

# 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

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 nutraceutical 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 post-exponential 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

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

facultative anaerobic. Although the majority is mesophilic and there are also psychrophilic 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,
3. 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,
4. 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 that of its prokaryotic competitor, *E. coli*,
6. 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 (Çalik 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.,  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{SO}_4^{2-}$ ), 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  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Cl}^-$ , 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	0.2
<i>rRNA</i>	0.16
<i>tRNA</i>	0.03
<i>mRNA</i>	0.01
DNA	0.03
Lipid	0.09
Lipopolysaccharide	0.03
<b>Total</b>	<b>1</b>

**Table 3b:** The elemental composition of a bacteria.

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):

1. Macronutrients are needed in concentrations higher than  $10^{-4}$  M, such as carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus,  $\text{Mg}^{2+}$ , and  $\text{K}^+$ . Table 4 lists the major macronutrients and their physiological functions
2. Micronutrients are needed in concentrations less than  $10^{-4}$  M. Trace elements such as  $\text{Mo}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ , vitamins, growth hormones and metabolic precursors are known as micronutrients.

**Table 4:** The major micronutrients and their physiological functions.

Element	Physiological Function	Required Concentration (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 chlorophylls 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,  $\text{KH}_2\text{PO}_4$ ,  $(\text{NH}_4)_2\text{HPO}_4$ , and  $\text{MgCl}_2$  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:

- (i) 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 & Dey 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 soy meal, 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 (Çalık 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 deal 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 compartment–environment 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).

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

Model	Normalized kinetic expression
Aiba	$\frac{C_s}{K_s + C_s} \exp(-C_s/K_i)$
Andrews	$\frac{C_s}{(K_s + C_s)(1 + \frac{C_s}{K_i})}$
Chen (toxic)	$\frac{C_s}{K_s + C_s - (K_{s2} C_s)^2} (1 - \frac{C_s}{K_i})$
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	$\frac{\sum_{i=1}^n (1 - \frac{C_i}{C_i^*})^{m_i} C_s}{C_s + C_M (\sum_{i=1}^n (1 - \frac{C_i}{C_i^*})^{m_i})}$
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	$\frac{C_s(1 + \frac{C_s}{K_i})}{K_s + C_s + \frac{C_s^2}{K}}$

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 metabolism-related 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** 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 alkalis. 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.

## References

- Amrane A, Prigent Y (1999) Analysis of growth and production coupling for batch cultures of *Lactobacillus helveticus* with the help of an unstructured model. *Process Biochem* 34:1-10.
- Amrane A, Prigent Y (1994) Mathematical model for lactic acid production from lactose in batch culture: model development and simulation. *J Chem Technol Biotechnol* 60:241-246.
- Aunstrup K. 1980. Proteinases. in: *Economic Microbiology: Microbial Enzymes and Bioconversions*, (Ed.) A.H. Rose, Vol. 5, Academic Press. New York, pp. 50-114.
- Banerjee R, Bhattacharyya BC (1992) Optimisation of multiple inducers effect on protease biosynthesis by *Rhizopus oryzae*. *Bioprocess Eng* 7:225-228.
- Bautista J, Chico E, Machado A (1986) Cell removal from fermentation broth by flocculation-sedimentation. *Biotechnol Lett* 8:315-318.
- Beg QK, Saxena RK, Gupta R (2002) De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. *Process Biochem* 37:1103-1109.
- Bell DJ, Hoare M, Dunnill P (1983) The formation of protein precipitates and their centrifugal recovery. *Adv Biochem Eng Biotechnol* 26:1-72.
- Bentley WE, Kompala DS (1989) A novel structured kinetic modeling approach for the analysis of plasmid instability in recombinant bacterial cultures. *Biotechnol Bioeng* 33:49-61.
- Bohdziewicz J (1996) Ultrafiltration of technical amylolytic enzymes. *Process Biochem* 31:185-191.
- Bohdziewicz J (1994) Ultrafiltration of technical proteolytic enzymes. *Process Biochem* 29:109-118.
- Bohdziewicz J, Bodzek M (1994) Ultrafiltration preparation of pectinolytic enzymes from citric acid fermentation. *Process Biochem* 29:99-107.
- Boyer EW, Byng GS. 1996. *Bacillus proteolyticus* species which produce an alkaline protease, US Patent No: 5518917.
- Çalık P, Bilir E, Çalık G et al (2003) Bioreaction operation parameters as tools for metabolic regulations in fermentation processes: influence of pH conditions. *Chemical Engineering Science* 58:759-766.
- Calik P, Calik G, Ozdamar TH (2000) Oxygen-transfer strategy and its regulation effects in serine alkaline protease production by *Bacillus licheniformis*. *Biotechnol Bioeng* 69:301-311.
- Çalık P, Çalık G, Özdamar TH (2001) Bioprocess development for serine alkaline protease production: A Review. *Reviews in Chemical Engineering* 17:1-62.
- Çalık P, Çalık G, Özdamar TH (1998) Oxygen transfer effects in serine alkaline protease fermentation by *Bacillus licheniformis*: Use of citric acid as the carbon source. *Enzyme Microb Technol* 23:451-461.
- Calik P, Calik G, Takacs S et al (1999) Metabolic flux analysis for serine alkaline protease fermentation by *Bacillus licheniformis* in a defined medium: effects of the oxygen transfer rate. *Biotechnol Bioeng* 64:151-167.
- Cazzador L, Mariani L (1988) A simulation program based on a structured population model for biotechnological yeast process. *Appl Microbiol Biotechnol* 29:198-202.
- Chaloupka J (1985) Temperature as a factor regulating the synthesis of microbial enzymes. *Microbiol Sci* 2:86-90.
- Chandrasekaran S, Dhar SC (1983) A low cost method for the production of extracellular alkaline proteinase using tapioca starch. *J Ferment Technol* 61:511-514.
- Chaphalkar S, Dey S (1994) Some aspects of production of extracellular protease from *Streptomyces diastaticus*. *J Microb Biotechnol* 9:85-100.
- Cheng SW, Hu HM, Shen SW et al (1995) Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. *Biosci Biotechnol Biochem* 59:2239-2243.
- Chu IM, Lee C, Li TS (1992) Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. *Enzyme Microb Technol* 14:755-761.
- Coppella SJ, Dhurjati P (1989) A detailed analysis of *Saccharomyces cerevisiae* growth kinetics in batch, fed-batch, and hollow-fiber bioreactors. *Chem Eng J* 41:B27-B35.
- Cruegar W, Cruegar A. 1984. *Biotechnology—A Textbook for Industrial Microbiology*, (Ed.) W.I. Madison, Science Tech Inc. London.
- Curien G, Ravanel S, Dumas R (2003) A kinetic model of the branch-point between the methionine and threonine biosynthesis pathways in *Arabidopsis thaliana*. *Eur J Biochem* 270:4615-4627.
- De Coninck J, Bouquelet S, Dumortier V et al (2000) Industrial media and fermentation processes for improved growth and protease production by *Tetrahymena thermophila*. *J Ind Microbiol Biotechnol* 24:285-290.
- Donaghy JA, McKay AM (1993) Production and properties of an alkaline protease by *Aureobasidium pullulans*. *J Appl Bacteriol* 74:662-666.
- Durham DR (1987) Utility of subtilisin GX as a detergent additive. *J Appl Bacteriol* 63:381-386.
- Dutta SK, Mukherjee A, Chakraborty P (1996) Effect of product inhibition on lactic acid fermentation: simulation and modeling. *Appl Microbiol Biotechnol* 46:410-413.
- El-Shanshoury AR, El-Sayed MA, Sammour RH et al (1995) Purification and partial characterization of two extracellular alkaline proteases from *Streptomyces corchorusii* ST36. *Can J Microbiol* 41:99-104.
- Esener AA, Roels JA, Kossen NW (1983) Theory and applications of unstructured growth models: Kinetic and energetic aspects. *Biotechnol Bioeng* 25:2803-2841.



- Fogarty WM, Kelly CT. 1990. *Microbial Enzymes and Biotechnology*. 2<sup>nd</sup> ed. Elsevier Applied Sciences, London, New York.
- Frankena J, Koningstein GM, van Verseveld HW et al (1986) Effect of different limitations in chemostat cultures on growth and production of exocellular protease by *Bacillus licheniformis*. Appl Microbiol Biotechnol 106:112.
- Frankena J, van Verseveld HW, Stouthamer AH (1985) A continuous culture study of the exocellular protease in *Bacillus licheniformis*. Appl Microbiol Biotechnol 22:169-176.
- Fredrickson AG (1976) Formulation of structured growth models. Biotechnol Bioeng 18:1481-1486.
- Fujiwara N, Yamamoto K (1987) Production of alkaline protease in a low-cost medium by alkalophilic *Bacillus sp.* and properties of the enzyme. J Ferment Technol 65:345-348.
- Fujiwara N, Yamamoto K, Masui A (1991) Utilization of a thermostable alkaline protease from an alkalophilic thermophile for the recovery of silver from used X-ray film. J Ferment Bioeng 72:306-308.
- Garcia-Ochoa F, Santos VE, Alcon A (1995) Xanthan gum production: an unstructured kinetic model. Enzyme Microb Technol 17:206-217.
- Giesecke UE, Bierbaum G, Rudde H et al (1991) Production of alkaline protease with *Bacillus licheniformis* in a controlled fed-batch process. Appl Microbiol Biotechnol 35:720-724.
- Grosz R, Stephanopoulos G (1990) Physiological, biochemical, and mathematical studies of micro-aerobic continuous ethanol fermentation by *Saccharomyces cerevisiae*. III: mathematical model of cellular energetics and catabolism. Biotechnol Bioeng 36:1030-1040.
- Gupta R, Beg QK, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15-32.
- Gusek TW, Wilson DR, Kinsella JE (1988) Influence of carbon source on production of a heat-stable protease from *Thermomonospora fusca* YX. Appl Microbiol Biotechnol 28:80-84.
- Hameed A, Keshavarz T, Evans CS (1999) Effect of dissolved oxygen tension and pH on the production of extracellular protease from a new isolate of *Bacillus subtilis* K2, for use in leather processing. J Chem Technol Biotechnol 74:5-8.
- Han J, Park CH, Ruan R (1995) Concentrating alkaline protease, subtilisin, using a temperature-sensitive hydrogel. Biotechnol Lett 17:851-852.
- Han K, Levenspiel O (1988) Extended monod kinetics for substrate, product, and cell inhibition. Biotechnol Bioeng 32:430-447.
- Hanlon GW, Hodges NA, Russell AD (1982) The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. J Gen Microbiol 128:845-851.
- Hjortso MA, Bailey JE (1984) Plasmid stability in budding yeast populations: dynamics following a shift to nonselective medium. Biotechnol Bioeng 26:814-819.
- Horikoshi K (1971) Production of alkaline enzymes by alkalophilic microorganisms. Part I. Alkaline protease produced by *Bacillus* No. 221. Agric Biol Chem 35:1407-1414.
- Hübner U, Bock U, Schügerl K (1993) Production of alkaline serine protease subtilisin Carlsberg by *Bacillus licheniformis* on complex medium in a stirred tank reactor. Appl Microbiol Biotechnol 40:182-188.
- Ikeda S, Tobe S, Niwa K et al (1974) Production of alkaline protease from acetic acid. Agric Biol Chem 38:2317-2322.
- Ikura Y, Horikoshi K (1987) Effect of amino compounds on alkaline amylase production by alkaliphilic *Bacillus sp.* J Ferment Technol 65:707-709.
- Kim BG, Shuler ML (1990) A structured, segregated model for genetically modified *Escherichia coli* cells and its use for prediction of plasmid stability. Biotechnol Bioeng 36:581-592.
- Kim W, Choi K, Kim Y et al (1996) Purification and characterization of a fibrinolytic enzyme produced from *Bacillus sp.* strain CK 11-4 screened from Chungkook-Jang. Appl Environ Microbiol 62:2482-2488.
- Kirk RE, Othmer DF. 1994. *Encyclopedia of Chemical Technology*. 4<sup>th</sup> ed. The Interscience Encyclopedia Inc., New York.
- Kitada M, Horikoshi K (1976) Alkaline protease production from methyl acetate by alkalophilic *Bacillus sp.* J Ferment Technol 54:383-392.
- Kole MM, Draper I, Gerson DF (1988) Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. J Chem Technol Biotechnol 41:197-206.
- Kompala DS, Ramkrishna D, Jansen NB et al (1986) Investigation of bacterial growth on mixed substrates: experimental evaluation of cybernetic models. Biotechnol Bioeng 28:1044-1055.
- Kumar CG, Tiwari MP, Jany KD (1997) Purification and characterization of two alkaline proteases from an alkalophilic *Bacillus sp.* Zeitschrift für Ernährungs-Wissenschaft 36:48-48.
- Kwon YT, Kim JO, Moon SY et al (1994) Extracellular alkaline protease from alkalophilic *Vibrio metschnikovii* strain RH530. Biotechnol Lett 16:413-418.
- Larcher G, Cimon B, Symoens F et al (1996) A 33 kDa serine proteinase from *Scedosporium apiospermum*. Biochem J 315 (Pt 1):119-126.
- Laskin AI, Lechevalier HA. 1973. *Handbook of Microbiology*. CRC Press, USA.
- Lee SB, Bailey JE (1984) Genetically structured models for lac promoter-operator function in the *Escherichia coli* chromosome and in multicopy plasmids: Lac operator function. Biotechnol Bioeng 26:1372-1382.
- Luedeking R, Piret EL (2000) A kinetic study of the lactic acid fermentation. Batch process at controlled pH. Reprinted from Journal of Biochemical and Microbiological Technology Engineering Vol. I, No. 4. Pages 393-412 (1959). Biotechnol Bioeng 67:636-644.
- Malathi S, Chakraborty R (1991) Production of alkaline protease by a new *Aspergillus flavus* isolate under solid substrate fermentation conditions for use as a depilation agent. Appl Environ Microbiol 57:712-716.
- Manachini PL, Fortina MG, Parini C (1988) Thermostable alkaline protease produced by *Bacillus thermoruber*—a new species of *Bacillus*. Appl Microbiol Biotechnol 28:409-413.
- Mao W, Pan R, Freedman D (1992) High production of alkaline protease by *Bacillus licheniformis* in a fed-batch fermentation using a synthetic medium. J Ind Microbiol 11:1-6.
- McKay AM (1992) Production of an alkaline protease by *Fusarium graminearum* grown on whey. Milchwissenschaft 47:147-148.
- Michalik I, Szabova E, Polakova A et al (1995) The selection of *Bacillus licheniformis* strains for protease production: Characterization of bacterial alkaline protease. Biologia 50:249-252.
- Moon SH, Parulekar SJ (1991) A parametric study of protease production in batch and fed-batch cultures of *Bacillus firmus*. Biotechnol Bioeng 37:467-483.
- Moon SH, Parulekar SJ (1993) Some observations on protease production in continuous suspension cultures of *Bacillus firmus*. Biotechnol Bioeng 41:43-54.
- Moraine RA, Rogovin P (1971) Xanthan biopolymer production at increased concentration by pH control. Biotechnol Bioeng 13:381-391.

- Mukhopadhyay A, Chakrabarti SK, Bajpai PK (1990) Treatment and clarification of fermented broth in bacterial enzyme production. *Biotechnol Techniques* 4:121-126.
- Nehete PN, Shah VD, Kothari RM (1986) Isolation of a high yielding alkaline protease variant of *Bacillus licheniformis*. *Enzyme Microb Technol* 8:370-372.
- Nielsen J, Emborg C, Halberg K et al (1989) Compartment model concept used in the design of fermentation with recombinant microorganisms. *Biotechnol Bioeng* 34:478-486.
- Nielsen J, Pedersen AG, Strudsholm K et al (1991) Modeling fermentations with recombinant microorganisms: formulation of a structured model. *Biotechnol Bioeng* 37:802-808.
- Nielsen J, Villadsen J. 1994. *Bioreaction Engineering Principles*. Plenum, New York.
- Okazaki N, Sugama S, Tanaka T (1980) Mathematical model for surface culture of koji mold. *J Ferment Technol* 58:471-476.
- Ong PS, Gaucher GM (1976) Production, purification and characterization of thermomycolase, the extracellular serine protease of the thermophilic fungus *Malbranchea pulchella* var. *sulfurea*. *Can J Microbiol* 22:165-176.
- Peek K, Daniel RM, Monk C et al (1992) Purification and characterization of a thermostable proteinase isolated from *Thermus* sp. strain Rt41A. *Eur J Biochem* 207:1035-1044.
- Phadataré SU, Deshpande VV, Srinivasan MC (1993) High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents. *Enzyme Microb Technol* 15:72-76.
- Pluschkell S, Hellmuth K, Rinas U (1996) Kinetics of glucose oxidase excretion by recombinant *Aspergillus niger*. *Biotechnol Bioeng* 51:215-220.
- Power SD, Adams RM. 1986. *Bacillus*. in: *Molecular Genetics and Biotechnology Applications*, (Eds.) A. Ganesain, J.A. Hoch, Academic Press. New York, pp. 101-108.
- Priest FG (1977) Extracellular enzyme synthesis in the genus *Bacillus*. *Bacteriol Rev* 41:711-753.
- Puri S, Beg QK, Gupta R (2002) Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. *Curr Microbiol* 44:286-290.
- Rahman RN, Geok LP, Basri M et al (2005) Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. *Bioresour Technol* 96:429-436.
- Rajendran A, Thangavelu V (2008) Evaluation of Various Unstructured Kinetic Models for the Production of Protease by *Bacillus sphaericus* MTTC511. *Engineering in Life Sciences* 8:179-185.
- Rao CS, Sathish T, Brahmaiah P et al (2009) Development of a mathematical model for *Bacillus circulans* growth and alkaline protease production kinetics. *J Chem Technol Biotechnol* 84:302-307.
- Rizzi M, Theobald U, Querfurth E et al (1996) In vivo investigations of glucose transport in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 49:316-327.
- Roy D, Leudy A, Goulet J (1987) Kinetics of growth and lactic acid production from whey permeate by *Lactobacillus helveticus*. *Can J Chem Eng* 65:597-603.
- Schuegerl K, Bellgardt KH. 2000. *Bioreaction engineering, modeling and control*. Springer, Berlin Heidelberg New York.
- Sen S, Satyanarayana T (1993) Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. *Ind J Microbiol* 33:43-47.
- Seo JH, Bailey JE (1985) A segregated model for plasmid content and product synthesis in unstable binary fission recombinant organisms. *Biotechnol Bioeng* 27:156-166.
- Shih YC, Prausnitz JM, Blanch HW (1992) Some characteristics of protein precipitation by salts. *Biotechnol Bioeng* 40:1155-1164.
- Shuler ML, Kargi F. 2008. *Bioprocess Engineering Basic Concepts*. 2<sup>nd</sup> ed. Prentice Hall Pvt. Ltd.
- Sinha N, Satyanarayana T (1991) Alkaline protease production by thermophilic *Bacillus licheniformis*. *Indian J Microbiol* 31:425-430.
- Strathmann H (1990) The use of membranes in downstream processing. *Food Biotechnol* 4:253-272.
- Strauch MA, Hoch JA (1993) Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential phase gene expression. *Molecular Microbiol* 77:337-342.
- Subba Rao C, Sathish T, Ravichandra P et al (2009) Characterization of thermo- and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. *Process Biochemistry* 44:262-268.
- Sullivan TGO, Epstein AC, Korchin SR et al (1984) Applications of ultrafiltration in biotechnology. *Chem Eng Prog* 80:68-75.
- Takagi H (1993) Protein engineering on subtilisin. *Int J Biochem* 25:307-312.
- Takagi H, Tsai YC, Nakamori S et al (1995) Improved production and recovery of alkaline elastase from alkalophilic *Bacillus* strain by a change of medium composition. *Biosci Biotechnol Biochem* 59:1591-1592.
- Takii Y, Kuriyama N, Suzuki Y (1990) Alkaline serine protease produced from citric acid by *Bacillus alcalophilus* subsp. *halodurans* KP 1239. *Appl Microbiol Biotechnol* 34:57-62.
- Thilakavathi M, Basak T, Panda T (2007) Modeling of enzyme production kinetics. *Appl Microbiol Biotechnol* 73:991-1007.
- Todar K. 2000. *Nutrition and Growth of Bacteria*, University of Wisconsin-Madison.
- Tsai YC, Juang RY, Lin SF et al (1988) Production and further characterization of an alkaline elastase produced by alkalophilic bacillus strain ya-B. *Appl Environ Microbiol* 54:3156-3161.
- Tsai YC, Yamasaki M, Yamamoto-Suzuki Y et al (1983) A new alkaline elastase of an alkalophilic bacillus. *Biochem Int* 7:577-583.
- Tsuchida O, Yagima Y, Ishizuka T et al (1986) An alkaline proteinase of an alkalophilic *Bacillus* sp. *Curr Microbiol* 14:7-12.
- Tsuchiya K, Sakashita H, Nakamura Y et al (1991) Production of thermostable alkaline protease by alkalophilic *Thermoactinomyces* sp. HS682. *Agric Biol Chem* 55:3125-3127.
- Tsujibo H, Miyamoto K, Hasegawa T et al (1990) Purification and characterization of two types of alkaline serine proteases produced by an alkalophilic actinomycete. *J Appl Bacteriol* 69:520-529.
- Varela H, Ferrari MD, Belobradjic L et al (1996) Effect of medium composition on the production by a new *Bacillus subtilis* isolate of protease with promising unhairing activity. *World J Microbiol Biotechnol* 12:643-645.
- Velkovska S, Marten MR, Ollis DF (1997) Kinetic model for batch cellulase production by *Trichoderma reesei* RUT C30. *J Biotechnol* 54:83-94.
- Venkatesh KV, Okos MR, Wankat PC (1993) Kinetic model of growth and lactic acid production from lactose by *Lactobacillus bulgaricus*. *Process Biochem* 28:231-241.
- Weiss RM, Ollis F (1980) Extracellular microbial polysaccharides I. Substrate, biomass and product kinetic equation for batch xanthan gum fermentation. *Biotechnol Bioeng* 22:859-873.
- Yamagata Y, Ishiki K, Ichishima E (1995) Subtilisin Sendai from alkalophilic *Bacillus* sp.: Molecular and enzymatic properties of the enzyme and molecular cloning and characterization of the gene, apr S. *Enzyme Microb Technol* 17:653-663.
- Yeh PLH, Bajpai RK, Iannotti EL (1991) An improved kinetic model for lactic acid fermentation. *J Ferment Bioeng* 71:75-77.