A review on production of serine alkaline protease by *Bacillus spp*.

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Abstract

In recent times, protease has gained considerable importance in the world market. Proteases are groups of proteins included in the subclass hydrolases, within the main class enzymes. Serine alkaline proteases (SAP) are one of the most important groups of industrial enzymes. They account for approximately 35% of the total microbial enzyme sales. Serine protease is produced by various types of fermentation techniques using microorganism. Among the proteases, bacterial proteases are more significant than animal and fungal proteases. Bacillus is the most invigorated species producing extracellular proteases among many bacterial species that have found tremendous application in pharmaceutical, leather, laundry and food processing industry. Mathematical modeling of fermentation process helps understand the relationship between protease production and bacterial growth to provide quantitative information on the behavior of the system. Therefore, high level production of protease in industrial scale should be made feasible. The focus of the present review is to provide an updated overview of fermentative production and the factors that influence production, growth kinetics and downstream processing of serine alkaline protease.

Key words: Fermentation, enzyme, biomass, purification, optimization, modeling.

Introduction

Microorganisms are being used for thousands of years to supply fermented products. The importance of microbes was increased significantly during World War I during which development of bioconversion and fermentation of many useful products such as enzyme, amino acids, nucleotides, vitamins, organic acids, solvents, vaccines and polysaccharides. A major segment of these segments is represented as enzyme. Enzyme is a major resource utilized by the

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Tel: 00 91 343 2755209, Fax: 0091 343 2547375; *Email: apurbadey.bt@gmail.com food, chemical, and allied industries to produce a wide range of biotechnology products and have already been recognized as valuable catalysts for various organic transformations and production of fine chemicals and pharmaceuticals (Gupta et al. 2002).

Among industrial enzymes, proteases have a pivotal role in application areas ranging from domestic to leather processing, environmental pollution abatement to neutraceutical applications, healthcare product to diagnostic kit development and value-added product production to clinical applications. (Rao et al. 2009). The estimated value of the worldwide sales of industrial enzymes was \$220 billion in 2009 (Subba Rao et al. 2009).

Serine alkaline proteases (SAP) are one of the most important groups of industrial enzymes that are widely used in detergent, leather and meat industries. They account for approximately 35% of the microbial enzyme sales. For enzyme production, there are some important criteria that must be taken into account in order to have high product yield and selectivity. These are (1) microorganism, (2) medium composition and (3) physicochemical parameters '(pH, temperature and oxygen transfer)'. Selecting the proper microorganism is important in order to obtain the desired product. The microorganism that is to be used should give adequate yields, be able to secrete large amounts of protein and should not produce toxins or any other undesired products. Serine alkaline proteases, like most of the commercial enzymes, are produced by organisms belonging to the Bacillus species since they are able to secrete large number of extracellular enzymes (Priest 1977).

Generally, proteases production from microorganisms is constitutive or partially inducible in nature. Under most culture conditions, Bacillus species produce extracellular proteases during postexponential and stationary phases. Extracellular protease production in microorganisms is also strongly influenced by media components, e.g. variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose (Beg et al. 2002), and metal ions (Varela et al. 1996). Protease synthesis is also greatly affected by rapidly metabolizable nitrogen sources, such as amino acids in the medium. Besides these, several other physical factors, such as aeration, inoculum density, pH, temperature and incubation, also affect the amount of protease produced (Hameed et al. 1999; Puri et al. 2002). In order to scale up protease production from microorganisms at the industrial level, biochemical and process engineers use several strategies to obtain high yields of protease in a fermentor. In recent years, there has been a great amount of research and development effort focusing on the use of statistical approach methods, using different statistical software packages during process optimization studies, with the aim of obtaining high yields of alkaline protease in the fermentation medium (De Coninck et al. 2000; Puri et al. 2002; Varela et al. 1996).

The production of an enzyme exhibits a characteristic relationship with regard to the growth phase of that organism. Production of protease is controlled by numerous complex mechanisms operative during the transition state between exponential growth and the stationary phase (Priest 1977; Strauch & Hoch 1993). The rate of enzyme production is variable with the specific organism. In several cases, the function of the enzyme is not very clear, but its synthesis is correlated with the onset of a high rate of protein turnover and often sporulation (Chu et al. 1992; Power & Adams 1986).

The analyses of huge amounts of fermentation data are very important to correlate with protease production and bacterial growth. Data from fermentation studies are highly complex in nature and the mathematical treatment for data analysis in bioengineering is cumbersome due to the complexity of living systems and the segregated nature of microbial life. By combining experimental work with mathematical modeling, it is possible to provide meaningful interpretations of the experimental results and analyze new aspects of microbial physiology. The model can then be used for designing new and more focused experiments (Hjortso & Bailey 1984; Kompala et al. 1986; Nielsen et al. 1991; Seo & Bailey 1985).

As per our knowledge concern no such report was found for serine protease production. Therefore, in the present review, we discuss the effect of nutritional and physicochemical parameter of fermentative production of serine alkaline protease. Additionally the kinetic production and purification of serine alkaline protease also have included in this review. Mathematical modeling of the fermentation process helps to understand the relationship among protease production and bacterial growth to provide quantitative information on the behaviour of the system. This information is essential for analysis of the fermentation process with respect to biological significance of each parameter and their levels with statistical consistency and to design and operate a fermentor for enhanced production

Fermentative Production of Serine Alkaline Protease

Microorganism Protease

Production is an inherent capacity of all microorganisms. A large number of bacterial species are known to produce serine-type alkaline proteases. But very few are recognized as commercial producers. In bioprocesses selecting the proper microorganism is important in order to obtain the desired product. The microorganism that is to be used should give adequate yields, be able to secrete large amounts of proteases and should not produce toxins or any other undesired products. Potential hosts should be suitable for industrial fermentations and produce large cell mass per volume quickly on cheap media (Kirk & Othmer 1994).

The Genus Bacillus

Bacillus is a genus of Gram-positive rod-shaped endospore forming bacteria and a member of the division Firmicutes. The endospores of the bacilli are more resistant than the vegetative cells to heat, drying, disinfectants, and other destructive agents and thus may remain viable for centuries. Cells stain Gram positive and are motile by peritrichous flagella. The genus Bacillus encompasses a great diversity of strains. Some species are strictly aerobic, others are

 Table 1. Commercial bacterial alkaline proteases, sources, applications and their industrial suppliers (Weiss and Ollis 1980)

| Supplier | Product trade name | Microbial source | Application |
|--------------------------------------|---|---|--|
| Novo Nordisk, Denmark | Alcalase Savinase Esperase Biofeed pro Durazym Novozyme 243 | Bacillus licheniformis Bacillus sp. B. lentus B. licheniformis Bacillus sp. B. licheniformis | Detergent, silk degumming Detergent, textile Detergent, food, silk degumming Feed |
| Genencor International, USA | Purafact Primatan | <i>B. lentus</i> Bacterial source | Detergent Leather |
| Gist-Brocades, The Netherlands | Subtilisin Maxacal Maxatase | B. alcalophilus Bacillus sp. Bacillus sp. | Detergent Detergent Detergent |
| Solvay Enzymes, Germany | Opticlean Optimase Maxapem HT- proteolytic | B. alcalophilus B. licheniformis Protein engineered variant of Bacillus sp. B. subtilis | Detergent Detergent Alcohol, baking, brewing, feed, food, leather, |
| | Protease | B. licheniformis | photographic waste Food, waste |
| Amano Pharmaceticals, Japan | Proleather Collagenase Amano protease S | Bacillus sp. Clostridium sp. Bacillus sp. | Food Technical Food |
| Enzyme Development, USA | Enzeco alkaline protease Enzeco alkaline protease-L | B. licheniformis B. licheniformis Bacillus sp. | Industrial Food Industrial |
| Nagase Biochemicals, Japan | FG Bioprase concentrate Ps. protease Ps. elastase Cryst. protease Bioprase Bioprase SP- | B. subtilis Pseudomonas aeruginosa Pseudomonas aeruginosa B. subtilis (K2) B. subtilis (bioteus) B. subtilis B. subtilis | Cosmetic, pharmaceutical s Research Research Research Detergent, cleaning Food |
| Godo Shusei, Japan | Godo-Bap | B. licheniformis. | Detergent, food |
| Rohm, Germany | Corolase 7089 | B. subtilis | Food |
| Wuxi Synder Bioproducts, China | Wuxi | Bacillus sp. | Detergent |
| Advance Biochemicals, India | Protosol | Bacillus sp | Detergent |

facultative anaerobic. Although the majority is mesophilic and there are also psycrophilic and thermophilic species. Some are acidophiles

while others are alkalophiles. Strains of some species grow well in a solution of glucose, ammonium phosphate and a few mineral salts, others need additional growth factors or amino acids, and still others have increasingly complex nutritional requirements (Laskin & Lechevalier 1973). Bacilli are well known for their ability to excrete enzymes such as amylases and proteases and are, therefore, excellent candidates for large-scale production of these enzymes (Moon & Parulekar 1991). Advantages of B. subtilis for production of foreign proteins can be stated as follows (Fogarty & Kelly 1990):

- 1. It is non-pathogenic,
- 2. It can be manipulated by current genetic engineering techniques,
- It lacks both endotoxins (a characteristic important in the production of proteins for medical or foodstuff application) and protein modification mechanisms which may create inactive enzyme forms,
- It can be grown more easily and has greater rates of protein synthesis than many eukaryotic systems,
- 5. Its ability to secrete a wide variety of proteins far exceeds than of its prokaryotic competitor, E.coli,
- The tendency of B.subtilis to produce several proteases is able to degrade foreign proteins.

Several products based on bacterial alkaline proteases have been launched in the market (Table 1).

Medium Design

The concentrations of media components are really important as they are tools for bioprocess medium design (Çalık et al. 2001). Culture medium supplies the microorganism with all the essential elements for microbial growth. Certain microorganisms are capable of synthesizing all of their cellular constituents from carbon and nitrogen sources. However, most of the microorganisms require some source of micronutrients (i.e., amino acids, trace elements, vitamins, etc.). The culture conditions that promote production of enzymes like proteases are significantly different from the culture conditions promoting cell growth (Moon & Parulekar 1991). Therefore optimization of media component is required for optimum cell growth and product formation.

Cell Composition

Bacterial cells are composed of high molecular weight polymeric compounds such as proteins, nucleic acids, polysaccharides, lipids, and other storage materials. Formation of macromolecules which constitute the major part of the cell mass requires production of the necessary building blocks followed by polymerization of the building blocks (Nielsen & Villadsen 1994). In addition to these biopolymers, cells contain other metabolites in the form of inorganic salts (e.g., NH_4^+ , PO_4^{3-} , K^+ , Ca^{2+} , Na^+ , SO_4^{2-}), metabolic intermediates (e.g., pyruvate, acetate), and vitamins. A typical bacterial cell is composed of 50% carbon, 20% oxygen, 14% nitrogen, 8% hydrogen, 3% phosphorus, and 1% sulfur, with small amounts of K^+ , Ca^{2+} , Na^+ , Mg^{2+} , $C\Gamma$, and vitamins (Table 2&3) (Shuler & Kargi 2008).

Most of the enzymes formed by organisms are produced as a result of their response to media components, such as nutrients, growth hormones, and ions. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation. Table 3a: The elemental composition of a bacteria.

| Species | Content (g/g of cell) |
|---------------------|-----------------------|
| Protein | 0.55 |
| RNA rRNA tRNA | 0.2 0.16 0.03 |
| mRNA | 0.01 |
| DNA | 0.03 |
| Lipid | 0.09 |
| Lipopolysaccharide | 0.03 |
| Total | 1 |

| Element | % of dry weight |
|------------|-----------------|
| Carbon | 50 |
| Oxygen | 20 |
| Nitrogen | 14 |
| Hydrogen | 8 |
| Phosphorus | 3 |
| Sulfur | 1 |
| Potassium | 1 |
| Sodium | 1 |
| Calcium | 0.5 |
| Magnesium | 0.5 |
| Chlorine | 0.5 |
| Iron | 0.2 |
| All others | ≈ 0.3 |

Nutrients required by the cells can be classified into two categories (Shuler & Kargi, 2008):

- Macronutrients are needed in concentrations higher than 10⁻⁴ M, such as carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg²⁺, and K⁺. Table 4 lists the major macronutrients and their physiological functions
- Micronutrients are needed in concentrations less than 10⁴ M. Trace elements such as Mo²⁺, Zn²⁺, Cu²⁺, Mn²⁺, Ca²⁺, Na⁺, vitamins, growth hormones and metabolic precursors are known as micronutrients.

Table 4: The major micronutrients and their physiological functions.

| Element | Physiological Function | Required Concentrati on (mol/L) |
|------------|--|---------------------------------------|
| Carbon | Constituents of organic Cellular material. Often the energy source. | > 10*2 |
| Nitrogen | Constituents of proteins, nucleic acids, and coenzymes. | 10-3 |
| Hydrogen | Organic cellular material and water. | - |
| Oxygen | Organic cellular material and water. Required for aerobic respiration | - |
| Sulfur | Constituents of proteins and certain coenzymes. | 10 ⁻⁴ |
| Phosphorus | Constituents of nucleic acids, phospholipids, nucleotides, and certain coenzymes. | 10 ⁻⁴ -10 ⁻³ |
| Potassium | Principle inorganic cation in the cell and cofactor for some enzymes. | 10 ⁻⁴ -10 ⁻³ |
| Magnesium | Cofactor for many enzymes and chlorophyls and present in cell walls and membranes. | 10 ⁻⁴ -10 ⁻³ |

Alkaline protease is comprised of 53.8 % carbon and 15.6 % nitrogen. Production of protease depends heavily on the availability of both carbon and nitrogen sources in the medium. Either an excess or a deficiency of carbon and nitrogen may cause repression of the synthesis of protease by prokaryotes (Moon & Parulekar 1991). Some carbon and nitrogen sources utilized by fermentation industry are summarized in Table 5.

Table 5. Some carbon and nitrogen sources utilized by fermentation industry.

| Carbon Sources | Nitrogen Sources | |
|---------------------------------|------------------------|--|
| Starch waste (maize and potato) | Soybean meal | |
| Molasses (cane and beet) | Yeast extract | |
| Whey | Distillers solubles | |
| n-Alkanes | Cottonseed extract | |
| Gas oil | Dried blood | |
| Sulfite waste liquor | Corn steep liquor | |
| Domestic sewage | Fish solubles and meal | |
| Cellulose waste | Groundnut meal | |
| Carbon bean | Casein | |

Types of Media

Bacterium can be cultured for any purpose in the presence of appropriate biochemical and biophysical environment. The biochemical or nutritional environment is made available as a culture medium. Depending upon the special needs of particular bacteria a large variety and types of culture media have been developed with different purposes and uses (Todar 2000). There are two major types of media depending on their composition or use. A chemically defined or synthetic medium is one in which the exact chemical composition is known. A complex or undefined medium is one in which the exact chemical constitution of the medium is not known.

Defined media are usually composed of pure biochemicals. A medium containing glucose, KH₂PO₄, (NH₄)₂HPO₄, and MgCl₂ is an example of a defined medium. Complex media usually contain complex materials of biological origin such as soybean, yeast extract, peptone, molasses or corn steep liquor, the exact chemical composition of which is obviously undetermined. In industry, complex media is preferred since the attainable enzyme activity and cell yields are much higher than that of defined media due to the presence of necessary growth factors, vitamins, hormones, and trace elements.

Optimization of Media component

For commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components in production media. Optimization helps minimizing the amount of unutilized components at the end of fermentation. Research efforts have been paying attention mainly toward:

- Evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes;
- (ii) Requirement of divalent metal ions in the fermentation medium; and
- (iii) Optimization of environmental and fermentation parameters such as pH, temperature, aeration, and agitation.

In addition, there are no defined medium established for the best production of alkaline serine proteases from different microbial sources. Each organism or strain has its own nutritional requirement for maximum enzyme production.

Effect of carbon source

Glucose is frequently used in bioprocesses for protease production. Studies have also indicated a reduction in protease production due to catabolic repression by glucose (Frankena et al. 1986; Frankena et al. 1985; Hanlon et al. 1982; Kole et al. 1988). Increased yields of alkaline proteases were also reported by several workers in the presence of different sugars such as lactose (Malathi & Chakraborty 1991), maltose (Tsuchiya et al. 1991), sucrose (Phadatare et al. 1993) and fructose (Sen & Satyanarayana 1993). However, a repression in protease synthesis was observed with these ingredients at high concentrations. In commercial practice, high carbohydrate concentrations repressed enzyme production. Therefore, carbohydrate was added either continuously or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited and thereby reduce the power requirements (Aunstrup 1980). Whey, a waste byproduct of the dairy industry containing mainly lactose and salts, has been demonstrated as a potential substrate for alkaline protease production (Donaghy & McKay 1993; McKay 1992). Similarly, maximum alkaline protease secretion was observed in the presence of pure cellulose (Solka-floc) as the principal carbon source (Gusek et al. 1988). Various organic acids, such as acetic acid (Ikeda et al. 1974), methyl acetate (Kitada & Horikoshi 1976) and citric acid or sodium citrate (Takii et al. 1990) have been demonstrated to increase production of proteases at alkaline pHs. The use of these organic acids was interesting in view of their economy as well as their ability to control pH variations.

Effect of nitrogen source

Most microorganisms can utilize both inorganic and organic forms of nitrogen which are required to produce amino acids, nucleic acids, proteins and other cell wall components. The alkaline protease comprises 15.6% nitrogen (Kole et al. 1988) and its production is dependent on the availability of both carbon and nitrogen sources in the medium (Kole et al. 1988). The complex nitrogen sources are usually used for alkaline protease production. The requirement for a specific nitrogen supplement differs from organism to organism. Low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Chandrasekaran & Dhar 1983; Chaphalkar & Dev 1994; Sen & Satyanarayana 1993). Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium (Cruegar & Cruegar 1984; Frankena et al. 1986; Giesecke et al. 1991). However, no repression in the protease activity was found in the presence of ammonium salts (Nehete et al. 1986). An increase in protease production was also observed in the presence of ammonium sulphate and potassium nitrate (Sinha & Satyanarayana 1991). Similarly, sodium nitrate (0.25%) was found to be stimulatory for alkaline protease production (Banerjee & Bhattacharyya 1992). Substitution of sodium nitrate in the basal medium with ammonium nitrate increased enzyme production (Phadatare et al. 1993). On the contrary, several reports have demonstrated the use of organic nitrogen sources leading to higher enzyme production than the inorganic nitrogen sources. Soybean meal was also reported to be a suitable nitrogen source for protease production (Chandrasekaran & Dhar 1983; Cheng et al. 1995; Sen & Satyanarayana 1993; Tsai et al. 1988). In addition, by using an acid hydrolysate of soybean in place of conventional soymeal, a

threefold increase in total enzyme activity was observed (Takagi et al. 1995). Corn steep liquor (CSL) was found to be a cheap and suitable source of nitrogen by some workers (Fujiwara & Yamamoto 1987; Malathi & Chakraborty 1991; Sen & Satyanarayana 1993). Besides serving as a nitrogen source, CSL also provides several micronutrients, vitamins, and growth-promoting factors. Tryptone and casein also serve as excellent nitrogen sources (Ong & Gaucher 1976; Phadatare et al. 1993) for protease production. The certain amino compounds were shown to be effective in the production of extracellular enzymes by *Bacillus* sp. (Ikura & Horikoshi 1987). However, glycine appeared to have inhibitory effects on protease production. Casamino acid was also found to inhibit protease production (Ong & Gaucher 1976).

Effect of metal ion and salt

Divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese, and molybdenum are required in the fermentation medium for optimum production of alkaline proteases. However, the requirement for specific metal ions depends on the source of enzyme. Potassium phosphate has been used as a source of phosphate in most studies (Fujiwara et al. 1991; Hübner et al. 1993; Mao et al. 1992; Moon & Parulekar 1991). This was shown to be responsible for buffering the medium. Phosphate at the concentration of 2 g/l was found optimal for protease production. However, amounts in excess of this concentration showed an inhibition in cell growth and repression in protease production (Moon & Parulekar 1991). Furthermore, the supplementation of salts viz., sodium chloride and phosphate ion was used as inducers for protease secretion. Owing to buffering action, phosphate ions may cause stabilization of the pH of the fermentation medium (pH homeostasis) which will indirectly favour protease synthesis. It was earlier established that bacteria utilizes the sodium-driven solute transport systems for their survival and adaptation in high pH environments. It was also reported that the growth of Bacillus sp. is regulated by sodium gradient. Therefore sodium ions are required for bioenergetic and metabolic processes of bacterium such as pH homeostasis and ATP synthesis.

Optimization of physical parameter

Bioreactor operation conditions such as oxygen transfer rate, pH and temperature show diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes (Çalık et al. 2001).

Effect of Temperature and pH

The reaction temperature and pH of the growth medium are important bioprocess parameter that is normally desired to keep both these variables constant and at their optimal values throughout the fermentation process. The influence of temperature and pH on a bioprocess can be very different, and since the growth process is the result of many enzymatic processes the influence of both culture parameters on the overall bioreaction is quite complex (Çalık et al. 2001).

The influence of temperature on the maximum specific growth rate of a microorganism is similar to that observed for the activity of an enzyme. Specific growth rate is gradually increased up to optimum temperature. But beyond that rapid decrease of specific growth rate was observed (Nielsen & Villadsen 1994). The mechanism of temperature control of enzyme production is not well understood (Chaloupka 1985). However, studies by Frankena et al. (Frankena et al. 1986) showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake.

Culture pH strongly affects many enzymatic processes and transport of several species across the cell membrane. Variation in pH alters acid-base equilibria and fluxes of various nutrients, inducers and growth factors between the abiotic and biotic phase (Moon & Parulekar 1991). The influence of pH on cellular activity is determined by the sensitivity of the individual enzymes to changes in pH. Enzymes are normally active only within a certain pH interval and the total enzyme activity of the cell is therefore a complex function of the environmental pH. Microbial cells have a remarkable ability to maintain the intracellular pH at a constant level even with large variations in the pH of the extracellular medium (Nielsen & Villadsen 1994). The intracellular aqueous (cytoplasmic) pH of alkalophilic *Bacillus* species (e.g. *B.firmus*) is 8.2-8.5, whereas for neutrophilic *Bacillus* species (e.g. *B.subtilis*, *B.licheniformis*) this value is 7.5 (Calık et al. 2001).

When ammonium ions were used, the medium turned acidic, while it turned alkaline when organic nitrogen, such as amino acids or peptides were consumed (Moon & Parulekar 1993). The decline in the pH may also be due to production of acidic products (Moon & Parulekar 1991). In view of a close relationship between protease synthesis and the utilization of nitrogenous compounds, pH variations during fermentation may indicate kinetic information about the protease production, such as the start and end of the protease production period. Temperature is another critical parameter that has to be controlled and varied from organism to organism.

Aeration and agitation

During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Different dissolved oxygen profiles can be obtained by: (i) variations in the aeration rate; (ii) variations in the agitation speed of the bioreactor; or (iii) use of oxygen rich or oxygen deficient gas phase (appropriate air-oxygen or air-nitrogen mixtures) as the oxygen source (Michalik et al. 1995; Moon & Parulekar 1991). The variation in the agitation speed influences the extent of mixing in the shake flasks or the bioreactor and will also affect the nutrient availability. Optimum yields of alkaline protease are produced at 200 rpm for *B. subtilis* ATCC 14416 (Chu et al. 1992) and *B. licheniformis* (Sen & Satyanarayana 1993). However, lowering the aeration rate caused a drastic reduction in the protease yields (Moon & Parulekar 1991). This indicates that a reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis.

Oxygen transfer shows diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes. According to cell growth conditions and metabolic pathway analysis some bioprocesses require high oxygen transfer rate conditions while others require controlled oxygen transfer rate conditions (Calik et al. 1999). It has been extensively investigated in defined (Calik et al. 2000; Çalık et al. 1998; Calik et al. 1999) and molasses based complex medium (Çalık et al. 2003) for serine alkaline protease production and medium oxygen transfer conditions were found to be favorable for SAP production.

Effect of inoculum percentage and incubation time

Biomass and enzyme production was also influenced by the initial inoculum concentration. Optimum inoculum size was required for protease production. The increase in protease production using small inoculum sizes was suggested to be due to the higher surface area to volume ratio resulting in increased protease production. If the inoculum size is too small, insufficient number of bacteria would lead to reduced amount of secreted protease. This may be reasoned due to the limitation in other fermentation medium components and reduced dissolved oxygen (Rahman et al. 2005).

The incubation also affects protease production. Normally protease is auto-degradable in nature. So protease yield can be increased by proper incubation time. This may be reasoned due to the limitation in other fermentation medium components.

Strain improvement

Strain improvement plays a key role in the commercial development of microbial fermentation processes. As a rule, the wild strains usually produce limited quantities of the desired enzyme to be useful for commercial application. However, in most cases, by adopting simple selection methods, such as spreading of the culture on specific media, it is possible to pick colonies that show a substantial increase in yield. The yield can be further improved by the use of mutagens or antibiotics and the adoption of special techniques or procedures for detecting useful mutants.

Kinetic Model

The modeling of enzyme production kinetics is very difficult and challenging job for model development, i.e., model formulation, identification and estimation of the parameters, and solution of the equations (Coppella & Dhurjati 1989). Models of single cells, cell populations, and cultures can be most useful in organizing information in a comprehensive system description, as well as in optimizing and controlling actual production operations (Curien et al. 2003; Grosz & Stephanopoulos 1990; Rizzi et al. 1996).

Classification of models

Many classes of models can be distinguished for microbial kinetics. In general, fermentation process models are either structured or unstructured depending on the metabolic analysis pattern. Structured models use the information on intracellular metabolic pathway reaction rates, while unstructured models utilize the knowledge of experimental reality and describe biomass and associated metabolite production. The unstructured model presumes the balanced growth, which is equivalent to fixed cell composition. Such assumption is valid primarily in single-stage, steady-state continuous culture and the exponential phase of batch culture, and it fails to discuss during any transient condition. If cell response is fast then external changes and if the magnitude of these changes is not too large, the use of unstructured models can be used because the deviation from balanced growth may be small. Structured models describe culture response to large or rapid perturbations satisfactorily. Structured models deals with the inner structure of cells and thus acknowledge only implicitly the changes of cellular physiological state with the environment. The unstructured models are relatively easy to build and are used extensively in simulating steady state or slowly changing systems (Amrane & Prigent 1999; Dutta et al. 1996; Okazaki et al. 1980; Pluschkell et al. 1996; Roy et al. 1987; Weiss & Ollis 1980). Structured models are superior to unstructured models because they can provide a measure of the quality of the cell population. These models incorporate biological knowledge by separating the biomaterial into compartments. The interactions between compartment-compartment and compartmentenvironment are described by equations on stoichiometry and kinetics, especially on mass and energy balances, on the rates of

transport, and on reactions (Thilakavathi et al. 2007). Structured models can be used in a broader domain of conditions and have better capacity to extrapolate than unstructured ones (Bentley & Kompala 1989; Cazzador & Mariani 1988; Fredrickson 1976; Grosz & Stephanopoulos 1990; Kim & Shuler 1990; Lee & Bailey 1984; Nielsen et al. 1989).

Unstructured model

Unstructured kinetic models are the most frequently employed for modeling microbial systems based on simplicity and technical robustness. However, the formulation of an unstructured model was based on Monod model and logistics model (Esener et al. 1983; Shuler & Kargi 2008). Unstructured models are simpler than the structured models. Thus, it is necessary to measure smaller numbers of important components. So, the model may describe at least the evolution of biomass; carbon sources; products; and often, another substrate, mainly nitrogen (Moraine & Rogovin 1971). In a typical fermentation process, a number of these rate processes are important, viz, the rate of change of the amount of biomass and its different components, the rate of consumption of nutrients, and the rate of production of products and metabolites (Thilakavathi et al. 2007). In general, the biomass growth is modeled using Monod's equation or logistics equation for the specific growth of cells. The coefficient of the microbial decay for the microbial growth is also included in the differential equation (Moraine & Rogovin 1971). The different types of models used for growth kinetics for a single substrate are given in Table 6 (Han & Levenspiel 1988; Schuegerl & Bellgardt 2000).

 Table 6. Growth kinetics for a single substrate (Weiss and Ollis 1980)

| Model | Normalized kinetic expression | Remarks |
|---------------------|---|--|
| Blackman | $\frac{C_s}{2K_s}$ | Additional rate-limiting step besides uptake |
| Contois | $\frac{C_s}{K_{sx}C_x + C_s}$ | Diffusion layer surrounding the cell |
| Mason and Millis | $\frac{C_S}{K_S + C_S} + K_I C_S^n$ | Parallel uptake by transport and diffusion |
| Monod | $\frac{C_s}{K_s + C_s}$ | Substrate uptake step rate limiting |
| Moser | $\frac{C_s^n}{K_s + C_s^n}$ | Substrate uptake with higher order of reaction |
| Powel | $\frac{C_s - K_I(C_s)\bar{r}}{K_s + C_s - K_I(C_s)\bar{r}}$ | Back diffusion of inner substrate |
| Tessier | $1 - \exp(-KC_s)$ | Empirical form |
| Vavilin | $\frac{C_S^N}{C_S^N + K_S^{N-M}C_{S(t=0)}^M}$ | Initial inactivation by toxic substrates |

Usually, substrate consumption is explained with the equation of either biomass growth and yield coefficient for biomass production or product production and its yield coefficient (Amrane & Prigent 1994; Venkatesh et al. 1993; Yeh et al. 1991). The substrate uptake kinetics including inhibition are given in Table 7 (Han & Levenspiel 1988; Schuegerl & Bellgardt 2000). The differential equation, concerning product concentration, is explained using the Luedeking and Piret equation (Garcia-Ochoa et al. 1995; Luedeking & Piret 2000; Velkovska et al. 1997).

| Model | Normalized kinetic expression |
|----------------------------|--|
| Aiba | $\frac{C_s}{K_s + C_s} \exp(-\frac{C_s}{K_l})$ |
| Andrews | $\frac{C_s}{(K_s + C_s)(1 + \frac{C_s}{K_s})}$ |
| Chen | C_s (1 C_s) |
| (toxic | $\frac{1}{K_{s} + C_{s} - (K_{s_{2}}C_{s})^{2}} (1 - \frac{1}{K_{I}})$ |
| Edwards | $\frac{C_s}{K_s + C_s} \exp(-K_e C_s)$ |
| Haldane (uncompetitive) | $\frac{C_s}{(K_s + C_s)(1 + \frac{C_s}{K_I})}$ |
| Han and Levenspiel | $\sum_{i=1}^{j} (1 - \frac{C_{ii}}{C_{ii}})^{ni} \frac{C_{S}}{C_{S} + C_{M}(\sum_{i=1}^{j} (1 - \frac{C_{ii}}{C_{ii}^{*}})^{ni})}$ |
| Hill (allosteric) | $\frac{C_s^n}{C_s^n + K_s}$ |
| Jerusalimsky | $\frac{C_s}{(C_s+K_s)(1+\frac{C_p}{K_p})}$ |
| Teissier-type | $\exp(-\frac{C_s}{K_I}) - \exp(-\frac{C_s}{K_s})$ |
| Webb | $\frac{C_s(1+\frac{C_s}{K_t})}{K_s+C_s+\frac{C_s^2}{K_s}}$ |

 Table 7.
 Substrate uptake kinetics including inhibition (Weiss and Ollis 1980).

A simplified, unstructured, nonsegregated, kinetic model has been proposed for the production of protease by Bacillus sphaericus MTCC511. This kinetic model assumes that the glucose was the limiting nutrient of the bacterial growth and protease production. The various unstructured models, i.e. the Monod model for microbial growth, the Monod incorporated Luedeking-Piret model for the production of protease and the Monod-incorporated modified Luedeking-Piret model for the utilization of substrate were analyzed to simulate the experimental values of microbial growth, protease activity and substrate concentration (Rajendran & Thangavelu 2008). An unstructured mathematical model was developed to understand information on the relationship between Bacillus circulans growth and metabolismrelated protease production using logistic and Luedeking-Piret equations respectively. This kinetic model assumes that the glucose was the limiting nutrient of the bacterial growth and protease production (Rao et al. 2009).

Purification of Alkaline Proteases

Crude preparations of alkaline proteases are generally used for commercial use. However, the purification of alkaline proteases is important from the perspective of developing a better understanding of the functioning of the enzyme (Takagi 1993; Tsai et al. 1988).

Recovery

After successful fermentation, broth was kept in low temperature (to below 4°C) to prevent microbial contamination as well as to maintain enzyme activity and stability. The removal of the cells, solids, and colloids from the fermentation broth is the primary step in enzyme downstream processing. The vacuum rotary drum filters and cold centrifuges are commonly used. To prevent the losses in

enzyme activity caused by imperfect clarification or to prevent the clogging of filters, it is necessary to perform some chemical pretreatment of the fermentation broth before commencing separation (Aunstrup 1980; Mukhopadhyay et al. 1990). Changes in pH may also be suitable for better separation of solids (Tsai et al. 1983). Furthermore, the fermentation broth solids are often colloidal in nature and are difficult to remove directly. In this case, addition of coagulating or flocculating agents becomes vital (Boyer & Byng 1996). Flocculating agents are generally employed to effect the formation of larger flocs or agglomerates, which in turn accelerate the solid-liquid separation. Cell flocculation (Bautista et al. 1986) can be improved by neutralization of the charges on the microbial cell surfaces, which includes changes in pH and the addition of a range of compounds that alter the ionic environment. The flocculating agents commonly used are inorganic salts, mineral hydrocolloids, and organic polyelectrolytes. In some cases, it becomes necessary to add a bioprocessing filter aid, such as diatomaceous earth, before filtration (Boyer & Byng 1996; Tsuchida et al. 1986).

Isolation and purification

When isolating enzymes on industrial scale for commercial purposes, the prime consideration has been the cost of production in relation to the value of the end product.

Precipitation Precipitation is the most commonly used method for the isolation and recovery of proteins from crude biological mixtures (Bell et al. 1983). It also performs both purification and concentration steps. It is generally affected by the addition of reagents such as salt or an organic solvent, which lowers the solubility of the desired proteins in an aqueous solution. Although precipitation by ammonium sulfate has been widely used for many years, ammonium sulfate found wide utility only in acidic and neutral pH values and developed ammonia under alkaline conditions (Aunstrup 1980). Hence, the use of sodium sulfate or an organic solvent formed the preferred choice. Despite better precipitating qualities of sodium sulfate over ammonium sulfate, the poor solubility of the salt at low temperatures restricted its use for this purpose (Shih et al. 1992). Many reports revealed the use of acetone at different volume concentrations: 5 volumes (Horikoshi 1971), 3 volumes (Kim et al. 1996; Tsujibo et al. 1990), and 2.5 volumes (Kumar et al. 1997), as a primary precipitation agent for the recovery of alkaline proteases. Precipitation was also reported by various workers with acetone at different concentrations: 80% (v/v) (Durham 1987; Kwon et al. 1994), 66% (v/v) (Yamagata et al. 1995); or 44, 66, and 83% (v/v) (El-Shanshoury et al. 1995), followed by centrifugation and/or drying. Precipitation of enzymes can also be achieved by the use of water-soluble, neutral polymers such as polyethylene glycol (Larcher et al. 1996).

Concentration

Because the amount of enzyme present in the cell-free filtrate is usually low, the removal of water is a primary objective. So, membrane separation processes have been widely used for downstream processing (Strathmann 1990). Ultrafiltration (UF) is one such membrane process that has been largely used for the recovery of enzymes (Bohdziewicz 1996; Bohdziewicz 1994; Bohdziewicz & Bodzek 1994) and formed a preferred alternative to evaporation. This pressure-driven separation process is inexpensive, results in little loss of enzyme activity, and offers both purification and concentration (Sullivan et al. 1984), as well as diafiltration, for salt removal or for changing the salt composition (Boyer & Byng 1996; Manachini et al. 1988; Peek et al. 1992). However, a disadvantage underlying this process is the fouling or membrane clogging due to the precipitates formed by the final product. This clogging can usually be alleviated or overcome by treatment with detergents, proteases, or acids and alkalies. Han and associates (Han et al. 1995) used a temperature-sensitive hydrogel ultrafiltration for concentrating an alkaline protease. This hydrogel comprised poly (N-isopropyl-acrylamide), which changed its volume reversibly by the changes in temperature. The separation efficiency of the enzyme was dependent on the temperature and was 84% at temperatures of 15°C and 20°C. However, at temperatures above 25°C, a decrease in the separation efficiency was observed.

Conclusion

Microbial metabolism is a complex process, differing in terms of biomass and associated metabolite production with only slight variation in physiological, fermentation and medium components. Hence its comprehensive quantitative and mechanistic evaluation for complete metabolic information of processes, mass balances, inhibitions and production processes is often very difficult or impossible in the case of normal fermentation process results. So, in the present article, an attempt has been made to describe the effect of nutritional and physicochemical parameters on bacterial serine protease production. This is important for obtaining higher alkaline protease production as well as for the reduction of process operating cost. Kinetic model was applied for enzyme production kinetics. For the treatment of the kinetics of microbial processes, more attention has been directed to unstructured models.

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