Large-scale production of an abalone probiotic, *Vibrio midae*, isolated from a South African abalone, *Halitotis midae* for use in aquaculture.

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Abstract

The South African abalone industry is under severe pressure due to illegal harvesting and poaching of this seafood delicacy. These abalone are harvested excessively and consequently, these animals do not have a chance to replace themselves, ensuing in a drastic decrease in natural abalone stocks. Abalone, has an extremely slow growth rate, and takes approximately four years to reach a size that is marketable. Probiotics offer a viable treatment solution to improve the growth, health and vigour of these animals. Two microorganisms, a yeast isolate *Debaryomyces hansenii* and a bacterial isolate *Vibrio midae*, from the gut of the South African abalone were characterised for their probiotic abilities. This work resulted in an internationally competitive concept technology that was patented. Thereafter research was focussed on developing a suitable bioprocess to enable commercial production of one the isolates, *V. midae*. During this development, production parameters such as temperature and pH, as well as alternative medium additives, such as the use of corn steep liquor (CSL) and high test molasses (HTM) was assessed. Once a high efficiency production process was developed at laboratory scale, this up- and downstream production technology was scaled up to manufacturing scale, which resulted in a final market ready probiotic product. The optimization of the key variables resulted in a 180% increase in cell concentration and a 5716-fold increase in cell productivity, in comparison to conventional growth conditions and parameters used at the beginning of the study.

1. Introduction

Abalone is one of the most valuable seafood species in the world, whereby demand far exceeds supply, especially in Asia (Reddy-Lopata et al., 2006; Stanford J, 2004). 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 destination of majority of the globally produced abalone is China, were abalone are used primarily as a celebration dish. As abalone take a long time to grow to market size in its natural habitat, they have been successfully cultivated in aquaculture operations around the world. This alleviates demand considerations without stimulating mass poaching and illegal harvesting. However, aquaculture systems also have challenges, including diseases, waste accumulation and deterioration of environmental conditions. The application of antimicrobial drugs is a common approach to dealing with animal health issues in the aquaculture industry.

However, the use of such treatments has led to the development of antibiotic resistant bacteria (Schwarz and Kehrenberg, 2001). 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). 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 (Saraswathy et al., 2018). 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, 2006).

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 (Ringø et al., 2010). Probiotics offers the aquaculturist 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 (Macey et al., 2005; Verschuere et al., 2000).

This study followed on from initial R&D efforts conducted by (Macey et al., 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 feed conversion (Macey et al., 2005).

Once the probiotic effects were elucidated, research efforts were centred on developing and delivering a viable and robust probiotic product of *Vibrio midae* to be incorporated into abalone feed. 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. To ensure that this probiotic product offered the desired effect to the cultivated abalone, a laboratory scale production process was first developed, and thereafter scaled up to manufacturing scale, thereby resulting in a market-ready abalone probiotic product.

2. Methods and Materials

2.1. Organism

Cryopreserved cultures were obtained from the University of Cape Town and contained *V. midae* (LMG P-27727), at a concentration of approximately 2×10^9 CFU.ml^{-1}. This was used as an inoculum source for all bioreactor cultivations conducted in this study.

2.2. Medium and inoculum culture conditions

For the temperature and pH study; one cryovial was used to inoculate 200 ml cultivation medium in a 1 L Erlenmeyer flask containing 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. 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.

For the Corn Steep Liquor (CSL) and High Test Molasses (HTM) study, one cryovial was used to inoculate 700 ml cultivation medium as listed; in a 1.8 L Fernbach. Flask media was pH adjusted to 6.5 and sterilized. Culture flasks were incubated at 30°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.

All materials used in this study were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

2.3. Effect of temperature and pH

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. Cultivation of *V. midae* was performed across a range of temperatures (10ºC, 20ºC, 25ºC, 27.5ºC, 30ºC, 35ºC, and 40ºC). The medium was prepared to a volume of 1500 ml, and pH adjusted to 7.0. The pH of the cultivation medium was maintained at this value post sterilization (121ºC, 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%. For the pH investigation, cultivation of *V. midae* was performed at pH 5, 6, 7, and 8 using the same parameters as in the temperature study, at 30ºC (optimum temperature).

Bioreactors were sampled hourly (20mL), until maximum growth (determined by measuring OD_{660nm}) was reached, and terminated once the organism entered the stationary phase. Data analysis included determination of overall growth rate, cell concentration and cell productivity. Growth rate was 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.

2.4. Evaluation of CSL and HTM as nutrient sources in laboratory studies

The growth of *V. midae* 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 laboratory studies using 15 L Biostat C fermenters. The salts, antifoam (1 $ml.l^{-1}$, Pluriol P2000, BASF, Ludwigshafen, Germany) and CSL of the media were added to the initial charge and made up to a volume of 9.3 L. Subsequent to the *in-situ* sterilization (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.

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). The sugar feed rate was adjusted using a gravimetric flow controller to maintain a glucose concentration of 2.0 to 5.0 $g.l^{-1}$. 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

For the HTM study, a range of concentrations (1$g.l^{-1}$, 5 $g.l^{-1}$, 15 $g.l^{-1}$, 25 $g.l^{-1}$ and 40 $g.l^{-1}$ using the optimum growth medium identified in the CSL study. 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) was added.

For both studies, reactors were sampled every 30 minutes. The fermentation temperature, pH and aeration was maintained at 30 ºC, 6.5 and 1 $v.v^{-1}.m^{-1}$ respectively. The stirrer speed was set at 500 rpm and ramped to 1000 rpm over 3 h, to maintain the dissolved oxygen above 30% saturation. A decrease in the oxygen utilization rate by ~10$mM.L^{-1}h^{-1}$, indicated the end of exponential growth, which served as the stop signals for each fermentation.

2.5. Data analysis

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). Each of the key responses (growth rate, cell concentration, cell productivity, cost of production and yields on protein, oxygen and carbohydrate), in response to change in CSL or HTM concentration, were statistically analysed to determine appropriate fits to quadratic or cubic models. Numerical optima integrating all

responses 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.

Cost of production was determined by calculating the cost of medium components (used for the production of one litre of 1×10^7 cells. This is the supplementation level per gram of abalone feed according to Macey et al., (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).

Yield co-efficients were 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). A comparative assessment of key responses was evaluated to assess the impact of each process parameter on production performance.

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.

2.6. Comparison of laboratory scale optimised technology to base case technology

An analysis of variance (ANOVA), t-test assuming equal variances, was conducted to evaluate the effect of laboratory scale optimization on key production performance indicators (cell concentration, cell productivity, cost of production, YPP, YPS and YPO) in comparison to the base case production technology.

2.7. Production at manufacturing scale

The manufacturing scale process consisted of four process unit operations similar to that of the HTM study. A 10 L bioreactor cultivation was used as a seed inoculum for the production vessel. Once an OD of ~25.0 was reached, culture was assessed for its monoseptic status, this inoculum (10 L) was transferred to the production vessel (200L Biostat Braun DS300) using a sterile transfer assembly (Sartorius BBI systems, Melsungen, Germany).

The culture medium in the bioreactor contained ingredients at concentrations used in the optimized HTM study. The salts, antifoam and protein sources of the culture medium were added to the initial charge and made up to a volume of 190 L. 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, pH and aeration was maintained at setpoints used in the HTM study. Agitation speed was 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 cultivation was stopped when a decline in the oxygen utilization rate was observed.

The *V. midae* cell culture from the bioreactor was harvested into a pre-disinfected storage tank with constant mixing at ambient conditions using an overhead mixer (Heidolph RZR 2102, Kelheim Germany). The third process step was cell separation, and was performed continuously using a disk stack centrifuge (Westfalia, SA1, GEA, Germany). Culture broth was fed into the centrifuge at a rate of 26.40 $l.h^{-1}$. 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. All biomass fractions were combined and reconstituted to form a homogenous

solution using 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).

3. Results and Discussion

Effect of temperature on production of *V. midae*

Overall growth rate, cell productivity and cell concentration were selected as marker responses to determine the optimum growth temperature of *V. midae*. In contrast to maximum intrinsic growth rate, overall growth rate is a better indicator of commercial cell production. Growth rate against temperature conformed to a cubic model ($R^2 = 0.96$) with maximum overall growth rate (0.65 h^{-1}) at 30^{o}C (Figure 1 a). Overall growth rate decreased at temperatures on either side of the optimum temperature (30^{o}C). This observation justifies the importance of temperature optimization studies, because outside of the optimum range, cell energy wastage is increased, as a result, the amount of available energy for metabolic processes related to cell replication is reduced (Bonaïti et al., 2004).

Productivity (rate 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 (Viniegra-González et al., 2003), but our interest was in the actual probiotic cells and therefore productivity and growth rate data followed a similar trend as they are both measures of cell replication rate (Figure 1 a and b). 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 1 b). Under extreme temperatures, productivity of the process is reduced, and in some instances population death occurs, resulting in negative productivity, which was clearly observable at the extreme cold and hot temperatures (10 and 40^{o}C respectively).

Cell concentration is also important as obtaining a high cell concentration positively influences the cost of production through volumetric efficiency in both up and down stream processes (Lalloo et al., 2009). In our case, a target cell concentration of 1×10^{10} cells.ml^{-1} was defined up-front based on commercial requirements (high concentration, stable liquid product for inclusion into abalone feed during extrusion). Higher cell concentrations, compensate for viability losses in downstream processes such as centrifugation, formulation, extrusion and drying. Cell concentration data against temperature also conformed to a cubic model ($R^2 = 0.98$). The cell concentration was optimum at 30^{o}C and met the target of 1.20×10^{10} cells.ml^{-1}. As noted in Figure 1 a and b, cell concentration was also compromised at sub-optimal temperatures, attributable to reduced growth at these conditions.

One method of understanding the fundamental growth kinetic in response to temperature, is by using the Arrhenius approach (Figure 2). Our data indicated high linearity in both the hot and cold domains ($R^2 > 0.96$). There was an exponential increase in maximum growth rate (μ_{max}) between 10^{o}C and 30^{o}C; and subsequent decline in μ_{max} above 30^{o}C which were both thermodynamically influenced (Ratkowsky et al., 1983). 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 all growth indicators presented in our study (Figure 2). The critical temperature point (inflection of the two linear regressions) correlates to the point of optimum growth and productivity (Figure 3). Apart from providing classical thermodynamic information, the presence of low and high temperature domains illustrated by the Arrhenius plot, can be used to infer functionality or survival of the probiotic organisms in product processing and the gut of abalone in response to temperature.

Effect of pH on production of *V. midae*

Overall growth rate data against pH conformed to a quadratic model ($R^2 = 0.97$), where growth was maximum (1.31 h^{-1}) at an optimum pH of 6.0 (Figure 3 a). Overall growth rates were lower on either side of the optimum, but *V. midae* grew at neutral and mildly alkaline pH conditions (pH 7.0 and 8.0), in contrast to pH 5.0 where growth was severely attenuated. Studies conducted by Hug et al. (1985), indicated that *V. cholerae* grew better at alkaline pH up to 8.5, but in their studies, growth at pH 6.5, was less favourable, possibly due to different pH optima for the different Vibrio species.

The optimization of pH resulted in higher cell productivity, thereby improving the techno-economics of our 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. (Figure 3 b). Target *V. midae* cell concentration of 1.00×10^{10} cells.ml^{-1} was achieved at pH 6.0, 7.0 and 8.0, but not at pH 5.0 (Figure 3). Viable culture checks (data not shown), confirmed that pH 5 was lethal to *V. midae*, also observed by Wang and Gu, (2005), wherein *Vibrio vulnificus* MP -2, showed no growth at pH 5.2. Cell concentration data conformed to a quadratic model ($R^2 = 0.93$), with a maximum cell concentration of 3.60×10^{10} cells.ml^{-1} observed at pH 6.0 (Figure 3).

Effect of corn steep liquor (CSL) as a nutrient source for the production of *V. midae*

CSL and HTM are preferred fermentation substrates due to cost and local availability as by-products from the corn and sugar processing industries respectively. Both these nutrients have been shown to support viable cell production, but supplementation concentration had to be optimised to obtain the best performance (Prabakaran et al., 2007; Srivastava et al., 2015). The cell concentration profile conformed to a second order polynomial function ($R^2 > 0.90$) when ploted against the range of CSL concentrations tested (Figure 4 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 a). Beyond 10 g.l^{-1}, there was a decreasing trend in cell concentration of *V. midae* with increasing CSL concentration (Figure 4 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 (Kona et al., 2001; Ramawat et al., 2007). It is known that CSL supplementation at high concentrations has an inhibitory effect on organism growth as it contains high concentrations of lactic, butyric and phytic acid that affect substrate metabolism (Treichel et al., 2009). Growth may have also been osmotically inhibited at higher CSL concentrations, due to increasing concentrations of ammonium sulphate, present in CSL.

Cell productivity 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 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, because productivity has a greater influence of production efficeincy (Téllez-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 c).

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%. Another investigation similarly showed an increase in productivity with increasing substrate up to a maximum, where after performance is compromised. (Kona et al., 2001). *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 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 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 observed for all yield co-efficients calculated in our study, wherein YPP, YPO and YPS decreased with an increase in CSL supplementation (Figure 5

a, b and c) with maximum yields at 5 $g.l^{-1}$ (Figure 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. All yield co-efficients were poor at a CSL concentration of 40 $g.l^{-1}$, due to poor growth and substrate wastage. (Figure 5 a, b, c).

Effect of High Test Molasses (HTM) as a suitable substrate for the production of *V. midae*

Key responses of cell concentration, cell productivity and cost of production were further analysed when plotted against the range of HTM concentrations tested (Figure 6). 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 6 a). Similarly, cell productivity was optimum (1.62×10^{13} $cells.l^{-1}.h^{-1}$) at a HTM concentration of 25 $g.l^{-1}$ (Figure 6 b).

An increased cost of production at the minimum and maximum HTM test concentrations (1 and 40 $g.l^{-1}$) was noted (Figure 6 c). This is expected as both cell concentration and productivity were low at these extremes. 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 the optimum HTM concentration of 25 $g.l^{-1}$ (Figure 6 c). The strategy to replace glucose with HTM drastically reduced production cost (2.3 fold). Cell yields based on protein and oxygen conformed to cubic model fits ($R^2 > 0.93$ and $R^2 > 0.97$ respectively) (Figure 7 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 substrate limitation or substrate overflow respectively. Both yield on protein and oxygen increased as HTM concentration increased from 5 $g.l^{-1}$ to 25 $g.l^{-1}$ (Figure 7). It was also observed that both YPO and YPP was sub-optimal at the minimum and maximum HTM supplementation levels, because cellular respiration and protein flux to cell production was inefficient at these conditions.

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 7 b). The data indicates that cell production efficiency on carbohydrate is at its maximum under near starvation conditions. This atypical trend is possibly attributable to the marine origin of the *V. midae*, where sugar concentrations in the surrounding environments are low. Although the yield of cells is highest, when the sugar concentration is lowest, there is a playoff between sugar conversion efficiency and more important commercial parameters such as productivity and cell concentration. Therefore, fermentation process optimization must explore various responses in an integrated way to derive commercially relevant optima.

Comparison of laboratory scale optimised technology to base case technology

An overall comparison of the laboratory scale technology to the base case technology resulted in substantial improvements in all key indicators of production efficiency. Percentage improvement in cell concentration, productivity, YPP, YPS and YPO were 122, 12, 252, 94 and 633% (Table 1). This data confirms the relevance of laboratory scale development studies, prior to scale-up into commercial production.

Production of *V. midae* at manufacturing scale

The cultivation of *V. midae* at 200 L scale resulted in a fermentation process time of 5.46 ± 0.05 h. 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 triplicate batches (Table 2). The coefficients of variations across

the replicate batches for all five key responses tested in this study were <10%, indicating excellent process reproducibility (Table 2). There was no significant difference in any of the key performance indicators ($p > 0.05$) between lab and production scale, which is a major achievement because process performance is generally expected to decline at larger scale due to mass transfer limitations. Contrastingly in our manufacturing process, the selected substrates used in the cultivation at optimized conditions were able to support similar efficiencies at both scales. The control of key operating variables such as temperature, pH and aeration were successfully scaled. Control of process parameters is vital in any bioreactor cultivation in order to maintain optimal conditions for product formation. Profiles of the key operating variables were evaluated across the replicate batches, and variations from set-points were always below 10% (data not shown).

Post cultivation, the *V. midae* culture broth was centrifuged using a two-step separation process, resulting in a mass balance closure of 96.6% and a corresponding cell recovery of 94.9%. The viable cells were successfully formatted in a saline phosphate buffer at a concentration of 1.00×10^7 cells.ml^{-1}, which was subsequently packaged into ready to use sachets, as specified by relevant stakeholders in both the abalone feed production industry and with abalone growers. Our study yielded a validated, stream-lined, industrially relevant bioprocess that encompassed reproducibility, scalability, standardization, robustness and safety considerations (Hambor, 2012). The probiotic product developed in this study further satisfied the key supply chain and application criteria as described by Satyanarayana et al., (2012).

4. Conclusions

This study resulted in a complete development of a laboratory scale technology for the production of *V. midae*, with significant improvement in all process parameters when compared to the original base case technology. The study further showed the efficiency and reproducibility of the technology at full manufacturing scale. While 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 of *V. midae* as a probiotic of benefit to the abalone industry.

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