determine D_{temp}, the following equation was used:

$$\mathbf{\log (D_{temp} \div D_{ref}) = (T_{ref} - T_{temp}) \div z}$$

where, T_{temp} = is the storage temperature

D_{ref} = is the associated D value at the reference temperature

Equation 7.11

To determine D_{ref} in Equation 7.11, a T_{ref} was chosen and the associated D_{ref} was determined from the plot of the logarithmic value of the D values at each temperature

against the temperature (done above). Using Equation 7.10, it was possible to project the shelf life of a particular product form at any given temperature.

Using the model generated from the kinetic assessment of the data in section 7.2.3, the performance of the liquid product prototypes were assessed at varying storage temperature conditions, viz., refrigeration temperature (4 ^{o}C), farm operation temperature (18 ^{o}C), ambient temperature (24 ^{o}C), optimum temperature for the growth of *V. midae* (30 ^{o}C) and maximum growth temperature for *V. midae* (37^{o}C).

7.3 RESULTS AND DISCUSSION

7.3.1. Measurement of the *V. midae* culture in the VBNC state

Upon conducting an extensive literature search of several published works, it was noted that during a certain stage of processing, Vibrios enter a VBNC state (Roszak and Colwell, 1987; Wong and Wang, 2004, Wong *et al*., 2004; Oliver, 2000a, Oliver 2000b, Oliver 2005). This state can easily be misunderstood, and cells can be incorrectly deemed dead. The LIVE/DEAD BacLight™ staining kit has been used in various applications, and has shown good correlation to culturable plate assays for a number of gram positive and gram negative bacterial species (Molecular Probes, February 2001).

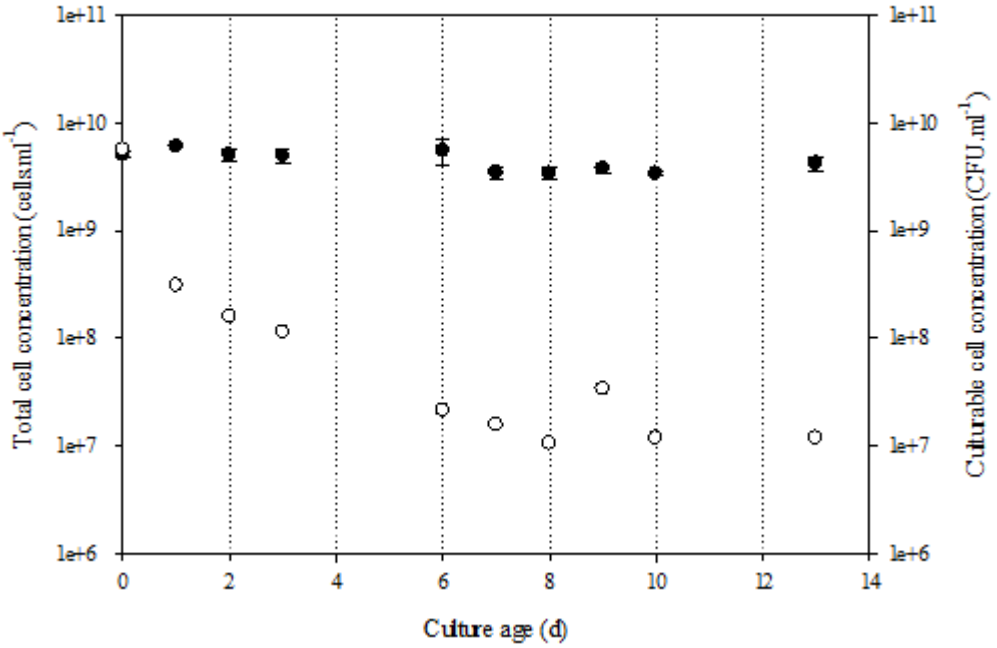

Figure 7.1. Assessment of the VBNC state of *V. midae* induced through nutrient limitation at 30ºC (based on total cell concentration (●) and culturable cell concentration (○).

Total cell concentration remained fairly stable over the 14 day period, however, the typically culturable cell population started to decline after 1 day of incubation (Figure 7.1). When conventional plate count assays were performed after 8 days of cultivation, a culturable cell concentration of 1.07×10^7 CFU.ml^{-1} was obtained. However, a total microscopic count of 3.47×10^9 cells.ml^{-1} was detected. When the same sample was stained, 89.45% of the cells stained green, indicating that the cells were still viable, although not culturable (Figure 7.2). To ensure that the stain was indeed accurately enumerating viable cells (n=5), we resuscitated the sample and obtained a correlation of 99.55% between viable cells determined from viability staining, and culturable counts obtained using standard microbial plate count assay post resuscitation (n = 9).

The standard deviation within counts was checked to ensure robustness of the correlation (SD < 10%).

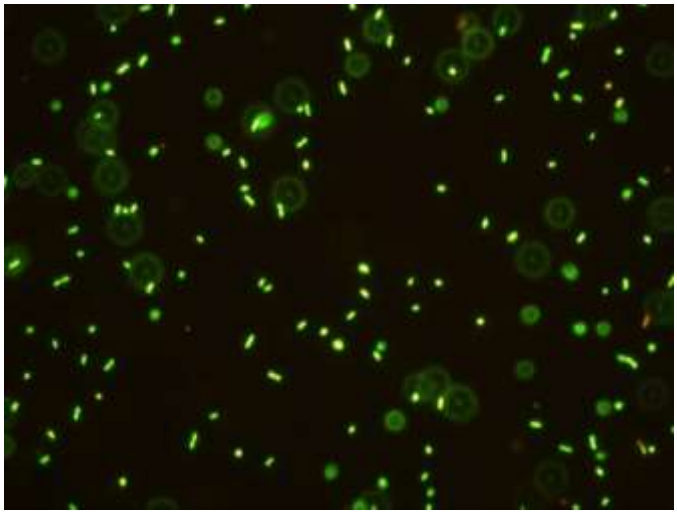

Figure 7.2. *V. midae* cells viewed using the LIVE/DEAD BacLight™ assay kit, whereby cells stained green was an indication of cell viability, and red was an indication of non-viable (dead) cells.

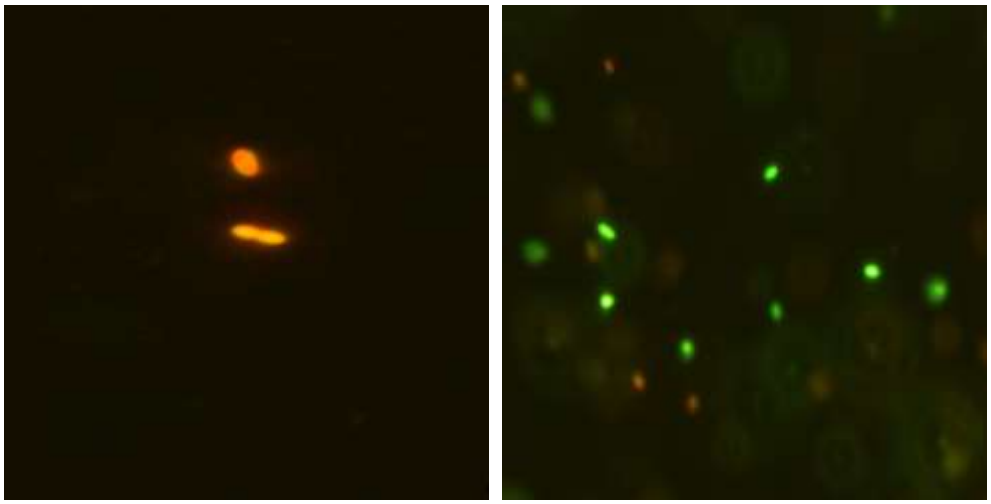

Figure 7.3. *V. midae* cells stained using the LIVE/DEAD BacLight^{TM} assay; (a) dead *V. midae* cells and (b) a mixed population of living and dead *V. midae* cells, whereby cells stained green was an indication of cell viability, and red was an indication of non-viable (dead) cells.

If the decline of culturable cell population was as a result of real cell mortality, the cells contained in the sample would have appeared red (Figure 7.3). In contrast our culture stained green (Figure 7.1), showing a very high level of viability. Due to the high correlation of the viability stain to actual viable cell counts in resuscitated cells from the VBNC state, we adopted the stain technique of viability assessment in all future studies.

7.3.2 Assessment of liquid product prototypes containing *V. midae*

Once the VBNC state was understood and the necessary assays evaluated, extensive microbiological testing was performed to assess the stability of the four chosen liquid product options over a 22-day trial period at the 3 pre-selected temperatures, 4^{o}C, 25^{o}C, and 30^{o}C. The four variants chosen included a phosphate buffer and an artificial salt water medium. Phosphate buffers (PB) are generally used in liquid product formulations, as they are non-toxic and maintain the pH balance of the solution. ASW was included as a formulation medium, due to it having the similar composition to the rearing waters of the abalone in the aquaculture farm. In addition, glycerol was added to both buffers to ascertain the impact of adding this cell humectant. The formulation containing only PB displayed a classical phosphate buffers formulation as viable cells remained fairly stable for the duration of the trial (Figure 7.4 a-d), and only minor fluctuations were observed for the duration of the trial. The addition of glycerol did not significantly improve cell viability, nor did the use of a marine ASW medium improve cell viability of the product formulation.

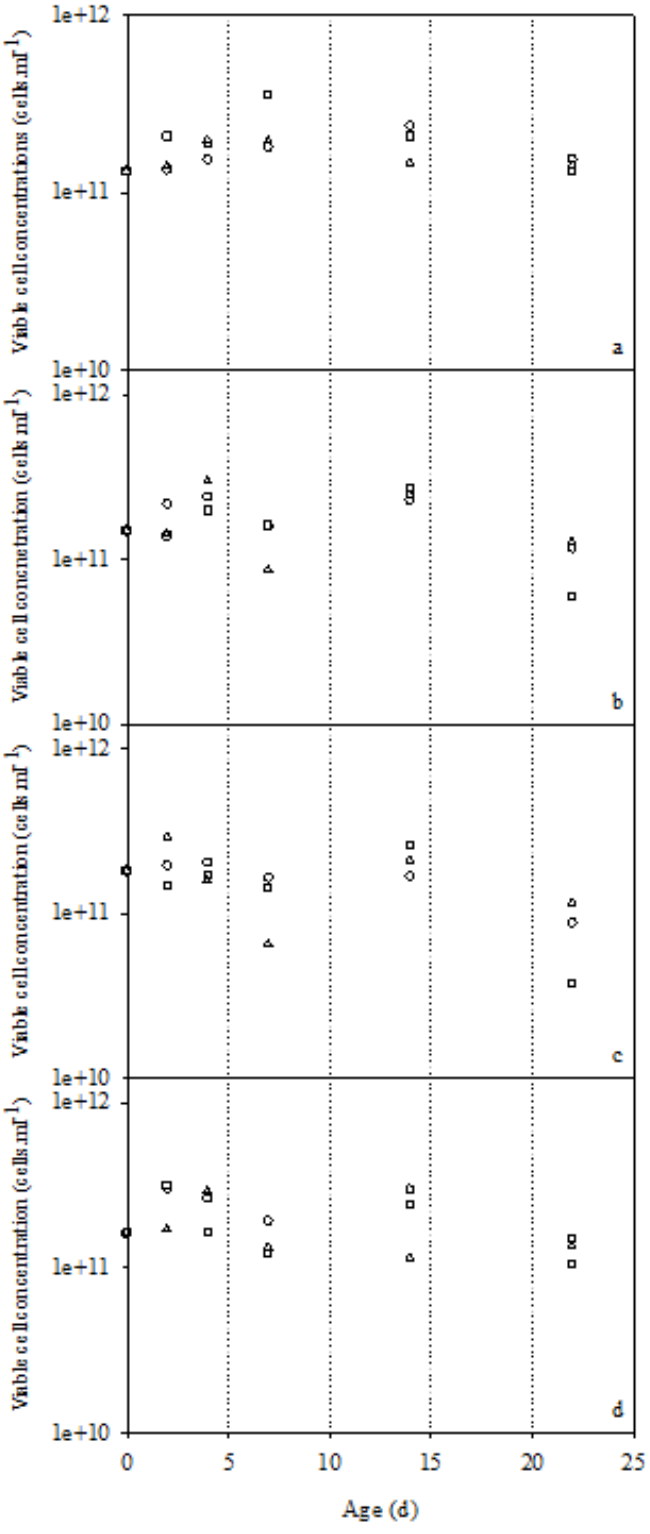

Figure 7.4. Performance of liquid product prototypes, (a) *V. midae* cells in a saline phosphate buffer; (b) *V. midae* cells in a saline phosphate buffer with glycerol; (c) *V.*

midae cells in artificial salt water medium and (d) *V. midae* cells in artificial salt water medium with glycerol at 4ºC (○), 25ºC (□) and 30ºC (Δ).

Statistical analysis of the data however revealed no significant difference between the initial and final viable cell numbers for each storage buffer tested ($p > 0.05$ in all cases). There were also no significant differences in stability between the storage buffers tested ($p > 0.05$; $n = 3$). We therefore used cost as a selection criterion for the stabilization buffer by calculating the sum of each individual cost component that constituted a specific buffer (Figure 7.5). The PBS liquid formulation was selected based on its lower material cost contribution and was the cheapest of the four alternatives tested (Figure 7.5).

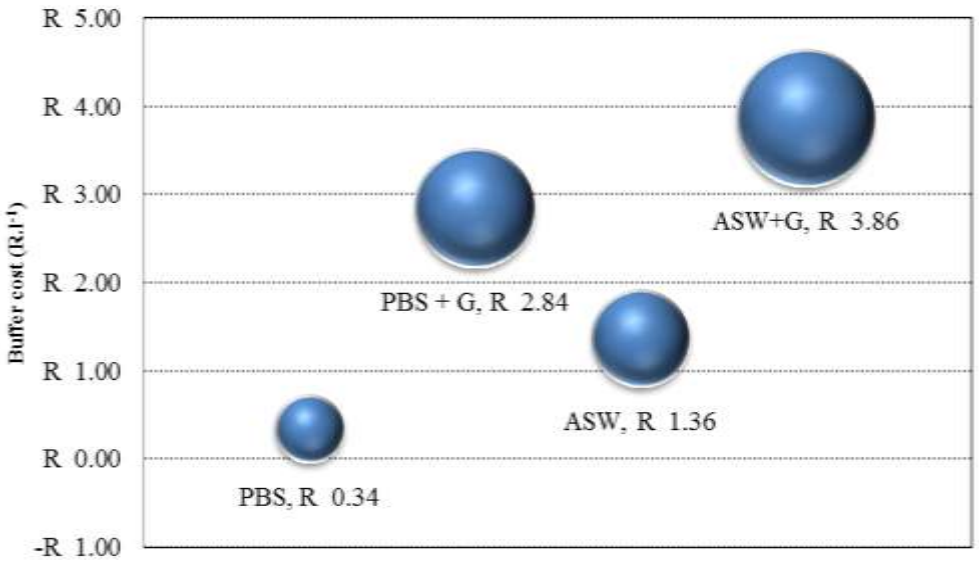

Figure 7.5. Cost distribution of buffers used in the initial stability testing which enabled the effective selection of a storage buffer system for a *V. midae* liquid product, where size of bubble is proportional to the cost.

During process development, it is vital to ensure that sensible, cost effective measures, such as the selection of a cheap yet effective storage medium, are in place to maintain process competitiveness. Similar to various other animal production facilities, formulated feed costs are a significant contributor to operations and efforts are focused on reducing feed costs by assessing alternate feed ingredients or by increasing feed conversion ratios thereby improving farm profitability (Sales and Britz, 2001). By selecting the minimum cost formulation of the liquid probiotic product, we influenced the cost attractiveness of the final abalone feed product and in so doing ultimately improved the yield of farm reared abalone and improved farm profitability.

7.3.3. Shelf life assessment of the selected *V. midae* liquid product prototype

In order to determine the shelf life of the PBS product, accelerated stability kinetic studies were performed by determining viable cell concentrations (determined by BacLight^{TM} analysis). This technique interprets the death patterns of a viable cell population, and with the use of mathematical curve fits, helps to model the death or stability of a product at a specified temperature.

The D-values of the PBS product decreased as temperatures increased (Figure 7.6). Heldman (2003) described the D-value as the time taken to reduce a microbial population by 90%. It can be observed from Figure 7.6, that as temperature increased from 40 to 60^{o}C, a decrease in D-values was evident. This was an indication that it took a shorter period of time to substantially reduce a microbial population by 90% at 60^{o}C in comparison to 40^{o}C. The D value is also a measure of heat resistance of a microorganism.

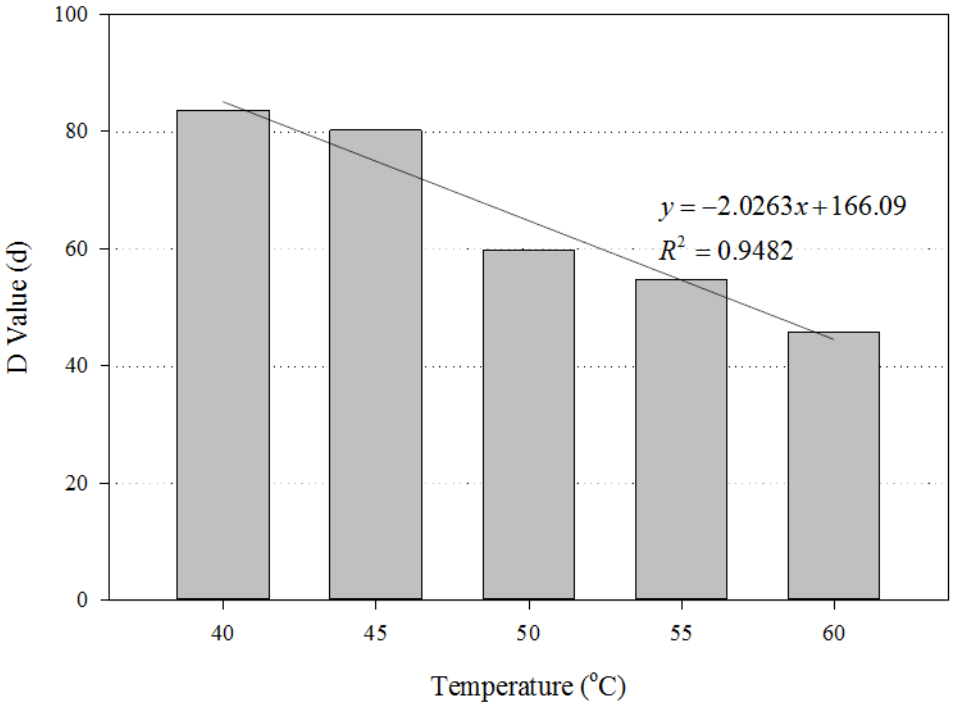

Figure 7.6. D-values obtained during the storage of PBS product prototypes containing *V. midae* at a range of elevated temperatures.

The D-values followed a linear trend and the model had a regression of 0.9482, which further highlighted the integrity of the data obtained from the experiment. Logarithmic D-values were then used to generate a plot against temperature (Figure 7.7). The inverse of the resultant gradient was then used to calculate the z value. This z-value reflects the temperature dependence of the reaction, and is defined as the temperature increase required in order to reduce the thermal death time by a factor of 10.

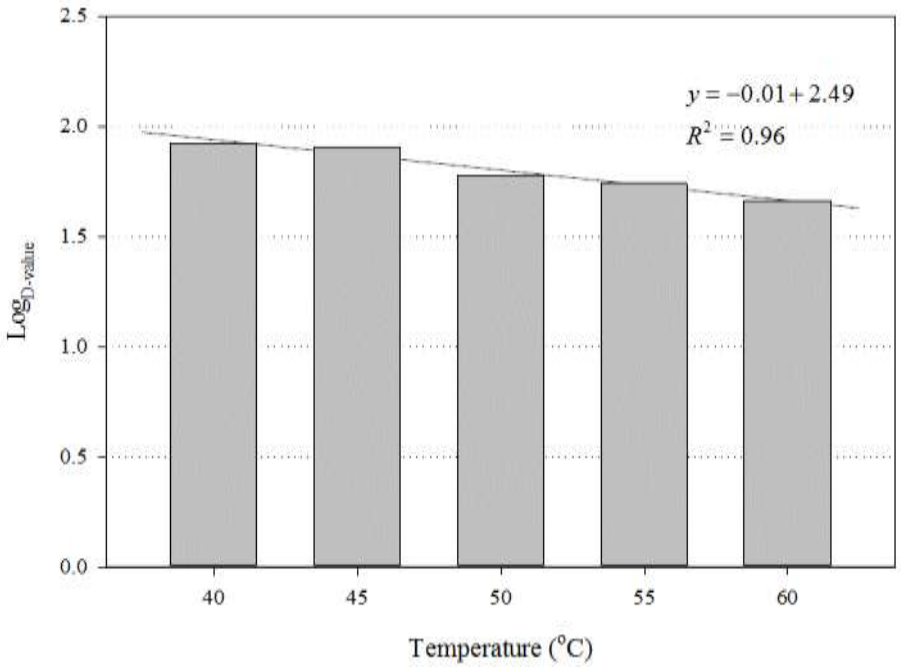

Figure 7.7. Logarithmic D-values obtained during the storage of PBS product prototypes at a range of elevated temperatures.

The D-value and z-value of a population differs, in that the D- value indicates the time that is required to kill an organism at a specified temperature, and the z-value represents the resistance of an organism to differing temperatures. *V. midae* demonstrated a z-value of 72.33ºC. This gives an indication of the relative impact of temperature on a microorganism. Smaller D-values indicates a higher heat sensitivity profile of the microorganism. Due to the fairly large z-value it can be ascertained that *V. midae* in a PBS buffer is stable. This stability is largely due to the VBNC state of the stabilized product.

The D-temp is the d-value at a specified temperature, and is used to calculate the shelf life of a product, which is represented in Equation 1 (section 7.2.3). The F-value is

the amount of time it takes to destroy a specific number of viable cells having a specific z-value, and is an indication of the shelf life of the product. The shelf life of *V. midae* in a PBS buffer was calculated to be ~335 d under refrigeration conditions.

7.3.4 Assessment of the performance of the *V. midae* liquid prototype at various storage conditions

The accelerated stability kinetic data model was used as a tool to confer information regarding the impact of storage temperature on the liquid product. Storage conditions such as farm environment, transport and supply chain conditions and during transit of the finalized product were modelled from the data (Figure 7.8).

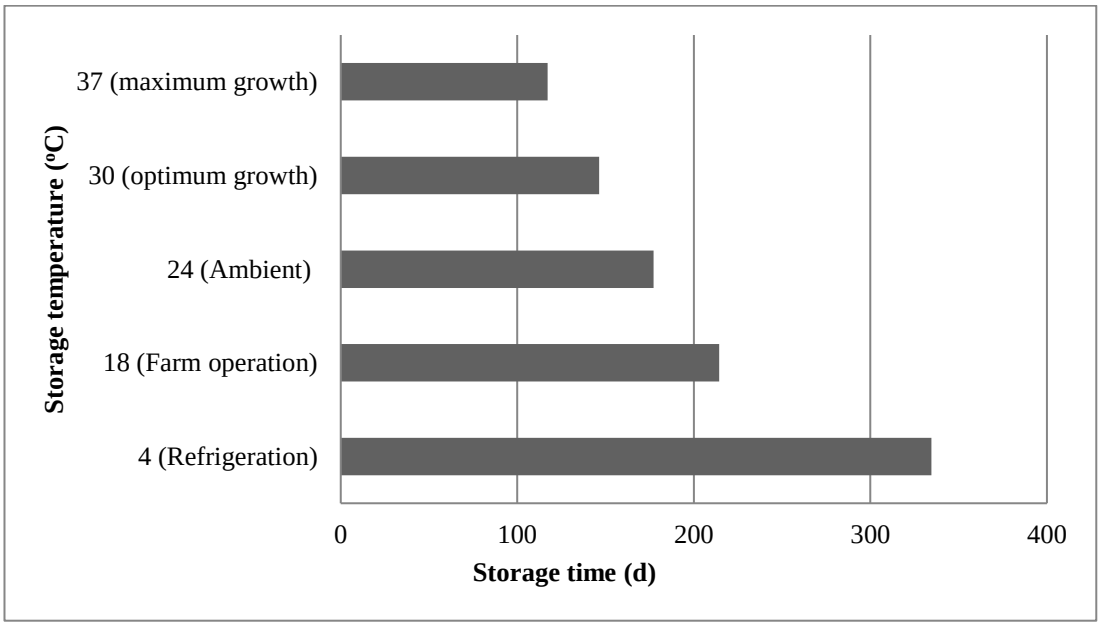

Figure 7.8. Performance of *V. midae* cells in a liquid product at various storage temperatures showing stability for duration of storage period (based on 1 log decline in viable cell number).

It was determined that storage at ambient temperature (24^{o}C) would allow for approximately 6 months of product stability, with a maximum decline of 1 log of cell concentration (Figure 7.8). This observation is significant in that it reduces the need for cold chain transportation of the product, which reduces the cost implications to facilitate the delivery of the product locally and abroad. In discussion with our local feed producers, the typical turnaround time for abalone feed from production to farm consumption is approximately 6 weeks. Our product formulation surpasses the local need of fourfold as it is stable for ~25 weeks.

Due to the nature of the *Vibrio* organism and its associated VBNC state, the LIVE/DEAD BacLight™ viability kit assay conferred more information regarding the suitability of product formulation and its resultant impact on cell viability. This was when the staining assay was used in combination with the other standard cell enumeration assays, such as microscopic counting of cells (to obtain total cell concentration) and viable plate counts (to obtain the concentration of cells that are still viable and culturable). This useful analytical tool will be included in all further assay methods in order to establish the suitability of a specific product prototype. The PBS product prototype stored at 4^{o}C demonstrated the highest suitability for storage to maximize viability. In addition, conventional thermal kinetic modeling conferred vital information regarding the storage of the *V. midae* PBS product during the entire supply chain from place of manufacture to the abalone farm environment.

CHAPTER 8: DEMONSTRATION OF COMPLETE PROCESS FOR PRODUCTION OF *V. midae* AT MANUFACTURING SCALE

8.1 INTRODUCTION

Microbial cell production technologies developed at small scale do not satisfy the need for commercial production of large volumes of probiotic as required by the aquaculture industry. Our technology required demonstration at production scale (200 L) to assess performance and reproducibility. It has been reported by Doble (2006), that the scale up correlations and relationships of chemical processes do not always apply to biological processes due to their complex nature. As a result, the design of the scale-up and optimization of cell production systems are sophisticated and difficult due to the complicated biochemical activities that are associated with microorganisms (Doble, 2006). It is therefore important that small-scale technologies are scaled up and validated at production scale, in order to demonstrate a commercially viable production technology. In process development it is necessary to show repeatable performance of all unit operations and to quantify the upstream production performance and the recovery through the various downstream process unit operations to the final product.

There are various factors to consider during the scale up of our abalone probiotic production technology. Scale up of bioreactor cultivations can be an empirical,

imprecise art. Traditionally, scale-up strategies have included attention to variables such as constant power to volume ratios, constant k_La, volumetric air flow rate and impeller tip speed. It is furthermore challenging to scale up bacterial cultivations, due to the complex nature of large scale vessel conditions (Shuler and Kargi, 1992). Stirred tank bioreactors are traditionally used to cultivate bacteria. It is common practice that classic stirred tank bioreactors used in laboratory scale development are then scaled up based on the small-scale design principles (Hambor, 2012). Challenges with scale up include: difficulty to maintain homogeneity in large systems, a change in surface to volume ratio and the cultivation itself may change due to longer culture times. The primary limitations of stirred tank bioreactors are gas supply and heat removal. The mass transfer (k_La) in the reactor is also a key concern, and is dependent on the type of reactor used, gas flow rate, agitator speed, as well as nature of the medium contained within the reactor (Shuler and Kargi, 1992). Due to these considerations, it is important to successfully transfer a laboratory scale process to manufacturing scale to reduce the scale-up risk for commercialization.

The technology for the production of *V. midae* has been demonstrated at laboratory scale using corn steep liquor (chapter 4) and high-test molasses (chapter 5) as nutrient and carbohydrate sources. In addition, a suitable cell separation strategy in order to maximize cell recovery at minimal operating cost; as well as the formulation of the probiotic into a market ready product was conducted (chapter 6 and chapter 7). The process performance achieved at smaller scale was sufficient to meet the cost of production targets for commercial manufacture and use of the probiotic in abalone production.

The success of any microbial production technology ultimately depends on the performance at manufacturing scale. Key measures of a bioprocess technology at full production scale include process performance indicators, such as productivity of the process, yield of product on protein, carbohydrate and oxygen, as well as the commercial feasibility assessment of the production process (Hambor, 2012). Many researchers in the field of bioprocess development face several challenges that pose risks to the application of their technologies. These risks include the lack of competitiveness of the technology due to poor techno-economics, inability to demonstrate process repeatability and robustness, poor stability of the produced cells as well as further challenges that may arise during scale-up of technologies.

Considering these challenges, outlined above, our research aimed to demonstrate the performance of each process unit operation (PUO) and sub-unit operation (SUO) for the production of *V. midae* at manufacturing scale. The full process was conducted in triplicate to demonstrate process performance, repeatability and robustness.

8.2 MATERIALS AND METHODS

The process summary of the *V. midae* abalone probiotic production technology is outlined in Figure 8.1. The upstream production process (PUO 1 and PUO 2) demonstration and validation was conducted across three batches.

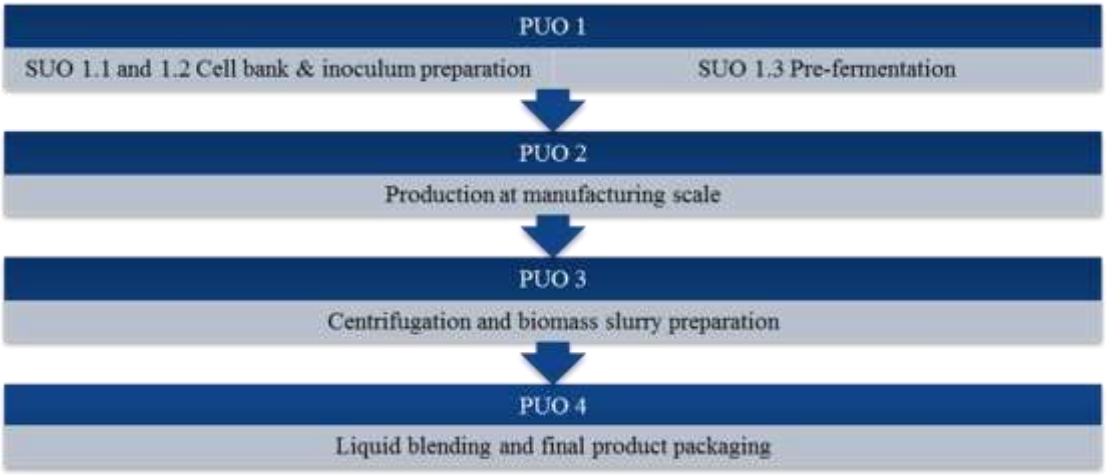

Figure 8.1. Process summary of the abalone probiotic production technology

8.2.1 Preparation of inoculum for production bioreactor (PUO 1)

8.2.1.1 Flask inoculum culture (SUO 1.2)

For each production batch, three cryovials (2mL) were removed from the -80^{o}C ultra-freezer (Thermo-Fischer Scientific, USA) and used to inoculate three Fernbach flasks (seed flasks) containing 700mL of medium under conditions specified in chapter 2, section 2.2.1.

8.2.1.2 Pre-fermentation (SUO 1.3)

Each seed flask (one was a back-up) were used to inoculate two 15 L Biostat Braun C bioreactors (Sartorius BBI systems, Melsungen, Germany) containing 9.3 L of medium prepared according to methods outlined in chapter 5, section 5.2.2.

The salts, antifoam (1 ml.l^{-1}, Pluriol P2000, BASF, Ludwigshafen, Germany) and protein sources of the culture medium were added to the initial charge and made up to

a volume of 9.3 L. Subsequent to the *in-situ* sterilization of the initial charge, a separately sterilized HTM solution was added (24 $g.l^{-1}$, 55% $m.m^{-1}$ TSAI) into the bioreactors. The fermentation temperature was maintained at 30°C with the stirrer speed set at 500 rpm and ramped to 1300 rpm over 2 h, to maintain the dissolved oxygen concentration above 30% saturation. The pH was maintained at 6.5 using 25%$v.v^{-1}$ NH_4OH and aeration in the vessel was set at 1 $v.v^{-1}.m^{-1}$. Cell culture broth (10 L from one reactor, the other was a back-up) was harvested after 4.5 h of cultivation at an OD_{660nm} of approximately 25.0 using an aseptic transfer assembly.

8.2.2 Production of *V. midae* at manufacturing scale (200L) (PUO 2)

The inoculum (10 L) was aseptically transferred into the 200L Biostat Braun DS300 production reactor (Sartorius BBI systems, Melsungen, Germany). The culture medium in the reactor contained ingredients at concentrations as listed in chapter 5, section 5.2.2. The salts, antifoam (1 $ml.l^{-1}$, Pluriol P2000, BASF, Ludwigshafen, Germany) and protein sources of the culture medium were added to the initial charge and made up to a volume of 190 L. Vitamins were not supplemented in the commercial scale demonstration studies as a previous study confirmed that this had no impact on growth. Subsequent to the *in-situ* sterilization of the initial charge, a separately sterilized HTM solution was added (24 $g.L^{-1}$, 50% $m.m^{-1}$ TSAI) into the bioreactor. The fermentation temperature was maintained at 30°C, with the stirrer speed set at 100 rpm and ramped to 450 rpm over 2 h, to maintain the dissolved oxygen concentration in the reactor above 30% saturation, the pH was maintained at 6.5 using 25% $v.v^{-1}$ NH_4OH and aeration was set at 1 $v.v^{-1}.m^{-1}$. The fermentation

process was stopped when a decline in the oxygen utilization rate measured on-line was observed. This was then confirmed by a decline in cell growth based on microscopic cell counts as described in chapter 4, section 4.2.6. The *V. midae* cell culture produced was harvested into a pre-disinfected storage tank with constant mixing at ambient conditions using an overhead mixer (Heidolph RZR 2102, Kelheim Germany).

8.2.3. *V. midae* cell separation using a Disk stack centrifuge and product formulation (PUO 3 and PUO 4)

Cell separation was performed continuously using a disk stack centrifuge (Westfalia, SA1, GEA, Germany) according to methods outlined in chapter 6. Culture broth was fed into the centrifuge at a rate of 26.40 $l.h^{-1}$ (SUO 3.1). The centrifuge was operated at a constant speed of 9000 rpm and the bowl pressure was maintained at 100 kPa by adjusting the back pressure valve, and the bowl was de-sludged at two minute intervals to collect the biomass paste. The biomass paste was thereafter reconstituted to 150 l in a saline phosphate buffer (PBS) (KH_2PO_4 0.11, K_2HPO_4 0.71, NaCl 2.91 $g.l^{-1}$ per litre of de-ionized water). The re-suspended pellet was re-centrifuged as a second pass using the same flow rate and de-sludge intervals (SUO 3.2). All biomass fractions were combined and reconstituted into a homogenous solution using PBS and analyzed as described in section 8.2.4. The biomass slurry was stored in sealable 20 L buckets at 4^{o}C for further use. The supernatant fractions were collected and analyzed as described in section 8.2.4.

Soon after harvest (<1 d) the biomass slurry was formulated by aseptic dilution with PBS to yield a final viable cell concentration of 1×10^7 cells.ml$^{-1.}$ This liquid product was packaged into sachets containing *V. midae* cells using a vertical fill, form and seal sachet pack machine (Alpha Packaging, Hong Kong, China). The completed products were placed in the refrigerator and assessed for product quality according to methods outlined in section 8.2.4.

8.2.4 Sampling, analysis and data collection

8.2.4.1 Flask inoculum culture (SUO 1.2):

Inoculum flasks were sampled at the end of the incubation period prior to transfer. Optical density measurements at 660nm were measured using a Beckman Coulter DU 800 Spectrophotometer (Beckman Coulter Inc, USA). Samples were the OD_{660nm}, exceeded 0.9, were appropriately diluted using distilled water, prior to measurement. Total microscopic cell counts were performed using a Thoma® counting chamber (Hawksley and Sons, London) according to methods outlined in chapter 7; section 7.2.2.2. Glucose concentrations were determined using an Accutrend® alpha glucometer according to methods described by the manufacturer (Boehringer Mannheim, Germany). Sugar analysis was conducted according to methods outlined in chapter 5, section 5.2.1.3 in order to calculate yield of cells on sugar. The viable cell concentration within the population was determined using the LIVE/DEAD BacLight^{TM} bacterial viability kit (Molecular Probe Inc., USA) according to methods outlined in chapter 7; section 7.2.2.2. Viable cell concentration was determined using this equation below.

$$Viable\ cell\ concentration = total\ microscopic\ cell\ concentration \times viability\ (\%)$$

Equation 8.1

8.2.4.2 Pre-fermentation (SUO 1.3):

The bioreactors containing the inoculum cultures were sampled (30 ml) immediately after inoculation and prior to harvesting. In addition, samples were extracted on an hourly basis to conduct optical density measurements, and to measure total microscopic cell concentration and the cell viability (%) using the LIVE/DEAD BacLight^{TM} bacterial viability kit (Molecular Probe Inc., USA) according to the methods outlined in chapter 7; section 7.2.2.2.

8.2.4.3 Production fermentation (PUO 2):

Samples (30 ml) were removed from the 200 L bioreactor immediately after inoculation and at 30 minute intervals until the batch was terminated. Analysis of all samples was carried out as described in chapter 7; section 7.2.2.2.

Cell productivity was expressed as the rate change in total cell concentration, determined from the microscopic enumeration of viable cells over time. Yield co-efficients based on protein (YPP), glucose substrate added (YPS) and oxygen consumed (YPO) were calculated and assessed for each of the production batches as described in chapter 5, section 5.2.5. Exhaust gas analysis was similarly carried out using an Uras 10E gas analyser (Sartorius BBI Systems, Melsungen, Germany). Oxygen utilization (OUR) and carbon dioxide evolution (CER) rates were calculated online using MFCS software, from carbon dioxide and oxygen concentration

measurements of the exhaust gases. Oxygen utilization rate was maintained below 200 $mM.l^{-1}.h^{-1}$ as this is the recommended safe maximum transfer rate for stirred tank bioreactors (Rivière 1977; Ozturk 1996). This was calculated by determining the volume corrected OUR at a specific time point in the cultivation based on the actual volume of the reactor and amount of air introduced into the fermenter.

8.2.4.4 Cell separation (PUO 3):

Samples obtained from the supernatant and biomass slurry fractions were analyzed for total viable cell concentration and optical density. Viability assessments were conducted using the LIVE/DEAD BacLight^{TM} viability kit (Molecular Probe Inc., USA). Process repeatability assessments were conducted on batches as described in chapter 6, section 6.2.3.

8.2.4.5 Formulation of the liquid product (PUO 4)

Quality assessment of the refrigerated samples (n = 9) were assessed for total cell concentration and cell viability according to methods outlined in section 8.2.4.

8.3 RESULTS AND DISCUSSION

8.3.1 Flask inoculum (SUO 1.2)

In this study, process unit operation (PUO) 1 consisted of three sub unit operations (SUOs) (Figure 8.1). The replicate growth data (n = 6) of *V. midae* cells in an inoculum flask (SUO 1.2) and the pre-fermentation stage of the technology (SUO 1.3) is listed in Table 8.1 and Table 8.2 respectively.

Table 8.1. Replicate data obtained during the cultivation of *V. midae* in a Fernbach flask – (*n=6)* (SUO 1.2)

Unit	Inoculum flask OD	Inoculum flask cell concentration
	#	cells.ml^{-1}
Flask A	5.03	9.73×10^9
Flask B	3.91	7.91×10^9
Flask C	4.44	2.88×10^9
Flask D	5.01	2.68×10^9
Flask E	3.40	3.81×10^9
Flask F	3.40	2.78×10^9
High performance level	5.03	9.73×10^9
Low performance level	3.40	2.68×10^9
Average performance level	4.19	4.41×10^9
Standard deviation	0.74	3.65×10^9
CV (%)	17.71	82.64

An average OD of 4.19 ± 0.74 was achieved for the replicate cultivations (Table 8.1). The highest OD obtained in this study for SUO 1.3 was 5.03, which also corresponded to the highest cell concentration 9.73×10^9 cells.ml^{-1} (Table 8.1). A much higher variation in cell concentration data was observed compared to OD (CV of 17.1 and 82.64% respectively). This is attributed to the sensitivity of the inoculum stage and for this reason, redundancy is used in the flask growth stage. This is done so that the variability in inoculum cell concentration can be reduced by eliminating flasks with

excessive or too little cell growth to provide a more consistent pre-fermentation batch performance.

8.3.2 Pre-fermentation (SUO 1.3)

Cultivation of *V. midae* in 10 L bioreactors (SUO 1.3) resulted in an average OD of 25.2 ± 2.87 (Table 8.2). The average cell concentration based on microscopic counts was $5.76 \times 10^{10} \pm 1.50 \times 10^{10}$ cells.ml^{-1} of which approximately 99.3% was deemed viable as confirmed by viability staining (Table 8.2). These studies resulted in a coefficient of variation of < 30% based on cell count data for this stage of the process.

In typical manufacturing processes, either flasks or batch fermentations in a bioreactor may serve as inoculum for the manufacturing scale cultivations. The advantages of using a flask to cultivate the inoculum, is that a reduced process time is achieved, as the culture is grown for a period of time, in this instance, ~ 4.5 h, and thereafter it is transferred aseptically into the reactor. In addition to the reduced process time, a process that uses a flask stage inoculum instead of a bioreactor has a lower capital cost as an additional bioreactor is not required for the growth of the culture.

A lower variation in the data was observed in the bioreactor cultivations in comparison to the flask cultivations. Data obtained during the pre-fermentation SUO identified the improved consistency of using a batch fermentation process in contrast to using a flask inoculum for the production PUO of the process (Table 8.1 and 8.2). Based on the variations experienced in the flask stage culture, and its associated implications, specifically at manufacturing scale, it was decided that a two stage inoculum was

required in order to minimize batch to batch performance. Although a slightly extended process time was obtained, it was deemed acceptable as the growth time for the *V. midae* culture in its inoculum stage is fairly short (~4 – 5 h).

Table 8.2. Replicate performance data of the pre-fermentation step (*n=6)* (SUO 1.3)

	Pre-fermenter OD	**Pre-fermenter cell concentration**	**Viability of the population**
Unit	#	cells.ml^{-1}	%
Batch A	22.0	5.66×10^{10}	100
Batch B	22.3	8.16×10^{10}	96.5
Batch C	29.0	6.86×10^{10}	100
Batch D	24.7	4.22×10^{10}	100
Batch E	28.0	5.13×10^{10}	99.3
Batch F	27.2	4.52×10^{10}	100
High performance level	29.0	8.16×10^{10}	100
Low performance level	22.0	4.22×10^{10}	96.5
Average performance level	25.2	5.76×10^{10}	99.3
Standard deviation	2.87	1.50×10^{10}	1.40
CV (%)	11.4	26.1	1.41

Our findings also indicate that the pre-fermentation SUO does not need redundancy (a back-up batch), due to the acceptable consistency in performance across six different pre-fermentation batches. However, there may be repercussions affecting production

scheduling if batch failure is experienced and the production reactor cannot be started due to the lack of a redundant pre-fermenter. These scheduling matters can be mitigated on the basis of the short growth periods of *V. midae* for SUO 1.2 and 1.3, and the rapid turn-around time and re-inoculation if necessary, with minimal negative impact on the production schedule. Once the target OD was reached, the inoculum from the 10L pre- fermenter stage was transferred, using a sterile transfer assembly, into the production vessel.

8.3.3 Cultivation of *V. midae* at a production scale

The cultivation of *V. midae* at 200 L scale resulted in a fermentation process time of 5.46 ± 0.05 h. Maximum optical density and a total cell concentration of 23.10 ± 2.17 and $8.05 \times 10^{10} \pm 1.05 \times 10^{10}$ cells.ml^{-1} were achieved respectively at the end of the batch cultivation (Figure 8.2).

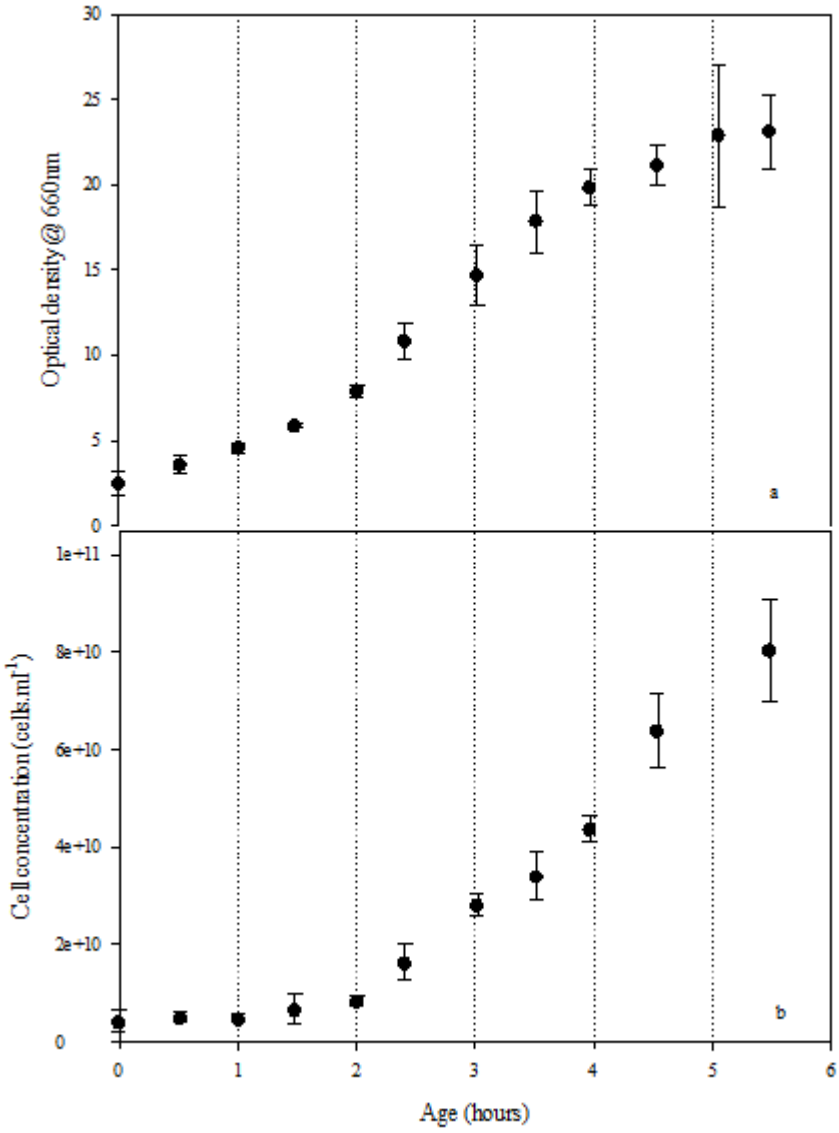

Figure 8.2. Average optical density (a) and cell concentration (b) profiles observed during the cultivation of *V. midae* at 200 L production scale (*n = 3).*

The volume corrected oxygen utilization rate (OUR) and carbon dioxide evolution rate (CER) were maximum at peak growth (4.5 h) and were 54.9 $mMol.l^{-1}.h^{-1}$ and 92.1 $mMol.l^{-1}.h^{-1}$ respectively (Figure 8.3). The oxygen requirements are well within the

mass transfer capability of stirred tank reactors as an average respiratory quotient (RQ) of 1.27 ± 0.17 was obtained. *Vibrio* organisms form part of the Proteobacteria phylum, and these organisms have been found to all converge around an RQ of 1.2 ± 0.17 for exponential growth. Although previous assumptions state that all organisms have an RQ of ~ 1.0 (Berggren *et al*., 2012) during normal cellular respiration, our findings further substantiate that this is indeed slightly different for *Vibrios.* When designing bacterial production processes, care must be applied such that a RQ of 2.0 must not be exceeded, as this is an indication of carbon flux to other waste metabolites, rather than cellular respiration and biomass synthesis, the latter being the key objective for the production of the probiotic organism. This cause of concern is usually most prevalent in the exponential phase of growth where metabolism is at its maximum.

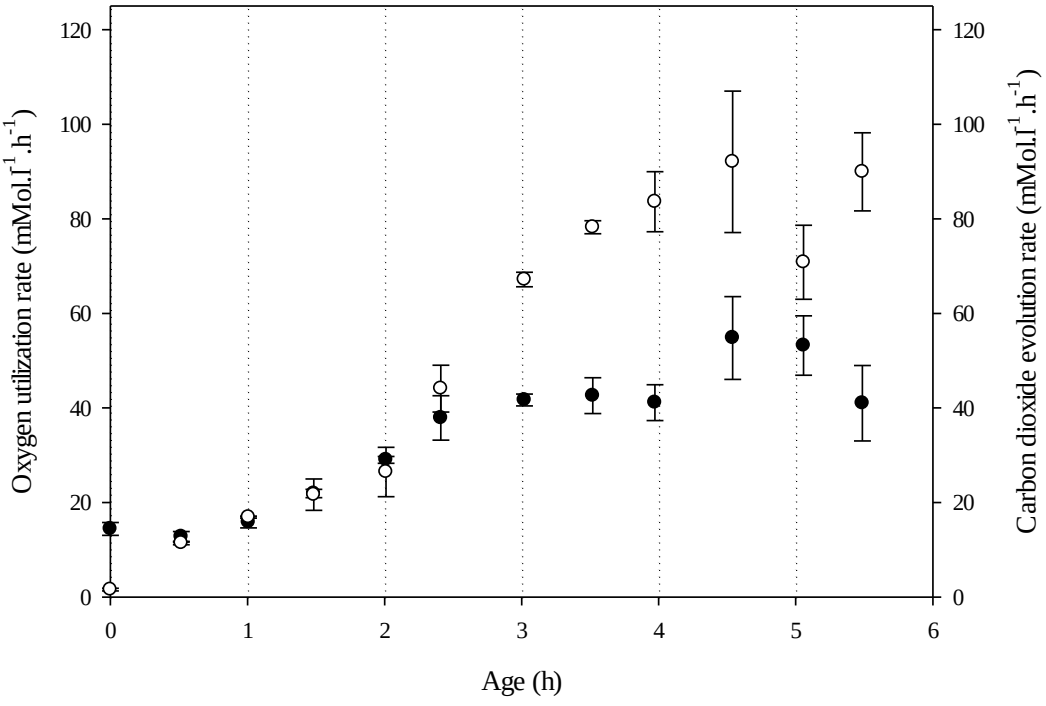

Figure 8.3. Oxygen utilization rate (●) (a) and carbon dioxide evolution rate (○) (b) profiles observed during the cultivation of *V. midae* at 200L production scale *(n = 3).*

The on-line gas rates form a useful tool which was used to monitor the production process on-line, allowing real time assessment of cell growth rate and the point at which maximum cells are produced. Other than tracking process performance, this data also informs the most suitable time for cell harvesting in real time.

In addition to assessing the performance of *V. midae* cultivation in the 200 l bioreactor, control of key operating variables such as temperature, pH and aeration were also assessed. Control of process parameters is vital in any bioreactor cultivation in order to maintain optimal conditions for product formation. Temperature, pH and aeration are the most commonly controlled process variables; however various other variables such as foam level, turbidity, and pressure are important in maximizing process performance. The use of probes in fermentation processes must be carefully selected and justified, as the insertion of probes into the reactor poses several operational and contamination risks. The benefits of the selected probes must outweigh the economic losses that may occur if process sterility is breached or sensor failure contributes to a loss in control. Control bar charts for the profiles of the key operating variables were evaluated across the replicate batches to assess process consistency. A control limit threshold of 10% deviation from the set-point is indicated in each of the control charts (Figure 8.4 a, b and c).

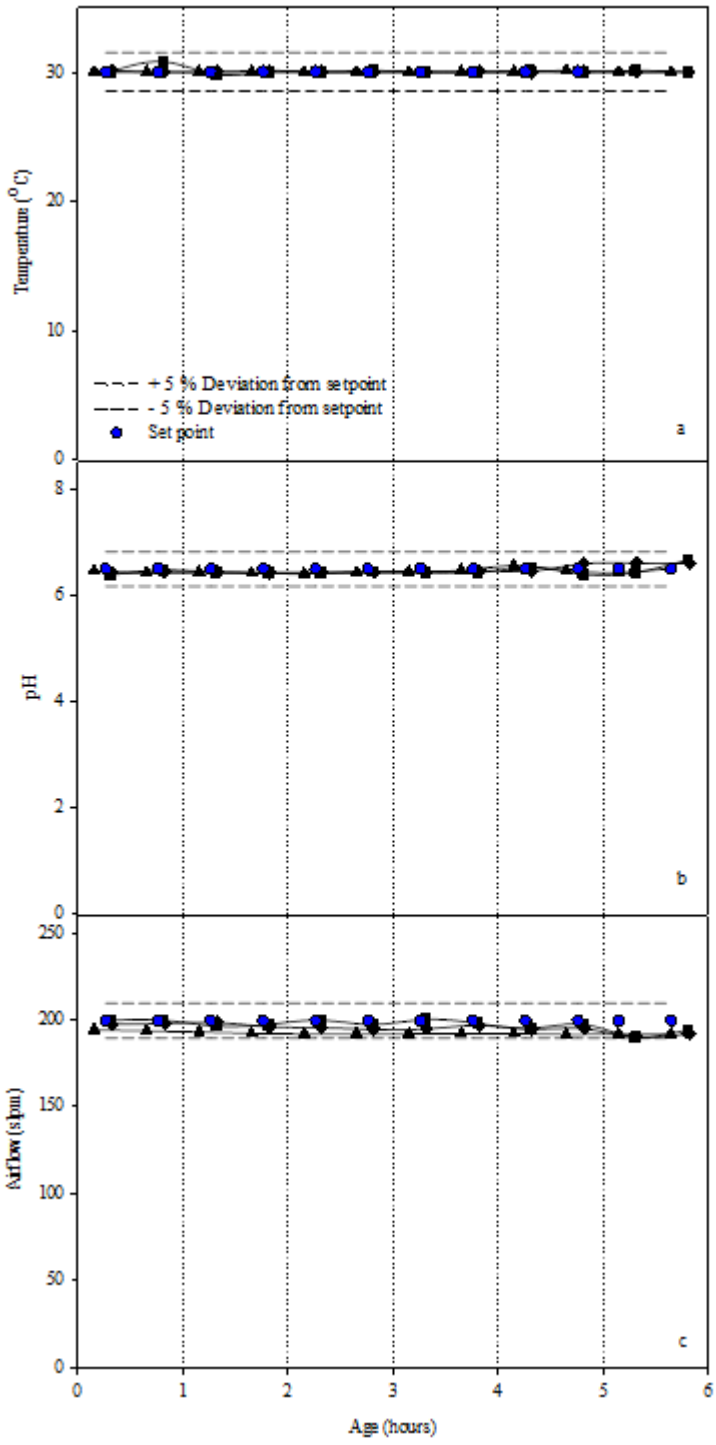

Figure 8.4. Profiles of key physical control parameters; (a) temperature, (b) pH and (c) aeration; representing process control within the 200 L bioreactor for replicate batches 1 (▲); 2 (■) and 3 (♦). Control levels are ± 10% of set-point value.

Operational consistency was achieved as each parameter in the respective batches did not exceed or drop below the 10% control limit threshold. Due to the short process time observed at manufacturing scale, a 10% control limit threshold was sufficiently adequate, as the reactor was not subject to long periods of deviation from set point.

An average viable cell concentration of 7.47×10^{10} cells.ml^{-1} and a corresponding cell productivity of 1.36×10^{13} cells.l^{-1}.h^{-1} were achieved across the triplicate batches (Table 8.3). The key bioprocess indicators YPP, YPS and YPO for the triplicate batches are reported in Table 8.3. The coefficient of variation for these five key responses were <10%, indicating excellent process reproducibility (Table 8.3).

Table 8.3. Key performance indicators of the fermentation process obtained during the cultivation of *V. midae* at a production scale (*n=3).*

Key response	Unit	High performance level	Low performance level	Average performance level	Standard deviation	CV (%)
Viable cell concentration	cells.ml^{-1}	8.17×10^{10}	6.92×10^{10}	7.47×10^{10}	6.42×10^{9}	8.60
Cell productivity	cells.l^{-1}.h^{-1}	1.49×10^{13}	1.25×10^{13}	1.36×10^{13}	1.24×10^{12}	9.10
Yield on protein added (YPP)	cells.g^{-1}	1.34×10^{13}	1.14×10^{13}	1.23×10^{13}	1.06×10^{12}	8.60
Yield on substrate added (YPS)	cells.g^{-1}	4.08×10^{12}	3.35×10^{12}	3.70×10^{12}	3.67×10^{11}	9.88
Yield on oxygen consumed (YPO)	cells.g^{-1}	1.38×10^{13}	1.19×10^{13}	1.25×10^{13}	1.12×10^{12}	8.97

Once the technology was demonstrated in triplicate batches, performance data obtained was compared to that achieved in laboratory scale demonstrations, to confirm that the scale-up was successful. The following observations were made (Table 8.4).

Cell productivity data revealed a high correlation between data obtained for both lab scale and manufacturing scale demonstrations. This was an indication that the efficiency of the technology was not hampered by the scale up. In addition, coefficients of variation of 0.61 and 8.75% were obtained for YPP and YPS. YPO data however, displayed a variation of 28.41% at manufacturing scale in comparison to actual data achieved at lab scale (Table 8.4). This decrease in YPO in the demonstration study can be attributed to differences in the absolute agitation tip speed between production and laboratory scale fermentations. The tip speed of laboratory scale impellers was 5.03 $m.s^{-1}$ when operated at the maximum speed of 1200 rpm. In the manufacturing scale demonstration study, a maximum impeller tip speed of 3.77 $m.s^{-1}$ was achieved under maximum agitation conditions. The variation of the YPO data can therefore be directly attributed to the differences in tip speeds achieved (~33%). The agitation controller in both instances was set on profile mode over 2 h. The lab scale profile differed in that the stirrer speed was set to increase from 500 rpm to 1300 rpm over 2 h. Due to the design considerations in the manufacturing scale reactor, the stirrer speed could only be set to increase from 100 to a maximum of 400 rpm over 2 h. In order to achieve an equal tip speed in both lab and manufacturing scale, an agitation speed of 534 rpm needed to be achieved, however, this value is beyond the maximum output of the manufacturing scale bioreactor. If classical scale up studies were undertaken, the tip speed of the reactors would have been made equal in both demonstrations in order to

reduce any potential factors of variation. However, due to the demonstration nature of this study, this was not done. This valuable learning can therefore be applied in all future cultivations at manufacturing scale.

Table 8.4. Comparison of key performance indicators obtained during the cultivation of *V. midae* at 10 L and 200L scale.

Key measure	**unit**	**Laboratory scale (10L)**	**Manufacturing scale (200L)**	CV %
Cell concentration	cells.ml^{-1}	7.52×10^{10}	7.47×10^{10}	0.51
Cell productivity	cells.l^{-1}.h^{-1}	1.37×10^{13}	1.36×10^{13}	0.31
Yield of cells on protein (YPP)	cells.g^{-1}	1.22×10^{13}	1.23×10^{13}	0.61
Yield of cells on substrate added (YPS)	cells.g^{-1}	3.28×10^{12}	3.71×10^{12}	8.75
Yield of cells on oxygen consumed (YPO)	cells.g^{-1}	1.88×10^{13}	1.25×10^{13}	28.41

8.3.4 Separation of *V. midae* cells from the fermentation broth

Three batches of *V. midae* culture broth, containing a starting viable cell concentration of 7.47 × 10^{10} ± 6.42 × 10^{9} cells.ml^{-1} were used to validate the cell separation unit operation (PUO 3) and the final product formulation (PUO 4). The resultant supernatant had an optical density of 2.80 and contained a cell concentration of 5.43 × 10^{9} cells.ml^{-1}, which equated to a 3.78 ± 0.51% loss of cells in SUO 3.1. The supernatant generated from SUO 3.2 contained a cell concentration of 7.54 × 10^{9} cells.ml^{-1}, which translated to a recovery of less than ~90%.

The amount of cells recovered during SUO 3.1 and 3.2 are reported in Table 8.5. SUO 3.1 demonstrated a higher mass balance recovery (103.5%) with a corresponding cell recovery (100.1%) in comparison to SUO 3.2. After the second centrifuge run, SUO 3.2, the centrifuge was once again dismantled, washed and reassembled, and a 96.6% mass balance closure and a corresponding cell recovery of 94.9% were observed. The data obtained in this validation study was compared to the data collected during the development stage (Table 8.5).

Table 8.5. Comparison of key performance indicators (%) obtained during the cell separation process unit operation *n=3* (PUO 3)

	Indicator	DSP development	DSP validation	Ave	SD	CV
	Cell recovery	68.7	100.1	84.4	22.2	26.3
SUO 3.1	**Mass balance closure**	72.5	103.5	88.0	21.9	24.9
	Loss to supernatant	3.8	3.3	3.6	0.3	8.87
	Cell recovery	103.6	94.9	99.3	6.2	6.2
SUO 3.2	**Mass balance closure**	105.7	96.6	101.1	6.4	6.3
	Loss to supernatant	2.1	1.7	1.9	0.2	11.8
	Cell recovery	71.2	95.0	83.1	16.9	20.3
Overall	**Mass balance closure**	75.2	100.1	87.6	17.6	20.1
	Loss to supernatant	5.2	5.1	5.1	0.1	1.6

A higher overall cell recovery as well as mass balance closure was obtained during the manufacturing scale validation study. This is expected as the disc stack centrifuge is designed for continuous operation and the quantity of unrecoverable material is proportionally much smaller when working with large batches compared to small

batches. Minimal losses were noted in the supernatant and as a result, excellent recoveries in terms of both cells and mass (94.9 and 96.6% respectively) were observed during the demonstration of the cell separation PUO at manufacturing scale. In addition to demonstrating the efficiency, robustness and repeatability of the cell separation process unit operation, it was also important to ensure that the viability of the cells were maintained during PUO 3.0 as well as during formulation of the product into market ready prototypes (Figure 8.5).

Cells present in the final biomass slurry obtained from PUO 3.0 were viewed microscopically, and the cell viability was determined. The biomass slurry was formulated in a saline phosphate buffer to contain viable cells at a concentration of 1.00×10^7 cells.ml^{-1}. This liquid product was packaged into sachets ready to be shipped via cold chain to the abalone feed production facility (Figure 8.5). Upon assessment of the cell viability in the packaged product, it was noted that a 30% variation was noted in the viable and total cell concentration (Table 8.6), however, all market ready samples conformed to the product standard specifications which stated that a concentration of 1.0×10^7 viable cells per ml of product was required.

Figure 8.5. Market ready samples containing *V. midae* cells

Variations in total viable cell concentrations are typical in biological products due to the nature of the material. Some factors that contribute to such variability include losses in viability during handling and packaging, but mostly the variability in cell enumeration method. As an example, the clumping of cells in concentrated products can cause variability in plate count assays and similarly depth of field issues can contribute to variability in microscopic counting. The biomass slurry used in the formulated product, although washed during the cell separation process, could still contain residual nutrients and as a result, could trigger the growth of the *V. midae* cells for a short period of time, until all residual nutrients were utilized. Prior research also revealed an increase in *V. midae* cell concentration for the initial period in all product formulations under various storage conditions. However, cell populations thereafter stabilized in terms of both cell population and cell viability (Table 8.6). This aspect completed PUO 4 of the production process (Figure 8.1).

Table 8.6. Key performance indicators of the formulation of *V. midae* into market ready products (PUO 4) *(n=9)*.

Key response	Unit	High performance indicator	Low performance indicator	Average performance indicator	Standard deviation	CV (%)
Total cell concentration	cells.ml^{-1}	2.77×10^{7}	1.24×10^{7}	2.10×10^{7}	6.37×10^{6}	30.28
Cell viability	%	90.95	79.23	84.09	4.54	5.27
Viable cell concentration	cells.ml^{-1}	2.35×10^{7}	1.01×10^{7}	2.33×10^{7}	1.77×10^{7}	30.90

It is imperative that small scale culture and associated protocols are developed into validated, stream-lined, industrially relevant bioprocess steps that can guarantee reproducibility, scalability, standardization, robustness and safety (Hambor, 2012). This study demonstrated the production of our *V. midae* abalone probiotic at production scale (PUO 2), the separation of the cells from the culture broth (PUO 3) and formulation thereof into a market ready abalone probiotic product (PUO 4). This study resulted in the development of a complete bioprocess technology resulting in an abalone probiotic product ready for use in the abalone feed production industry.

The end product was suitably developed after continuous engagements with the relevant stakeholders in both the abalone feed production industry and with abalone growers. Satyanarayana *et al*., (2012) stated that liquid product formulations should bear the following characteristics, (i) contain stable microorganisms throughout the supply chain, (ii) be easily applicable to the field, (iii) protect the organism against adverse environmental factors, and (iv) enhance the activity of the organism/s by increasing its viability, reproduction and resultant efficacy when applied to the target host. In the instance of our *V. midae* abalone probiotic, the stability and viability of the

V. midae probiotic under typical farm and feed production environments had been demonstrated in chapter 7.

During the development phase of the final formulated product, a visit was made to an abalone feed production facility based in Hermanus, Western Cape. It was then observed that abalone feed production occurs on a batch process, whereby dry ingredients are weighed and mixed together using a limited amount of water. Thereafter, the wet "dough" is extruded and dried at a constant temperature and humidity. It was during this visit that the point of inclusion of the probiotic into the feed production process was identified. It was decided by the research team that the liquid product would be added with the water component, prior to the mixing and extrusion, in order to obtain even dispersion of the probiotic product into the feed. Additionally, the packaging of the final *V. midae* probiotic product was deemed suitable and the product addition integrated seamlessly into feed production operations.

This study concluded the manufacturing scale performance and final product formulation of the *V. midae* abalone probiotic. In many instances, data and information describing process performance at manufacturing scale is limited and maintained as an industry secret. This study provides vital insight into the commercial production process and implementation of this technology at full commercial scale and is of benefit to the abalone industry.

CHAPTER 9: CONCLUDING REMARKS

The primary objective of this study was to develop a new bioprocess technology to produce a novel abalone probiotic product containing a marine organism, *V. midae*. The aim was met by increasing the productivity of the fermentation process through optimisation of a number of fermentation parameters such as the development of a robust inoculum, optimising temperature and pH and optimization of protein and carbohydrate concentrations in the growth medium. In addition, specialized detection methods were applied in this study in order to detect cells that were in the VBNC state and an appropriate recovery procedure was developed for such dormant cells. Apart from developing an upstream production process, an efficient downstream production process was developed, which included cell harvesting and formulation of the probiotic into a market ready product. This product addressed the needs of the end user and delivered on the need for a probiotic organism which was ready for use in the abalone aquaculture industry as an abalone feed additive. A further objective of the project was to fulfil all aims through innovative research and bioprocess development, which addressed existing technology gaps and the generation of new knowledge regarding the cultivation of this novel marine microorganism, *V. midae*.

9.1.1 Cell banking and inoculum development

The success of any bioprocess technology is dependent on a well-developed and well characterized inoculum. In this study, an inoculum containing a pure culture of *V. midae* cells, with a fast growth rate and short transfer times was developed. By following the protocol outlined in the cryovial and inoculum preparation, a robust and

reproducible inoculum stage of cultivation was obtained thus reducing the likelihood of batch variation or batch failure due to inconsistent inoculum cultures.

9.1.2 The impact of temperature and pH on the growth of *V. midae*.

The growth of *V. midae* was evaluated across a range of temperature and pH conditions. The optimum temperature and pH for the growth of *V. midae* was determined to be 30^{o}C and 6.5 respectively. The effect of temperature and pH on intrinsic growth was elucidated, and the information obtained will be used to successfully cultivate and apply the probiotic in industry. The cultivation of *V. midae* at these optimum conditions resulted in increased cell numbers and cell productivities, faster growth rates and shorter process times.

9.1.3 The assessment of CSL as a suitable nutrient for use in large scale cultivation of *V. midae.*

The high cell density cultivation of *V. midae* was successfully achieved through appropriate development of the key nutrient sources in the fermentation process. This aspect focussed on the use of an industrially relevant protein rich nutrient source, which had a positive impact on the cost of the production process. Corn steep liquor (CSL) was shown to be a better nutrient supplement when compared to conventionally used laboratory based nutrient substrates such as yeast extract and meat peptones. The CSL concentration was optimized by mathematically modelling commercially relevant responses such as cell concentration, cell productivity and material cost of production. The modelled data correlated well with actual fermentation data (CV < 15%). The mathematical optimum for the concentration of CSL, expressed as protein,

was 6.4 $g.l^{-1}$. An increase in cell productivity (2.8-fold) and a corresponding decrease in material cost of production (78 %) were observed when the optimum CSL concentration was tested in replicate fermentations in comparison to the originally used marine broth growth media.

9.1.4 The assessment of use of HTM as a suitable carbohydrate source for use in large-scale cultivation of *V. midae.*

In comparison to conventionally used carbohydrate sources such as glucose, HTM was assessed for its suitability of use in the production of *V. midae* due to its local availability and cost competitiveness. When HTM was used as a sole carbohydrate source overall increases in cell concentration and cell productivity, as well as cell yields on protein, carbohydrate and oxygen were observed in comparison to the previously used medium containing glucose and conventionally used protein sources. In addition, a further 48% decrease in MCOP was noted when HTM concentration was optimized in the MVM medium containing CSL. The resultant fermentation process using locally available agro-industrial "waste" by-products such as CSL and HTM improved production efficiency, which champions the acceptance of the technology due to reduced cost.

9.1.5 Development of a suitable cell separation technology for the *V. midae* abalone probiotic cells

Effective separation and recovery of *V. midae* cells from the fermentation broth was an essential aspect of the *V. midae* probiotic production process. Once cells were harvested from the bioreactor, a disk stack centrifuge was used in order to continuously

separate cells from the cultivation medium. The feed rate of broth into the centrifuge was determined in order to achieve maximum recovery of cells at the lowest cost of operation. In addition, the de-sludge interval of the centrifuge bowl was established in order to boost process efficiency. A mass balance of all process streams (biomass pellets and supernatants) was conducted, as well as a cell recovery determination. A flow rate of 26.40 $l.h^{-1}$ and a corresponding de-sludge time of two minutes were established as key process parameters for efficient cell separation. An overall cell recovery of 71.17% and a final overall mass balance closure of 75.21% were achieved. The data contained in chapter 6 is valuable in terms of assessing overall process efficiency of the cell separation process unit operation (PUO 3.0). The biomass slurry produced at the end of this unit operation was used to formulate the *V. midae* abalone probiotic into market ready products.

9.1.6. Formulation of the probiotic into a product

Once the biomass slurry was prepared it was essential to formulate the slurry into a market ready probiotic product. The information about the known VBNC state of *Vibrio* cells was exploited to develop four liquid product prototypes to meet end user requirements. *Vibrio* cells are known to enter a viable but non-culturable state, and this non-typical growth state alters the "normal" growth characteristics of the isolate. Upon assessment of the viable cells in the liquid product preparation for 22 d, all product options were rendered as feasible storage options. However, only the formulation containing phosphate buffer (PB) was selected for further analysis based on lowest cost. Suitability of the PB product prototype for long term storage was demonstrated using accelerated stability kinetics. The refrigerated *V. midae* PB product option

indicated a superior shelf life (334 d at 4 ºC) in comparison to all other temperatures tested. In addition, this study conferred vital information regarding the viability of the *V. midae* abalone probiotic product under various feed production and abalone farm storage conditions.

9.1.7. Technology demonstration at a production scale

A fully defined upstream process (USP) and downstream process (DSP) technology which was developed at laboratory scale, was successfully demonstrated at manufacturing scale (200 L), in order to demonstrate process robustness and repeatability. Studies were performed in triplicate batches at 200 L scale, and key process indicators such as cell concentration, productivity, yield of product on protein (YPP), substrate (YPS) and oxygen consumed (YPO) were evaluated for the USP aspect of the demonstration. In addition, performance of the technology at manufacturing scale was compared to laboratory scale demonstrations, in order to assess the impact of scale up on the production technology. All key performance indicators were statistically similar to the performance data achieved on a 10L scale, indicating a successful demonstration of the technology at full manufacturing scale. Batch to batch variability for 10 L and 200 L scale on all performance indicators were less than 10% (n= 3), with the exception of YPO (28.4%). The DSP aspect of the study demonstrated the cell separation PUO 3.0, as well as the formulation of the produced cells into market ready products (PUO 4). The viability of the *V. midae* cells through the DSP PUOs as well as the conformance of the final product to customer expectations was demonstrated. Overall mass balance and cell recovery assessments of 95.0 and 100.1% were observed during the demonstration of PUO 3.0 at

manufacturing scale respectively. This study completed the finalization of the bioprocess technology encompassing the development of an abalone probiotic production process at a manufacturing scale.

9.2 IMPLICATIONS OF STUDY

The successful completion of this study has resulted in the following implications, contributions and overall impact:

i. This investigation demonstrated a high degree of novelty for the production of *V. midae* which was patented (PCT/IB2014/058523).

ii. Knowledge generation surrounding the cultivation of a novel marine microorganism, *V. midae* (First page of publication attached).

iii. This is the first study optimizing key parameters for commercial production of this probiotic as no bioprocess for the production of *V. midae* has been reported in the literature.

iv. Development of a *V. midae* cultivation medium containing industrially relevant nutrient sources (CSL and HTM) which resulted in an efficient and cost effective production process.

v. A cell separation process using a disk stack centrifuge was developed resulting in an efficient and cost effective cell harvesting process.

vi. The VBNC state of the *V. midae* organism was suitably demonstrated, and novel viability enumeration techniques for this marine probiotic isolate were developed.

vii. A commercial product was produced which was robust based on a long shelf-life under industrially appropriate storage conditions.

viii. The overall contributions of this study boosts the commercial attractiveness of this novel technology because of a thorough demonstration of the complete process at manufacturing scale. The rigor of the process data also facilitates easier transfer and uptake by a bio-manufacturing entity that would produce the abalone probiotic product.

It is envisaged that the development of successful bioprocess technologies using these agricultural wastes from agro-industries in South Africa, will create an opportunity in the field of industrial biotechnology. These biotechnology-based production technologies would not only develop SA's emerging bioeconomy, but also contribute towards the sustainability of both the corn and sugar processing industries. In addition to the commercially sound production process of the abalone probiotic, the use of the product would further positively impact on the competitiveness of the abalone mariculture industry and much needed job creation in South Africa. Indirectly this could boost export volumes of farmed abalone and reduce the poaching of natural abalone.

9.3 FUTURE WORK

In order to enable the smooth commercial implementation of this technology at a production scale, a full technology package would need to be compiled and the necessary technology transfer completed. Furthermore, the efficacy of abalone

probiotic may be elucidated by extending this probiotic technology on other mollusc species. Another consideration for further technology development could be to determine the impact of the probiotic addition on the cost of production of the abalone feed itself by conducting a full cost benefit assessment.

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