6

Bioprocess engineering approaches for the production of marine enzymes

Sreyashi Sarkar, Sayani Mitra, Arnab Pramanik, Jayanta Debabrata Choudhury, Anirban Bhattacharyya, Malancha Roy, Kaushik Biswas, Anindita Mitra, Debashis Roy and Joydeep Mukherjee, Jadavpur University, Kolkata, India

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Abstract: This chapter focuses on the attempts made to translate novel marine enzymatic activities to commercial bioprocesses. Cultures can be suspended or immobilized in the production medium. The cylindrical tank is the most common reactor and alternatives to the stirred reactor include vessels with no mechanical agitation. There are three principal modes of bioreactor operation: batch, fed-batch and continuous. Solid-state fermentation denotes cultivation of microorganisms on solid, moist substrates. Bioreactors with novel design elements have been applied for studying and enriching marine microbes in bioreactors to attain good control of the environmental factors. Three different strategies can be distinguished for (i) mimicking the natural environment, (ii) stimulating the uncultured microbes or producing metabolites of interest and (iii) controlling redox conditions on the sediment/water interface. Some examples of laboratory reactor-scale production of marine enzymes are: protease produced by Antarctic *Bacillus*, immobilization of *Teredinobacter turnirae* and biofilm cultivation of an intertidal gamma-Proteobacterium. Xylanase was produced by the hyperthermophilic *Pyrodictium abyssi* and L-glutaminase by the marine fungus *Beauveria bassiana* in a packed-bed reactor. Continuous cultivation of *Pyrococcus furiosus* produced saccharification enzymes, while quinol oxidase was obtained from a

barophilic *Shewanella* sp. grown in a pressurized vessel. Pyruvate carboxylase was obtained from hyperthermophilic *Methanococcus jannaschii*.

Key words: bioprocess, bioreactor, marine microbe, solid state fermentation, packed bed reactor, continuous cultivation, biofilm.

6.1 Introduction

Competition amongst microorganisms for space and nutrients in the marine environment is a powerful selective force, which has led to evolution. Evolution prompted the marine microorganisms to generate multifarious enzyme systems to adapt to the complex marine environments. Therefore, marine microbial enzymes can offer novel biocatalysts with extraordinary properties (Ghosh et al., 2005; Zhang and Kim, 2010).

Examples of potential commercialization of marine enzymes may be cited such as enzyme-based antifouling paints (Kristensen et al., 2010), saccharification of marine microalgae using amylase from marine bacteria in saline conditions (Matsumoto et al., 2003) and enzymatic degradation of biofilms of both Gram-positive and Gram-negative bacteria for which a US patent has been filed (Manyak et al., 2010; Nijland et al., 2010).

This chapter focuses on the attempts made to translate the novel marine enzymatic activities to commercial bioprocesses. Thus, laboratory studies carried out in bioreactors have been described in substantial detail to make interested investigators seek details from the references cited. The chapter begins with the current state of bioreactor technology for marine cultivations followed by the enzymes where such technology has been applied.

6.2 Traditional cultivation, bioreactor configuration and modes of operation

Conventionally cultures can be suspended (free) or immobilized in the culture medium. The restriction of cell mobility in a fixed space is known as cell immobilization. Immobilized cell cultures have certain advantages over suspension cultures such as attainment of high cell concentration, reuse of cells and allowing a combination of high cell concentration and

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high flow rates; thus, high volumetric productivities can be achieved (Shuler and Kargi, 2003).

Among the various bioreactor configurations, the cylindrical tank is the most common reactor in bioprocessing. Much of the challenge in reactor design lies in the provision of adequate mixing and aeration for the large proportion of bioprocesses that require oxygen. Reactors for anaerobic culture are usually very simple in construction without sparging or agitation, while alternatives to the stirred reactor include vessels with no mechanical agitation. In bubble-column reactors, aeration and mixing are achieved by gas sparging, which requires less energy than mechanical stirring. Mixing in airlift reactors is accomplished without mechanical agitation and shear levels are significantly lower than in stirred vessels. Packed-bed reactors (PBR), consisting of a tube packed with catalyst, are used for immobilized or particulate biocatalysts. The medium can be fed either at the top or bottom of the column and forms a continuous liquid phase between the particles. Damage due to particle attrition is minimal in packed beds compared with stirred reactors. When packed beds are operated in an upflow mode with catalyst beads of appropriate size and density, the bed expands at high liquid flow rates due to upward motion of the particles. This is the basis for the operation of fluidized-bed reactors (Doran 1995).

The mode of bioreactor operation is an important factor affecting reactor performance and there are three principal modes of bioreactor operation: batch, fed-batch and continuous. The choice of operating strategy has a significant effect on substrate conversion, product concentration, susceptibility to contamination and process reliability.

Batch processes operate in closed systems, substrate is added at the beginning of the process and products removed only at the end. The classic mixed reactor is the stirred tank; however mixed reactors can also be of bubble column, airlift or other configuration. Batch culture is a common mode for commercial fermentation, but in addition to fermentation time, however, the production cycle also includes turnaround time (needed for sterilization, inoculation, etc.) which leads to a reduction in overall productivity and adds to production costs. The 'draw-and-fill' culture represents a potential alternative mode of fermentation in which all or part of the medium is drawn out and fresh medium refilled periodically, and here the turnaround time is avoided.

In a fed-batch operation, intermittent or continuous feeding of nutrients is used to supplement the reactor contents and provide control over the substrate concentration. By starting with a relatively dilute solution of substrate and adding more nutrients as the conversion proceeds, high

growth rates are avoided. This is important in cultures where the oxygen demand during fast growth is too high for the mass-transfer capabilities of the reactor.

In continuous cultures, if the vessel is well mixed, the product stream has the same composition as the liquid in the reactor. Therefore, when continuous reactors are used with freely-suspended cells, the catalyst is continuously withdrawn from the vessel in the product stream. Growth supplies additional cells to replace those removed (Doran 1995).

Solid-state fermentation (SSF) denotes cultivation of microorganisms on solid, moist substrates in the absence of a free aqueous phase; that is, at average water activities significantly below 1.0. In a broader definition, SSF can be seen as including processes during which microorganisms are cultivated in the presence of a liquid phase at maximal substrate concentrations or on inert carriers. SSF, an environmental-friendly bioprocess, offers numerous advantages over submerged processes for the production of bulk chemicals and enzymes such as simplified downstream operation, reduced energy requirements, less waste water produced, high yields of products, increased volumetric productivity, enhanced product recovery and simplicity of bioreactor design. The reasons against the application of SSF are engineering problems, the low compliance of the processes to standardization and the limited reproducibility of the results.

6.3 Specialized bioprocess technologies

Microorganisms living in marine sediments usually have a very small niche for survival, under defined physico-chemical conditions. Only under these specific conditions is the thermodynamic energy gained from their metabolic reactions sufficient to sustain them. For maintaining or cultivating these organisms in the laboratory, temperature, pressure and fluid/gas flux should be given due consideration. The temperature/pressure dependency of marine prokaryotes and fungi, in terms of their growth behavior as well as their potential to produce new metabolites or enzymes, was reviewed by Lang et al., (2005). Advanced shake-flask cultures and controlled bioreactor cultivations following the batch-type, fed-batch-type and/or continuous-type operations were also described. Values on maximal biomass, specific growth rates, and (sub) optimal production yields were presented. The application of mesophilic microbes to intensify bioprocess engineering studies was the goal of many bioprocesses. Isolation of cold-active enzymes and thermostable enzymes

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from psychrophilic and hyperthermophilic microbes were the targets of many experiments. A special challenge to bioengineers is also provided by barophilic strains originating from depths of, say, nearly 11 000 m, or from hydrothermal vents (Lang et al., 2005). In addition to the traditional approaches described, the bioreactors with novel design elements have been employed for studying and enriching marine microbes in bioreactors to attain good control of the environmental factors (Zhang et al., 2011). The processes that have already been simulated *in vitro* by a reactor system are listed in Table 6.1 following Zhang et al., 2011. The choice of the most suitable system is situation-dependent.

Depending on the research objective, three different strategies can be distinguished for (i) mimicking the natural environment, (ii) stimulating the uncultured microbes or producing metabolites of interest and (iii) controlling redox conditions on the sediment/water interface. Zhang et al., (2011) enumerated six major classes of conventional reactors suited for marine ecosystem research as summarized in Table 6.2.

The design of an efficient bioreactor for the cultivation of marine microbes would take into account the physical conditions such as temperature, pressure and materials of construction.

Table 6.1 ***In vitro*** **simulations of marine microbial processes and niche environments. The simulation of an environment by a particular reactor type has been indicated by reference(s).**

Processes and environments	Reactors	References
Intertidal estuarine	Rotating disk bioreactor	Sarkar et al., 2008
Sinking from surface water to deep ocean	Pressurized microcosm	Grossart and Gust, 2009
Cold water seeps	Continuous flow-through reactor, continuous high-pressure reactor, membrane bioreactor	Girguis et al., 2003; Meulepas et al., 2009; Zhang et al., 2010
Hydrothermal vents	Continuous high-pressure reactor, gas lift bioreactor, fermenter	Imai et al., 1999; Mukhopadhyay et al., 1999; Houghton et al., 2007; Postec et al., 2007
Hyperbaric environment	Diamond anvil cell	Aertsen et al., 2009

Table 6.2 **Conventional bioreactor configurations employed for the cultivation of marine microorganisms. The application of the particular reactor configuration has been indicated by suitable reference(s).**

Bioreactor configuration	Characteristics	References
Stirred tank reactor	Batch and continuous mode cultivation	Muffler and Ulber, 2008; Sarkar et al., 2008
Rotating disk/ drum bioreactor	Biofilm formation on disk/ drum, collect/remove fast growing biofilm forming biomass from disk, mimicking environments with intervals of oxic/anoxic	Konopka, 2000
Air lift reactor	Gaseous substrate is fed from the bottom to induce mixing	Meulepas et al., 2009
Membrane reactor	Selective membrane is applied to retain biomass	Meulepas et al., 2009
Continuous high-pressure reactor	High-pressure pump needed to induce high hydrostatic pressure, high pressure gas or compressor can be combined to induce high gas pressure	Wright et al., 2003; Pradillon et al., 2004; Parkes et al., 2009; Zeng et al., 2009; Deusner et al., 2010; Zhang et al., 2010
Bioelectrochemical system	Separation of bioelectrochemical reactions in space and time	Logan et al., 2006; Zuo et al., 2008; Clauwaert and Verstraete, 2009

6.3.1 Temperature

The development of cultivation protocols for hyperthermophilic microorganisms presents some interesting problems that are not encountered when working with more conventional organisms growing at mesophilic temperatures. Probably the most significant problem is the relatively little information generated to date on the growth and metabolism of hyperthermophiles. Biochemical and enzymological research is often limited by the low biomass yields that can be attained for many hyperthermophiles. Along these lines, production of large

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amounts of biomass presents the unfavorable prospect of very poor volumetric efficiency of fermenters with the additional problem of dealing with the hazards and corrosivity associated with high levels of biologically generated hydrogen sulfide. Thus, difficulties with cultivation of hyperthermophiles represent the key technological roadblock (Andrade et al., 2001).

Despite the low global temperature in the deep sea, the hydrothermal vents are hotspots, where the temperature can reach up to 1000 °C. To maintain the correct incubation temperature of microbes sourced from hydrothermal vents in the laboratory, either the reactor is placed inside a thermally controlled incubator or surrounded by a layer filled with temperature-controlled water. These methods can normally control the temperature in a range of 1 to 80 °C. If the incubation temperature has to be even higher, which is not feasible by hot water, a hot air incubator can be used (Zhang and Kim, 2010).

6.3.2 Pressure

Marine micro-organisms can live up to about 110 MPa hydrostatic pressure which is three orders of magnitude higher than the atmospheric pressure. Regarding the design of a high-pressure reactor, both high hydrostatic pressure and high gas pressure need to be considered. For the piezophilic bacteria whose substrates are easily dissolved at atmospheric conditions, hydrostatic pressurization is sufficient to alter their gene expression to achieve maximum cell growth. Depending on the research purpose, different types of high-hydrostatic-pressure reactors have been constructed: the pressurized chemostat, pressurized thermal gradient block and continuous mode high hydrostatic pressure reactor. For certain piezophilic microorganisms, their main substrates (such as methane or hydrogen) are in gaseous form and as a consequence in most cases poorly soluble under atmospheric pressure. Hence an *in vitro* high gas pressure is needed to enhance their metabolic activity and growth rate (Zhang and Kim, 2010). Mitsubishi Heavy Industries provides an onshore facility that cultivates microorganisms living in deep-sea mud. This facility permits deep-sea microorganisms to be taken into the same environment (pressure and temperature) as the deep sea where those organisms are living without being exposed to the onshore environment (*http://www.mhi.co.jp/en/products/expand/high-temperature_and_high-pressure_tank_supply_result_03.html#anchorPagetop*).

6.3.3 Materials of construction

Some marine microorganisms specifically require high concentrations of the sodium ion for their growth. High salt levels in combination with aeration may cause corrosion problems on austenitic steels, so molybdenum is additionally added for pitting resistance. Denitrification and sulfate reducing processes influence the corrosion of iron and stainless steel by using the cathodically produced hydrogen when iron is immersed in anoxic aqueous liquids. For high-pressure incubations, materials like PEEK (polyether ether ketone) plastic and stainless steel have been used for the vessels and the tubing. Surface attachment and biofilm formation are known to influence metabolite and enzyme production by many marine microorganisms and specialized reactors is required for organisms that produce desired products at highest rates when attached to surfaces. Inside the reactors, carrier materials, for example different polymer materials, have been tested to provide sufficient surface to form a biofilm (Sarkar et al., 2011; Zhang et al., 2011).

6.4 Bioprocess engineering data on specific marine enzymes

This section describes the data available on specific bioprocessing methods adopted for the production of marine enzymes derived mainly from microbial sources. By referring to the United States and the European patent databases (the freely accessible parts) as well as research journals, this section focuses on a wide spectrum of enzymes. Interesting examples of extremophiles producing unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes are also provided.

6.4.1 Protease

Vibrio harveyi was used to produce extracellular proteases in a seawater/Zobell based medium, supplemented with skim milk. Three agitation speeds (300, 500 and 700 rpm) and three air flow rates (0.2, 0.5 and 0.8 l/l/min – litres of air per litre volume of medium per minute) were investigated to determine the optimal process condition in a 1.5 l jar-fermenter (Applikon, Holland). Foam was controlled by adding a few

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drops of sterilized sunflower oil. The growth curve was defined as the logarithm of the biomass [$y = \ln (X/X_0)$] as a function of time (t), μ determined by the Gompertz model (Eq. 6.1):

$$y = A\exp\left\{-\exp\left(\frac{\mu e}{A}(\lambda - \iota) - 1\right)\right\} \qquad [6.1]$$

where μ is the specific growth rate, λ the lag time and the asymptote A. The addition of skim milk to Zobell medium enhanced the extracellular enzyme production fivefold. A significant amount of activity was not detected at 300 rpm, whereas at 500 and 700 rpm the activity increased appreciably when the aeration was 0.8 l/l/min (Estrada-Badillo and Marquez-Rocha, 2003).

Kumar et al., (2004) investigated batch cultivation of an alkaline protease secreted by *Bacillus clausii* and stable towards oxidizing agents and sodium dodecyl sulfate (SDS) under a range of process conditions in a 5l bioreactor (Model KF5L, Kobiotech, Inchon, Korea). The culture was agitated at 400 rpm without control of pH during the operation and the air flow-rate was kept at 1.5 v/v/min. Antifoam A (Sigma) was used to minimize foam formation. The enzyme activity increased with an increase in fermentation time and the rate of agitation. The increase in the protease yields may be due to efficient mass transfer coefficients which were dependent on optimal aeration and agitation rates.

Narinx et al., (1997) grew the Antarctic *Bacillus* strain TA39 at 8 °C for six days in a LH2000 fermenter (10l). The production of cells at the stationary phase was half at 25 °C compared to that obtained at 4 °C whereas the protease secretion hardly reached one-third of that recorded at 4 °C. After a lag phase of about 20 h, the doubling time at 4 °C during the exponential growth was 9 h compared with 2 h at 25 °C (Figure 6.1).

Submerged cultivation of *Hyphomonas jannaschiana* producing a metalloprotease was conducted aerobically with stirring with a mechanical agitator or air spargers (Weiner et al., 1996). A productive cultivation typically was in the range of 20–50 hours (Figure 6.2). Optimally, exoenzymes were detectable in the culture supernatant after about 17 hours of the start of the bioprocess. Cells could also be harvested at the early stationary growth phase and resuspended in a reduced volume of media to induce production of exoprotease in the culture fluid.

Elibol and Moreira, (2003) immobilized whole cells of marine shipworm bacterium *Teredinobacter turnirae* in calcium alginate beads which were used to produce alkaline protease. The cell suspension was aseptically added to sterile sodium alginate solution to achieve the required cell/alginate ratio. The mixture obtained was then extruded dropwise into

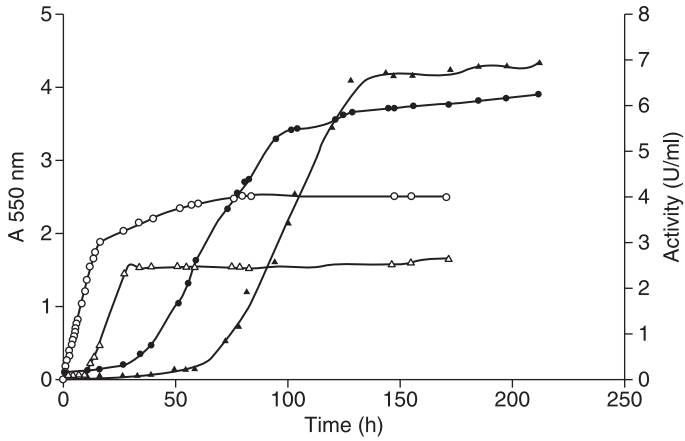

Figure 6.1 **Growth of the Antarctic strain *Bacillus* TA39 at 4 °C (closed circle) and at 25 °C (open circle) associated with the excretion of subtilisin S39 at 4 °C (closed triangle) and at 25 °C (open triangle). Reprinted with permission from Oxford University Press.**

$CaCl_2$ solution and hardened in this solution. The possibility of multiple use of *T. turnirae* cells immobilized within calcium alginate matrix was investigated and the beads were used for eight successive batches each lasting 72 hours. Protease production increased as the number of cyclic cultivations increased and reached a maximum after three cycles with a concomitant decrease in process time and an overall 3.5 fold increase in volumetric productivity.

In the next study by Beshay and Moreira, (2003) the authors chose optimal conditions for immobilization of *T. turnirae* cells on different inorganic matrices and evaluated the immobilized biocatalysts in repeated batch cultivation for production of the alkaline protease. Alkaline protease activity produced by immobilized whole cells was about 2.3 times higher than that produced by freely suspended cells under the same cultivation conditions. The possibility of the multiple use of *T. turnirae* cells was studied by repeated batch cultivation for 16 days (seven cycles). The activity of alkaline protease produced by immobilized cells increased gradually and reached a steady state after four cycles after which it remained constant until the end of the seventh cycle (Figure 6.3).

Physicochemical parameters affecting the keratinase production by *Penicillium* spp. Morsy1 in SSF was studied by El-Gendy, (2010).

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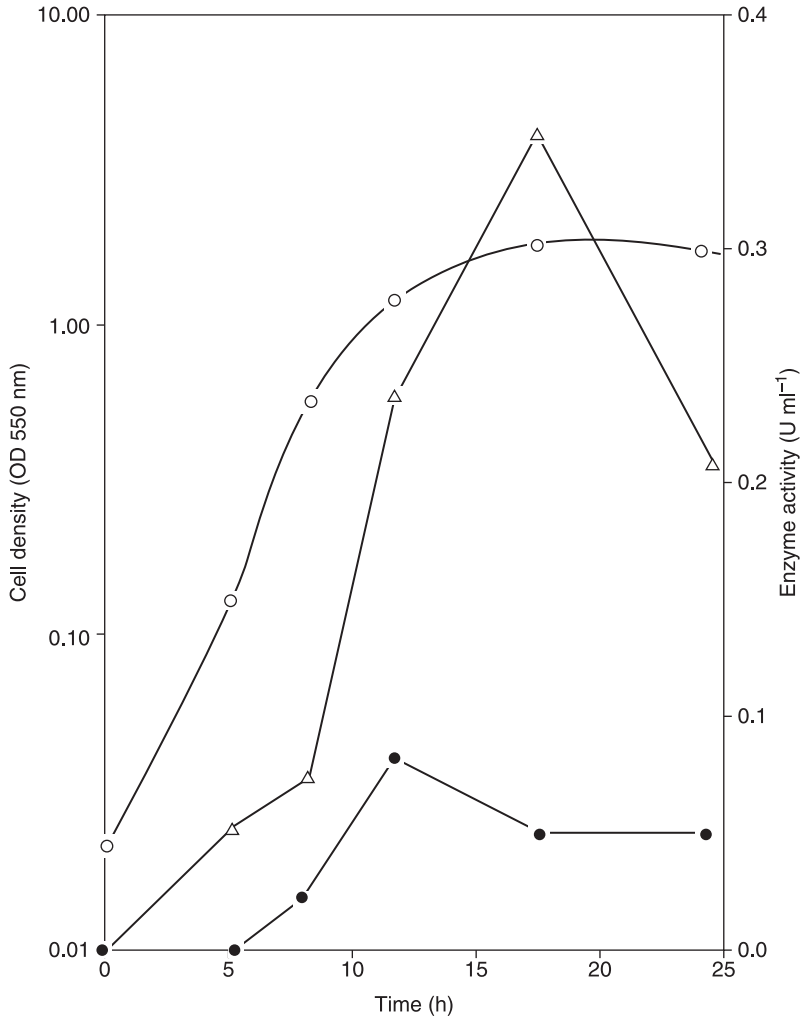

Figure 6.2 Thermostable alkaline metalloprotease produced by *Hyphomonas jannaschiana*. Open circle–cell growth as measured by optical density at 550 nm, open triangle–extracellular protease activity, solid circle–cytoplasmic protease activity. Protease activity was determined by the azocasein assay and expressed as units of activity per milliliter of sample (U/ml). Reprinted with permission from the United States Patent and Trademark Office.

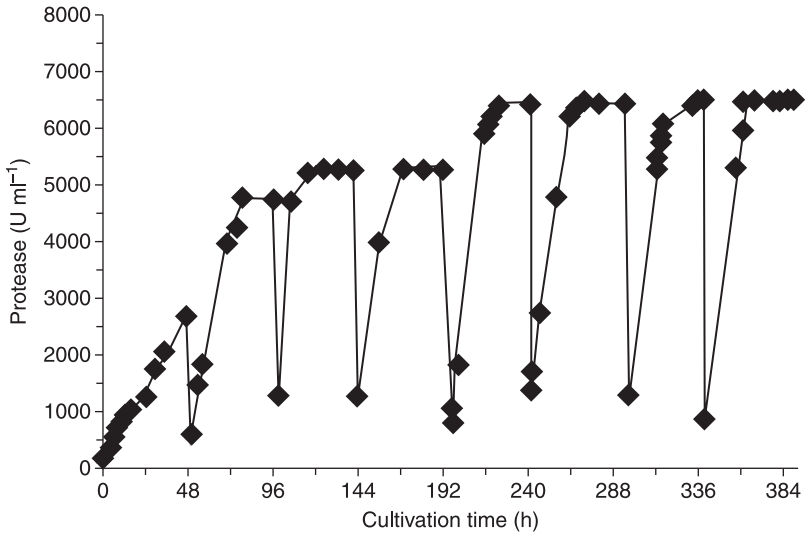

Figure 6.3 **Time course of repeated batch production of alkaline protease by immobilized *Teredinobacter turnirae*. Reprinted with permission from Elsevier.**

Keratinase activity under SSF with different agriculture and poultry wastes were tested and *Penicillium* spp. Morsy1 yielded maximum keratinase production at pH 6.0 but declined thereafter. Fungal extracellular enzymes were produced in high titer when the pH optimum was the growth pH, while on incubation temperature of 26 °C resulted in maximal enzyme production by Morsy1.

A polymethylmethacrylate (PMMA) conico-cylindrical flask (CCF) with an inner arrangement consisting of eight equidistantly spaced rectangular strips mounted radially on a circular disk to provide additional surface area for microbial attachment (Figure 6.4) was employed for protease production by a biofilm-forming intertidal γ-Proteobacterium. The flask design allowed comparison of protease production during cultivation with a hydrophilic (glass) or hydrophobic (PMMA) surface. Compared to the Erlenmeyer flask, the CCF with a hydrophobic surface allowed higher protease production. This investigation pioneered the application of a vessel beyond the traditional shake-flask for enhancing protease production by biofilm-formers (Sarkar et al., 2011).

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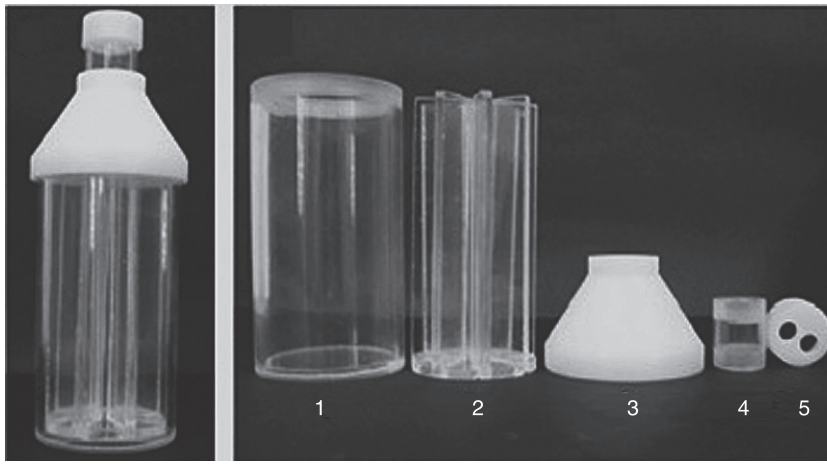

Figure 6.4 (a) Conico-cylindrical flask (CCF) and (b) the components of the CCF. 1: lower cylindrical portion; 2: inner arrangement; 3: upper funnel section; 4: neck for joining top lid; 5: top lid for provision for aeration. Reprinted with permission from Elsevier.

6.4.2 Xylanase and cellulase

The hyperthermophilic archaeon *Pyrodictium abyssi* grows optimally at 97 °C and is a prospective source of highly thermostable xylanases. The studies reported by Andrade et al., (2001) were undertaken to better characterize some parameters involved in the cultivation of the *P. abyssi*. The cultures were grown in serum bottles at 97 °C for 48 h and used as inoculum for a 16-l bioreactor (Bioengeneering, Wald, Switzerland) with a working volume of 13 l. The kinetic parameters of the Monod, Contois, and Tessier models were estimated using a nonlinear method. The cell yield was shown to be very low owing to incomplete substrate utilization, but a very high maximal specific growth rate was determined. The specific activity of xylanases produced by *P. abyssi* in batch culture was lower than 0.1 mU/l for β-xylosidase and arabinofuranosidase. The low specific activities could be owing to the yeast extract concentration in the medium that was required to obtain acceptable growth yield in the absence of elemental sulfur. Smaller variability for Contois estimates than for the

Monod and Tessier models was obtained. The physical significance of its parameters and its acceptability for high cell density cultures pointed to the choice of the Contois model as most indicated to predict the growth of *P. abyssi* on xylan. The cultivation of *P. abyssi* in a fermenter without stirring was successful. Shearing forces may disrupt the fragile microbial cell network of *P. abyssi* and could have a marked influence on xylanase production.

The alkaline tolerant fungus *Chaetomium* sp. (NIOCC 36) was tested for production of alkaline cellulases (β-endoglucanase, β-exoglucanase, β-glucosidase) using agricultural and industrial wastes as substrates in SSF processes. Cotton seed under SSF conditions showed maximum enzyme production at high alkaline pH, while cellulase enzymes produced under alkaline cotton seed SSF conditions at 50 °C had higher stability and activity than under non-alkaline conditions (Ravindran et al., 2010).

The polymethylmethacrylate conicocylindrical flask described earlier (Sarkar et al., 2011) was employed for production of cellulase by *Chaetomium crispatum* and xylanase by *Gliocladium viride*. The design allowed comparison of production between CCFs with hydrophobic surface (PMMA-CCF), hydrophilic glass surface (GS-CCF) and 500-ml Erlenmeyer flask (EF). Endo-β–1,4-glucanase, FPase (filter paper degradation) activities as well as growth of *C. crispatum* were highest in the PMMA-CCF. Xylanase production and growth of *G. viride* were highest in the EF (Figure 6.5). Temporal pattern of biofilm development based on two-channel fluorescence detection of extracellular polymeric substances and whole cells in a confocal laser scanning microscope demonstrated increase in the biofilm architectural parameters (Mitra et al., 2011).

6.4.3 Chitinase

Three-liter jacketed bench-top stirrer tank reactors controlled by means of a microcomputer controller (Applikon Dependable Instruments, Schiedam, The Netherlands) filled with 2.0 l of medium were used for the production of chitinase by *Penicillium janthinellum* (Fenice et al., 1998). The enzyme activity appeared related to the mycelial growth.

Chitinase fermentations with strain *Paenibacillus* sp. CHE-N1 was carried out in a 5-l stirred tank bioreactor (BTF-A5L, Bio-Top Inc., Taiwan) with 3-l culture medium by Kao et al., (2007a). The volumetric mass transfer coefficient, $K_L a$, mixing time and culture viscosity were determined. The chitinase level at an agitation rate of 100 rpm was lower

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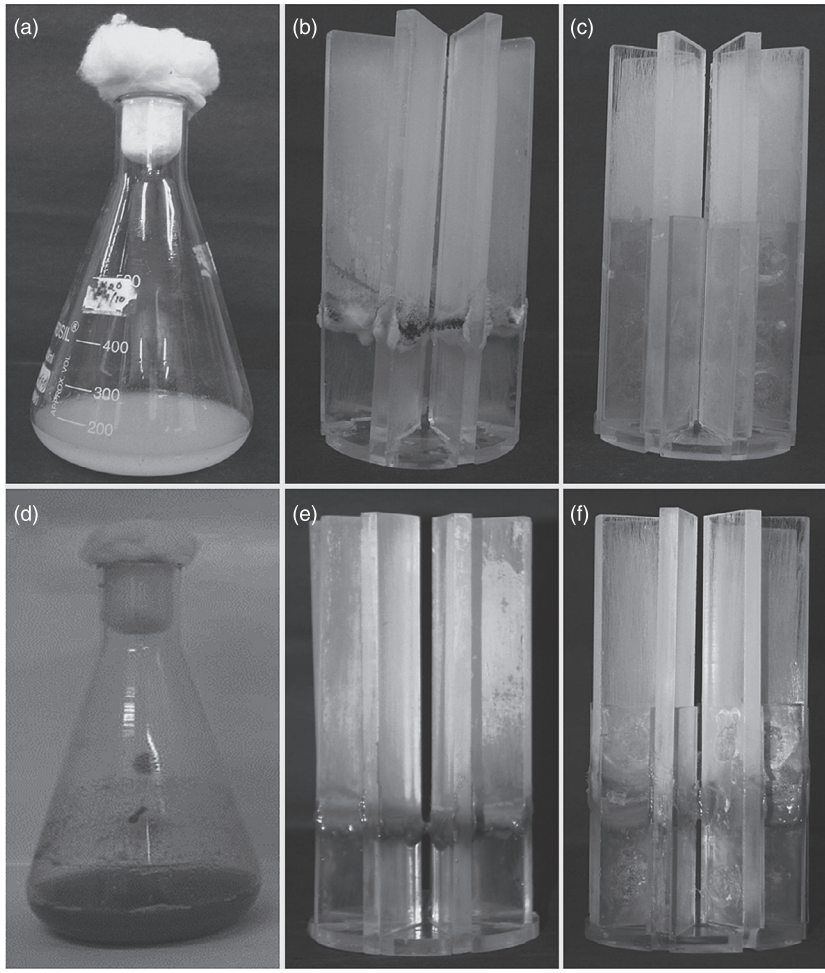

Figure 6.5 Appearance of (a) EF, (b) the inner arrangement of PMMA-CCF and (c) the inner arrangement of GS-CCF after cultivation of *C. crispatum* for 7 days; appearance of (d) EF, (e) the inner arrangement of PMMA-CCF and (f) the inner arrangement of GS-CCF after cultivation of *G. viride* for 7 days. Reprinted with permission from Springer.

than that at 200 rpm and may be due to incomplete mixing and/or oxygen transfer resistance at the lower agitation rate. However, the lowest chitinase level was observed at an agitation rate of 300 rpm, a result of high shear stress. The $K_L a$ value at 100 rpm was lower than those at higher agitation rates, hence it is reasonable to believe that oxygen transfer limitation predominated at the lower agitation rate. The $K_L a$ value of 35.5 per hour could be set as the basis requirement suitable for cell growth and chitinase production in the process. Figure 6.6 shows the rheology graph of the fermentation culture. The fermentation broth could be regarded as a non-Newtonian Bingham fluid, i.e. the shear stress was linearly correlated to the shear rate with a slope equal to the apparent viscosity (η, g/cm s) and an intercept of τ_0 (1.8 dyn/cm^2). The shear stress was calculated as a product of viscosity and shear rate plus τ_0. The amount of spores increased with the agitation rate up to 200 rpm at a constant cell level, whereas, at 300 rpm, more spores and fewer cells were found. From these results, a shear stress higher than 5.8 dyn/cm^2 was supposed to be harmful to both cell growth and chitinase production.

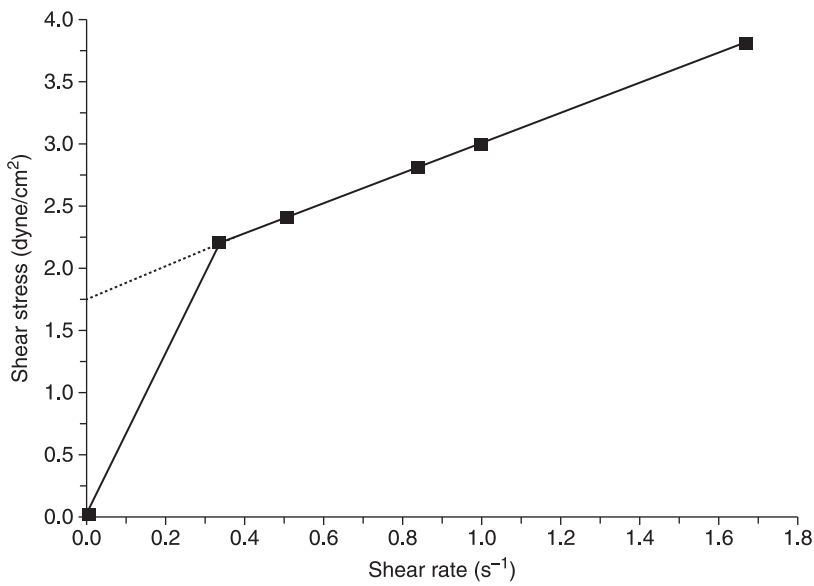

Figure 6.6 The rheology graph of fermentation broth of *Paenibacillus* sp. CHE-N1 with shear stress, τ (dyn/cm^2) expressed as a function of the shear rate γ (per sec). Reprinted with permission from Elsevier.

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The effects of submerged cultivation parameters on the production of chitinase by *Verticillium lecanii* in bioreactors were investigated by Liu et al., (2003). In a 5-l, stirred tank reactor (STR), high chitinase activity was obtained under optimal cultivation conditions (Figure 6.7).

The influence of fermentation medium pH was also studied. The chitinase activity varied as medium pH increased from 2 to 9, the optimal being 4.0. The STR adapted with a baffle could afford better mass/gas transfer efficiency. However, the baffle also caused an aggregation of *V. lecanii* during the cultivation and the growth of fungus and synthesis of enzyme were hindered. The agitation also caused morphological changes in the bioreactor that resulted in different productivity of target enzymes.

The effect of aeration was also investigated by comparing performance at three aeration rates, namely 0.6, 0.9, and 1.2 vvm (volume of air per volume of culture medium per minute) in 30-l airlift bioreactor incorporated with 24-mesh net-draft tube. Figure 6.8 shows the chitinase activity and DO (dissolved oxygen) time course for the cultivation of *V. lecanii* in a 30-l fermenter. Aeration slightly influenced the DO

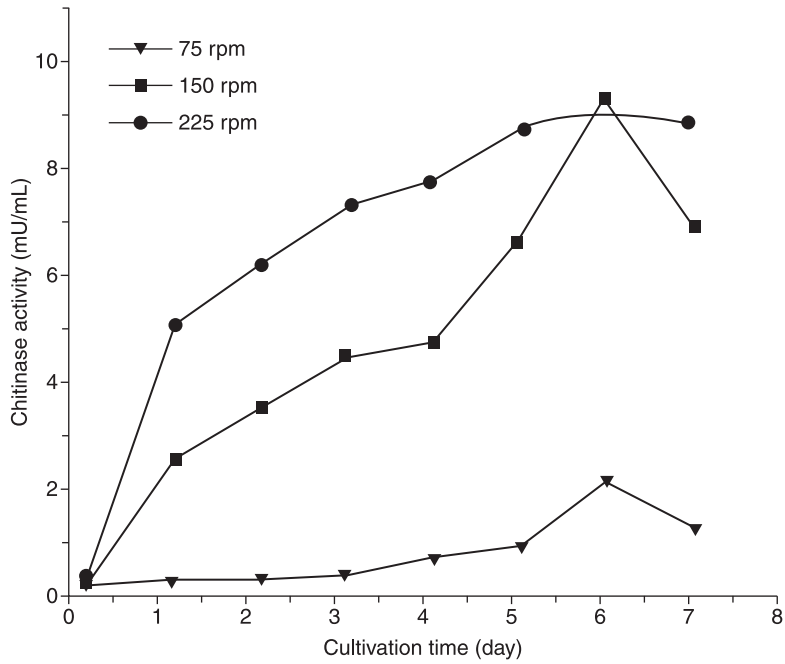

Figure 6.7 **Time course of chitinase activity of *Verticillium lecanii* F091 under different agitation rates. Reprinted with permission from Elsevier.**

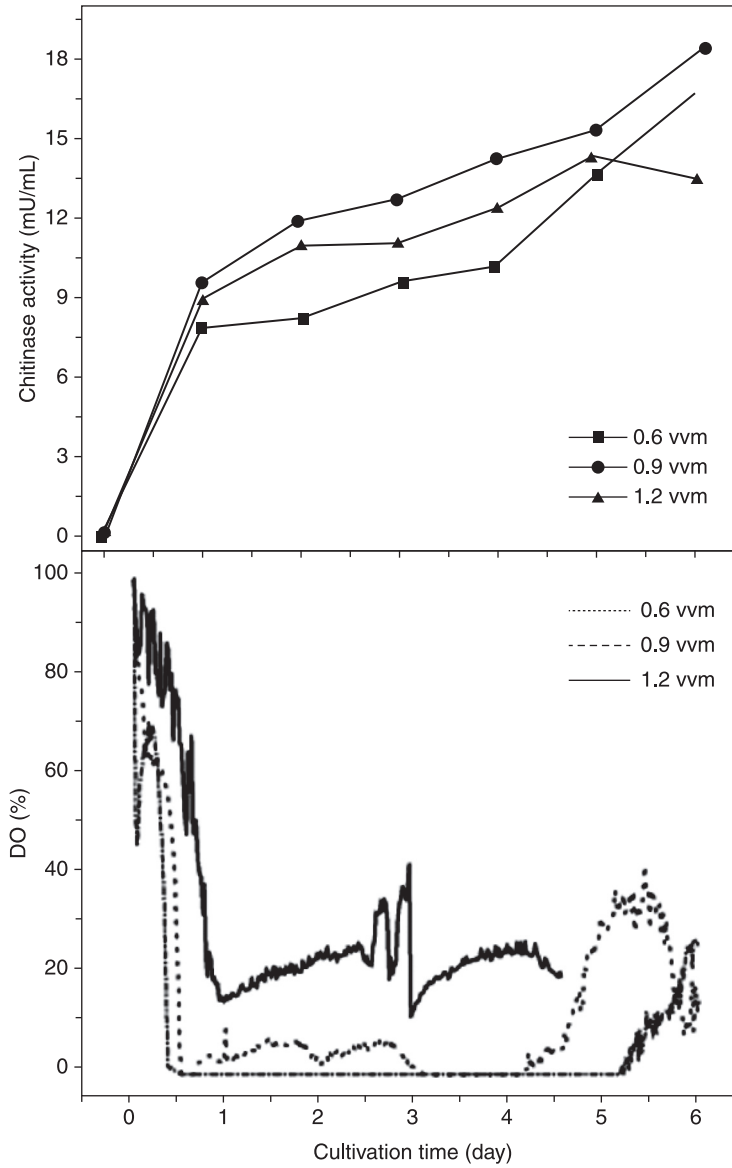

Figure 6.8 **Time course of chitinase activity of *Verticillium lecanii* F091 (top panel) and dissolved oxygen level (lower panel) under the different aeration rates. Reprinted with permission from Elsevier.**

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concentration, which in turn affected cell growth, enzyme activity and substrate utilization. When DO concentration fell below a critical level, cell respiration shifted from DO to the gaseous form. However, this phenomenon occurred only if a high aeration rate was employed. Otherwise, a linear growth proceeded which indicated a deficiency of both gaseous and DO. This concept suggested that depletion of DO and concomitant increase in CO_2 partial pressure could stimulate a respiration shift and a start of metabolism or form changes. This study indicated that the effects of pH and agitation rate on fermentation were more significant as compared with aeration rate.

The feasibility of using membrane mode fermentation operations for the continuous chitinase production by *Paenibacillus* sp. CHE-N1 was investigated by Kao et al., (2007b). The bioreactor with a membrane outer recycling loop was used to evaluate the effect of membrane pore size on cell retention efficiency, permeate flow rate, fouling, and chitinase recovery in permeate. The results showed that at a transmembrane pressure of 0.9 kg/cm^2, M 9 microfiltration column with a nominal pore size of 300 kDa exhibited the best microfiltration characteristics and was used for the membrane mode operation. The total chitinase activity obtained in membrane operation was about 78% higher than that obtained in batch mode operation (Figure 6.9). Further improvement was obtained by feeding chitin every 3–4 days.

6.4.4 Glutaminase

Sabu et al., (2002) reported the continuous production of extracellular L-glutaminase by the marine fungus *Beauveria bassiana* BTMF S–10 in a packed-bed reactor. Parameters influencing bead production and performance under batch mode were optimized. Diffusional limitations were encountered at higher alginate concentrations, since it was known that an increase in alginate concentration resulted in a tighter crosslinking. Parameters optimized under batch mode for L-glutaminase production were incorporated into the continuous production studies and the effect of flow rate of the medium, substrate concentration, aeration and bed height on continuous production of L-glutaminase was studied. Continuous production of the enzyme by Ca-alginate-immobilized spores was well suited for *B. bassiana* because higher yield of enzymes within a shorter time was obtained.

A marine *Pseudomonas* sp BTMS–51, immobilized by Ca-alginate gel entrapment, was used for the production of extracellular L-glutaminase

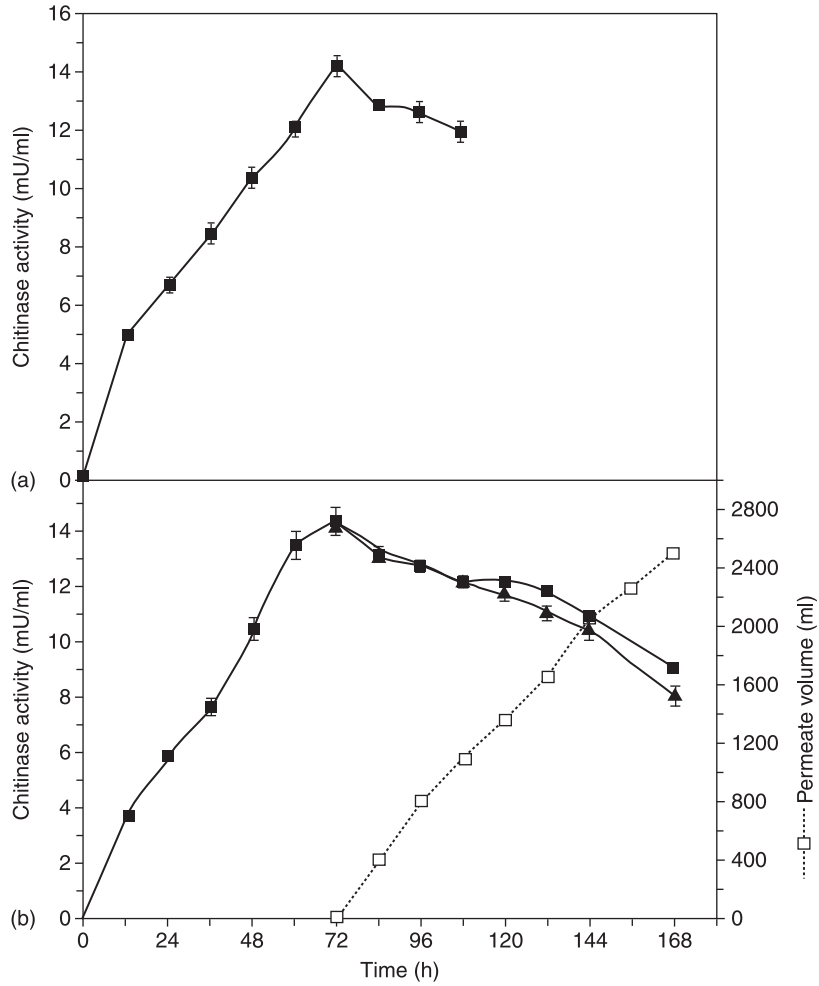

Figure 6.9 Time course of chitinase production (a) by *Paenibacillus* sp. during batch mode operation and (b) in the membrane mode operation. Solid square–activity level in the broth, solid triangle–activity level in the permeate, open square–total permeate volume in the broth. The working volume in the reactor was maintained at 2 l. Reprinted with permission from Elsevier.

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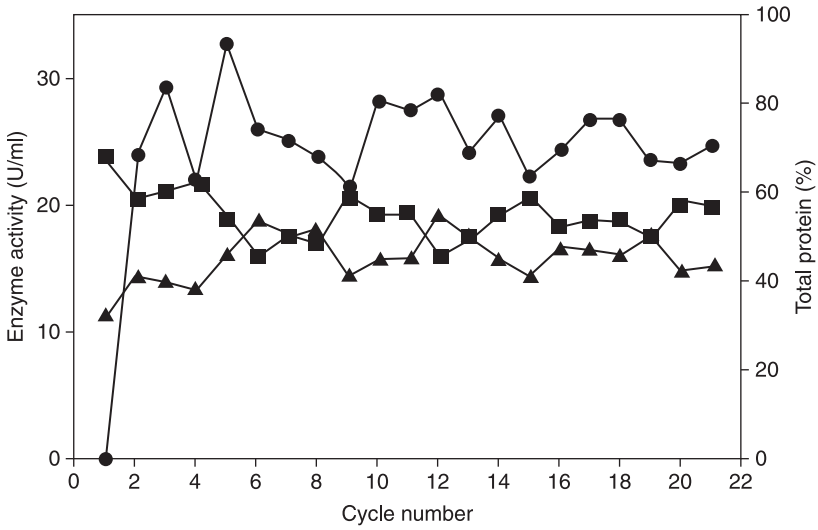

Figure 6.10 **Repeated batch production of L-glutaminase by *Pseudomonas* sp BTMS–51 by Ca-alginate immobilized cells. Open triangle–biomass in beads, open square–biomass in medium, closed circle–enzyme activity. Reprinted with permission from Elsevier.**

under repeated batch process and continuous process employing a PBR (Kumar and Chandrasekaran 2003). Immobilized cells after 20 cycles of repeated batch operation did not show any decline in production upon reuse. The enzyme yield correlated well with the biomass content in the beads (Figure 6.10).

Furthermore, continuous production of the enzyme in PBR was studied at different substrate concentrations and dilution rates. In general, the volumetric productivity increased with increased dilution rate and substrate concentrations and the substrate conversion efficiency declined. The system could be operated for 120 h without any decline in productivity.

6.4.5 Inulinase

The INU1 gene encoding exo-inulinase cloned from *Kluyveromyces marxianus* CBS 6556 was ligated into the expression plasmid pINA1317 and expressed in yeast *Yarrowia lipolytica* (Cui et al., 2011). The fermentation was carried out in a Biostat B2 2-l fermenter (B. Braun, Germany) equipped with baffles, a stirrer, alkali pump, heating element,

oxygen sensor, and temperature sensor. Seed culture was transferred into production medium containing 4.0% (w/v) inulin or 8.0% meal of Jerusalem artichoke tubercles. The fermentation was performed under the conditions of agitation speed of 250 rpm, aeration rate of 10 l/min, temperature of 28 °C and fermentation period of 80 h. The activity of the secreted inulinase was found to be 43.1±0.9 U/ml after cell growth for 80 h. When the engineered yeast cells were grown in the medium containing 8.0%, the meal of Jerusalem artichoke tuber in 2–l fermenter, crude protein and cell mass values obtained were higher than in the medium containing 4.0% inulin (Figure 6.11).

The inulinase gene cloned from the marine-derived yeast *Pichia guilliermondii* strain 1 was expressed in *Pichia pastoris* X–33 and the conditions for overexpression of the inulinase were optimized. For high cell density fermentation of the transformant *INU1* carrying plasmid pPICZaAINU1, a Biostat B2 2–1 fermenter (B. Braun, Germany) was used. The agitation rate, aeration rate and temperature were 160 rpm, 4 l/min and 28 °C, respectively. After 48 h of the fermentation, 1.5% methanol was added to the medium for induction of the recombinant inulinase. Then, 1.5% methanol was added to the medium every 24 h. After the optimization of the conditions, fermentation efficiency of 13.04 mg ± 0.4 of protein/ml/d for production of the recombinant inulinase was attained (Zhang et al., 2009).

6.4.6 Agarase

The optimum culture conditions for the production of agarases by *Vibrio* sp. strain JT0107 were determined in a 5–l jar fermenter (Marubishi Eng. Co., Tokyo) containing 3 l medium by Sugano et al., 1995. The effect of changes in the oxygen concentration on cellular growth and agarase production was examined with and without aeration. The results are shown in Figure 6.12.

The maximum agarase activity of the aerated culture was seven times more than that of the non-aerated culture. Presence of dissolved oxygen significantly enhanced both cell growth rate and agarase production.

6.4.7 Amylase

A system was developed which allowed continuous cultivation of hyperthermophilic archaebacteria at temperatures approaching 100 °C.

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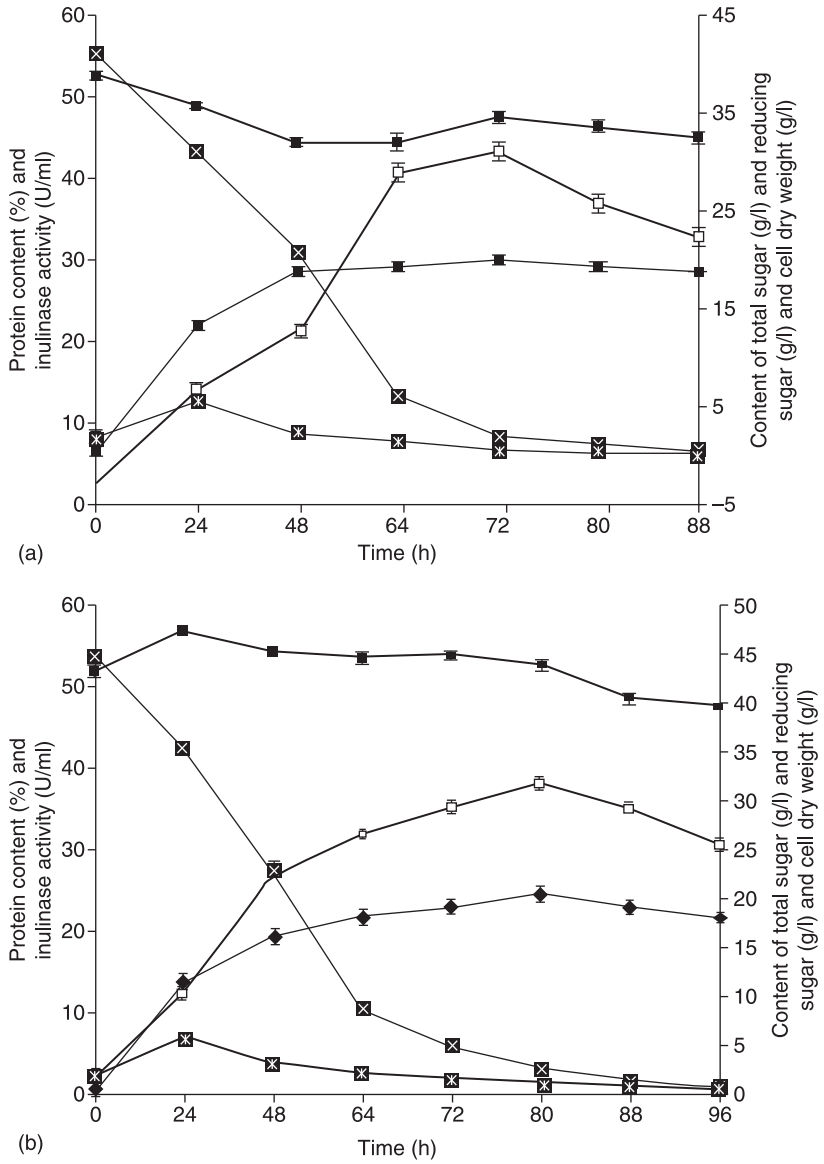

Figure 6.11 Time course of cell growth (diamond), single cell protein (closed square), inulinase activity (open square), amount of total sugar (cross) and reducing sugar (dot) during the 2-l fermentation by the transformant C55 grown in (a) 4.0% inulin and (b) 8% of the meal of Jerusalem artichoke tuber. Reprinted with permission from Elsevier.

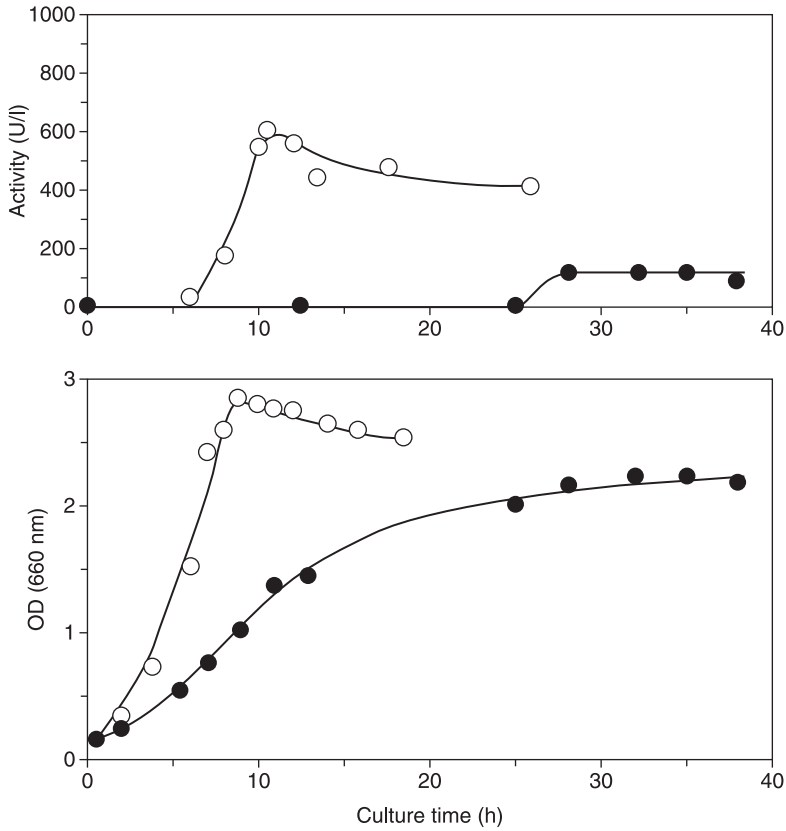

Figure 6.12 **Top panel: relationship between agarase activity of *Vibrio* sp. strain JT0107 and incubation time. Open circle–0.5 vvm aeration, closed circle–no aeration. Bottom panel: relationship between growth (OD_{660}) and incubation time. Open circle–0.5 vvm aeration, closed circle–no aeration. Reprinted with permission from Elsevier.**

Using this system, continuous cultivation *Pyrococcus furiosus* producing saccharification enzymes was carried out and the resulting dilution rate and gas production profiles were determined (Brown and Kelly 1989; Kelly et al., 2002). *Pyrococcus furiosus* is an obligatedly anaerobic heterotroph and grows in both the presence and absence of elemental sulfur. When sulfur is present, H_2S and CO_2 are produced as a consequence of growth, along with trace amounts of H_2. In the absence of sulfur, only CO_2 is produced, and the H_2 eventually becomes inhibitory to cell growth.

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Furthermore, addition of appropriate carbohydrate substrates to cultures of *P. furiosus* was found to induce enhancement of both extracellular and intracellular production of saccharification enzymes, usually those activities involved in degradative metabolism of the particular added substrate. *P. furiosus* could reach cell densities of over 10^8 cells/ml, which was relatively high for this class of organisms.

Penicillium sp NIOM–02 grown by submerged fermentation and SSF produced pigment and amylase and the production of these metabolites varied. Amylase activity during SSF was less compared with submerged fermentation and indicated the role of dissolved oxygen and continuous agitation. Oxygen transfer was a major concern of SSF and diffusion of oxygen was hampered by mycelial mat on and inside the substrate (Dhale and Vijay-Raj 2009).

6.4.8 DNA polymerase

Recombinant Tma (*Thermotoga maritima*) DNA polymerase was purified from *E. coli* strain DG116 containing plasmid Tma12–3 (Gelfand et al., 1997). The volume of seed culture inoculated into the fermenter was calculated such that the bacterial concentration was 0.5 mg dry weight/l. Foaming was controlled by the addition of propylene glycol. Airflow was maintained at 2 l/min. The culture was grown at 30 °C and then the growth temperature was shifted to 35 °C to induce the synthesis of recombinant Tma DNA polymerase. The temperature shift increased the copy number of the Tma12–3 plasmid and simultaneously derepressed the promoter controlling transcription of the modified Tma DNA polymerase gene.

6.4.9 Esterase and lipase

Sun et al., (2009) applied for a patent related to a method for preparing calcium-alginate-immobilized marine bacterium MP–2 esterase. Sodium alginate and marine bacterium MP–2 esterase were dissolved in a buffer solution of glycine-NaOH and then mixed evenly; the mixture was dripped into $CaCl_2$ solution for balling, standing, immobilization, washing and drying so as to obtain the immobilized marine bacterium MP–2 esterase.

The 32 kDa or 40 kDa lipase produced by *Tetrasphaera* sp. could be immobilized on an anion exchange resin or a hydrophobic resin through

adsorption and could be used as an immobilized enzyme (Nakao et al., 2011). When filled into a column, the immobilized enzyme allowed a continuous reaction in which source materials were passed through the column. Moreover, the immobilized enzyme could be readily removed from the reaction solution for reuse.

6.4.10 Ligase

Pyrococcus furiosus has a fermentative type of metabolism and produces organic acids, CO_2 and H_2 as final products. H_2 production inhibits growth, so cultures have to be sparged with argon to remove H_2. Alternatively, elemental sulfur may be added. The reductant that would otherwise be used to generate H_2 was used to reduce elemental sulfur to H_2S. The addition of elemental sulfur was convenient for small–scale cultures in glass vessels, but its reduction could not be used to remove inhibitory H_2 in 500l stainless steel fermenters because of the corrosive nature of H_2S (Mathur et al., 1997). A two liter flask was inoculated with two 100 ml cultures and sparged with argon. The two liter culture was used as an inoculum for a 20l culture. Two 20l cultures were used to inoculate a 500l culture. The culture was maintained at 88 °C, bubbled with argon and stirred at about 50 rpm.

6.4.11 Oxidase

The respiratory chain system of a deep-sea barophilic bacterium, *Shewanella* sp. strain DB–172F was investigated by Qureshi et al., 1998. A membrane-bound ccb-type quinol oxidase was obtained from cells grown in a pressurized vessel. The bacterium was cultivated in pre-autoclaved bags containing Marine Broth 2216 in the presence as well as the absence of oxygenated fluorinert. The bags containing the medium were placed in titanium pressure vessels (manufactured by HiP, *http://www.highpressure.com*) and kept at atmospheric pressure (0.1 MPa) or pressurized at 60 MPa. The cells were collected in the early exponential phase of growth. This enzyme was specifically induced under conditions of elevated hydrostatic pressure and high levels were expressed in cells grown at 60 MPa. Results suggested the presence of two kinds of respiratory chains regulated in response to pressure in the deep-sea bacterium DB–172F. Quinol oxidase was partially purified from the same wet weight of cells in each instance. Cells grown at 60 MPa contained the

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highest amount of quinol oxidase and this enzyme was not detected in cells grown at atmospheric pressure (0.1 MPa).

6.4.12 Peroxidase

A superior method for producing lignin peroxidase from the marine fungus *Caldariomyces fumago* was developed (Irvine and Venkatadri 1994). The microorganism was grown on one side of an oxygen-permeable surface in an aqueous medium while the opposite side of the surface was supplied with oxygen. The inventors found that by immobilizing the organism on an oxygen-permeable surface and by supplying oxygen to the organism through the oxygen-permeable surface, the production of the extracellular enzyme by the microorganism reached the highest level in eight batch production periods. When the organism was suspended and air or oxygen supply to the organism was provided by being bubbled through the aqueous medium, decreased activity of lignin peroxidase was obtained. When the organisms were attached to an oxygen permeable membrane but oxygen was supplied by flushing it through the headspace of the reactor and not through the membrane, no enzyme activity was observed after the initial addition of production medium. It was necessary to stress *C. fumago* in order to stimulate the production of peroxidase. Typically this was accomplished by reducing the supply of nutrients to the organism. When the nutrient supply was reduced and the fungus began to starve, it initiated the production of lignin peroxidase from some of its own protoplasm. After a period of lignin peroxidase production, it was necessary to again feed the fungus and stimulate its growth. Thus, production typically proceeded through alternating cycles of growth and peroxidase production which were controlled by supplying the fungus with a growth-producing medium followed by an enzyme-producing medium which was deficient in certain nutrients. After a number of cycles, the microorganism began to show signs of exhaustion and its rate of enzyme production fell. Rejuvenation of the microorganism could be accomplished by hosing down the membrane to remove older fungi and permit the growth of the younger fungi (Figure 6.13).

6.4.13 Pyruvate carboxylase

Pyruvate carboxylase was obtained from *Methanococcus jannaschii*, a hyperthermophilic, strictly hydrogenotrophic, autotrophic marine

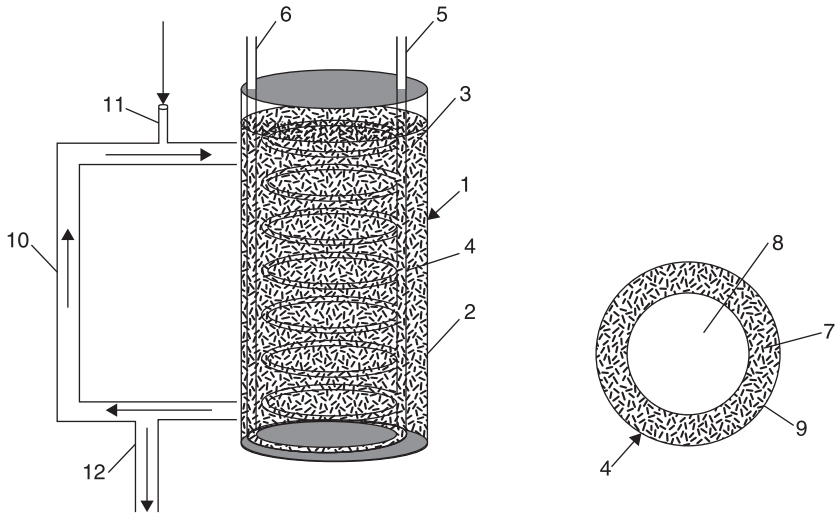

Figure 6.13 (a) Diagrammatic view of the apparatus that illustrates the process of invention (Irvine and Venkatadri 1994). Reactor (1) consisted of a tank (2) containing an aqueous medium (3) in which was immersed tubing (4) having an inlet (5) and an exhaust (6). (b) Cross section of the oxygen-permeable tubing shown in (a). Tubing (4) was made of an oxygen-permeable material (7). Oxygen-containing gas flowed through lumen (8) and a film (9) of an aerobic microorganism that grew on the outside of the tube. Aqueous medium was recirculated through reactor (1) through loop (10). Nutrients were supplied into the loop through input (11) and the enzyme product was removed through outlet (12). Reprinted with permission of the United States Patent and Trademark Office.

methanarchaeon. The organism was grown in a STR (Mukhopadhyay et al., 1999). The reactor-scale experiments were carried out in a 16-l (12 l working volume) stainless steel constantly stirred tank reactor (model Microgen; New Brunswick Scientific Company, New Brunswick, N.J.). For making anaerobic and sterile additions (manual or automatic) to the

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culture, two of the additional ports on the head plate of the vessel were fitted with rubber stoppers. All gases were supplied to the culture at the bottom of the vessel through a single-hole sparger situated directly below the agitator shaft. The N_2, CO_2, and H_2 streams were made oxygen free by passage through a common heated bed of copper turnings; the flow of hydrogen ensured continuous regeneration of oxidized copper and the heating and cooling of reactor contents were accomplished by using flows of steam. The sterilized medium was cooled to 30 °C under nitrogen, then the flows of hydrogen and CO_2 were initiated and the nitrogen flow was discontinued. At this stage an anaerobic sterile solution of salt nutrients was added to the medium. After the redox potential reading for the medium stabilized, a continuous flow of a gas mixture of N_2 and H_2S was initiated. At this stage the agitation speed and gassing rates were adjusted to desired values and the medium was inoculated with 25 ml of a mid-log-phase culture. Throughout the cultivation period the vessel was maintained at a positive pressure and the culture temperature was maintained at 85 °C. Whenever needed, foaming in the culture was suppressed by addition of a 0.2-ml anaerobic and sterile aqueous solution of Sigma Antifoam 289.

6.5 Conclusion

Production of enzymes synthesized by marine microorganisms employing different traditional bioreactor configurations as well as specialized reactor systems is described in this chapter. These include submerged cultures (SDS–stable alkaline protease, metalloprotease, chitinase, exo-inulinase, agarase, pyruvate carboxylase, saccharification enzymes, etc.), immobilized whole cell cultivations (alkaline protease, extracellular L-glutaminase, esterase, lipase, lignin peroxidase), combination of submerged and surface-attached cultures (protease, thermostable xylanases and cellulase) and solid state fermentation (saccharification enzymes).

Marine-derived enzymes have reached the market, an example being Aquabeautine XL™, a skin-care product of Aqua Biotechnology (*http://aquabiotechnology.com*). Biotec Pharmacon (*http://www.biotec.no/*) is in the midst of a broadening of its portfolio of cold-adapted enzymes whose top-selling product is shrimp alkaline phosphatase. The company introduced Cod-UNG (uracil-DNA N-glycosylase), which is an ideal enzyme for DNA/RNA analysis that already has been adopted by renowned international diagnostics companies. Zymetech (*http://www.zymetech.com*), established in 1996, is primarily involved in development, production and marketing of

marine enzymes and their derived products. It has developed proprietary patented technology on marine enzymes for pharmaceutical and cosmetic use.

The progress achieved in the past 10–15 years in the area of bioreactor design and operation has opened up numerous opportunities for the production of the many interesting marine enzymes discovered so far. It is expected that the successful integration of bioprocess engineers in the multidisciplinary team of oceanographers, biologists and chemists will eventually bridge the gap between discovery and commercialization of marine enzymes.

6.6 Acknowledgement

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