Chapter 7

Application of Biological Agents in Abalone Aquaculture: A South African Perspective

Ghaneshree Moodley, Lethabo Mashigo,
Rajesh Lalloo and Suren Singh

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1. Introduction

1.1. 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 [1]. 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 [2]. Fisheries itself, cannot provide sufficient amounts of aquatic products to fulfil the demands of the consumer; therefore, aquaculture provides a crucial alternative resource [1,4]. 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 [1,3].

Modern aquaculture involves the intensive production of finfish, crustaceans, molluscs, and algal plants under controlled conditions [5]. Aquaculture yields far exceeds that of natural fishing, and provides an effective means for a constant, year round supply for good quality seafood and seafood products [6-8]. The practice of aquaculture not only provides local food security, but also improves the livelihoods of people in many poorly developed coastal regions [2].

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 [8,9]. 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 destina-

tion markets (6-8,10). Abalone is used primarily as a celebration dish, especially during weddings and other special occasions such as the Chinese New Year [11]. On account of the ever growing demand of live, dried and canned abalone, already high prices of this seafood delicacy continue to escalate.

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 [11, 12]. The abalone family consists of about 56 species all belonging to the genus *Haliotis* [13,14]. Many members of this family have achieved commercial status as fishery and/or aquaculture species, and are of major economic importance (Table 1). 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 [15]. 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.

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

Table 1. Globally farmed abalone species and their location adapted from [16,17].

Cultivation of abalone is widespread in many countries, including USA, Mexico, South Africa, Australia, New Zealand, Japan, Taiwan, China, Ireland, Chile and Iceland [17-20] China is the largest producer in the world with over 300 farms and a total production of approximately 4500 metric tonnes [9]. China, Taiwan and South Africa are considered as the key production powerhouses in the abalone industry (Table 2). China is the highest contributor of live product annually, and is still the major market for abalone produced world-wide [8]. This occurrence is closely related to the economic growth and the increase in personal wealth exhibited by the

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Chinese population as well as the growth of the Chinese middle class population [4,15]. It has been reported that the total abalone produce reaching markets through harvesting, illegal poaching and natural supply, does not meet demand for this seafood delicacy [9,18].

COUNTRY	CULTURE	LEGAL HARVEST	ILLEGAL HARVEST
China	4500	-	-
Taiwan	3000	-	-
South Africa	600	237	1850
Japan	200	2200	536
USA	170	0	250
Australia	290	5128	1000
Chile	200	-	-
Mexico	50	1066	550
New Zealand	3	1078	400
Other	30	442	110
Total	9 043	10 151	4696
Grand total in 2005: 24 040 tonnes live weight			

Table 2. Global production of abalone indicating world leaders in abalone production, quantity of produce legally harvested and sold, and quantity of product illegally harvested; 2004-2005 data [20].

The South African abalone industry 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 [15]. One of the main challenges faced by the SA industry is the loss in revenue experienced due to poaching. Reports suggest that approximately 2000 tonnes are lost to the economy [4]. 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 [17]. Abalone rearing facilities employs an intensive system in which abalone is reared at high densities in shore-based aquaculture systems [21].

The South African abalone, *Haliotis midae*, locally known as "perlemoen" is the only one of six indigenous species that is of commercial importance [8,22]. The abalone *H. midae*, takes over 30 years to reach a maximum size of 200mm (shell length) in natural habitats [21]. Even under farmed conditions, abalone growth is slow and often varies with size and age [23]. *H. midae* takes approximately 4 to 5 years to reach a marketable size of 100 mm (shell length) before it can be sold between US$ 34 to 36 per kg on international markets [23,24]. Mariculture of abalone is thus important to ensure market supply 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 required.

Figure 1. 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), and commercially produced abalone has almost completely replaced the wild harvested product [4]. In 2010 the output of all facets of abalone harvest totalled 1015.44 metric tons.

The former status of abalone aquaculture in South Africa is outlined in Table 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, a gross turnover of approximately R200 million per annum was achieved [26]. 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 [27]. 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 [4]. 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. Global

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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 [15]. 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 [15].

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

Table 3. The status of abalone aquaculture and total investment in the South African abalone industry between 2004 and 2006 [26].

2. Challenges faced in abalone mariculture and conventionally used mitigation strategies

Many aquaculture farmers, including those in the abalone mariculture sub-sector are faced with a myriad of challenges [28]. 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 of abalone, the outbreak of diseases, waste accumulation and deterioration of environmental conditions within the culture system [29,30]. 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 [31]. 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 [23].

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 [5]. 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 [1]. 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 [23,32,33]. When pathogenic bacteria or viruses are detected, farmers usually apply antimicrobial compounds to the feed and the rearing water [34]. 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 [23]. 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 resistance and as a result leads to the emergence of more virulent pathogens [28,35]. 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 [36]. 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, and thereby poses further challenges. Consequently, the indiscriminate use of antibiotics confers a negative effect on the health of aquatic host species, the environment and consumers of food products [37]. Due to these concerns, more stringent regulation of antibiotic use in aquaculture has been imposed by the European Union [38]. Since the application of antibiotics is problematic, a strong demand for alternative methods of disease control is required in abalone mariculture.

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 results in different growth rates [39]. 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 [40]. In abalone production systems abalones are fed either formulated diets or seaweed/kelp, and in some instances, a combination of both [25]. 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 [21]. 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% [15].

Incorrectly formulated diets, may also lead to the accumulation of waste in the culture system which 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

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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.

In conventional mariculture operations, due to the high stocking densities, the generation of elevated stressful conditions in the culture environment is a frequent occurrence [41]. 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 [42]. Most operations employ land-based cultivation systems and use pump ashore technology which is energy intensive and costly [15]. 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.

3. Biological agents as an option to address the challenges in abalone 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 [43]. 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 [44]. Biological agents are emerging as a significant microbial supplements in the field of prophylaxis [36]. Many studies to date have revealed the potential of these beneficial organisms to combat disease in an aquaculture environment [5, 45-51].

In aquatic ecosystems there is an intimate relationship between microorganisms and other biota in the environment [47]. 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, whereby a biological agent is used 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, 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" [47]. Some studies have shown that as a result of intensification of aquaculture farms a negative impact has been conferred on the composition of the different protective microbial flora interacting with the host [5]. 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 [5].

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 [52]. In abalone aquaculture, potential probionts listed to date include, *Vibrio* spp., [23,53-55] *Debaryomyces sp*., *Cryptococcus sp*., and *Pseudoalteromonas sp*., [23,24,30], *Lactobacillus* and *Enterococcus sp*., [56]; *Pediococcus* sp. Strain Ab1 [57], *Agarivorans albus* F1-UMA [58] and *Shewanella sp* [51].

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 [24] 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 additional 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 [47]. 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 [47,59-61]. In addition, these biological agents compete with pathogens based on intrinsic growth rate and spacial 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 [62]. It is common knowledge that for a pathogen to be active and replicate in a host system, it requires attachment to these surfaces [62]. 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 [62].

Host nutrition is improved, as the applied probionts secrete high levels of hydrolytic enzymes such as amylases, proteases and lipases; as well as the provision of growth factors such as fatty

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acids, amino acids and vitamins [63,64]. Some isolates also have the ability to break down potentially indigestible components of the feed thus reducing toxicity and improving feed conversion efficiency [23,63]. Abalones are in most instances, fed a diet consisting mainly of kelp, which is a complex macroalgal polysaccharide deficient in many essential nutrients [64]. 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 [23]. Many bacteria displaying these properties have been found to exist throughout the digestive tract of *H. midae* [23,40]. 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 [40].

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 [65]. 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 day period [58]. 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 days in juvenile and adult abalone after cessation of feeding with a probiotic supplemented diet. Authors, [40] and [66] also 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.

An inaugural application of probiotics in abalone aquaculture was demonstrated by [23]. 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 probionts were able to breakdown complex proteins and starches, hence making the subsequent assimilation by abalone easier. Studies conducted by [23] 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 (SS1, AY1 and SY9) respectively for abalone. The probiont 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 [30].

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, was isolated by [56], and were shown to inhibit the growth of three abalone pathogens *viz*., *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* [57].

Studies conducted by [51] 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 may be used as a dietary probiotic supplement to improve innate immunity and disease resistance in abalone.

Studies on feed probiotics for abalone aquaculture show much promise, however the use of water bioremediation bacteria has been neglected. With intensification of abalone culture activities, increased energy costs of pumping sea water and stricter regulation on environmental pollution, the need for such biological agents will become obvious in the near future. A study based on carp was done by [67] where a consortium of three *Bacillus* isolates demonstrated the ability to reduce diseases and improve water quality. Additionally, studies revealed that when a three organism consortium was added to a culture environment, a decrease in the prevalence of pathogenic bacteria was observed. Moreover it was found that nitrate, nitrite and ammonium concentrations were significantly lower as compared to the control treatments and that the applied treatment did not alter the health, growth and oxygen sufficiency of the test systems negatively. The attractive nature of *Bacillus* spp. as biological agents should be considered for application in abalone mariculture.

4. Rationale used for the production of probiotics and biological agents

The use of biological agents in aquaculture has over the years gained momentum. It is thus, imperative that these micro-organisms be commercially produced in order to meet market needs. A comprehensive production process needs to be developed and optimised for each biological agent. This will facilitate the commercial roll-out of probiotic products of this nature, but will be 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 [69]. Important criteria influencing the commercial use of biological products are cost, efficacy, shelf life and convenience to the end user [70,71]. 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 [72]; each of which have to be optimized to ensure a cost effective production process.

The growth medium that is used to support high productivities in commercial bioprocesses is predominantly formulated with inexpensive nutrient sources [73]. The choice of medium to be used in production is an essential aspect of process development as it influences the

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economic competitiveness of the bioprocess technology [74]. 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 [75]. It has been suggested that economical and commercially available medium options be investigated in order to reduce production costs [76,77]. The growth medium used can be either a defined or 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 low cost [74].

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 [78-80]. Several studies have indicated that high cell yields and productivities have been obtained with the use of yeast extract in various production processes [81-83]. However, the disadvantage of using this nutrient source is that it results in high-priced production processes due to its associated cost [78]. The use of yeast extract in a production process is therefore regarded as a major technical hurdle that should be overcome in order to successfully minimize production costs [79]. 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 [80]. 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.

CSL has been identified as a lower cost, more effective growth medium that can be used in production, in comparison to conventional nutrient substrates such as yeast extract, peptone and casamino acids [50]. It is produced by immersing corn into dilute sulphur oxide during the starch-manufacturing processes and is a major by-product of the corn-starch processing industry [84]. It has also been shown to be a supplementary source of vitamins and nitrogen to the culture medium [85,86]. The use of CSL has had numerous successes in diverse industrial fermentation processes [76] with high cell yields and productivities being major benefits [87]. Other than the assessment of a suitable nitrogen source, alternative carbohydrate sources also need to be reviewed 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 [75]. 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 high subsequent production costs [88]. When developing efficient bioprocesses, attempts are made to obtain economical and commercially available carbohydrate sources such that the production costs are minimised [74,76,77,89,90]. High test molasses (HTM) is a

valuable carbohydrate used commercially due to its local availability and cost competitiveness. It has been applied extensively as an alternative carbohydrate source in various production processes [74,76,77,80,90,91]. HTM, unlike conventional molasses, is a purer product form that enhances mass transfer and reduces impurities in a production process. 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 [80,92].

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 [93]. Vitamins are growth factors required by most microorganisms for the production of enzyme cofactors [74,94]. 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 [74,95]. Supplementation of exotic trace elements and vitamins can be costly and are therefore avoided if cheaper nutrient sources can satisfy the essential requirements for growth of biological agents in production processes.

Other than an influence on growth, the type of growth medium used in a production process also influences physical parameters such as mass transfer and the formation of foam. Growth media rich in nitrogen sources usually result in increased foam formation [96]. In addition the sparging of gas through the growth medium and agitation at high speeds results in excess foam formation, in oxygen intensive processes. This reduces the efficiency of gaseous exchange at the surface of the culture, as a barrier is formed between the culture and the gases present in the headspace of the vessel [97,98]. Additionally, cells and the culture medium 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 of the process performance [98].

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 control the activities and growth rates of many microorganisms as it governs all the 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 [99-102]. 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 micro-organisms, which is highly undesirable when designing an efficient bioprocess strategy [104]. 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 [105]. pH homeostasis is another important factor that needs to be considered during the growth of microorganisms [106]. 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

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between the optimum and the maximum pH value [107]. It is well known that pH is important in controlling initiation of growth by microorganisms [108]. 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 [108-111]. 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 titres [112]. The method of oxygenation must be given a high degree of consideration, as excessive rates of agitation and sparging will encourage foam formation and initiate cell sheer. 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 [96,112]

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 they also confer information of the functionality of the probiotic once it enters the host environment [50,90]. Therefore, 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 are met to ensure that biological agents can be affordably adopted for use in abalone aquaculture.

5. Processing of probiotics into market acceptable products

Once the relevant biological agents have been successfully cultivated at a large scale, the resultant fermentation broth needs to be recovered efficiently to be utilized in subsequent processing and formulation steps [113,114]. The downstream process has a major influence on product commercialization as major constraints in most processes are embedded in harvesting and formulation costs [115,116]. 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 [117]. In addition, it is also vital to ensure that the final product to be administered to the host aligns with end user requirements such as stability, consistency, easy application, efficacy and affordability [115,118,119]. As a consequence; 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 [115].

The main objective for downstream processing is to minimise the number of unit operations involved in the process, thus reducing overall process and validation costs, while also simplifying ease and economy of process automation [115]. An additional consideration is the final anticipated form of the end product which has implications on the choice of process options while meeting customer expectations (Figure 2) [120]. The downstream process unit operations, completes the process chain; from the upstream fermentation to the end product. It is therefore considered to be an extremely important prerequisite for commercialization of

probiotic technologies. Regrettably, published literature regarding downstream processing and formulation for commercially available products is very limited [115,119].

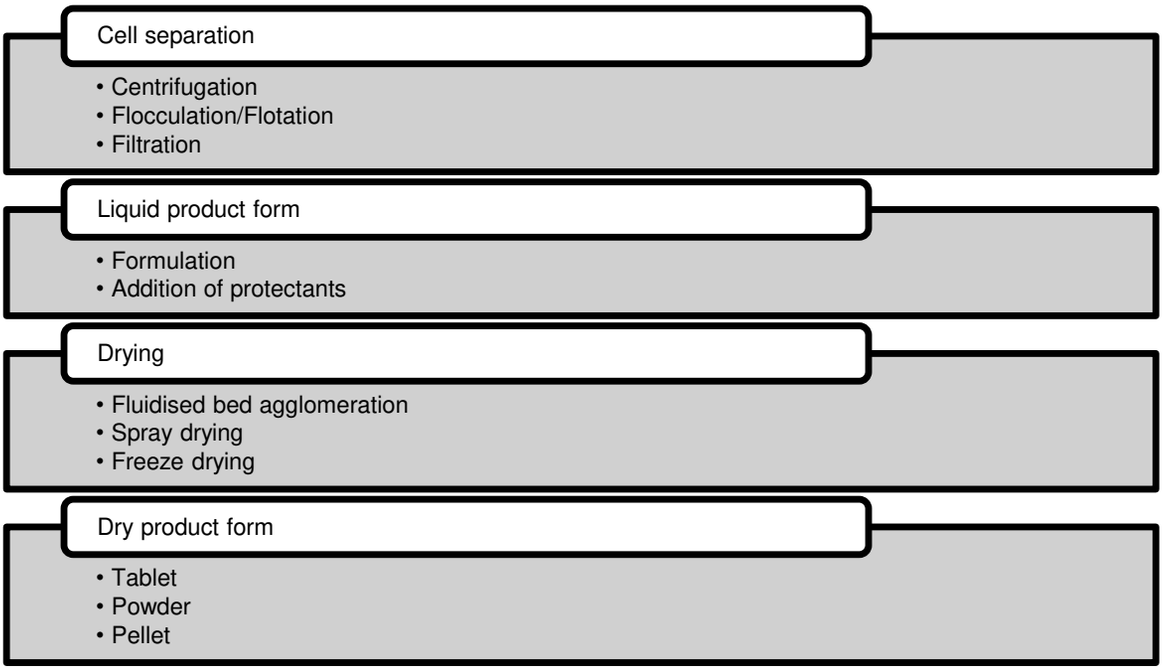

Figure 2. Schematic illustration of potential downstream process unit options [121].

The harvesting efficacy of a unit operation governs the marketability of a product, as it affects the potency and aids in any potential 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 [120]. Process options for cell harvesting from fermentation broth include microfiltration, sedimentation, flocculation and ultrafiltration (Figure 2) [116,122]. Flocculation and flotation using surface action or electrical charge have been reported to be inefficient in the separation of bacterial cells [120]. Although there have been some positive reports for harvesting using ultra-filtration [116], the most widely used process remains centrifugation [123-125]. Among all options, centrifugation appears to be the most viable alternative for cell harvesting resulting in recoveries of ~ 99% [115,126]. Usually, product intermediates are anticipated to be a high cell concentration paste containing the biological agent. 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 [127,128].

Subsequent to cell separation, formulation generates a crucial link between production and application of probiotics (Figure 2). This crucial 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. Intelligent formulations will allow innovation in application techniques using unique combinations of active ingredients, adjuvants or inerts and the end product

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should seamlessly integrate into standard food production and farming activities [115,129]. Formulations of biological agents can be broadly classified into either dry solids in various forms, or liquid suspensions or emulsions [130]. The inclusion of additives that enhance process-ability eco-friendliness and customer acceptance of the final product are also important considerations [115]. Any potential impact to the host, environment and even end product consumer, must be thoroughly investigated prior to application [131].

In the case of a probiotic product, the formulation needs to encompass ingredients that aid viability and growth of the cells in its intended application. Sugars and proteins are normally the key nutrients that support the stability of cell preparation. It also further provides a protective layer for the cells, preventing death and assists in the recovery of injured cells during processing [115,132,133]. The addition of nutrients was also shown to improve storage of a *Pseudomonas fluorescens* F113 strain [134] and a *Bacillus megaterium* [135] 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 [70].

Processing options for abalone biological agents, will include, both dry and liquid product forms (Figure 2). Due to the intended use of the selected isolates as a living cell preparation, product options with a high stability are considered to be most appealing. The application of fresh cells that need to be routinely produced is not attractive as there is significant risk in ensuring consistent inclusion into the abalone feed [135]. This complicates the processing segment of the technology to a large extent, as innovative ways of ensuring and maintaining cell viabilities are required. Potential options for commercial processes to stabilize these probiotic products include refrigeration, freezing, freeze drying, spray drying and low temperature fluidised bed agglomeration. 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 [117,136,137]. An alternate approach is to concentrate the product into a convenient dosage quantity and form that reduces the bulk logistics burden for products that are not amenable to drying. Low temperature drying processes such as freeze drying are suitable for higher value, heat labile bio-products, but is costly, time consuming and discontinuous for bulk production compared with moderate temperature drying processes [131,138]. Spray drying processes are widely used for large scale drying of products; however, higher drying temperatures decrease the viability of microbes faster than lower drying temperatures [131,139]. Spray drying requires high temperatures to facilitate water evaporation, which can cause irreversible changes to structural and functional integrity of the intended biological product and reduce viability and activity of the organisms itself [140,141]. Spray drying also has a high energy demand requiring 2500 to 10 000 $J.g^{-1}$ of evaporated water and is therefore not the most attractive process option for drying of abalone probiotics [122].

There are several reports on the use of agglomeration as a commercially viable process option for moderate temperature drying of biological material, mainly due to excellent mass and heat transfer characteristics [133,139,142]. During agglomeration, a mixture is atomised to form droplets at lower temperature (typically 30-40^{o}C) which results in coating of the probiotic cells

on the surface of suitable carrier particles. Probiotic cell cultures are subjected to evaporative cooling during the warming up and constant-rate drying periods and therefore have a substantially lower temperature than the drying air, resulting in increased viability [139.]. Advantages of fluidised bed drying over freeze and spray drying include lower investment and maintenance costs, ease of large scale continuous production, rapid exchange of heat, minimising heat damage, rapid mixing providing near isothermal conditions and uniform end product [122,139,143]. Due to these reasons fluidized bed drying has become an accepted method for large scale production of heat labile biological materials, however, viability losses have still been reported [142].

In addition to production and formulation of user-friendly product through a downstream process, the stability and consistency of product intermediates and the end product itself are crucial requirements for successful commercialization [144]. 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 production cost [141]. 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 [145].

The problems of stability during processing, storage and after application have stalled development of biologically based products [146]. 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 [147]. Temperature dependant half-life plots can be generated to predict stability of the probiotic product intermediates as well as the end product. This approach has however only been used to a limited extent [121,123,135].

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 [44,47]. 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 [148]. The method of incorporation selected must overcome challenges faced in feed production and the mariculture system itself; such as major losses of viability, 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 [148]. Incorporation of the probiotic into the feed is almost always 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 [56,150,151] 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

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[117]. The soaking method uses preformed feed pellets, which are soaked in a saline broth containing the probiotic organism at a desired concentration [57,149]. 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 [58]. 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 [153]. In addition, [154] described methods of spraying dried feeds with cells that were placed onto plastic trays. Lastly, the bathing option of probiotics involving the application of living cells directly to the rearing water has been used [155]. 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.

6. 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 [29,52,156]. 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 [68].

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. 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 [157]. These critical factors have sometimes been overlooked, and have consequently led to the failure of probiotic technologies in some instances [41]. 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 *Carnobacterium* families [158]. 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 [159]. 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 the technology, thereby hindering its uptake by the aqua-culturists.

7. 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 [47]. 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 [160]. 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 [29].

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 [160]. 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 [158]. 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 [160].

The commercial aquaculture sector will make a notable difference in terms creating jobs and economic development in most developing countries by 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 [27], 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 [9]. 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.

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Author details

Ghaneshree Moodley[1,2*], Lethabo Mashigo[1], Rajesh Lalloo[1] and Suren Singh[2]

*Address all correspondence to: GMoodley@csir.co.za

1 CSIR Biosciences, Pretoria, South Africa

2 Department of Biotechnology & Food, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa

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