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THESIS

IMPROVEMENT OF SILK DEGUMMING PROTEASE PRODUCTION FROM *Bacillus* sp. C4 SS-2013

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Bacillus sp. C4 SS-2013 isolated from the wastewater of a silk factory is a promising silk degumming protease producer. The optimization of the aeration and agitation rates for protease production by batch culture using a central composite design and response surface methodology was investigated. Both the agitation and aeration rates significantly affected the protease production, specific protease production rate and K_La. The maximum protease activity of 1,890 U/mL and a specific production rate (q_p) of 21,412 U/g/h were obtained at the agitation and aeration rates of 400 rpm and 2 vvm which corresponded to a K_La value of 182.16 h^{-1}. These experimental values of protease production, q_p, μ and K_La were coincident with the predicted values and the models were proved to be adequate with the determination coefficient (R^2) of 0.950, 0.919, 0.931 and 0.881, respectively. The unstructured models provided the good approximation of cell growth, protease production and substrate concentration kinetic profile of the batch fermentation. The data fitted the proposed mathematical model very well. The R^2 values were found to be 0.908, 0.887 and 0.990 by Logistic model for cell growth, Luedeking-Piret model for protease production and Luedeking- Piret like model for substrate utilization, respectively. The improvement of protease production with pH-stat fed-batch culture resulted in an increase of the silk degumming protease production to 4,437 U/mL (2.35-fold) and qp to 36,820 U/g/h (1.72-fold). Degumming of raw silk yarn by the enzyme obtained caused the weight loss of raw silk about 25.31±2.98% which corresponded to 92.76±1.16% sericin removal. The addition of 10 mM $CaCl_2$ into the crude protease and storage at 4°C for 8 weeks could maintain its activity at about 64.25% of the initial activity. According to these data, the improvement of production and stabilization during storage of silk degumming protease were achieved in our study and has potential for industrial applications.

Student's signature Thesis Advisor's signature ___ / ___ / ___

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LIST OF ABBREVIATIONS

K_La	=	Volumetric oxygen transfer coefficient (h^{-1})
C*	=	Saturated dissolved oxygen concentration in the broth (mmol O_2/ L)
C_L	=	Dissolved oxygen concentration in the broth (mmol O_2 L^{-1})
dX/dt	=	Cell concentration rate (g/L/h)
dP/dt	=	Protease production rate (U/L/h)
dS/dt	=	Glucose consumption rate (g/L/h)
X	=	Cell concentration (g/L)
P	=	Protease activity (U/mL)
S	=	Glucose concentration (g/L)
μ	=	Specific growth rate (h^{-1})
μ_{max}	=	Maximum specific growth rate (h^{-1})
K_s	=	Saturation constant (g/L)
X_{max}	=	Maximum cell concentration (g/L)
$Y_{X/S}$	=	Yield of cell (g. cell/g total sugar consumed)
$Y_{P/S}$	=	Yield of protease (kU/g total sugar consumed)
m_s	=	Maintenance coefficient (g/g/h)
q_p	=	Specific protease production rate
α	=	growth-associated constant for protease production (U/g)
β	=	non -growth-associated constant for protease production (U/g)

IMPROVEMENT OF SILK DEGUMMING PROTEASE PRODUCTION FROM *Bacillus* sp. C4 SS-2013

INTRODUCTION

Proteases execute a large variety of functions and have important biotechnological applications. Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes (Anwar and Saleemuddin, 1998; Gupta *et al.*, 2002). *Bacillus* strains are the main source of industrial proteases among microorganisms (Gupta *et al.*, 2002; Joo *et al.*, 2005; Kirk *et al.*, 2002; Manachini and Fortina, 1998; Masui *et al.*, 2004).

Raw silk from silkworm cocoons consists of double-stranded filaments of protein called fibroin (70-78%), held together by gum-like protein termed sericin (22–30%), water, and mineral salt (Rajasekhar *et al*., 2011; Yuksek *et al*., 2012; The National Bureau of Agricultural Commodity and Food Standards (ACFS), 2014). Degumming is the process where sericin, i.e. the silk gum gluing the fibroin filaments, is totally removed in order to obtain a typical shiny, soft handle and other desirable properties (Freddi *et al.*, 2003). In recent years, various studies have dealt with the removal of sericin from the raw silk fibre by using proteolytic enzymes, which is milder than soap or alkaline treatments (Lotz and Cesari, 1979). Moreover, it saves expenses in terms of water, energy, chemicals and effluent treatment. Alkaline and neutral proteases effectively degummed silk fabrics. Almost complete sericin removal was obtained when raw silk fabric was the substrate (Freddi *et al.*, 2003). Accordingly, the percents weight loss of papain enzymatic, alkaline or soap degummed fibers were not significantly different (Nakpathom *et al.*, 2009). Senatham *et al.* (2005) screened and selected bacteria which exhibited of protease activity for silk degumming. In the same way, the bacteria were shown to specifically digest sericin better than fibroin and skim milk (Kaeyanon and Wongsaengchantra, 2005).

The production of extracellular proteases by microorganisms in a bioreactor is greatly influenced by medium components and physical factors such as aeration, agitation, temperature, inoculum density, dissolved oxygen and incubation time (Gupta *et al.*, 2002; Çelik and Çelik, 2004; Jasvir *et al.*, 2004). Oxygen shows diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes of microorganism (Çelik *et al*., 1998). The dissolved oxygen (DO) concentration becomes a rate limiting factor in many aerobic fermentation processes. Therefore, it is very important to investigate the appropriate dissolved oxygen for protease production in a stirred tank bioreactor. Moreover, the use of enzymes for industrial purposes usually depends on their stability during isolation, production, purification and storage. Thus, it is very important to obtain enzymes with high stability and activity. The stability of proteases in aqueous solutions has been greatly improved with the addition of stabilizers such as calcium ions (Kembhavi *et al.*, 1993; Ghorbel *et al.,* 2003), organic solvent (Ogino *et al.*, 1995, 1999) and polyols compounds (Berry, 1974; Beg and Gupta, 2003; Joo *et al.* 2005).

A kinetic model describes relationships between principal state variables and explains quantitatively the behavior of the system. The mathematical model provides useful suggestions for analysis, design and operation of a fermenter. Two kinds of kinetic models are available; structured and unstructured (Constantinoides *et al.*, 1970; Jian *et al.*, 2002; Murat *et al.*, 1999; Sinclair *et al.*, 1987). The unstructured model is simple and useful to monitor fermentation process of interest adequately with out complication.

Fed-batch culture has been widely employed for the production of various products including primary and secondary metabolites, proteins, and other biopolymers. Fed-batch is generally superior to batch processing and is especially beneficial when changing nutrient concentrations affect the productivity and yield of the desired product (Yamane, T., Shimizu, 1984; Lee *et al.,* 1999). Thus, it is important to develop a cultivation method that applied to achieve a high cell density, which is necessary for a high productivity and yield.

In previous study, the crude protease was produced from *Bacillus* sp. C4 SS-2013 which was isolated in our laboratory from the wastewater of a Thai silk factory. It is capable of removing sericin very well, that is, about 26.5 % of the total dry weight of the raw silk yarn which entails 94% of the total sericin after incubation under mild conditions at pH 8 and 50^{o}C for 2 h (Suwannaphan, 2010). The morphology analysis of silk fibrin investigated under an electron microscope after degumming by the active protease revealed it to be clean and smooth without any damage to the filaments (Fufeungsombut, 2009). The optimization of medium composition and the culture conditions for the protease production in shake flasks by central composite design (CCD) and response surface methodology (RSM) was investigated using cheap and abundant raw materials in Thailand, for example, cassava starch and soy flour in order to reduce the cost of production (Romsomsa, 2010). However, hydrolyzed cassava starch and soy flour were also tested in a Newtonian system for process scale up (Converti, 1999). Hence, the agitation and aeration rates are the most critical parameters and they play significant roles in determining the productivity of the process (Jüsten *et al*., 1996; Felse and Panda; 2000). The aim of present study was to investigate the optimization of agitation and aeration on silk degumming protease in stirred tank bioreactor from *Bacillus* sp. C4 SS-2013, the kinetic modeling, the improvement of silk degumming protease production by fed batch fermentation. The effect of stabilizers on silk degumming protease stability during storage was also investigated.

OBJECTIVES

1. To optimize agitation and aeration rate for silk degumming protease production in stirred tank bioreactor by batch culture from *Bacillus* sp. C4 SS-2013.

2. To develop a kinetic model for silk degumming protease production by *Bacillus* sp. C4 SS-2013 in batch culture.

3. To improve silk degumming protease production from *Bacillus* sp. C4 SS-2013 by pH- stat fed batch culture.

4. To study an effect of stabilizers on silk degumming protease activity from *Bacillus* sp. C4 SS-2013.

5. To determined the efficiency of crude protease from *Bacillus* sp. C4 SS-2013 for silk degumming.

LITERATURE REVIEW

1. General characteristic of Proteases

Proteases, also know as peptidyl-peptide hydrolase (EC 3.4.21-24 and 99) are industrially useful enzymes which catalyze the hydrolysis of a peptide bond in a protein molecule and break them down into polypeptides or free amino acids via the addition of water across peptide bonds (Sookkheo *et al.,* 2000; Beg *et al*., 2003). The hydrolysis of peptide bonds by proteases as shown in Figure 1 is shown proteolysis process by protease; the products of proteolysis are protein and peptide fragments, and free amino acids.

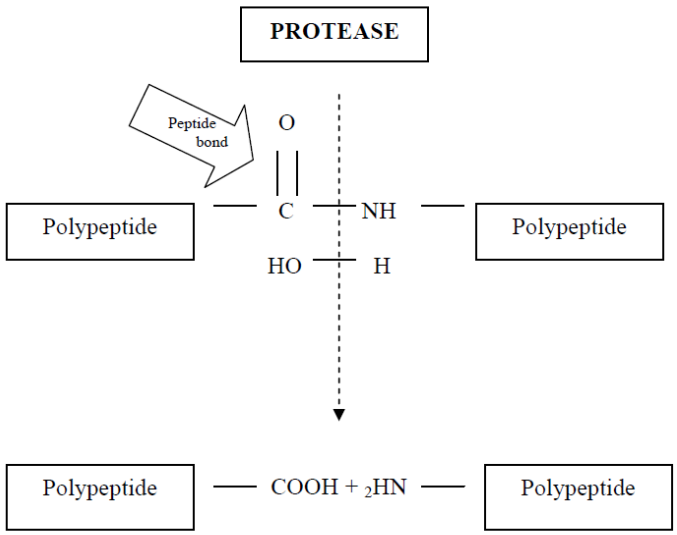


Figure 1 Protease catalysis of peptide bonds (Proteolysis).

Source: Gençkal (2004)

Proteases represent one of the three largest groups of industrial enzymes that constitute 59% of the global market of industrial enzymes, which is expected to exceed $ 2.9 Billion by 2012 (Deng *et al*., 2010). They have got a wide range of commercial application in detergents, leather, food and pharmaceutical industries,

silk, and recovery of silver from photographic films (Anisworth, 1994; Outtrup *et al.*, 1995; Inhs *et al*., 1999, Bhaskar *et al.,* 2007 and Jellouli *et al*., 2009).

Proteases are obtained from plants, animal organs and microorganisms, with the majority obtained from bacteria and fungi. Currently, a large proportion of commercially available proteases are derived from *Bacillus* strains (Mehrato *et al*., 1999). *Bacillus* proteases are predominantly extracellular and can be concentrated in the fermentation medium and produce extracellular protease during the post-exponential and stationary phases (Gupta *et al*., 2002). The most studies focused on screening proteases with a criterion set only to increase activity levels (Kim *et al*., 1994). Selection of the right organism plays a key role in high yield of desirable enzymes. They constitute a very large and complex group of enzymes with different properties of substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles. Industrial proteases have application in a range of process taking advantage of the unique physical and catalytic properties of individual proteolytic enzyme types (Ward, 1991). This vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Rao *et al*., 1998).

1.1 Classification of Proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 hydrolases. Proteases can be classified according to three major criteria. There are; the reaction catalysed, the chemical nature of the catalytic site and the evolutionary relationship, as revealed by the structure (Rao *et al*., 1998). Proteases as shown in Table 1 are broadly classified as endo- or exoenzymes on the basis of their site of action on protein substrates (Rao *et al.,* 1998). They are categorized as serine proteases, aspartic proteases, cysteine proteases, or metalloproteases depending on their catalytic mechanism. They are also classified into different families depending on their amino acid sequences and evolutionary relationships. Based on the pH of

their optimal activity, they are classified to as acidic, neutral, or alkaline proteases (Rao *et al.*, 1998).

Table 1 Classification of proteases.

Protease	EC no.
Exopeptidases	3.4.11
Aminopeptidases	3.4.14
Dipeptidyl peptidase	3.4.14
Tripeptidyl peptidase	3.4.16-3.4.18
Carboxypeptidase	3.4.16
Serine type protease	3.4.17
Metalloprotease	3.4.18
Cysteine type protease	3.4.15
Peptidyl dipeptidase	3.4.13
Dipeptidases	3.4.19
Omega peptidases	3.4.19
Endopeptidases	3.4.21-3.4.34
Serine protease	3.4.21
Cysteine protease	3.4.22
Aspartic protease	3.4.23
Metallo protease	3.4.24
Endopeptidases of unknown catalytic mechanism	3.4.99

Source: Rao *et al*. (1998)

1.1.1 Exoproteases

The exopeptidases act only near the end of polypeptide chains. Based on their sites of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively (Rao *et al.*, 1998).

1.1.1.1 Aminopeptidases

Aminopeptidases catalyze the cleavage of amino acids from N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. They are widely distributed throughout the animal and plant kingdoms and are also found in a wide variety of microbial species including bacteria and fungi. In general aminopeptidases are intracellular enzymes, but there has been a single report on an extracellular peptidase produced by *Aspergillus oryzae* (Rao *et al.*, 1998).

1.1.1.2 Carboxypeptidases

Carboxypeptidases a catalyze the cleavage of peptide bond at the carboxy-terminal (C-terminal) end of a protein or peptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallo carboxypeptidases, and cysteine carboxypeptidases, based on the nature of the amino acid residue at the active site of the enzymes (Rao *et al*., 1998).

1.1.2 Endopeptidases

Endopeptidases are a type of enzyme that breaks peptide bonds of nonterminal amino acids (i.e. within the molecule). The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanisms (Rao *et al*., 1998).

1.1.2.1 Serine proteases

Serine proteases are enzymes that cleave peptide bonds in proteins, in which a serine group in their active site. These proteases can be found in viruses, bacteria, archae, fungi and yeast (Rao *et al.*, 1998). Although serine alkaline proteases are produced by several bacteria such as Arthrobacter, Streptomyces, and Flavobacterium spp., subtilisins produced by *Bacillus* spp. are the best known ones (Rao *et al*., 1998). Two different types of alkaline proteases, subtilisin Carlsberg, and subtilisin Novo or bacterial proteases Nagase (BPN'), have been identified. Subtilisin Carlsberg produced by *Bacillus licheniformis* was discovered in 1947 by Linderstrom, Lang and Ottesen at the Carlsberg laboratory and is widely used in detergents. Serine proteases can be classified into three groups based mainly on their primary substrate preference: (i) trypsin-like, which cleave after positively charged residues; (ii) chymotrypsin-like, which cleave after large hydrophobic residues; and (iii) elastase-like, which cleave after small hydrophobic residues (Rao *et al.*, 1998). Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. Their molecular masses range between 18 to 35 kDa. The isoelectric points of serine proteases are generally between pH 4 and 6. Serine proteases are inhibited by irreversible inhibition such as diisopropylfluorophosphate (DFP), phenyl methyl sulfonyl fluoride (PMSF) but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or TLCK (Rao *et al.*, 1998).

1.1.2.2 Aspartic proteases

Aspartic acid proteases or acidic proteases depend on the aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin, retropepsin and enzymes from pararetroviruses. Most aspartic proteases show maximal activity at low pH and have isoelectric points in the range of pH 3 to 4.5. Their molecular weights are in the range of 30 to 45 kDa. The aspartic proteases are inhibited by pepstatin (Rao *et al.,* 1998).

1.1.2.3 Cysteine proteaeses

Cysteine proteases catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate (Rao et al., 1998). In catalytic mechanism of cysteine peptidases, the thiol group of a single cysteine residue plays an essential role. This group is susceptible to oxidation and can react with a variety of reagents; heavy metals, iodoacetate, N-ethyl-maleimide etc. (Kenny, 1999). Based on their side-chain specificity, they are broadly divided into four groups including papain-like, trypsin-like, and specific to glutamic acid and others. Examples of enzymes that are cysteine proteases include: acitinidain, papain, bromelains and clapains.

1.1.2.4 Metalloproteases

Metalloproteases are characterized by the requirements for a divalent metal ion for their activity and can be inactivated by dialysis or by the addition of chelating agents such as EDTA but not by sulfhydryl agents or DFP. Based on the specificity of their action, metalloproteases can be divided into 4 groups inclucing neutral, alkaline, Myxobacter I and Myxobacter II.

1.2 Factors affecting for protease production

Since protease production is an inherent capability of all microorganisms, the microbial proteases may be produced either intracellularly and extracellular. Extracellularly proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to adsorb and utilize hydrolytic products whereas intracellular proteases are important for various cellular and metabolic processes (such as sporulation and differentiation, protein turnover, the maturation of enzymes and hormones and maintenance of cellular protein pool) (Gupta *et al*., 2002). The production of extracellular proteases by microorganisms in a bioreactor is greatly influenced by medium components and physical factors such as aeration, agitation,

temperature, inoculum density, dissolved oxygen and incubation time (Gupta *et al*., 2002; Çelik and Çelik, 2004; Jasvir *et al.*, 2004). The factors affecting for protease production is presented as follow.

1.2.1 Medium component

1.2.1.1 Source of carbon

A carbon source is necessary to provide the cell with energy as well as the material which to grow and synthesis arrange of primary and secondary metabolites. The pathway by heterotrophs metabolize substrate carbon is important in determining the amount of carbon converted into cell material. It is found that facultative organisms incorporate around 10% of substrate carbon in cell material when metabolizing anaerobically, but 50-55% of substrate carbon is converted into cells with fully aerobic metabolism. Since 50% of the dry cell weight of cells is carbon, it is possible to calculate how much carbon must be supplied in the medium in order to give any particular weight of cells (Mcneil and Harvey, 2008). Glucose is frequently used in bioprocesses for protease production. Increased yields of alkaline proteases were also reported by several researchesers in the presence of different sugars such as lactose (Malathi and 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. Studies have also indicated a reduction in protease production due to catabolite repression by glucose (Frankena *et al*., 1986; Frankena *et al*., 1985; Hanlon e*t al.*, 1982; Kole *et al.*, 1988). 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).

1.2.1.2 Source of nitrogen

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 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. Low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Chandrasekaran and Dhar, 1983; Chaphalkar and Dey, 1994; Sen and Satyanarayana, 1993). An increase in protease production was also observed in the presence of ammonium sulphate, potassium nitrate (Sinha and Satyanarayana, 1991) and sodium nitrate was also found to be stimulatory for alkaline protease production (Banerjee and Bhattacharyya, 1992). 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 and Dhar, 1983; Sen and Satyanarayana, 1993, Cheng *et al.*, 1995; Tsai *et al.,* 1988). In addition, by using an acid hydrolysate of soybean in place of conventional soymeal, a three fold increase in total enzyme activity was observed (Takagi *et al.*, 1995). However, enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion in the medium (Cruegar and Cruegar 1984; Frankena *et al.* 1986; Giesecke *et al.* 1991).

1.2.1.3 Source of elements and trace elements

Trace elements are regarded as micronutrients, being required only in tiny amounts, usually microgram quantities per litre. The functions of the trace elements within the cell are many and varied but are usually associated with enzyme activity. It forms part of the enzyme or functions as catalyst for the biochemical reaction the enzyme is involved with. Typical trace elements include iron (Fe, probably one of the more important elements considering its role in cellular

respiration), copper (Cu) cobalt (Co), manganese (Mn), molybdenum (Mo), chromium (Cr, animal cells only) calcium (Ca, stabilize cell walls, and is particularly important if the cells form the endospores), magnesium (Mg, often acts as a cofactor for the activity of enzymes, can play a significant role in membrane structure and function), phosphorus (P, the production of phospholipids, nucleic acids and the generation of energy (ATP, ADP)), Potassium (K, involved in many rections within cells and is required by all cells, animals or microbes) (Mcneil and Harvey, 2008). There are several elements that are essential for the growth of microorganisms. Each element must be provided, generally in the form of an inorganic salt, in order for the cell to grow and metabolite (Mcneil and Harvey, 2008).

Table 2 show the range of concentration ranges of some elements found in bacteria, fungi and yeast.

Table 2 Inorganic constituents of different microorganism.

Element	Bacteria	Fungi	Yeast
		(g/ 100 g dry weight)	
Phosphorus	2.0-3.0	0.4-4.5	0.8-2.6
Sulphur	0.2-1.0	0.1-0.5	0.01-0.24
Potassium	1.0-4.5	0.2-2.5	1.0-4.0
Megnesium	0.1-0.5	0.1-0.3	0.1-0.5
Sodium	0.5-1.0	0.02-0.5	0.01-0.1
Calcium	0.01-1.1	0.1-1.4	0.1-0.3
Iron	0.02-0.2	0.1-0.2	0.01-0.5
Copper	0.01-0.02		0.002-0.01
Manganese	0.001-0.01		0.0005-0.0007
Molybdenium			0.0001-0.0002
Total ash	7-12	2-8	5-10

Source: Aiba *et al.* (1973)

1.2.1.4 Growth factors

Growth factors, like trace elements, are required only in tiny quantities by cell. Then can be often manufactured by the cell itself. However, some cells can not synthesize the certain key growth factors and must be supplied to the cell in the medium. They are invariably organic compounds, such as vitamins, purines, pirimidines, and amino acids. A very good example of a such a substrate is yeast extract. It contains a number of growth factors, especially B vitamins. Vitamins are the most commonly used growth factors in cell culture media and supplied them in the form of yeast extract, or malt extract and beef extract, which is cheap and convenient (Mcneil and Harvey, 2008).

1.2.2 Oxygen mass transfer

Oxygen transfer rate (OTR) is the most important parameter dependent on agitation speed and aeration rate in bioreactors, and plays a significant role in determining the productivity of fermentation process (Thiry and Cingolani, 2002; Kao *et al*., 2007). Oxygen has diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes (Çalik *et al*., 2000), with a reported correlation between the increase of productivity with the increase of oxygen availability to cells (Ramachandra Murty *et al*., 2002; Dominguez *et al*., 2005; Amaral *et al*., 2007). Oxygen transfer rate can be the controlling step in industrial bioprocesses, scale-up of aerobic biosynthesis systems (Al-Masry, 1999; Elibol and Ozer, 2000; Flores *et al*., 1997; Gibbs and Seviour, 1996; Weuster-Botz *et al*., 1998). The OTR could be affected by several factors, such as air flow rate, the stirrer speed, mixing, geometry and characteristics of the vessels, liquid properties (viscosity, superficial tension, etc.), the dissipated energy in the fluid, biocatalyst properties, concentration, and morphology of microorganisms (Bandaiphet and Prasertsan, 2006). Oxygen transfer in a fermentor depends on microbial physiology and bioreactor efficiency. Most laboratory-scale stirred-tank fermentation systems are fitted with high speeds which impart a high shear stress on the medium, and this is known to reduce metabolite yields (Çalik *et al.*, 2000).

Therefore, oxygen supply into the medium becomes one of the crirical factors in scaling up of aerobic biosynthesis system.

The dissolved oxygen (DO) concentration becomes a limiting nutrient in processes of high oxygen demand (fast growing microorganisms, high biomass, and production of biopolymer) or when the rheological properties of broth offer a high resistance to the mass transfer, such as xanthan gum production (Çalik *et al*., 1998; Casas *et al*., 2000; Lo *et al.*, 2001). However, the DO in the broths is limited by its consumption rate of cells or the oxygen uptake rate (OUR), as well as by its oxygen transfer rate. On the other hand, the oxygen uptake rate is limited by increase in viscosity resulting from polymeric property (Çalik *et al*., 2000; Eickenbusch *et al*., 1995; Kobayashi *et al.*, 1994; Kwon *et al.*, 1996). Control of dissolved oxygen tension in bacterial cell fermentation involves the manipulation of the agitation speed and the gas supply rate in order to optimize the volumetric mass transfer coefficient k_La. This approach gives satisfactory results since k_La is a function of the power dissipation by impeller per unit volume. The concentration of dissolved oxygen may be controlled by manipulating the partial oxygen pressure in the liquid phase and keeping the gas flow rate and stirring rate constant in the bioreactor (Simon and Karim 2001).

The importance of aeration and agitation in aerobic fermentation process is well recognized by laboratory and industrial fermentation technologists. The primary objective is to achieve the proper metabolic activities. There are also many factors affecting aerobic process and scale-up which are concerned with aeration and agitation such as shear stress, mixing, growth of microorganism, product concentration and viscosity, which are important directly on oxygen transfer rate in different scales and on k_La determination (Hsu and Wu, 2002).

1.2.2.1 Agitation

Agitation rate is one of the important parameter for proper oxygen transfer and homogeneous mixing of the nutrients in fermentation system. Agitation rate has a direct effect on mixing that reduces the effects of high viscosity and increases the oxygen solubility and transfer, including enhancing the cells'assimilation of dissolved oxygen and product formation at optimal stirring rate. Mechanically agitated aerated vessels are widely used rather than aerated only vessels which can be inadequate to promote the liquid turbulence necessary for small air bubble generation. Although the agitation could maintain available dissolved oxygen in the fermentor, but the inappropriate speed of agitation results is poor oxygen transfer especially in high viscous broths. At too high and too low speeds, the dissolved oxygen diffusion into the viscous broth was worse than optimal speed (Bandaiphet and Prasertsan, 2006). Since the distance between some parts of the culture broth volume and the impellers was large, the homogeneous mixing was difficult to achieve in the high viscous culture broth from the impeller (Kouda *et al.*, 1997). Turbulent flow at high agitation rate with high viscosity in impeller zone is more as compare to the in the bulk zone (Wernersson and Trägårdh, 1998). The high agitations reduced the cell growth and alkaline protease production due to sheer stress and heterogeneous mixing effects. The effects of higher agitation rates on cell growth had been documented by some other researchers (Gibbs and Seviour, 1996; Hewitt *et al*., 2000; Demirtas, *et al.,* 2003). Therefore, the type of impeller may impact on various transport phenomena throughout the fermentor, for example, disc turbines having high velocity heads that give good small bubble formation but poor mixing in highly viscous systems.

Shear rate is an important factor affecting the system viscosity, consequently vigorous mixing at high agitation speed is always used in viscous cultivation to increase the oxygen transfer and cause the inhibition of cell growth and activities with shear effect. High shear fields resulting from the fluid physical properties and hydrodynamics cause damage to fragile microorganism and reduced product formation (Al-Masry, 1999), which causes a flooding phenomenon around the

impellers. Gibbs and Seviour (1996) reported that dry cell weight values decreased at high shear speed giving high shear stress that had influences on both the oxygen transfers and cell activities in the system.

1.2.2.2 Aeration

The aeration efficiency depends on oxygen solubility and diffusion rate into the broths in the bioreactor capacity to satisfy the oxygen demand of microbial population (Galaction *et al*., 2004). Aeration only affects retention time of oxygen flow with corresponding increase or decrease of gas velocity in the broth at both high or low rates of aeration, respectively. Efficiency of aeration depends on oxygen solubilization, diffusion rate into broths, and bioreactor capacity to satisfy the oxygen demand of microbial population. However, a decrease in cell biomass concentration was noted at high aeration rate that might inappropriate transfer of oxygen in the growth medium. It has been reported that higher flow rates with low agitation speed increased the air flow up in the vessel along the stirrer shaft. This phenomenon, known as impeller 'flooding' has been recommended to be avoided, because an impeller surrounded by air column, no longer contact the liquid properly, resulting in poor mixing, reduced air dispersion and diminished oxygen transfer rates (Doran, 1995). These findings indicate that improper air dispersion and nutrients mixing in the fermentation medium at higher aeration rates can reduce the growth of microorganism in the fermentor.

1.2.2.3 The volumetric oxygen transfer coefficient (k_La)

The oxygen volumetric mass transfer rate is generally considered to be a critical parameter in aerobic cultures of microorganisms, due to its parch solubility and the need for a constant supply. The selection, design, and scale-up of biochemical reactors and the accurate estimation of mass transfer rates for different scales and different operational conditions are of critical importance in bioprocesses (Galaction *et al.*, 2004). The efficiency of agitation and aeration was evaluated through the oxygen volumetric mass transfer coefficient (k_La). The

availability of oxygen, determined by OTR, is also governed by K_La and the concentration of DO in the growth medium (Kapat *et al*., 2006). The K_La values ensure a mass transfer capability of the process that can cope with oxygen demand of the culture and often serve to determine the efficiency of bioreactors and mixing devices as well as an important scale-up factor from small cultivation to large scale production (Bandaiphet and Prasertsan, 2006). Fixing of k_La values has been commonly used criteria for scale-up of aerobic fermentations (Garc´ia-Ochoa *et al*., 2000; Gibbs and Seviour, 1996; Miura *et al*., 2003).

There are many methods for k_La determination and most k_La values are considerably affected by the geometry of the system.

- Direct Measurement

This can be accomplished at any instant during any fermentation provided sparged air is employed as the source of oxygen. The instruments required are accurated gaseouse oxygen analyzer and accurate flow meters, pressure gauges, and temperature measuring devices. For a well- mixed fermentor the concentration should be independent of position within the fermentor. In this case, only one measurement is required. On the other hand, for large-scale fermentors, especially those where non- Newtonian broth characteristics may exist, one cannot assume that fermentor content is well mixed.

The equilibrium dissolved oxygen concentration (C*) must also be evaluated in order to calculated k_La. Considering both cases, the mass transfer coeffient can be calculated for small-scale fermentors as

$$k_L a = \frac{N_A}{C^*_{out} - C_L} \qquad (1)$$

And for large-scale fermentors as

$$k_L a = \frac{N_A}{(C^* - C_L)_{\log mean}} \tag{2}$$

or

$$k_L a = \frac{N_A}{\frac{(C^*_{in} - C_L) - (C^*_{out} - C_L)}{\ln \frac{(C^*_{in} - C_L)}{(C^*_{out} - C_L)}}} \tag{3}$$

where C^*_{in}, C^*_{out} is the dissolved oxygen concentration in equilibrium with gaseous oxygen at fermenter inlet and outlet in mM dm^{-3}

- Dynamic Measurement

The dynamic method for measuring oxygen transfer coefficient was introduced by Taguchi and Humphrey (1996). Measurements are made over short intervals during transient conditions in a batch fermentation. When a material balance on the dissolved oxygen is performed during batch, one obtains.

$$\frac{dC_L}{dt} = k_L a(C^* - C_L) - rX \tag{4}$$

Where r is the specific oxygen uptake rate per unit weight of cells in mMO_2/g-h, and X is the dry weight of cells per volume in g/L.

Equation 4 can be rearranged to give

$$C_L = C^* - \frac{1}{k_L a}(rX + \frac{dC_L}{dt}) \tag{5}$$

One of the advantages of this method lies in the fact that only one dissolved oxygen probe, is needed. From the response of the oxygen probe it is possible to obtain the mass transfer coeffient k_La, with no other measurement necessary. If one plots the dissolved oxygen versus time, the response obtained is illustrated in Figure 2. The air to the fermentor is turned off at a selected time during fermentation. This also corresponds to a conditions where k_La becomes zero. The decrease in the dissolved oxygen concentration should be linearly related to time curve which yields an explicit value of the volumetric oxygen demand rate of the organisms, rX. The air is then turned on and the its dissolved oxygen concentration will rise accordingly.

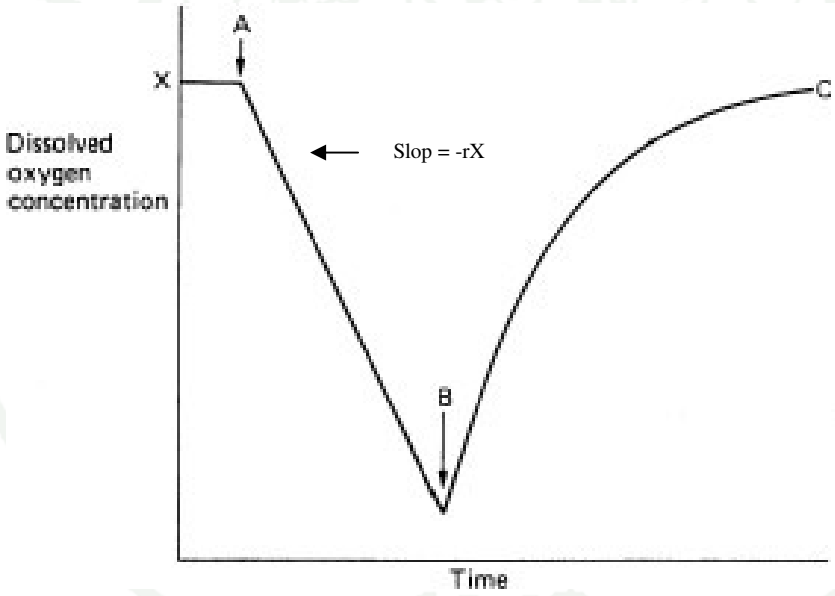
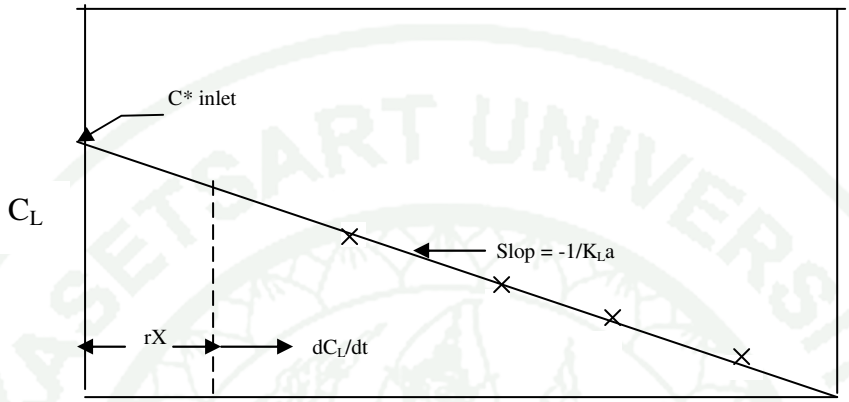


Figure 2 Relationship between dissolved oxygen concentration and time in dynamic gassing out method: point A shows level of DO before it was consumed (air supply off), point B represent air is pumped into the culture and the dissolved oxygen concentration increase as a function of time, point C represent steady- state value.

Source: Wang *et al*. (1979)

Figure 3 shows a typical trace of dissolved oxygen concentration versus time, plotted in accordance with Equation 4. In this plot the slopes is equal to the reciprocal of the mass transfer coefficient, k_La.



$rX + dC_L/dt$

Figure 3 Estimation of mass mass transfer coefficient using the dynamic technique.

Source: Wang *et al*. (1979)

- Sodium sulfide oxidation

This method was used by Cooper *et al.* (1944) to evaluate oxygen transfer performance in various types of gas-liquid contactors measuring the mass transfer coefficient employs the oxidation of sodium sulfite. It is based on the oxidation of sodium sulfite to sodium sulfate in the presence of catalyst (Cu^{++} or Co^{++}) according to

$$Na_2So_3 + \frac{1}{2}O_2 \xrightarrow{Cu^{++}orCo^{++}} Na_2SO_4$$

To determine the rate of oxygen absoption, a 1N sodium sulfite solution in the presence of 10^{-3} M Cu^{++} ion is allowed to react with the sparged air in the presence of mechanical agitation. The unreacted sulfite is placed in excess iodine solution and back titrated with standard sodium thiosulfate solution. Since the kinetics of the oxidation are extremely rapid, the dissolved oxygen will be zero at all times in the fermentor. One is therefore able to calculate the mass transfer coefficient according to

$$k_L a = \frac{N_A}{(C^* - C_L)} \tag{6}$$

where N_A is the oxygen absorption rate of the sodium sulfite solution in mM/h.

Some reports concerning with K_La, agitation and aeration are emphasized that the growth and oxygen consumption rates by microorganisms were highly dependent on the aeration and agitation (Demirtas *et al.*, 2003). The oxygen transfer rate and K_La value in the fermentor increased with increased aeration and agitation rates and led to give maximum cell concentration and product formation (Volpato *et al.*, 2009). The most pronounced effect on improvement of product formation by aeration and agitation rates; such as lipase (Alonso *et al.,* 2005), exopolysaccharide (Bandaiphet and Prasertsan, 2006), alkaline protease production and protease yield (Potumarthi *et al.*, 2007; Nadeem *et al.*, 2009), transglutaminase (TGase) production and cell sporulation (De Souza *et al.*, 2009). In addition, the level of dissolved oxygen concentration in the fermentation broth affected cell density as well as delta-endotoxin synthesis by *B. thuringiensis*. At 80% oxygen saturation in the medium throughout the fermentation conducted a high biomass, but low delta-endotoxin production. However at lower aeration level of 60% oxygen saturation led to lower growth and higher delta-endotoxin production (Ghribi *et al.*, 2007).

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2. Silk

2.1 Structure of silk

Silk is a continuous strand of two- filaments cemented together forming the cocoon of silkworm, *Bombyx mori*. Silk filament is a double stand of fibroin, which is a composite material formed by two fibroin filaments surrounded by a cementing layer of sericin. Both fibroin and sericin, which account for about 75 and 25 wt.%, respectively, are proteins (Freddi *et al*., 2003). Silk adapts various secondary structures, including α- helix, β- sheet, and crossed β- sheet (Komatsu, 1996).

Silk fibroin is a glycoprotein that forms the filament of silkworm and gives its unique physical and chemical properties (Cook, 1964; Iizuka, 1964). Fibroin composed of two equimolar protein subunits of 370 and 25 kDa covalently linked by disulphide bonds whose primary structure is rich in glycine, alanine, and serine amino acids(≅85 mol%) in the molar ratio 3:2:1, which form typical -(ala–gly)n- repeating motifs (Lotz and Colonna Cesari, 1979; Zhou *et al*., 2000). In the fibre, fibroin chains are aligned along the fibre axis, held together by a close network of interchain hydrogen bonds, with adjacent -(ala–gly)n- sequences forming the well known β-sheet crystals (Takahashi *et al.,* 1991). The β- sheet form (silk II or β- silk) and crystalline form (silk I) have been reported for silk fibroin, having relative molecular masses of 350-415 kDa (Lesile *et at*., 1996; Magoshi, 1996). Anti- parallel β- sheet structure forming microfibrils is responsible for the crystalline nature of the silk fibre. The microfibrils are organized into fibril bundles, with several bundles leading finally to a single silk thread (Voegli *et al*., 1993).

2.2 Sericin

Sericin, the silk gum gluing the fibroin filaments, is a complex mixture of 5-6 polypeptides widely differing in size (40–400 kDa), chemical composition, structure and properties, such as: solubility, hydrophylicity, and stickiness (Gamo *et*

al., 1977; Couble *et al.,* 1987). Sericin contains 18 amino acids including essential amino acid and is characterized by the presence of 32 percent of serine. The total amount of hydroxyl amino acid in serine is 45.8 percent. There are 42.3 percent of polar amino acid and 12.2 percent of nonpolar amino acid residues. Sericin contributes about 20-30 percent of total cocoon weight. Their main role is to envelope the fibroin. In the presence of sericin the fibres are hard and tough and become soft and lustrous after its removal. Sericin occurs mainly in an amorphous random coil and to a lesser extent, in a β- sheet organized structure. The randomly coiled structure easily changes to β- sheet structure, as a consequence of repeated moisture absorption and mechanical stretching (Shaw and Smith, 1951; Voegeli *et al*., 1993).

2.2.1 Form of sericin

Sericin can be classified into three fractions, depending on their solubility as sericin A, sericin B, and sericin C. Sericin A is the outermost layer and insoluble in hot water. It contains about 17.2 percent of nitrogen and amino acid like serine, threonine, glycine, and aspartic acid. Sericin B is the middle layer contains 16.8 percent of nitrogen. On acid hydrolysis it yields amino acid of sericin A with tryptophan. Sericin C is the innermost layer, which is adjacent to fibroin and is insoluble in hot water and can be removed from fibroin by hot dilute acid or alkali. On acid hydrolysis it yields proline in addition to amino acids of sericin B. It also contains sulphur and 16.6 percent of nitrogen (Shaw and Smith, 1951; Sprange, 1975). Sericin has been divided into various species based on relative solubilities. Various fractions of sericin are also designated by other researchers depending on their dissolutions behavior as sericin A and B, or sericin I, II, III, and IV, or S1, S2, S3, S4, and S5, and as α, β, and γ modification (Komatsu, 1996; Voegeli *et al*., 1993). The major molecular conformation of esily soluble sericin is random coil, the β - sheet structure is more difficult to dissolve. The repeated moisture absorption makes molecular crystalline structure, which is having reduced silubility.

The γ-ray study shows the three layers in the sericin structure. The outer layer contains some fibre direction filaments, middle layer exhibits cross-fibre direction filaments, and the inner layer shows longitudinal filaments (Wang *et al*, 1985). The structure of sericin also depends on the casting temperature. Lower the casting temperature more the sericin molecules assume β-sheet structure rather than random coil (Tsukada, 1980; Ayub *et al.*, 1993).

2.2.2 Properties of sericin

Sericin contains random coil and β-sheet structure. Random coil structure is soluble in hot water and as the temperature lowers the random coil structure converts to β-sheet structure, which results in gel formation (Zhu *et al*., 1998; Huddar, 1985). Sericin has easily dissolves into water at 50-60°C and again returns to gel on cooling (Zhu *et al*., 1996). The solubility of sericin increases by addition of Sodium polyacrylate and decreases by the addition of polyacrylamide, formaldehyde, or resin finishing agents (Kataoka, 1977; Ishizaka and Kakonoki; 1997; Zhu *et al*., 1995). As there are more acidic than basic amino acid residues the isoelectric point of sericin is about 4.0 (Voegeli *et al*, 1993).

2.2.3 Isolation of silk sericin

2.2.3.1 Isolation with aid of heat

The removal of gum from crude silk is based entirely upon its solubility in hot water. The number of methods illustrated by researchers for removing gum are as follows; the removal of gum by diluted solution of sodium carbonate, the extractions of silk for 1 h or simply heating in water at 100°C or autoclaving at 118°C or autoclaving for 3 h under 2.5-3 atmosphere pressure. In addition silk degumming by extraction with aqueous solution of urea at 100°C from cocoons (Tsubouchi *et al*., 1999), performed at 50-60°C for 25 days to avoid the decomposition (Shelton and Johnson, 1925) and silk fibres can be completely

degummed in boiling solutions of pH 11 containing 5-6 percent bentonite (Buadze, 1999). When sericin is extracted from cocoons of *Bombyx mori* by heating on water bath and autoclaving at different temperatures the satisfactory yield is obtained by autoclaving at 105°C for 30 min with good gelling property and yield. Further increase in temperature increases the yield but looses its gelling property (Padamwar and Pawar, 2003).

2.2.3.2 Extraction of silk sericin using enzymes

Several extraction processes were carried out by using enzyme alkylase (Iida, 1999) or with 2-2.5 g/L alkaline protease at 60°C for 90 min at pH 10 (Pak, 1997). Krysteva *et al*., 1981 hydrolysed sericin with trypsin at different concentatrations, temperatures and treatment times. The result revealed that at 1 percent of trypsin solution, silk sericin was almost completely extracted within 10 and 32 h at 37 and 20□C, respectively. While sericin obtained by 4 h treatment with 1 and 8 percent of trypsin solution were 26.4 and 28.7 percent, respectively.

2.2.4 Application of silk sericin

The small sericin peptides are soluble in cold water and can be recovered at early stages of raw silk production. The larger sericin peptides are soluble in hot water and can be obtained at the latter stages of silk processing or from processes for silk degumming. Because of its properties, sericin is particularly useful for improving artificial polymers such as polyesters, polyamide, polyolefin, and polyacrylonitrile and some additional properties like, gelling ability, moisture retention capacity, and skin adhesion. Thus it is widely applicable in medical practice such as silk sericin membranes. They are good bandage materials and water absorption properties promote smooth cure for defects in the skin under regeneration when detached from the skin (Tsubouchi, 1999). Silk sericin is also used in pharmaceutical; like silk sericin as a biomaterial (Kurioka, 1998) and has the potential to find application in the development of contact lenses (Nakamura *et al*., 2001; Wei *et al*., 1989). In case of silk fibroin has been used in skin, hair, and nail cosmetics

while sericin is use in the form of lotion, cream and ointment due to its ability to increase skin elasticity, antiwrinkle, and anti aging effects (Voebeli *et al*., 1993; Yamada *et al*., 1998; Ogawa and Yamada, 1999; Henne and Hoppe, 1986). Sericin powder in the form of sericin hydrolysate coated talc, mica, titania, iron oxide, and nylon have been used to formulate foundation cream and eyeliners (Yamada and Yuri, 1998). Sericin in sunscreen composition enhances the light screening effect of UV filter like triazines, and cinnamic acid ester (Yoshioka *et al*., 2001).

3. Degumming of silk

Degumming is a key process during which sericin is totally removed and silk fibres gain the typical shiny aspect, soft handle, and elegant drape highly appreciated by the consumers. The industrial process takes advantage of the different chemical and physical properties between fibroin and sericin. Fibroin is water-insoluble owing to its highly oriented and crystalline fibrous structure, while sericin is readily solubilized by boiling aqueous solutions containing soap, alkali, synthetic detergents, or organic acids (Svilokos Bianchi and Colonna, 1992; Freddi *et al*., 1996).

In recent years, various studies have dealt with the removal of sericin by using proteolytic enzymes. Several acidic, neutral, and alkaline proteases have been used on silk yarn as degumming agents. Enzyme degummed silk fabric displayed a higher degree of surface whiteness, but higher shear and bending rigidity, lower fullness, and softness of handle than soap and alkali degummed fabric, owing to residual sericin remaining at the cross over points between warp and weft yarns (Chopra *et al.,* 1996). Alkaline proteases performed better than acidic and neutral ones in terms of complete and uniform sericin removal, retention of tensile properties, and improvement of surface smoothness, handle, and lustre of silk (Gulrajani *et al.,* 1996, 1998, 2000). The development of an effective degumming process based on enzymes as active agents would entail savings in terms of water, energy, chemicals, and effluent treatment (Freddi *et al*., 2003).

Many researchers have studied on degumming with enzyme are described as follows:

Freddi *et al*. (2003) reported that a crêpe silk fabric was treated with different alkaline (3374-L, GC 897-H), neutral (3273-C), and acid (EC 3.4 23.18) proteases with to study their effectiveness as degumming agents. Proteases were used under optimum conditions of pH and temperature, while enzyme dosage (0.05–2 U/g fabric) and treatment time (5–240 min). Degumming loss with soap and alkali was 27%. The maximum amount of sericin removed in 1 h was 17.6, 24, and 19% for 3374-L (2 U/g fabric), GC 897-H (1 U/g fabric), and 3273-C (0.1 U/g fabric), respectively. Acid protease was almost ineffective as a degumming agent. The morphological analysis showed that sericin was completely removed from the warp yarns of the crêpe fabric, while the highly twisted weft yarns still exhibited the presence of sericin deposits within the most internal parts of the close fibre texture.

Nakpathom *et al*. (2009) observed that Thai *Bombi mori* silk fibers were degummed with a papain derived from local papyrus latex and compared with a traditional alkaline method. The percents weight loss of papain enzymatic and alkali/soap degummed fibers were not significantly different, i.e., 20-22%. The decreases in tensile strength were 46%, 40.2% and 28% for silk fiber degummed with 5% owf papain at high temperature (65-70°C for 1 h), alkali/soap and 10% owf papain at room temperature for 24 h, respectively. The surface morphological of the degummed fibers by SEM analysis confirmed the removal of sericin from silk fibroin.

Sasithorn and Luepong (2011) have reported that chooses papain enzyme form dried latex of *Carica payapa Linn*. to degum the raw silk. The result was revealed; the appropriate conditions for silk degumming be recommended as follows: the amount of dried latex solution of 4 % owf at 75°C for 30 minutes at neutral condition, in this condition was not harm to strength and fiber surface lustrous, soft and smooth surface.

More *et al*. (2013) investigated degumming of Chinese bivoltine silk with alkaline proteases from various microbial sources and compared with commercial enzymes. Among the proteases tested, two fungal and two actinomycete proteases

were promising, which showed weight loss similar to conventional method (19.58% to 21.78%). *Conidiobolus brefeldianus* and BOA-2 proteases were the best enzymes, which showed weight loss similar to conventional method with low enzyme concentrations and in shorter time. No significant differences were found in tensile strength or elongation at break by enzymatic degumming indicating that there was no damage to the fiber.

Sumana *et al*. (2013) found that the isolate SM1 (*Bacillus thuringensis*) isolated from Mandarnmani coastal region in West Bengal, India could perform degumming of raw silk fabric in significant amount. After the enzymatic treatment, texture of the fabric became shiny and the volume of the yarn increased. The other properties of the fabric like tensile strength, yarn count, colour fastness to water either improved unchanged after the enzymatic treatment.

4. Storage and stability of protease

4.1 Denaturation of enzyme

Enzymes comprise of protein, are often unstable when not in their native environments. Enzymes undergo a variety of denaturation reactions during production, storage and application in industry. Enzymes may be easily denatured by slightly changing of the environmental conditions such as temperature, pressure, pH or ionic strength (Michiaki *et al*., 1997) because proteins can loose activity as a result of proteolysis, aggregation (Pierce, 2005).

Denaturation is the unfolding of the enzyme tertiary structure to a disordered polypeptide in which key residues are no longer aligned closely enough for continued participation in functional or structure stabilizing interactions. Unfolding of the protein structure takes place under thermal stress or chemical changes, due to disruption of the hydrogen bonds and the other interactions, result in loss of activity involves changing in the three-dimensional structure of the protein molecule, which disassembles the active site of the enzyme (Schmid, 1979; Fagain, 1995). Unfolding

of the protein molecule is often the rate limiting step, a general approach to enzyme stabilization is inhibition suppression of unfolding with the residual groups in the protein backbone is lost, and interaction of the water molecules with the newly exposed regions occurred, resulting in protein denaturation (Noriko, *et al*., 1999; Yuji *et al*., 1984). Figure 4 shows the description of the denaturation process.

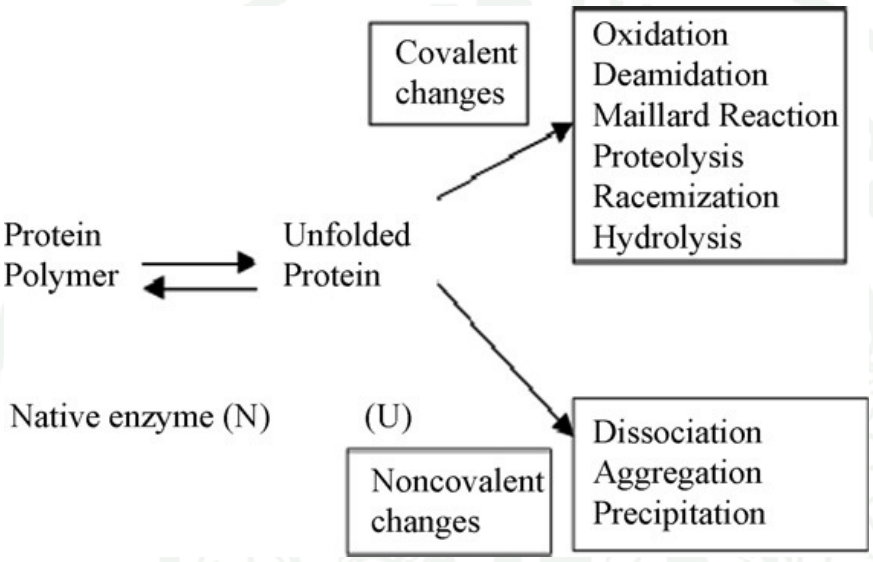


Figure 4 A schematic description of the denaturation process.

Source: Misset and van Dijk (1998).

An overview of the physical and biological factors affecting protein stability (Table 3) and the effect of chemical denaturants on protein structure are shown in Table 4.

Table 3 Effect of various physical and biological denaturants with their target sites and the resulting denatured forms of the protein.

Denaturant type	Target	End product
Physical denaturants		
Heat	Hydrogen bonds	Highly disordered structure
		Aggregates
Cold	Hydrophobic bonds, solvated groups	Aggregates
	Solvated groups	Inactive monomers
Mechanical forces	Solvated groups	Highly disordered structure
	Void volume	Inactive monomers
Radiation	Functional groups like cystein, peptide bonds	Highly disordered structure
Biological denaturants Proteases	Peptide bonds	Oligopeptides, amino acids

Source: Schmid (1979)

Table 4 Effect of chemical denaturants with their target sites on protein molecule.

Denaturant type	Target	End product
Acids	Buried uncharged group (e.g., histidine, peptide bonds)	Random coil
Alkali	Buried uncharged groups (e.g., tyrosine, cysteine, cystine)	Random coil
Organic H-bond formers	Hydrogen bonds	Random coil
Salts	Polar and non-polar groups	Highly disordered structure
Solvents	Non-polar groups	Highly disordered peptide with large helical regions
Surfactants	Hydrophobic domains (all surfactants) and charged groups (ionic surfactants)	Incompletely disordered structure with large helical regions
Oxidants	Functional groups like cysteine, methionine, tryptophan, etc.	Inactivated enzyme sometimes disordered structure
Heavy metals	Functional groups (e.g., cysteine, histidine and others)	Inactivated enzyme
Chelating agents	Cations important for structure or function	Inactivated enzyme

Source: Schmid (1979)

4.2 Stabilization of enzymes

The stability of the catalytic activity of an enzyme is important for its application in industry and which is basically on a function of the active conformation of the protein. The goal of stabilization is suppression of least one of the enzyme inactivation processes. The stability of enzymes and proteins in vitro remains a critical issue in biotechnology. Both storage and operational stabilities affect the usefulness of enzyme-based products. Storage stability, or shelf life, refers to an enzyme's maintaining its catalytic abilities in the period between manufacture and eventual use. The stability of an enzyme can be divided into two main categories: the native stability (defined as the ability of the protein to withstand a given stress independent of any solutes or cofactors) and the apparent stability, which is the stability measured in a different environment (Bowers, 1986). The hydrophobic interactions, the intrapeptide hydrogen bonds, and the ability to regain the original configuration during dehydration-hydration processes (re-folding) are the major parameters that contribute to the conformation stability of the enzyme. In general, stabilization of the enzymes could be achieved in several ways, i. e., screening for more stable ones (thermophiles, extremophiles), the addition of stabilizers, chemical modification, protein engineering, and immobilization or using stabilizing additives (Costa *et al*., 2001; Rocha *et al*., 1998). The limited stability of enzymes during long-term storage has been attributed to the deleterious effects of environmental moisture and microbial contamination (Rainer, 2000; Rajini and Mattiasson, 1993). The effect of stabilization may be interpreted as the preferential hydration of the protein or exclusion of the stabilizer from the protein surface which forces the protein to take up the conformation with minimal surface energy, increasing viscosity of the solvents, the protection of the enzyme from the attack the proteolytic enzymes (Bryjak, 1995).

The shelf life of a protein depends on both the intrinsic nature of the protein and the storage conditions. The shelf life can vary from a few days to more than a year and is dependent on the nature of the protein and the storage conditions used. Optimal conditions for storage are distinctive to each protein. Proteins (especially enzymes) must be stored at an appropriate temperature and pH range to retain activity

and prevent aggregation (Simpson, 2010). This is general considerations for protein storage.

4.2.1 Factors affecting protein stability during storage

4.2.1.1 Temperature

Temperature is an important consideration when designing a buffer system for protein stabilization. Generally, proteins stored in solution at 4°C can prevent microbial or proteolytic degradation; such proteins may not be stable for more than a few days or weeks. Freezing at -20°C or -80°C is the more common form of frozen protein storage and adding 50% glycerol or ethylene glycol will prevent solutions from freezing. By contrast, repeated freeze-thaw cycles should be avoid, which due to decrease protein stability while storage at room temperature often leads to protein degradation and/or inactivity, commonly as a result of microbial growth (Pierce, 2005; OPS Diagnostics, 2007).

Lyophilization allows for long-term storage of protein with very little threat of degradation, this method involves adding the protein solution dropwise (about 100 μL each) into a pool of liquid nitrogen, then collecting the drop-sized frozen beads and storing them in cryovials under liquid nitrogen (Pierce, 2005). Common conditions for protein storage are summarized and compared in Table 5. Generally, there is a trade off associated with each method.

Table 5 Comparison of protein storage conditions.

	Storage Condition			
Characteristic	Solution at 4°C	Solution in 25-50% glycerol or ethylene glycol at -20°C	Frozen at -20° to -80°C or in liquid nitrogen	Lyophilized (usually also frozen)
Typical shelf life	1 month	1 year	Years	Years
Requires sterile condition or addition of antibacterial agent	Yes	Usually	No	No
Number of times a sample may be removed for use	Many times	Many times	Once; repeated freeze thaw cycles generally degrade proteins	Once; it is impractical to lyophilize a sample multiple times

Source: Pierce (2005)

Züchner (2011) reported that the maximum storage time in solution at 4°C is highly dependent on type of protein (Table 6). Enzymes and antibodies can be very sensitive and some may already significantly loose activity after 2 h at 4°C.

Table 6 Comparison of storage time in different storage conditions

Storage condition	in solution, 4°C	-20°C, -80°C or liquid N_2	Lyophilized, 4°C or -20°C	In solution (with 50 % glycerol), -20°C
Maximum storage time	1- 2 week	Several years	Several years	6-12 months
Number of times sample can be brought to room temperature	many	One time (if necessary twice)	Several times	many
Antibacterial agent	required	if possible, yes	none	none
Protease inhibitor	required	if possible, yes	none	none

Source: Züchner (2011)

4.2.1.2 Protein concentration

Dilute protein solutions of less than 1 mg/mL are easy to inactivated and loss their activities. Therefore, the best condition is to store proteins in more concentrated form. However, the addition of a carrier protein, such as purified BSA (final concentration of 10-15 mg/mL), to dilute protein solutions helps to protect against such degradation and loss (Pierce, 2005).

4.2.2 Stabilizing additives

Additives, which stabilize the native conformation of enzymes, can be classified into the following categories: substrates, products, inhibitors, cofactors, allosteric effectors and other ligands, metal ions, ionic species, organic solvents; proteins, polymers, lipids (Bryjak, 1995). Most of the reports on enzyme stabilization are focused on the effect of additives on protein stability showing that it has been the most popular method of enzyme stabilization. The additions of low-molecular weight additives exert stabilizing effects by inducing preferential hydration of proteins, i.e. the additive tends to be excluded from the district of the protein molecule. Interaction between the protecting additive and the peptide backbone is unfavorable (Qu *et al.*, 1998).

A brief overview of the various additive classes that can be employed for enzyme stabilization is given below.

4.2.2.1 Addition of salts:

Salts are used mainly as cryoprotectants, this protection is achieved using the same ions as those used for salting out compounds and proteins. The effect of the addition of salts have been includes specific ion effects of divalent cations on thermostability that these ions being employed in concentrations of ≤0.1 M and second includes non-specific ion effects of salts being employed at higher concentrations of ≥0.1 M.

In order to protect the enzyme against denaturation during the freezing process, the addition of certain salts such as sodium chloride, sodium formate, and sodium borate, are used mainly as cryoprotectants and also have been proved useful due to their preferential hydration of the proteins (Eilertson *et al.,* 1985; Aunstrup, 1980). In accordance with the Hofmeister lyotropic series, the stabilizing effect of cations and anions decreases in the following order with anions and cations being additively effective (Klibanov, 1983).

$(CH_3)_4N^+ > NH_4^+ > K^+$; $Na^+ > Mg^{+2} > Ca^{+2} > Ba^{+2} > SO_4^{-2} > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$

The addition of these salts aims at dealing with the protein stabilization by controlling the water activity around the enzyme and increase the surface tension had been shown destabilize proteins (Arakawa and Timasheff, 1982). The addition of KCl, crown ethers, PEG and sugars during lyophilization of enzymes, enhanced stability and activity of enzymes (Iyer and Ananthanrayan, 2008). Thermolysin, a thermostable Zn protease from *B. stearothermophilus* has been shown to contain four Ca^{+2} ligands bound to asparagine and glutamine carboxyl groups. Bridging function of calcium increases molecular rigidity (Schmid, 1979).

4.2.2.2 Addition of polyols and sugars

The addition of sugars and polyols to aqueous solutions of enzyme employed strengthens the hydrophobic interactions among non-polar amino acid residues leading to protein rigidification and resistance to thermodeactivation. Stabilization effect of these compounds has been attributed to a positive effect on the water activity of the medium and a decreased possibility of microbial contamination. Polyols like sorbitol, mannitol, glycerol have been found to stabilize extremophilic enzymes like xylanase from alkalothermophilic *Thermomonospora* sp. while other additives like trehalose, gelatin, xylan and trehalose-gelation mixture marginally improved its stability at 80°C (George *et al.*, 2001). The stabilization of enzymes could be enhanced by the addition of polyols which the protective effect increased with increasing glycerol and propylene glycol concentrations, while it decreased with increasing PEG concentration (Haddar *et al.*, 2010). Glycerol was used as a cryoprotectant gives good results (Schmid, 1979).

4.2.2.3 Addition of Dimethyl sulfoxide (DMSO)

The addition of 40% (v/v) DMSO led to retain of 100% residual malate dehydrogenase activity after 9 months. Lactate dehydrogenase has been protected from damage during freeze–thawing using addivies including sugars, polyols, amino acids, methylamines, and lyotropic salts. These solutes are preferentially excluded from contact with the protein surface, thereby accounting for their cryoprotective action (Carpenter and Crowe, 1988).

4.2.2.4 Addition of polyethylene glycols (PEG)

Cryoprotectants such as 25-50% glycerol or ethylene glycol to help to stabilize proteins by preventing the formation of ice crystals at -20°C that destroy protein structure. Polyols, the preferential hydration arises from an increase in the surface tension of the solvent water (Kaushik and Bhat, 1998).

The increase in the thermal stability by adding polyols was probably due to the reinforcement of the hydrophobic interactions among nonpolar amino acids inside the enzyme molecules, and thus increased their resistance to inactivation, since it had been reported that polyhydric alcohols modify the structure of water and/or strengthen hydrophobic interactions among nonpolar amino acids inside the protein molecules (Back *et al*., 1979). The half-life of an alkaline protease from *B. mojavensis* was increased by 2.2 and 2.3 fold by adding PEG 6000 and glycerol at 60°C, respectively (Beg and Gupta, 2003).

Modification with PEG is also useful for making various enzymes soluble and active hydrophobic organic solvents such as benzene, toluene or chlorinate hydrocarbons (Kodera, *et al*., 1994). The increased solubility results from modification of the enzyme- surface characteristics with amphipathic PEG or protein modification (PM) molecules. Advantages of PEG or PM modifed enzymes include; reverse hydrolysis, carried out in hydrophobic media; increased catalysis of hydrophobic substrates, stereospecific synthesis in hydrophobic media; synthesis of compounds that are unstable in aqueous media and increased thermostability in both

hydrophobic and aqueous media (Inada *et al*., 1995). Modification with PEG can also increase the solubility and activity of enzymes in organic solvents, thus extending their potential for application in organic syntheses and biotransformation processes.

Joo *et al*. (2005) have reported that the evaluation of the protective effect of propylene glycol and glycerol on the inactivation of *Bacillus clausii* I-52 alkaline protease by sodium dodecyl sulfate (SDS) and hydrogen peroxide. The half-life of the enzyme was increased by 43-fold and more than 105-fold by the addition of 10% (v/v) propylene glycol to the enzyme preparations containing 5% (w/v) SDS and 5% (v/v) hydrogen peroxide at 50°C, respectively.

Padma and Ananthanarayan (2008) found that native catalase preparations isolated from *Bacillus* sp. were formulated with different additives like polyethylene glycol, glycerol, BSA, casein, glutaraldehyde, n-butylamine, ethylenediamine, 1,6-diaminohexane, BSA/glutaraldehyde and casein/glutaraldehyde for storage stabilization and better performance at high temperature and pH.

Haddar *et al*. (2010) observed that the addition of 10% (w/v) PEG 4000, 10% (v/v) glycerol and propylene glycol caused an increase in the stability of crude protease against SDS, these protective effects may be explained by the strengthening of the hydrophobic interactions among non-polar amino acids inside protein molecules, and thus rendering them resistant to denaturation by SDS and the stability of the crude protease against the strong anionic surfactants (SDS) was improved by the addition of polyols. The residual activity of the crude enzyme was about 76%, 93% and 124% by the addition of 10% (v/v) PEG 4000, glycerol and propylene glycol, respectively.

4.2.2.5 Addition of miscellaneous additives

Polymers also can stabilize proteins. Polyethyleneimine (PEI) is a cationic polymer with numerous uses, including protein stabilization (Andersson and Hatti-Kaul, 1999). Both high- and low-molecular weight fractions of

PEI, when included at 0.01–1% (w/v) concentrations, greatly increased the shelf lives of dehydrogenases and hydrolases stored at 36°C. 0.05% (v/v) Tween 20 has been found show effective stabilization of β-amylase (Schmid, 1979). On the other hand, protease inhibitors prevent proteolytic cleavage of proteins (Table 7).

Table 7 Common Protease Inhibitors.

Protease Inhibitor	Target Protease	Working Concentration
PMSF (Phenylmethylsulfonyl fluoride)	Serine proteases	0.1 – 1 mM
Benzamidine	Serine proteases	1 mM
Pepstatin A	Acid proteases	1 μg/mL
Leupeptin	Thiol proteases	1 μg/mL
Aprotinin	Serine proteases	5 μg/mL
Antipain	Thiol proteases	1 μg/mL
EDTA and EGTA	Metalloproteases	0.1 – 1 mM

Source: Pierce (2005)

Züchner (2011) reviewed that many compounds may be added for stabilization of enzyme to lengthen shelf life. For example, anti-microbial agents such as sodium azide (NaN_3) at a final concentration of 0.02-0.05% (w/v) or thimerosal at a final concentration of 0.01 % (w/v) inhibit microbial growth, metal chelators such as EDTA at a final concentration of 1-5 mM avoid metal-induced oxidation of –SH groups and helps to maintain the protein in a reduced state and reducing agents such a dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) at final concentrations of 1-5 mM also help to maintain the protein in the reduced state by preventing oxidation of cysteines.

4.2.2.6 Addition of surfactants

Surfactants can have potent effects on both the stabilities and activities of enzymes. The cationic surfactant benzalkonium chloride (0.01 or 0.1%) maintained the activity of bovine lactoperoxidase stored at 37°C, pH 7 (Spreti *et al*., 2001).

4.2.2.7 Addition of metal ion

Calcium ions are known to play a major role in enzyme stabilization at higher temperatures in many reports. Calcium has been shown to increase the activity and thermal stability of alkaline proteases at higher temperatures (Durham *et al.*, 1987; Kumar *et al.*, 1999; Rahman *et al*., 1994). The improvement in protease thermostability in the presence of calcium may be explained by the strength of interactions inside protein molecules and by the binding of calcium to autolysis sites (Ghorbel, 2003). The half-life of alkaline proteases was increased by 2.3-fold at 60°C, 10-fold at 60°C and 2.5-fold at 50°C by adding calcium for alkaline proteases from *B. mojavensis*, *Bacillus* spp. GX6638 and *B. sphaericus*, respectively (Beg and Gupta, 2003; Durham *et al.*, 1987; Singh *et al*., 1999). Similar pattern of increase in thermostability by calcium was also observed in alkaline proteases from *B. cereus* BG1, *Aspergillus oryzae* and *Condiobolus coronatus* (Ghorbel *et al*., 2003; Bhosale *et al.*, 1995; Ikegaya *et al*., 2002). Furthermore, many studies showed that contain Ca^{2+} binding sites which play an important role in stabilizing the enzyme against thermal denaturation and autodegradation (Kidd *et al.,* 1996; Smith *et al.*, 1999) and the calcium ions increased significantly the proteolytic activity of the purified proteases (114% of the control) (Haddar *et al*., 2010).

The examples of solvent additive that stabilize or destabilize proteins in solution are summarized in Table 8.

Table 8 Solvent additives that stabilize or destabilize proteins in solution.

Compounds	Mode of action	Working concentration
Osmolytic stabilizers[a]		
Polyols and sugars glycerol, erythritol, arabitol, sorbitol, mannitol, xylitol, mannisdomannitol, glucosylglycerol, glucose, fructose, sucrose, trehalose, isofluoroside	These stabilize the lattice structure of the water, thereby increasing surface tension and viscosity. In addition, they stabilize hydration shells and protect against aggregation by increasing the molecular density of the solution without changing the dielectric constant.	10%-40%
Polymers Dextrans, levans, polyethylene glycol	Polymers increase molecular density and solvent viscosity, thus lowering protein aggregation in a single-phase system. At high polymer concentration, a two-phase system develops, and the protein aggregates in the phase in which its concentration is highest.	1%-15%
Amino acids and their derivatives Glycine, alanine, proline, taurine, betaine, octopine, glutamate, sarcosine, γ-aminobutyric acid, trimethylamine *N*-oxide (TMAO)	Small amino acids with no net charge, such as glycine and alanine, have weak electrostatic interactions with proteins. Octopine is a derivative of arginine that is less denaturing to proteins. TMAO stabilizes proteins even in the presence of denaturants such as urea. Most of these compounds increase the surface tension of water.	20-500 mM

Table 8 (Continued)

Compounds	Mode of action	Working concentration
Ionic stabilizers[b]		
Salts citrate, sulfates, acetate, phosphates, quaternary amines	Larger anions shield charges and can stabilize proteins at low concentrations. At high concentrations, they lead to precipitation due to competition for water molecules.	20-400 mM
Denaturants (chaotrophs) Urea, guanidinium salts, trichloroacetates, acetylmethyl-ammonium salts, organic solvents	Denaturants either stabilize the unfolded state of proteins (urea) or perturb protein structure by interfering with hydrogen bonding or disturbing the hydration shell.	0.2-8 M
[a]In general, the osmolytic stabilizers have little direct interaction with proteins, but affect the bulk solution properties in water.		
[b]These affect enzyme reactions. Their stabilizing effects on proteins occur within a concentration.		

Source: Simpson (2010)

5. Experimental design

The classical "one parameter at a time" approach is laborious, time consuming, and inefficient to explain interaction effects among the variables. Conversely, the physicochemical parameters and the factors influencing the fermentation processes can be obtained through rapid statistical approach with limited number of planned experiments (Chandrashekar *et al.*, 1999; Chakravarthi and Sahai 2002; Abdel-Fattah 2002; Aravindan and Viruthagiri, 2007). Statistical design allows efficient evaluation of the key variables for further process improvement (Arifin *et al.*, 2012).

Process optimization is a topic of central importance in industrial production process with particular regard to biotechnology (Reddy *et al.*, 2008). Optimization of medium by the classical method involes changing one independent variable while maintaining all others at a fixed level is extremely time consuming and expensive when a large number of variables are evaluated.

5.1 Central composite design (CCD)

The model parameters can be estimated most efficiently if proper experimental designs are used to collect the data (Montgomery, 2000). The central composite design (CCD), statistical experimental design developed by Box and Wilson (1951) is the most frequently used Response surface methodology (RSM) design. This design is very efficient design for fitting the second –order model. A CCD can be broken down into three parts; two –level full or 2^k-factional design (the core), center points and axial points (outside the core), as shown in Figure 5.

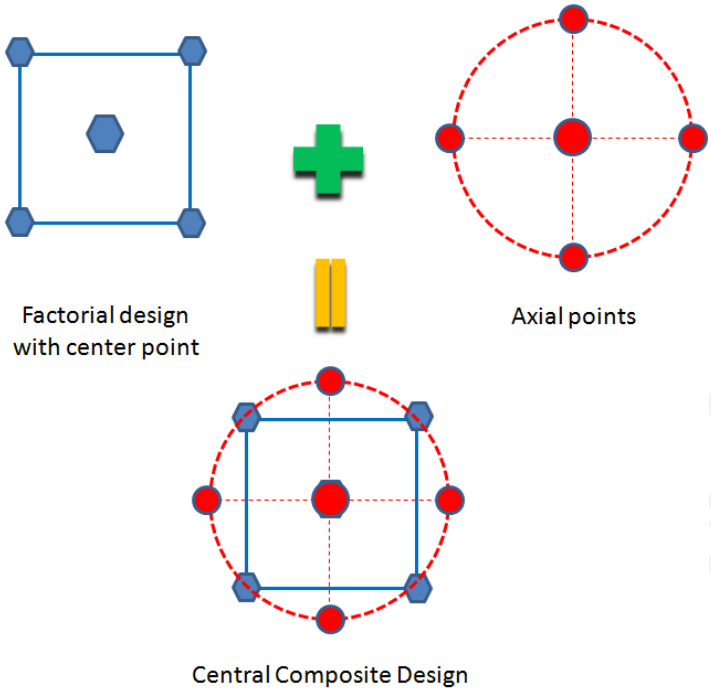


Figure 5 Generation of central composite design for two factors.

Source: Quality Invention Library (2014)

The two-level factorial part of the design consists of all possible combinations of the plus or minus one level of the factors. Axial points, often represented by the stars, emanate from the center point, with all but one of the factors set to 0. The coded distance of the axial points is represents as a plus or minus alpha (α). For a two-factor, the axial points are: (-α, 0), (+α, 0), (0, -α) and (0, +α). Center points are usually repeated to get an estimate of experimental error (pure error). It's desirable to set alpha at a level that creates "rotatability" in the design. To maintain rotatability, the value of α depends on the number of experimental runs in the factorial portion of the central composite design.

$$\alpha = [2^k]^{1/4} \text{ ; where k is number of factors.}$$

Designs with this property, such as the two-factor CCD with an alpha value of 1.414, exhibit circular contours on the standard error plot. The design matrix is shown in Table 9. There are two parameters in the design must be specified: the distance α of the axial run from the design center and number of required runs, and they are either rotatable or nearly rotattable.

Table 9 The central composite design matrix of two factors with triplication of center points.

X_1	X_2
-1	-1
-1	+1
+1	-1
+1	+1
0	0
0	0
0	0
-1.414	0
0	-1.414
+1.414	0
0	+1.414

Rajendran and Thangavelu (2010) reported that statistical evaluation of fermentation conditions and nutritional factors by Plackett–Burman two-level factorial design followed by optimization of significant parameters using response surface methodology for lipase production by *Bacillus brevis* was performed in submerged batch fermentation. Maximum lipase activity of 5.1 U/mL and cell mass of 1.82 g/L at 32 h were obtained at the optimized conditions of temperature, 33.7 °C; initial pH, 8; and speed of agitation, 100 rpm, with the optimized medium. The lipase productivity and specific lipase activity were found to be 0.106 U/mL/h and 2.55 U/ mg, respectively. Unstructured kinetic models and artificial neural network models

were used to describe the lipase fermentation. The kinetic analysis of the lipase fermentation by *B. brevis* shows that lipase is a growth-associated product.

5.2 Response surface methodology (RSM)

Response surface methodology (RSM) is the collection of statistical and mathematical techniques useful for the modelling and analysis of problem. It has eliminated the drawbacks of classical methods and proved to be more powerful for the optimization of the target metabolite productions which are influenced by several variables (Deepak *et al*., 2008; Liu and Wang, 2007; Montgomery, 2001, Sayyad *et al*., 2007). Second – order models like Central Composite, Box-Behnken and Doehlert designs are widely used in RSM as they can take a wide variety of functional forms. This flexibility allows them to closely approximate the true response surface (Srinivas *et al*., 1994; Carvalho *et al*., 1997; Adinarayana and Elliah 2002; Rahman and Gomes, 2003; Li *et al*., 2007; Xiao *et al.,* 2007). Moreover, it is easy to determine the parameters in a second-order model using the method of least squares. At present, RSM has been recently used for the modeling and optimization of several bioprocesses, including enzyme production (Kaushik *et al*., 2010; Cheng *et al*., 2012; Vijayaraghavan and Vincent; 2014) product recovery (Karvela *et al*., 2011) enzyme immobilization techniques (Zhao *et al.,* 2007; Chang *et al.,* 2007). The application of experimental design and response surface methodology in fermentations process can result in improved product yield, reduced process variability and development time and over all costs (Rao *et al*., 2000).

The three stages of optimization study using RSM as follow;

1) The determination of the independent parameters and their levels.

Since parameters have different units or ranges in the experimental design. Each of the coded variables is forced to range from -1 to +1, so that they all affect the response more evenly, and the units of the parameters are irrelevant. Commonly used equation for coding is seen below.

$$X = \frac{x - [x_{max} + x_{min}]/2}{[x_{max} - x_{min}]/2} \quad (7)$$

Where x is the actual variable, X is the coded variable and x_{max} and x_{min} are the maximum and minimum values of the actual variable.

2) The selection of the experimental design and the prediction and verification of the model equation.

After selection of the design, the model equation is defined and coefficients of the model equation are predicted. The model used in RSM is generally a second-order equation which is very flexible. It can take on a wide variety of functional form and often works well as an approximation to the true response surface. It is easy to estimate the parameters in the second-order model and there is considerable practical experience indicating that second-order models work well in solving real response surface problems (Myers and Montgomery, 1995).

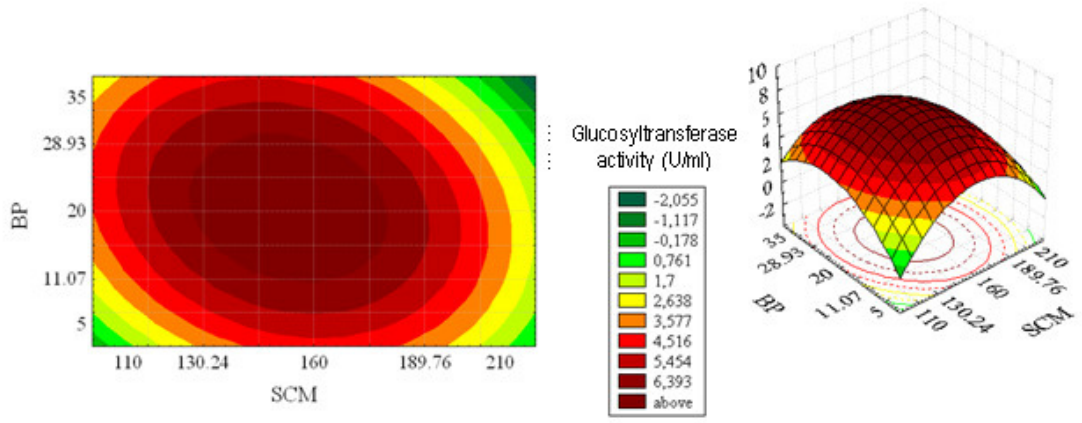
The second order model can be written as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_1 X_1 + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum \sum_{i<j} \beta_j X_i X_j + \varepsilon \quad (8)$$

Where β_0, β_i, β_{ii}, β_{ij} are regression coefficients for intercept, linear, quadratic and interaction coefficients respectively and X_i and X_j are coded independent variables.

3) The plots of the response surface plot and contour plot for the response as a function of the independent parameters and determination of optimum points.

The visualization of the predicted model equation can be obtained by the response surface plot and contour plot. The response surface plot is the theoretical three dimensional plot showing the relationship between the response and the independent variables. The contour plots help to visualize the shape of a response surface. When the contour plot displays ellipse or circles, the center of the system refers to a point of maximum or minimum response. Sometimes, contour plot may display hyperbolic or parabolic system of the contours. In the case, the stationary point is called a saddle point and it is neither a maximum nor a minimum point. These plots give useful information about the model fitted but they may not represent the true behavior of the system (Myers and Montgomery, 1995). Figure 6 is shown the response and the contour plot.



(A) (B)

Figure 6 Contour plot (A) and Response surface plot (B) as a function of independent variables which are used to express the predicted model equation.

Source: Kawaguti *et al*. (2006)

6. Kinetics model

Kinetic models facilitate to design and control microbial in fermentation process. In predicting the behavior of these processes, mathematical models make it possible to evaluate the behavior of systems more rapidly than with solely laboratory experiments. Mathematical modelling of the fermentation process helps in understanding the relationship among principal state variables to provide quantitative information on the behaviour and predict the effect of changes to the system (Gaden, 2000). This information is essential for analysis of the fermentation process with respect to biological significance of each parameter and their levels with statistical consistency. Moreover, this information is useful for fermenter design and operation for enhanced production.

Generally, a model proposed for a simulation study is a mathematical model developed with the help of simulation software. In general, simulation models are

stochastic and dynamic (Maria, 1997). By combinding experimental work with mathematical modelling, it is possible to provide important interpretations of the experimental results to analyze new aspects of microbial physiology. The model can then be used for designing new and more focused experiments (Hjortso and Bailey 1984; Kompala *et al*. 1986; Nielsen *et al*. 1991; Seo and Bailey 1985). A model should be a close approximation to the real system and incorporate most of its most important features. Moreover, it should not be so complex that it is impossible to understand and experiment with it. Model validation techniques include simulating the model under known input conditions and comparing model output with system output (Maria, 1997). 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. Unstructured models utilize the knowledge of experimental reality and describe biomass and associated metabolite production. In other words, only cell mass is employed to describe the biological system.

Unstructured kinetic models are the most frequently employed for modeling microbial systems based on simplicity and technical robustness which are quiet satisfactory in many situations when balanced growth condition is accomplished or in many control and optimization problems in fermentation process with minimum mathematical complexity. It can provide useful information for the analysis, design and operation of a fermentation process (Aravindan and Viruthagiri, 2008). Extensive experimental analyses have been made in batch, fed-batch, and continuous cultures to support the model. Basically, an unstructured model for cell growth, product formation, glucose consumption, and dissolved oxygen was found to be convenient to characterize the fermentation process. While, structured models use the information on intracellular metabolic pathway reaction rates which take into account for some basic aspects of cell structure, function, and composition. Studies have revealed that both model types are equally useful and descriptive in terms of experimental reality (Liu *et al*., 2003; Vazquez and Murado, 2008).

The applications of unstructured models to explain the behavior are outlined as in the following sections.

6.1 Microbial growth

The unstructured models which are more used for description of growth processes are the Monod and the logistic equations (Parente and Hill; 1992; Mercier *et al*., 1992). Under ideal conditions for growth, when a batch fermentation is carried out, it can be observed experimentally that the quantity of biomass, and therefore also the concentration, increases exponentially with respect to time. Thus the overall rate of biomass formation is described by a first order rate expression as

$$r_x = kX \tag{9}$$

where r_x is the rate of cell growth (g/L.h), X is the cell concentration (g/L) and k is a kinetic growth constant (1/h). For a batch system, this is equivalent to,

$$\frac{dX}{dt} = kX \tag{10}$$

where dX/dt is the rate of change of cell concentration with respect to time (g/L.h).

6.1.1 Monod equation

The Monod kinetic model is the most widely used empirical model that describes microbial growth under substrate-limiting conditions (Lobry *et al*., 1992; Dette and Melas, 2003). The exponential and limiting regions can be described by a single relation, μ equal to a function of substrate concentration. It is observed experimentally that μ is at a maximum when the particular limiting substrate

concentration S is large, and for low concentration μ is proportional to S. Over the whole range from low to high S, μ is described by the following;

$$\mu = \frac{\mu_m S}{K_S + S} \tag{11}$$

where μ_{max} is the maximum specific growth rate (1/h) and K_S is the saturation constant (g/L).

The feature of this relation is shown in Figure 7.

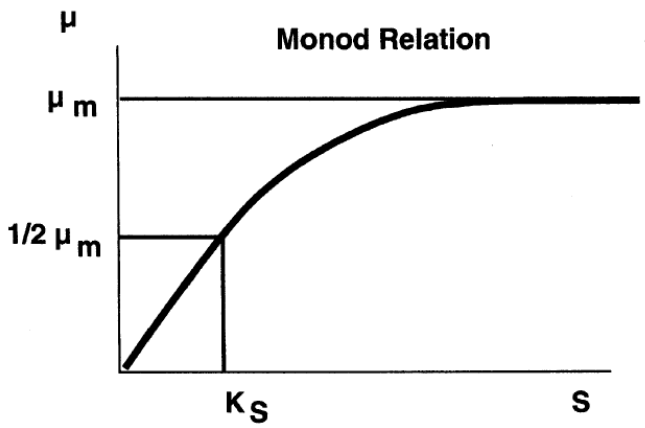


Figure 7 Specific growth rate versus limiting substrate concentration according to the Monod relation.

Source: Dunn (2003)

6.1.2 The Logistic Equation

Microbial processes, especially with a modified medium for the prolonged and high production of metabolites, do not necessarily follow the classical kinetic model of substrate-limited biomass growth and product formation proposed by Monod. Therefore, a logistic equation is used as an alternative empirical function

(Luedeking and Piret; 1959). A logistic equation allows simple calculation of fermentation parameters of biological and geometrical significance using sigmoid profiles independent of substrate concentration. In many fermentation systems, cell growth has already been characterized using a logistic equation which can be described as follows:

$$\frac{dX}{dt} = \mu_m (1 - \frac{X}{X_m}) X \qquad (12)$$

where X_m is the maximum cell concentration (g/L).

6.2 Substrate Uptake Kinetics

The rate of uptake of substrate by micro-organisms is generally considered to be either related to that of growth or to that required for cell maintenance. This can be expressed as:

$$r_s = \frac{-r_x}{Y_{X/S}} - mX \qquad (13)$$

where rs is the rate of substrate uptake by the cells (g substrate/L.h). YX/S is the stoichiometric factor or yield coefficient and relates mass cells/mass substrate (or g.cell/ g. substrate).

In general, most heterotrophic microorganisms require a carbon source to maintain cellular function, biomass formation and metabolite production (Pirt *et al*., 1965; Srinivasulu *et al*., 2002; Rao *et al*., 2004; Rao *et al*., 2006). The bacterial strain responds differently with respect to protease production according to the type of carbon source and its concentration indicating that carbon source concentration is one of the controlling factors for cellular maintenance leading to biomass and enzyme production (Prakasham *et al.*, 2006). Therefore the utilization of glucose (*S*)

throughout the fermentation process was modeled using the Luedeking–Piret equation.

$$\frac{-dS}{dt} = (\frac{1}{Y_{X/S}})\frac{dX}{dt} + m_s X \qquad (14)$$

where m_s is the maintenance coefficient and $Y_{X/S}$ is cell yield coefficient.

6.3 Product Formation

Most proteases produced by bacterial cells are extracellular in nature and help in the processing of complex proteinaceous compounds present in the fermentation medium in order to provide the amino compounds required by growing cells. These growth-promoting metabolites are produced during the exponential phase of the growth curve (Prakasham *et al.*, 2006; Subba *et al.*, 2008). Hence the kinetics of protease production was studied based on the Luedeking–Piret model. This model has great utility, with modifications that allow the incorporation of other effects in the description and specifications of the metabolites produced by the microbes. According to the Luedeking–Piret equation (a mixture of growth associated and non-growth associated terms), the rate of protease production is

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \qquad (15)$$

where α and β are parameters obtained from the following equations:

$\frac{dP}{dt} = \alpha\frac{dX}{dt}$ for product formation kinetics of growth associated.

$\frac{dP}{dt} = \beta X$ for product formation kinetics of non-growth associated.

Simulation models were performed by software packages which consist of the following steps: system entities, input variables, performance measures, and functional relationships. In a simulation study, human decision making is required at all stages, namely, model development, experimental design, output analysis, conclusion formulation, and making decisions to alter the system under study (Maria, 1997). Figure 8 is a schematic of a simulation study.

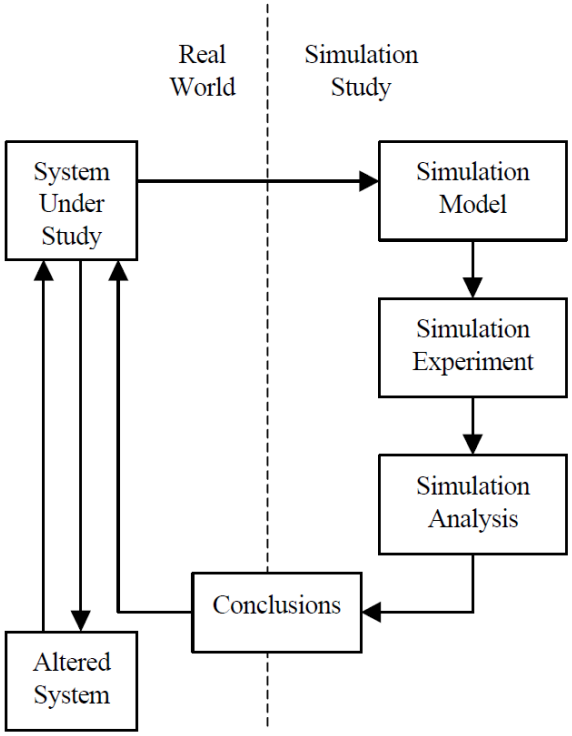


Figure 8 Simulation study schematic.

Source: Maria (1997)

The example of researches by using unstructured model to explain the behavior are as follows.

Baeil *et al.* (2008) studied the fermentation kinetics of citric acid by *Aspergillus niger* in a batch culture. A simple model was proposed using the logistic equation for growth, the Luedeking-Piret equation for citric acid production and

Luedeking-Piret-like equation for glucose consumption. The model appeared to provide a reasonable description for each parameter during the growth phase and the production of citric acid by *A. niger* is growth-associated.

Rajendran and Thangavelu (2008) analyzed various unstructured models to simulate the experimental values of microbial growth, protease activity and substrate concentration. The 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 capable of predicting the fermentation profile with high coefficient of determination (R^2) values of 0.9967, 0.9402 and 0.9729, respectively. The results indicated that the unstructured models were able to describe the fermentation kinetics more effectively.

Rao *et al.* (2009) developed an unstructured mathematical model to understand information on the relationship between *Bacillus circulans* growth and metabolism-related protease production (using logistic and Luedeking–Piret equations respectively) in a batch reactor with respect to glucose consumption and fermentation time. With the *B. circulans* strain used, an economic protease yield ($2,837 \times 10^3$ U/g) with respect to biomass and glucose ratio was achieved at low substrate concentration (10 g/L). The developed model could be effectively utilized for designing, controlling and up-scaling the protease production process using high-density fermentation in selected bioreactor with statistical consistency.

Shafaghat *et al.* (2009) reported that the growth kinetic parameters were determined with Malthus, Monod and Logistic rate equations. The obtained maximum specific growth rate, μ_{max} and the Monod constant (Ks) of various substrates for glucose, fructose and sucrose were 0.65, 1.35, 1.85 h^{-1} and 11.39, 39.19, 97.82 g/L, respectively.

Pansuriya and Singhal (2011) reported the kinetic model of the fermentation process of serratiopeptidase (SRP) production from *Serratia marcescens* NRRL B-23112. The resulting logistic and Luedeking-Piret models provided an effective description of the SRP fermentation, where the correlation coefficients for cell growth, SRP formation, and substrate consumption were 0.99, 0.94, and 0.84, respectively, revealing a good agreement between the model-predicted and experimental results. The kinetic analysis of the batch fermentation process for the production of SRP demonstrated the SRP production to be mixed growth associated.

7. Fed- batch fermentation

Fed-batch operation technique has been extensively applied in industrial fermentation processes for enhancing cell density and productivity. The major advantage of fed-batch application is the possibilities to control both reaction rate and metabolic reactions by substrate feeding rate, because the introduction of appropriate feed rate strategies can minimize by-product production, substrate inhibition and catabolite repression. The limitations caused by oxygen transfer and cooling can be avoided by controlling the reaction rate (Enfors and Häggström, 2000). The improvement more than 100 percent of cell growth rate and approximately two times higher final cell concentration can be achieved by fed-batch culture as compare to batch culture (Desmazeaud, 1996; Dong *et al.*, 2003; Korz *et al.*, 1995).

Different types of substrate limitations can be used in the fed-batch processes. The repression of the growth rate in batch culture by various substrates such as sugar, nitrogen or phosphate sources can be overcome. The metabolism control with the fed-batch process is useful also for the production of the secondary metabolites such as antibiotic syntheses are repressed during the unrestricted growth (Enfors and Häggström, 2000). In fed-batch technique almost every key variable is changing as the process progresses. In order to give the best possible growing conditions the pH and temperature levels are usually kept constant (Gregersen and Jorgensen, 1999).

Nutrient feeding strategies in fed-batch culture include simple indirect feedback methods such as pH-stat (Suzuki *et al*., 1990; Kim, 2002) or DO-stat (Kim *et al*., 2005), predetermined feeding strategies (exponential feeding) (Kim *et al*., 2004, Kim *et al*., 2006), feeding according to glucose uptake or demand (Kim *et al*., 1994), and other methods i.e., using feed-back control strategies for glucose feeding to maintain the dissolved oxygen concentration in a certain range (Cutayar and Poillon, 1989; Mori *et al*., 1979) and to keep the specific growth rate approximately constant (Riesenberg *et al*., 1991).

Many researchers have studied fed batch fermentation to improve protease production which are described as follows:

Mao *et al.* (1992) reported that alkaline protease production of 9,016 U/mL by *Bacillus licheniformis* were achieved by using a synthetic medium and a fed-batch operation controled. A 49 percent increase in production was achieved by the method used as compared with a batch culture.

Beg *et al.* (2003) found that protease production from *Bacillus mojavensis* was adopted to enhance protease synthesis by using intermittent de-repression and induction during the growth of the organism in fed-batch strategy. Feeding of casamino acids at this stage resulted in a 2.8-fold (1,219 U/mL) increase in protease yield after 36 h compared to the protease yield in batch culture. This protease production was enhanced further to 4 fold (1,770 U/mL) in the second fed-batch operation on the onset of the second stationary phase by a glucose feed (5 mg/mL) at 33 h followed by a casamino acids feed (5 mg/mL) at 36 h.

Beshay and Moreira (2005) reported that fed-batch culture was superior to batch fermentation of *Teredinobacter turniraein* producing alkaline protease. The maximum protease production rate of 158 U/mL/h was nearly 2.6-fold greater than values observed in batch operations.

MATERIALS AND METHODS

Materials

1. 2 L stirred tank bioreactor (Biostat B fermenter, B. Braun Biotech. Inc. Allentown. PA)

2. DO probe (In pro ® 6800 series O_2 sensors, Mettler Toledo, Switzerland)

3. pH probe (405 DPAS SC K8S/225 Combination pH, Mettler Toledo, Switzerland)

4. Water- bath (WBC -1510 W, Jeio Tech, Korea)

5. Visible Spectrophotometer (Genesys ® 20 Thermo spectronic, Taylor Scientific, US)

6. Autoclave (Sanyo Labo autoclave)

7. Digital scale (Denver Instrument,China)

8. pH meter (Consort C830, Consort bvba, Belgium)

9. peristaltic pump (Alitea U 1 M, Alitea, Sweden)

10. Rotary incubator shaker (Sanyo Gallenkamp PLC, UK)

11. Water bath shaker (MM-10 water bath shaker, Taitach)

12. Centrifuge (Spectrafuge 24D, Labnet International Inc.)

13. Silicone tube (Dura EZ - TG106)

14. Glassware for analysis

15. Scanning electron microscope (SEM) (Philips Model XL30, USA).

Methods

1. Preparation of hydrolyzed starch

Dissolve 250 g of cassava starch with 1 L of tab water. It was mixed with 50 mg of $CaCO_3$ and adjusted to 6.5 with 1 M NaOH and boiled for 10 min. The starch slurry was liquefied by 0.15 % (v/v) Termamyl (120L type LS, 120 KNU/g, Novo Nordisk, Denmark) at 95°C for 2 h after pH adjustedment to 4.3 by 1 M H_3PO_4. The liquefied starch was then saccharified by 0.1 % (v/v) dextrozyme, 225 AUG/mL, 75 PUN/mL, Novo Nordisk, Denmark) and incubated in water bath shaker at 60°C for 24 h (Sirisansaneeyakul, 2000) to obtain 70-75 % dextrose equivalent (DE). Glucose content of hydrolyzed starch was determined by using the modified dinitrosalicylic acid (DNS) method described by Prakasham *et al.* (2007).

2. Microorganism

Bacillus sp. C4 SS-2013 or strain C4, a strain from Laboratory of Assist. Prof. Dr. Patoomporn Chim- anage was used in this research, which was isolated from waste water of a Thai silk factory, and select for its potential in producing protease for silk degumming process. Based on morphological, biochemical, and physiological characteristics and the 16S rDNA sequence (Fufeungsombut *et al*., 2009), this strain showed 99.8% similarity *to Bacillus subtilis* subsp. *inaquosorum* (KCTC13429) with 99.8% and was given the accession number of AB841263. The culture was maintained at -20°C in modified Davis minimal medium with raw silk (Fufeungsombut *et al*., 2009) containing 20% (v/v) glycerol.

3. Inoculum preparation

Inoculum was prepared by transferring preserved culture into 100 mL pre-culture medium in 500 mL Erlenmeyer flask. The pre-culture medium (BMSM broth) was made up of 1 % (w/v) glucose, 0.5 % (w/v) yeast extract, 0.1 % (w/v) K_2HPO_4, 0.2 % (w/v) $MgSO_4 \cdot 7H_2O$, and 1.0% (w/v) skim milk adjusted pH to 7.5 with 5 N HCl/NaOH before sterilization (121°C, 15 min). The pre-culture medium was incubated on a shaker at 200 rpm at 30°C for 12 h and the process was repeated one more time. The starter absorbance at 660 nm was adjusted to 0.3 ($A_{660} \cong 0.3$) before use.

4. Effect of agitation and aeration on silk degumming protease production from *Bacillus* sp. C4 SS-2013 in a stirred tank bioreactor.

The batch cultivations were performed in a Biostat B (B. Braun Biotech. Inc. Allentown. PA) fermentor with a 1.5-L cuture medium. The fermentor was inoculated with 2% (v/v) of a 12 h *Bacillus* sp. C4 SS-2013 seed culture in previously optimized medium. The medium composition for the batch culture was: hydrolyzed cassava starch (total sugar 2 % (w/v)); yeast extract (0.25 % (w/v)); soy flour (2 % (w/v)); K_2HPO_4, (0.1 % (w/v)); $MgSO_4 \cdot 7H_2O$, (0.002 % (w/v)) and skim milk (0.1 % (w/v)). All the fermentation runs were conducted in batch mode at controlled temperature 30°C. pH was automatically controlled using a sterile pH electrode (405 DPAS SC K8S/225 Combination pH, Mettler Toledo, Switzerland) at 7.5±0.05 by adding 10 N NaOH. The dissolved oxygen (DO) concentration was measured using a sterilizable polarographic electrode (In pro ® 6800 series O_2 sensors, Mettler Toledo, Switzerland). The effects of agitation and aeration rate were studied, which varied according to the experimental design. The response surface approach involving a Central Composite Design (CCD) for 2 factors was adopted for improving protease production. A total of 11 sets of experiments including three central points were employed to determine the significant factors affecting the protease production. The variables range and the full experimental plan are listed in Table 10. The volumetric

oxygen transfer coefficient (K_La, h^{-1}) of cultures was measured according to the static method of gassing out described by Wise, 1951.

Table 10 The effects of aeration and agitation on the protease production were evaluated by using Central Composite Design.

Treatment	Codes values		Actual values	
	X_1	X_2	Agitation (rpm)	Aeration (vvm)
1	-1	-1	200	1
2	1	-1	600	1
3	-1	1	200	3
4	1	1	600	3
5	0	0	400	2
6	0	0	400	2
7	0	0	400	2
8	-1.414	0	118	2
9	0	-1.414	400	0.6
10	1.414	0	683	2
11	0	1.414	400	3.4

For the statistical model, Y is the measured response and denotes units of protease activity. The quadratic model for predicting the optimal points is expressed as follow:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (16)$$

Where Y represents the response variable, β_0 is the interception coefficient, β_1 and β_2 are the linear terms, $\beta_{11}$and β_{22} are the quadratic terms, and X_1 and X_2 represent the studied variables. The statistical analysis of the model was performed as analysis of variance (ANOVA) using SPSS for Windows Version 12. The significance of the

regression coefficients and the associated probabilities (*p*), were determined by Student's t-test; the second-order model equation was determined by Fischer's test. The variance explained by the model was given by the multiple coefficient of determination, R^2. Response surface methodology combined with CCD was established using Statistica (Version 5).

5. Development of a kinetic model for protease production to characterize the fermentation of *Bacillus* sp. C4 SS-2013.

This research attempted an unstructured kinetic model with cell growth, substrate consumption and protease production of *Bacillus* sp. C4 SS-2013 in batch fermentation.

For cell growth; logistics model

A logistic equation allows simple calculation of fermentation parameters of biological and geometrical significance using sigmoid profiles independent of substrate concentration and there is a limit to the maximum attainable cell mass concentration.

$$\frac{dX}{dt} = \mu_{\max}(1 - \frac{X}{X_{\max}})X \qquad (16)$$

For Product formation; Luedeking–Piret equation

In the exponential phase of the Luedeking–Piret equation which combines growth and non-growth associated contributions towards product formation. Thus, product formation depends upon both the growth and instantaneous biomass concentration. The rate of formation of product is given by

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta x \qquad (17)$$

Where α and β are the Luedeking Piret equation parameters for growth and non-growth associated product formation, respectively.

For substrate utilization

The glucose consumption equation given below is a Luedeking–Piret-like equation in which the amount of carbon substrate used for product formation is assumed to be negligible.

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{dX}{dt} - m_S X \qquad (18)$$

5.1 Estimation of model parameters

In order to describe the true behavior of the system, it is important to obtain accurate estimates of the kinetic parameters in these models (Okpokwasili and Nweke, 2005). Several parameters in the kinetic models have always been obtained from experimental data. For optimal estimation of model parameters, a non-linear regression technique was used by using Berkeley Madonna program (Trial version), which minimized the deviations between the model predictions and the experimental batch results. The sums of squares error between model predictions and experimental data for residual sugar concentration, total biomass concentration, and total protease production were minimized. Because minimization of the sum of squares for a general non-linear problem is quite dependent on the initial guesses for the parameters, various starting points were provided and the final parameter set that had the lowest sum-squared error was chosen. The initial conditions were given according to the experimental data in batch cultures of *Bacillus* sp. C4 SS-2013. The values of the optimized parameters are as follow $\mu_{max,}$ X_{max}, m_s, $Y_{X/S}$, α, β.

5.2 Simulation

The maximized model parameter served as input to the Berkeley Madonna program and output was the simulation of stirred tank bioreactor experiments. To test the validity of the established model parameters, model predictions were compared with experimental data, which were examined in order to formulate the basis of a kinetic model of the process.

6. Improvement of silk degumming protease production from *Bacillus* sp. C4 SS-2013 by pH-stat fed batch fermentation.

To improve the protease production, pH-stat fed batch fermentation were carried out in a 2 L fermentor by using the optimal medium and under the following conditions: temperature, 30°C; agitation rate, 400 rpm; aeration rate, 2 vvm; pH 7.5. Dissolved oxygen was maintained at greater than 30% of air saturation by supplementing with oxygen gas if necessary. The cells were initially grown in batch mode until the sugar was almost completely consumed (about 11 h) and addition of feed medium consisting of hydrolyzed cassava starch (total sugar 3.6 % (w/v)), yeast extract (0.5 % (w/v)), soy flour (4 % (w/v)) and skim milk (0.2 % (w/v)) was started and continued till the pH of the culture broth was above 7.5., feeding nutrient solution was automatically added by acid-supplying pump.

7. The effect of stabilizers on silk degumming protease activity from *Bacillus* sp. C4 SS-2013.

The optimization of medium and cultivation condition were previously investigated by Plackett – Burman, Central Composite Design (CCD) and Response surface methodology (RSM) in shake flask (Romsomsa *et al.*, 2010) The appropriate result was obtained as follows; 2% (w/v) soy flour and 0.1% (w/v) skim milk. The enzyme was investigated for its stability during storage.

The effect of additives on protease stability was investigated. The supernatant of the culture broth of *Bacillus* sp. C4 SS-2013 fermented for 24 h was stored in various chemical solutions to obtain the final concentration as follows: 10 mM $CaCl_2$, 0.02 % (w/v), sodium azide, 25 % (v/v) glycerol, 10% (v/v) polyethylene glycol (PEG 400) and 10 mM $CaCl_2$ combined with 10% (v/v) PEG 400 were added to crude enzyme, pH 8 (50 mM phosphate buffer). The reaction mixtures were kept variously at room temperature, 4°C and -20°C, respectively. Residual enzyme activities were measured after storage for 0, 1, and 3 weeks.

In addition, the effect of different concentrations of additives on protease stability was studied as descripted: 1, 10, 100 mM $CaCl_2$, 10% (v/v) PEG 400 and 10 mM $CaCl_2$ combined with 10% (v/v) PEG 400 were added to crude enzyme, pH 8 (50 mM phosphate buffer). The reaction mixtures were kept variously at 4°C. Residual enzyme activities were measured after storage for 8 weeks.

8. The effective crude protease from *Bacillus* sp. C4 SS-2013 for silk degumming.

The efficiency of the crude protease obtained when growing in the optimized medium and cultivation condition was studied. Approximately 100 mg of silk yarn hank was dried in hot air oven at 105°C for 90 min and kept in desiccators for 1 h. The dried sample was weighed with an analytical balance. The silk yarn was subsequently degummed in a 20 mL tight-capped screw cap bottle and carried on at different dilution of crude protease (undiluted; 1,560 U/mL, 2x; 780 U/mL and 5X; 312 U/mL) in 50 mM phosphase buffer pH 8 for 60 min in water bath shaker. The liquor to material (LR) ratio was kept at 50:1. At the end of the degumming process, the silk yarn was rinsed with 5 mL warm water, then subsequently with the same volume of tap water; the rinsing process was repeated three times. The degumming process was repeated twice. The yarn was air dried and then dried at 105°C for 90 min. The degummed sample was kept in a desiccator for 1 h and then weighed with an analytical balance. Triplicate samples were used for each treatment.

The degumming ratio was determined from the sample weight changes before and after the degumming process. The degumming ratio (*i.e.* weight loss) of the silk yarn was was calculated by the following equation.

$$\text{Degumming Ratio (\%)} = \frac{100(W_0 - W_1)}{W_0} \quad (19)$$

W_0 and W_1 are the sample weights of the completely dried silk yarn before and after degumming.

Analytical Method

During the fermentation, samples were withdrawn at 3-h intervals for the analysis. The samples were centrifuged at 9,200 g, for 10 min and each supernatant was used for determination of protease activity and reducing sugar.

Cell growth was determined as log CFU/mL by viable cell count. Correlation between log CFU/mL and dry cell weight (DCW) was estimated from several batch experiments. One unit of log CFU/mL was approximately equivalent to 0.51 g/L of cells (Appendix B). Spore yields were determined by heating the culture broth at 80°C in a water bath for 20 min and the numbers of endospores were determined by the spread plate method.

Protease activity was determined by modified method of (Ferrero, 1996) (Appendix B). Total sugar was determined by phenol–sulfuric acid method (Dubois *et al*., 1956) and reducing sugar was estimated using the modified dinitrosalicylic acid (DNS) method described by Prakasham *et al.* (2007) (Appendix B).

To ensure the crude enzyme obtained in the culture broth was protease, Native polyacrylamide gel electrophoresis (native PAGE) zymography impregnated with a protein substrate (García-Carreño *et al*., 1993) was performed.

RESULTS AND DISCUSSION

1. Effect of agitation and aeration on silk degumming protease production from *Bacillus* sp. C4 SS-2013 in a stirred tank bioreactor.

Since the oxygen is the critical factor and its requirement should be corresponded with its significant substrate, the preliminary study was carried out by using 3^2 factorial designs experiments in shake flasks by growing *Bacillus* sp. C4 SS-2013 in optimized medium (Romsomsa *et al.*, 2010). A clear interaction effect between hydrolyzed cassava starch and the shaker speed on silk degumming protease production was found, with increased hydrolyzed cassava starch in the medium beyond 8 g/L at 280 rpm leading to a decrease in protease production (data not shown). These results emphasize the necessity of oxygen supplementation which is related to the aeration and agitation rates in the fermenter. Table 11 shows the results obtained by using the central composite experiment to study the effects of these two parameters on silk degumming protease production in a stirred tank bioreactor. The maximum protease production (1,880 U/mL), maximum specific protease production rate (27,637 U/g/h) and maximum specific growth rate (0.086 h^{-1}) were achieved by agitation at 400 rpm and aeration rate of 2 vvm (Treatment 5, 6, and 7), where the average K_La was 171.48 h^{-1}.

Table 11 Experimental design showing the optimization of agitation rate and aeration rate on protease production and K_La

Treatment	Factors		Maximum protease activity (U/mL)		Specific growth rate (μ; h^{-1})		Specific production rate (q_p;U/g/h)		K_La (h^{-1})	
	Agitation rate (rpm)	Aeration rate (vvm)	Observed*	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	200	1	296.47 ± 14.11	353.00	0.058	0.065	3,154	2,952	25.56	6.50
2	600	1	178.12 ± 1.17	438.97	0.047	0.041	1,564	6,214	257.76	228.64
3	200	3	290.00 ± 16.67	67.25	0.019	0.016	3,687	564	122.40	89.85
4	600	3	697.04 ± 2.23	678.62	0.070	0.053	7,339	9,069	163.80	134.22
5	400	2	1880.00 ± 4.71	1,813.18	0.086	0.082	27,637	26,016	145.80	171.50
6	400	2	1,787.06 ± 4.12	1,813.18	0.074	0.082	27,389	26,016	183.24	171.50
7	400	2	1,776.64 ± 10.93	1,813.18	0.087	0.082	23,022	26,016	185.40	171.50
8	118	2	70.32 ± 0.19	195.69	0.044	0.039	885	3,552	23.76	56.65
9	400	0.6	558.33 ± 11.66	341.77	0.061	0.057	4,336	1,507	36.36	66.87
10	683	2	852.5 ± 10.00	689.06	0.033	0.047	16,069	11,877	225.72	254.42
11	400	3.4	130.74 ± 0.84	309.18	0.019	0.031	535	1,837	37.08	68.25

* The experiments were performed in duplicate and the results were expressed as mean ± SD.

Regression analysis (Table 12) of the experimental data showed that results of using the probability (p) values were used as a tool to check the significance of each of the coefficients. The smaller the magnitude of the p value, the more significant was the correlation with the corresponding coefficient and the very low probability value (p value ≅0) demonstrated a very high significance for the regression model, which was found in all responses. Among the test variables used in this study, the agitation and aeration rates had a strong positive linear effect (X_1, X_2) but there was also a negative effect of X_1X_1 and X_2X_2 (except X_1X_1 of K_La). In this study, the quadratic terms were significant, which suggested considerable curvature in the model. However the model terms X_1X_2 of maximum protease production and specific protease production rate (q_p) were not significant while the term X_1X_1 of K_La was also not significant.

The models fitting the above response variables were as followed:

Protease production (Y_1)

$$Y_1 = -3{,}701.93 + 13.25X_1 + 2{,}702.09X_2 - 0.0171X_1^2 - 744.08X_2^2 + 0.66X_1X_2$$

Specific growth rate (Y_2)

$$Y_2 = 0.001542 + 0.002X_1 + 0.036X_2 - 4.898e^{-7}X_1^2 - 0.019X_2^2 + 7.638e^{-5}X_1X_2$$

Specific protease production rate (Y_3)

$$Y_3 = -60{,}123.6 + 184.4X_1 + 46{,}198.1X_2 - 0.2X_1^2 - 12{,}175.6X_2^2 + 6.6X_1X_2$$

Volumetric oxygen transfer coefficient (Y_4)

$$Y_4 = -399.85 + 0.99X_1 + 303.82X_2 - 51.98X_2^2 - 0.24X_1X_2$$

where X_1 is the agitation rate and X_2 is the aeration rate.

Table 12 Estimated regression coefficients for protease production, specific protease production rate and K_La along with their significances.

Model	Coefficient	Standard error	t- value	p- value
Protease production (U/mL)				
Intercept	-3,701.93	395.21	-9.37	0.000
X_1	13.25	1.24	10.66	0.000
X_2	2,702.09	248.73	10.86	0.000
X_1X_1	-0.0171	0.00	-13.03	0.000
X_2X_2	-744.078	52.61	-14.14	0.000
X_1X_2	0.66	0.31	2.10	0.052
Specific growth rate (μ)				
Intercept	0.001542	0.023883	0.06	0.949
X_1	0.000254	0.000075	3.38	0.004
X_2	0.036453	0.015032	2.43	0.028
X_1X_1	0.00000	0.000000	-6.17	0.000
X_2X_2	0.000038	0.000009	4.04	0.001
X_1X_2	-0.019093	0.003180	-6.00	0.000
Specific protease production rate (q_p)				
Intercept	-60,123.6	7,169.74	-8.39	0.000
X_1	184.4	22.54	8.18	0.000
X_2	46,198.1	4,512.46	10.24	0.000
X_1X_1	-0.2	0.02	-9.59	0.000
X_2X_2	-12,175.6	954.52	-12.78	0.000
X_1X_2	6.6	5.66	1.16	0.265
Volumetric oxygen transfer coefficient (K_La)				
Intercept	-399.85	73.36	-5.45	0.000
X_1	0.99	0.23	4.27	0.001
X_2	303.82	46.17	6.58	0.000
X_1X_1	0.00	0.00	-0.82	0.426
X_2X_2	-51.98	9.77	-5.32	0.000
X_1X_2	-0.24	0.06	-4.11	0.001

The significance of the fit of the second-order polynomial for protease production, the specific growth rate, the specific protease production rate and K_La were assessed by carrying out analysis of variance (ANOVA) (Appendix table C2-C5). The resultant R^2 values were 0.950, 0.919, 0.931 and 0.881, respectively. In the case of protease production, an R^2 value of 0.950 means that 95% of the variability was explained by the model and only 5 % was as a result of chance. Likewise the specific growth rate, the specific protease production rate and K_La, it means that 91.9%, 93.1% and 88.1% of the variability were explained by the model. The results indicated that the model showed a good fit and explained the variability of these values very well.

Two-dimensional response surfaces were plotted on the basis of the model equation in order to investigate the interaction between the two variables (the aeration rate and agitation rates) and to determine the optimum level of each factor for maximum protease production by *Bacillus* sp. C4 SS-2013. The effect of agitation and aeration rate on protease production (Y_1) is represented in Figure 9. There was an increase in the protease production with the increase of agitation and aeration rate. The maximum protease production was obtained with an agitation rate in the range 320 to 520 rpm and an aeration rate from 1.5 to 2.5 vvm. A further increase in the agitation rate over 520 rpm and the aeration rate over 2.5 vvm resulted in decreased protease production. Similarly in Figure 10, agitation and aeration rate also have a profound effect on specific growth rate of *Bacillus* sp. C4 SS-2013. The specific growth rate increases from 0.019 to 0.087 h^{-1} when agitation rate increases over 280 rpm and aeration rate increases over 1.2 vvm and then increasing agitation rate (more than 520 rpm) and aeration rate (more than 2.4 vvm) caused the reduction of specific growth rate. Figure 11 clearly shows that the maximum specific protease production rate can be achieved with an increase in the agitation rate from 310 to 480 rpm and in the aeration rate from 1.6 to 2.5 vvm. However, increasing the agitation and aeration rates beyond these values resulted in a decrease in the specific protease production rate. The values of K_La often serves to compare the efficiency of bioreactors and mixing devices. It is also an important scale-up factor to indicate the oxygen demand of a culture. Thus, the aeration and agitation rates are often selected to achieve the

desired K_La, since this is the controlling parameter in most fermentation systems (Blakebrough and Moresi, 1981). As shown in Figure 12, increasing the agitation rate clearly increased the K_La value and the maximum K_La occurred with agitation greater than 630 rpm, while increasing the aeration rate to more than 2 vvm, led to a decrease in K_La. However, at the maximum K_La in this study both the activity and biomass dropped. Similar pattern was observed in case of dextransurase production (Chauhan *et al*., 2013) chitinases (Fenice *et al.*, 2012). In all operative conditions, contour plots represent the elliptical and saddle contour plots suggest that there are major interactions between agitation rate and aeration rate corresponding to the four responses. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar *et al.*, 2001). In the case of saddle contour plots, the optimum values are obtained at the point of intersection of lines which are formed by joining the locus (Murthy *et al*., 2000).

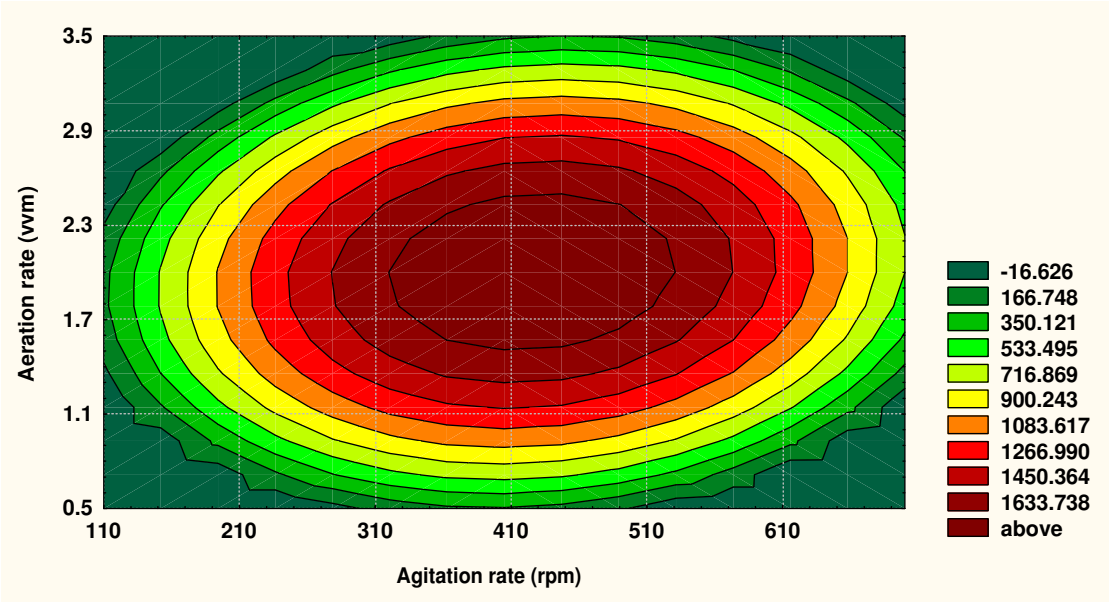


Figure 9 Contour plot for the effects of agitation and aeration rate on silk degumming protease production by *Bacillus* sp. C4 SS-2013 in stirred tank bioreactor.

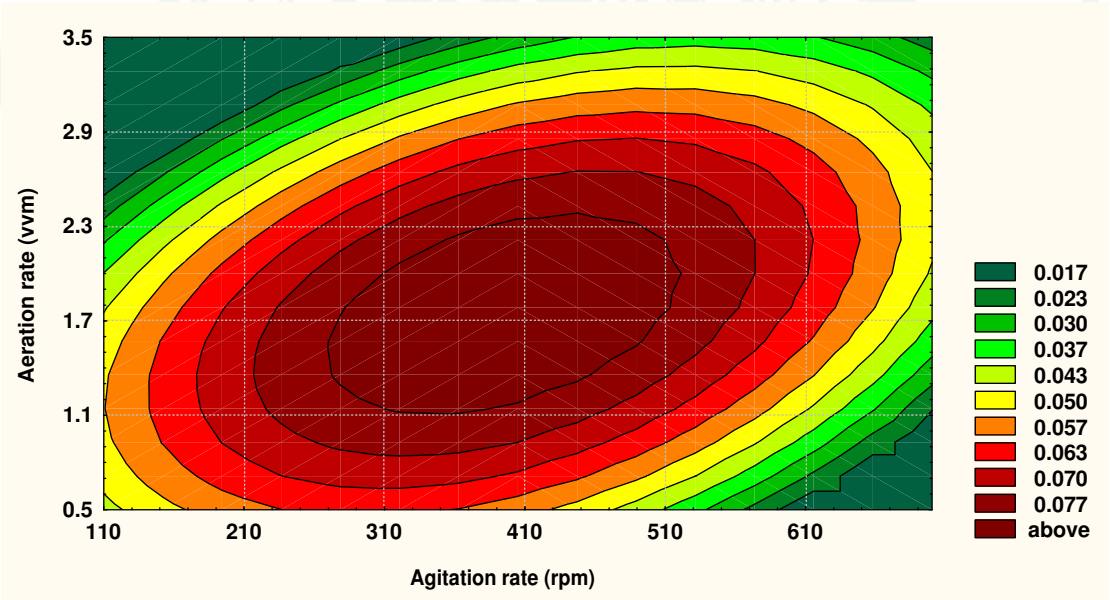


Figure 10 Contour plot for the effects of agitation and aeration rate on specific growth rate by *Bacillus* sp. C4 SS-2013 in stirred tank bioreactor.

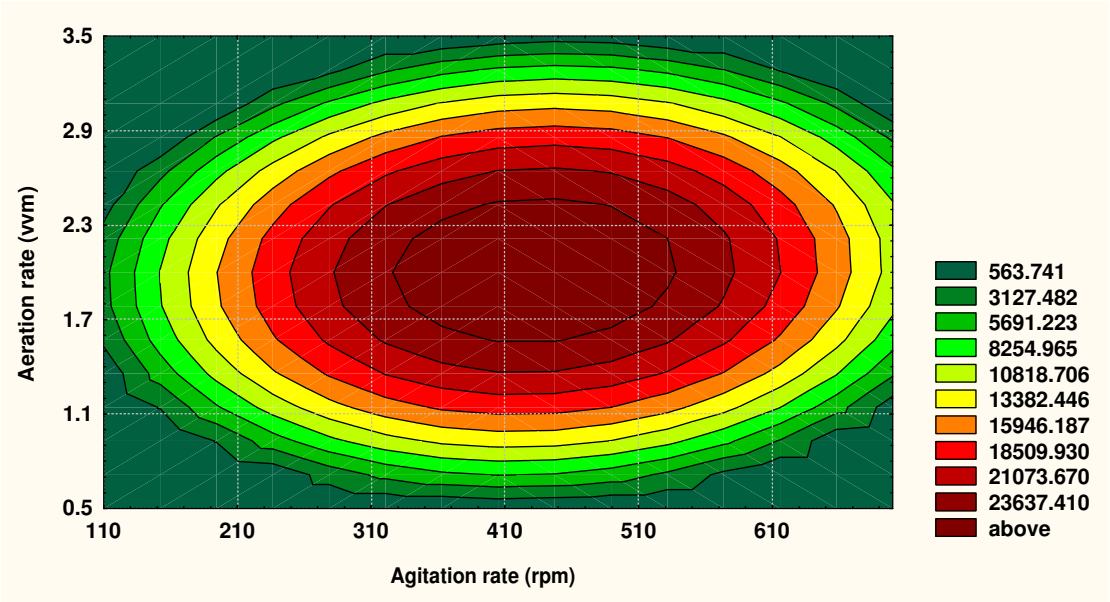


Figure 11 Contour plot for the effects of agitation and aeration rate on specific production rate for protease by *Bacillus* sp. C4 SS-2013 in stirred tank bioreactor.

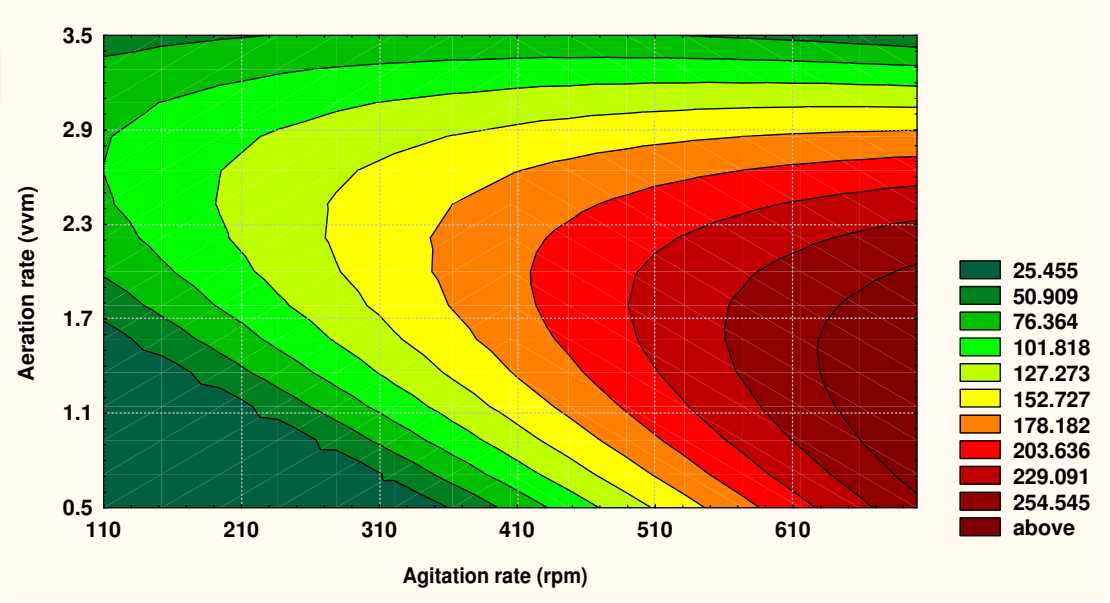


Figure 12 Contour plot for the effects of agitation and aeration rate on K_La in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 for silk degumming protease production.

Morever, the effect of K_La on specific growth rate and specific production rate is also considered as shown in Figure 13. As it is observed, specific growth rate increased with increasing K_La afterward decreased with further increase in K_La, demonstrating that growth was dependent on oxygen supply. Likewise protease production, initially specific production rate increase with increase in K_La up to a limit and then decreased. Many researches have reported similar pattern in case of protease production (Ducros *et al*. 2009), dextransurase production (Chauhan *et al*., 2013) chitinases (Fenice *et al.*, 2012).

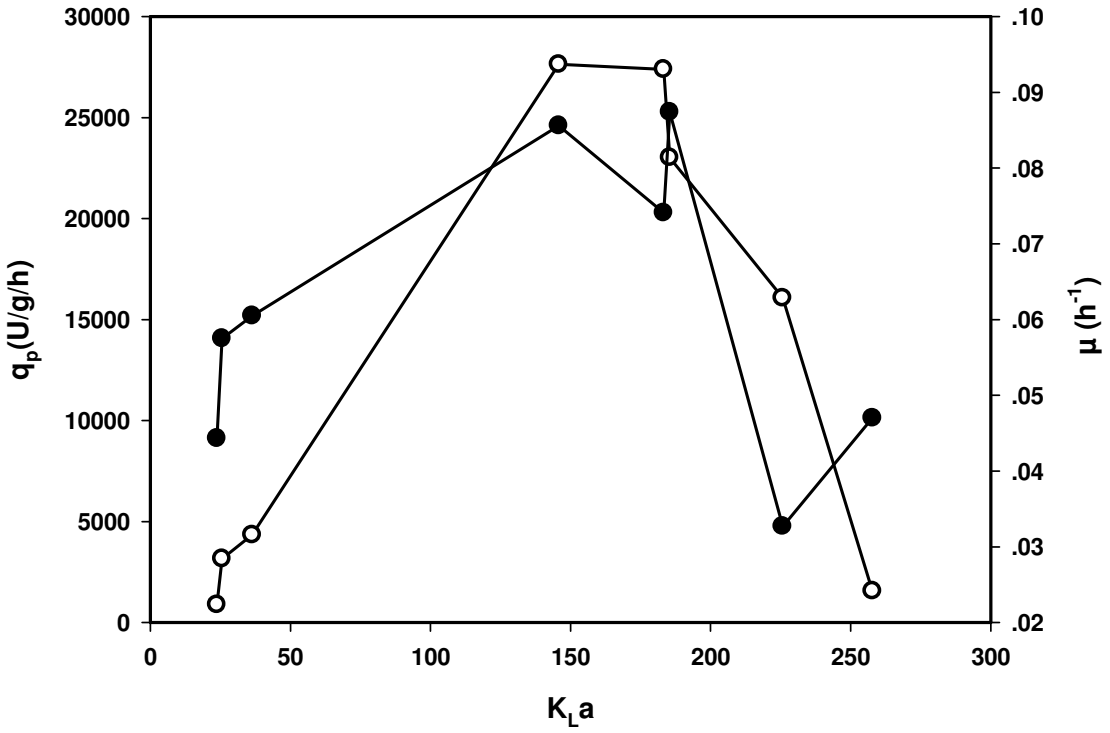


Figure 13 Effect of K_La on specific growth rate(●) and specific production rate (○) for protease by *Bacillus* sp. C4 SS-2013.

According to these above results, it was clear that agitation and aeration rates had significant effects on cell growth, protease production and the specific protease production rate and K_La. At a low aeration and agitation rate, incomplete mixing and/or oxygen transfer resistance probably caused insufficient oxygen supply for the bacterial growth and subsequent enzyme production (Nadeem *et al*., 2009). This indicates reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis (Nascimento and Martins, 2004). Therefore, increasing the agitation rate and aeration rate to appropriate values led to an improvement in the cell growth and protease production due to the better air supply in terms of dissolved oxygen concentration to the cells. The increased rates also provided good mixing of the fermentation broth, thus helping to maintain a concentration gradient between the interior and the exterior of the cells and thus affect the nutrient availability to microorganisms (Moon *et al*., 1991, Chu *et al*., 1992, Rosma and Ooi, 2006). This is especially important for the high biomass of *Bacillus* sp. C4 SS-2013 which is an aerobic microorganism. Moreover, the higher agitation rate may inhibit the growth and activity of microorganisms due to a higher shear stress and may also block the transfer of nutrients and their consumption by disturbing the cells (Bandaiphet and Prasertsan, 2006) and influencing both oxygen transfers and cell activity in the system (Gibbs and Seviour, 1996). Furthermore, the higher aeration rates inhibited cell growth by creating an air dispersion problem in the fermentation system which in turn affected the biomass concentration in the fermenter (Nadeem *et al*., 2009) lead to a drastic reduction in the protease yields (Roychoudhury *et al.* 1988). However, lower and higher agitation or aeration rates resulted in a reduction in alkaline protease productivity which coincided with those values reported from the mutant of *Bacillus licheniformis (*Nadeem *et al*., 2009). This indicates reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis (Nascimento and Martins, 2004). A similar trend was also observed by Silva-Santisteban and Filho (2005), who reported that innulinase production was affected by higher agitation and aeration rates.

Validation of the experimental model was tested by carrying out point prediction and contour plot generation. The optimum value of the combination of the three fermentation variables was determined for maximum protease production. There was high degree of similarity between predicted and experimental values, showing the validity of the RSM. The model was validated by further fitting the best optimized values of agitation rate (400 rpm) and aeration rate (2 vvm). The results demonstrated that maximum protease production (1,890 U/mL) obtained from experimental value as shown in Figure 14, which was close to the predicted value (1,831.94 U/mL). The excellent correlation between the predicted and measured values from these experiments indicates validity of response model. The growth of cells increased after 6 h of cultivation and reached the maximum value of 4.94 g/L in the stationary phase. Protease activity increased gradually until achieved the highest production at 27 h. However, the production dropped after 30 h which could be due to autolysis of the protease that was ensuing upon accumulation of the enzyme in the production media (Olajuyigbe, 2013). The results also revealed that the maximum protease production was obtained at the stationary phase of growth. The correlation of growth, enzyme production and sporulation of *Bacillus* sp. C4 SS-2013 was also investigated. The bacterial growth reached its maximum value after 24 h of cultivation and the protease production substantially increased with the maximum protease activity being obtained at 27 h during the stationary phase which revealed that the enzyme is partially associated with growth. The spore production was started after 24 h and reached its value of 3.4×10^5 spores/mL at 30 h cultivation. The depletion of glucose and oxygen limitation was concomitantly observed and made it possible to synchronize the sporulation (Park, 1992; Monteiro *et al*., 2005). It is known that beside carbon and oxygen, sporulation in *Bacillus* spp. depends on nitrogen and phosphorus in the medium (Dawes and J. Mandelstam, 1970, Anonymous., 2002). Moreover, sporulation is initiated by the accumulation of factors called extracellular differentiation factor (EDF-1) or external pheromone such as sigma E in *Bacillus subtilis* (Jones *et al.*, 1992). This substance is responsible for sporulation by inhibiting vegetative growth and derepress the spore genome. The very early stages of sporulation are reversible and transfer to fresh culture medium results in resumption

of vegetative growth (germination), provided the specific germinants such as L-alanine, inorganic ions asparagines, glucose, fructose, KCl. (Albert G. Moat *et al*., 2002), glutamic acid (Siegenthaler and Hermier, 1964) and L-tryptophan (Grover, 1964) etc. are required. Therefore, the addition of some limiting substrates before nutritional deprivation to prevent sporulation of *Bacillus* sp. C4 SS-2013 by fed-batch culture is a good method to improve the protease yield.

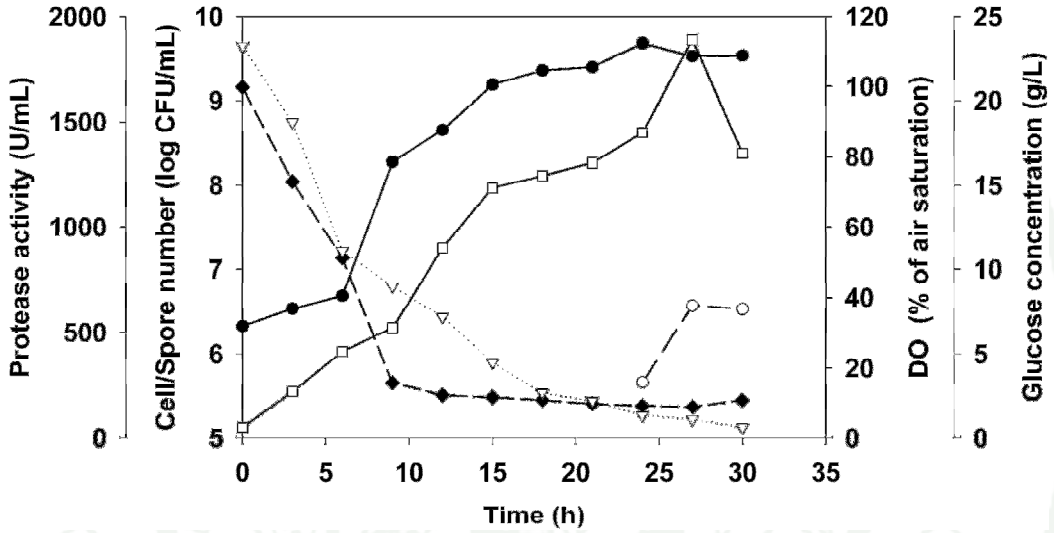


Figure 14 Batch culture of silk degumming protease production in the optimized medium at 400 rpm and 2 vvm in a fermenter. Symbols: cell growth (●), protease activity (□), spore (○), glucose concentration (▽) and dissolved oxygen (◆).

2. Development of a kinetic model for protease production to characterize the fermentation of *Bacillus* sp. C4 SS-2013.

The experimental results for biomass growth-related (μ_{max} and X_{max}), protease production-related (α and β) and substrate consumption-related ($Y_{X/Smax}$ and m_s) parameters were evaluated in the stirred tank bioreactor at optimum condition (400 rpm, 2vvm) using the proposed mathematical model equations, i.e. Eqns (16), (17) and (18). The experimental results and corresponding fits to the model equations are shown in Figure 15. The estimated parameters are summarized in Table 13. The kinetic parameters m_s and $Y_{X/Smax}$ of the obtained and fitted data are higher than the estimated of the simulation by model and the estimated $\mu_{max,}$ X_{max}, α and β are lower than that of this work.

(A)

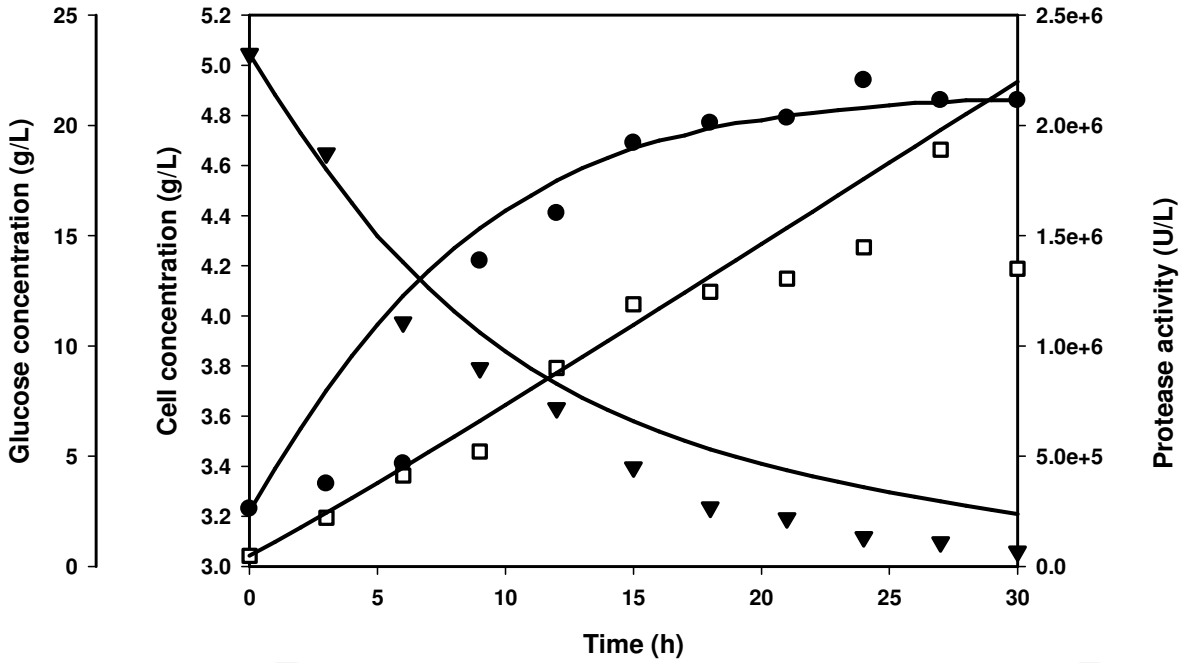


Figure 15 Experimental and model predictions of fermentation kinetics (A); microbial growth (●) using the Logistic model (B), protease activity (□) using the Luedeking–Piret model (C) and glucose concentration (▼) using the Luedeking–Piret like model (D).

(B)

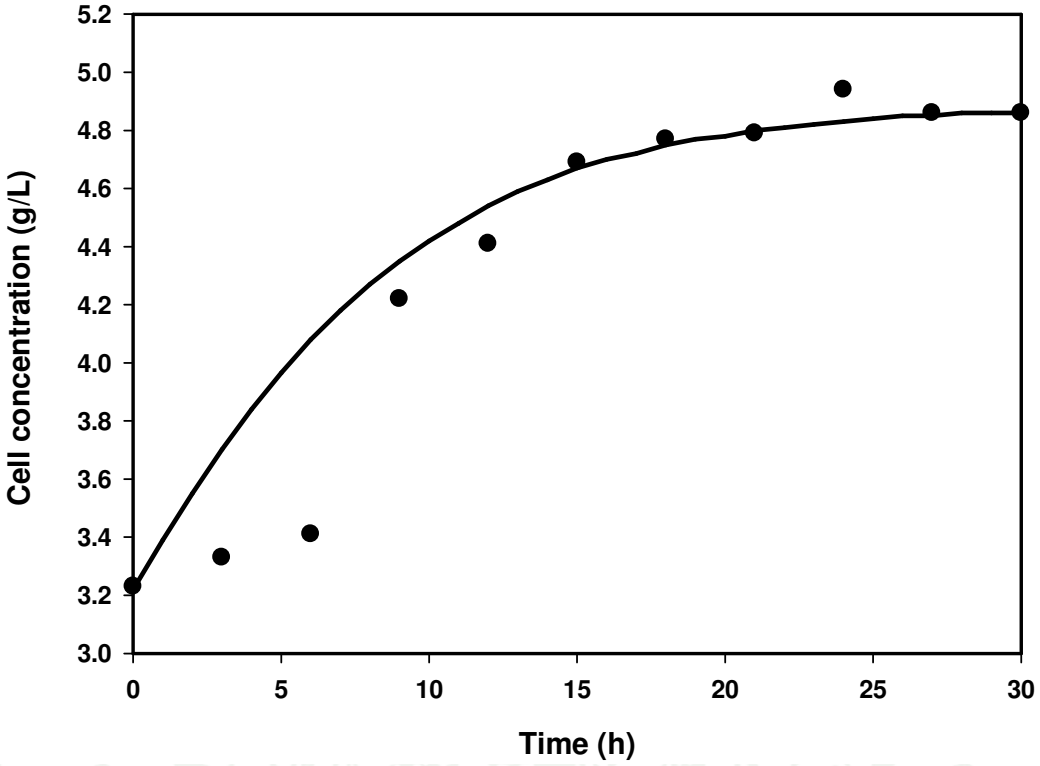


Figure 15 (Continued)

(C)

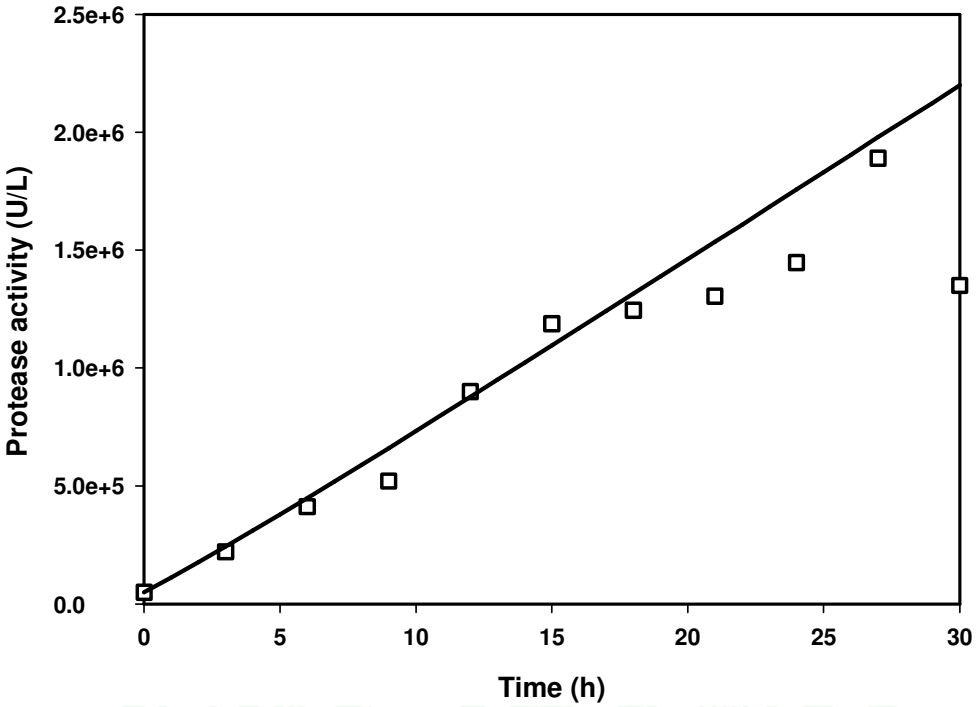


Figure 15 (Continued)

(D)

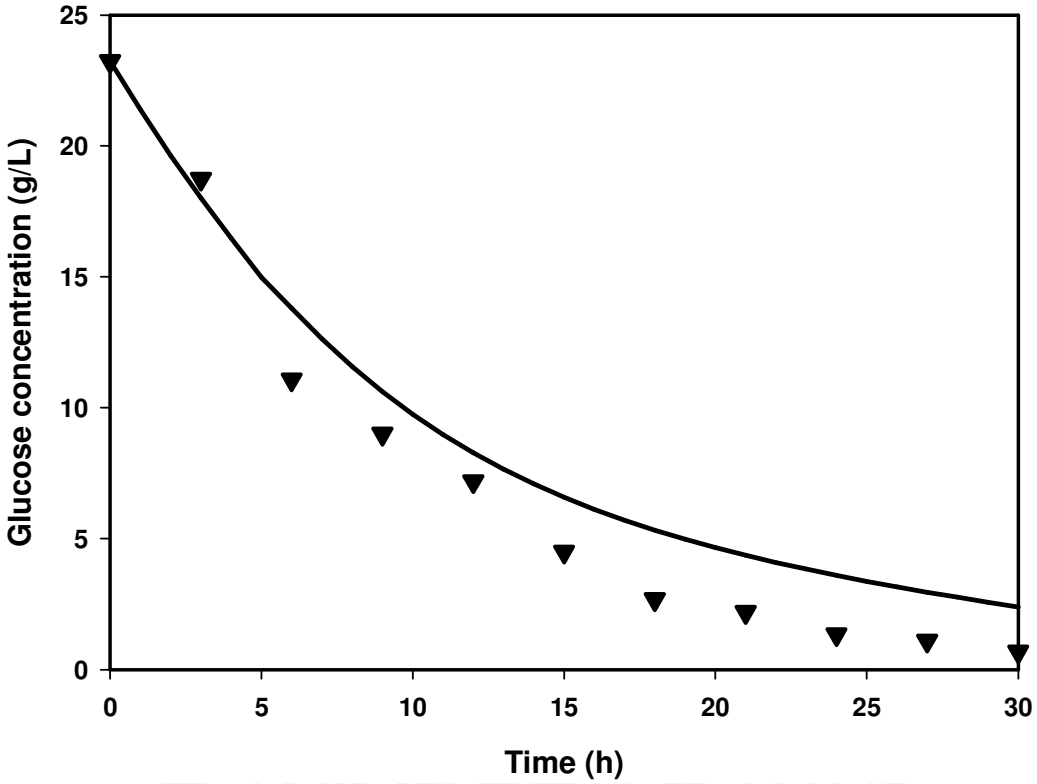


Figure 15 (Continued)

Table 13 Comparison of six parameters obtained from the models and the experiments of protease production by batch culture.

Parameters	Experiment	Estimated
μ_{max}	0.094	0.018
Xmax	4.94	4.89
m_s	0.012	0.03
$Y_{x/s}$	0.095	0.27
α	179,427	176,900
β	22,075	15,095.5

The unstructured models provide a good approximation of the fermentation profile. The data fitted the proposed mathematical model very well (Figure 16). The predicted and the experimental values are demonstrated by the R^2. It shows the strength of the linear relationship between the experimental and predicted values and to find the fitness of these models for cell growth, protease production and substrate concentration kinetics profile of the batch fermentation. In this study, the R^2 values were found to be 0.908, 0.887 and 0.990 for Logistic model to describe the cell growth, Luedeking–Piret model for protease production and Luedeking- Piret like model for glucose utilization, respectively (Appendix table C7, C9 and C11). Figure 15A reveals a typical sigmoid growth trend involving a lag phase, an exponential phase, and a stationary phase, which is similar to previous observations by logistic equation for cell growth (Rajendran *et al*., 2009: Bhunia *et al*., 2012). The glucose concentration was sharply reduced while the cell growth entered exponential phase. The rate of protease production started when the cells entered the exponential phase and the maximum rate of protease was found at about 24 h of cultivation and the Luedeking- Piret model shown the good fit for the protease production. It is indicated that protease production by strain C4 is partially associated with cell growth. Similar results of protease production by other microbial strains were reported (Hernández-Delgadillo and Ruiz-Cruz, 1994; Ladeira *et al*., 2010), however some protease

productions were grouped as growth associated (Rao *et al*., 2009; Bhunia *et al*., 2012).

3. Improvement of silk degumming protease production from *Bacillus* sp. C4 SS-2013 by fed- batch fermentation

To improve the protease production by supplying the main necessary substrates to overcome nutrient starvation and to prolong the protease production, a fed-batch culture was carried out using pH-stat feeding strategy. It is known that the respiration rate of aerobic microorganism is independent of DO above a certain critical level. However, below that level, a small change in DO concentration may cause a physiological alteration in cell metabolism. Therefore, a supply of oxygen to the growing cells is the rate limiting step in many aerobic fermentation processes (Ducros *et al*., 2009). According to the batch culture (Figure 14), the cell growth as well as protease tended to increase at lower rate when DO value was less than 20% air saturation. Thus the fed-batch fermentation was conducted at controlled conditions of agitation 400 rpm and aeration at 2 vvm and the DO concentration was controlled to be greater than 30% of air saturation throughout the fed-batch fermentation. Figure 16 depicts the time courses of fed-batch culture data. After 11 h of batch culture, feed medium was added to the culture broth, the pH of culture broth increased with nutrient consumption and the feed medium was added when pH rose above 7.5. The cell concentration increased up to 5.31 g/L at 42 h. A specific growth rate of 0.012 h^{-1} was obtained and remained almost constant throughout the fermentation. The protease production increased with feeding and reached maximum value of 4,437.34 U/mL at 45 h, resulting in a 2.35-fold increase compared to the batch culture.

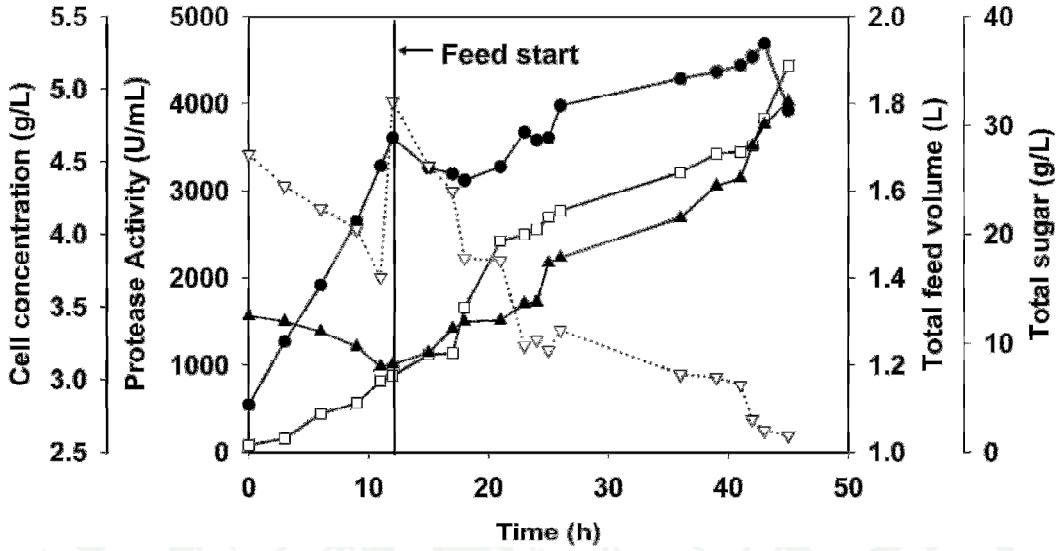


Figure 16 pH stat fed-batch cultures of silk degumming protease production from *Bacillus* sp. C4 SS-2013 in stirred tank bioreactor. Symbols: cell growth (●), protease production (□), total volume culture broth (▲), total sugar (▽).

In summarizing the growth kinetic data on protease production in batch fermentation under different cultivation conditions, it is interesting to notice that variation of the agitation and aeration rates also affected the substrate consumption and product formation rates as it directly affected the cell growth and enzyme formation. The maximum yield of protease production from substrate (Yp/s; 83.48 kU/g. total sugar) was achieved under optimized agitation and aeration rates in the fermenter (400 rpm 2 vvm), which was an improvement of 1.15-fold over the un-optimized medium (BMSM, Fufeungsombut, 2010) in a shake flask and pH-stat fed batch culture. The protease yield based on total sugar (Yp/s) registered a significant improvement from 72.90 to 140.22 kU/g. total sugar (Table 14). Therefore, pH-stat feeding strategy is effective for silk degumming protease production by *Bacillus* sp. C4 SS-2013 and these results were in agreement with other reports by which pH-stat feeding strategy was performed (Li *et al*., 2008; Kuo *et al*., 2009; Cho *et al*., 2010).

Table 14 Comparison of parameters of silk degumming protease production by *Bacillus* sp. C4 SS-2013 in batch fermentation under different cultivation conditions.

Treatment	Maximum protease production (U/mL)	μ	q_p	$Y_{x/s}$	$Y_{p/s}$	Reference
Unoptimized medium*	729	0.074	3,037.5	0.560	72.90	Romsomsa *et al.* (2010)
Optimized medium in shake flask **	1,537	0.071	16,604	0.076	82.19	Romsomsa *et al.* (2010)
Optimized agitation and aeration rate in stirred tank bioreactor (400 rpm 2 vvm)	1,890	0.084	21,412	0.073	83.48	This study
pH-stat fed batch culture	4,437	0.012	36,820	0.125	140.22	This study

* BMSM medium in shake flask with shaker speed was controlled at 200 rpm

** Optimized medium (2% w/v of total sugar content in hydrolyzed starch) in shake flask with shaker speed was controlled at 280 rpm (previous study)

μ: Specific growth rate (h^{-1}); q_p: Specific production rate for protease (U/g/h); Yx/s: yield of cell (g. cell/g. total sugar consumed); Yp/s: yield of protease (kU/g total sugar consumed).

Furthermore, the production of protease in optimized medium was confirmed by native-PAGE activity gels which were obtained from fermentation broth samples taken at different fermentation time intervals (Figure 17). No protease activity was detected at the beginning of fermentation (Lane 1). However, crude enzyme from the 21, 22, 24 h culture broth showed clear zones of protease activity after staining with Coomassie blue.

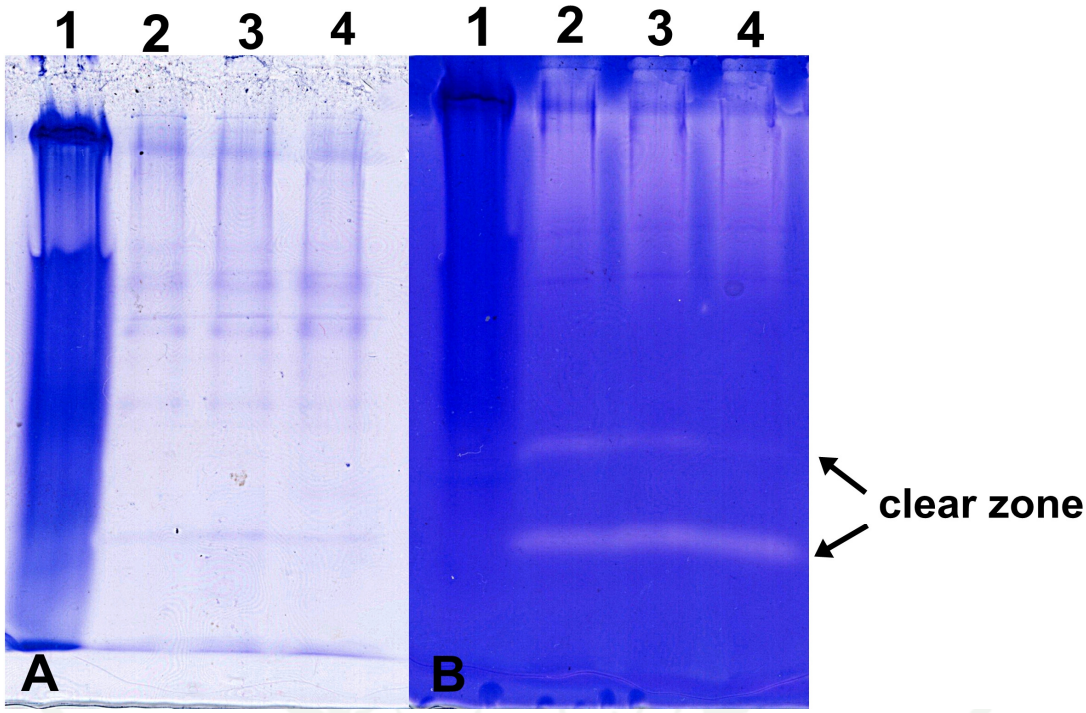


Figure 17 Gelatin (A) and Native-PAGE (B) zymogram analysis showing bands of different fermentation times. Lanes (left to right). Lane 1–4: 0, 21, 22 and 24 h old culture of crude protease, respectively.

In the present study, *Bacillus* sp. C4 SS-2013 provided higher protease production in batch fermentation when compared with other researches as shown in Table 15. The cessation in protease production was observed during batch process due to autoproteolysis (Jang *et al*, 2001) and degradation by some proteolytic activity on the cell surface of nitrogen starved cells have been proposed (Chu *et al*., 1992; Beg *et al*, 2003). However, the protease activity and product yield of fed-batch culture in this

experiment resulted in higher values as compared to other report (Tabandeh *et al*., 2011). It would be expected to gain even the higher production if repeated fed-batch culture was employed consecutively. Moreover the optimized medium used here consisted of cheap raw materials (cassava and soybean flour) which are abundantly available in Thailand to lower the cost of production. Recently we improved the protease production approximately 6-fold compared with un-optimized medium (BMSM) in a shake flask (729 U/mL) and it would worthwhile to scale up to the industrial level.

Table 15 Comparison of the batch fermentation data for protease production by various microorganisms.

Microorganism	Protease production (U/mL)	Reference
B. subtilis	9.6	ul-Haq and Mukhtar (2006)
B. licheniformis UV-9	1,270	Nadeem *et al.* (2009)
Bacillus sp.	896.5	Irfan *et al*. (2010)
B. *megaterium* IBRL MS 8.2	155.38	Darah *et al*. (2013)
Bacillus sp.	3.56	Prasad *et al.* (2014)
Pseudomonas putida SKG-1	882	Singh *et al.* (2011)
Rhizopus oryzae CH4	258.2	M'hir *et al.* (2012)
Bacillus subtilis C4	1,890	This study

4. The effect of stabilizers on silk degumming protease activity from *Bacillus* sp. C4 SS-2013.

Though *Bacillus* sp. C4 SS-2013 can produce high protease activity in the optimized medium, it is not stable during storage. To improve the stability of crude protease, a preliminary study was undertaken on the effect of several additives, namely $CaCl_2$, sodium azide, glycerol, and polyethylene glycols (PEG), which were evaluated under different temperatures, namely, room temperature (30 to 35°C), 4°C, and -20°C. As shown in Figure 18, among the additives examined, the highest relative activity was obtained in the presence of 10 mM $CaCl_2$ combined with 10% (v/v) PEG 400 at 4°C which remained at 67.05% of the initial activity after storage for 3 weeks. In the presence of 10 mM $CaCl_2$, 25% glycerol, 0.02% sodium azide and 10% PEG 400 the activity remained at 66.27, 47.47, 42.11% and 55.28%, respectively, after storage at 4°C for 3 weeks. Generally, proteins are best stored at ≤4°C, which this condition can prevent microbial or proteolytic degradation. The crude protease stored at -20^{O}C showed very low activity (10.38 %) and addition of 25% glycerol could not much improve the shelf-life (18.46%). Glycerol acts as a cryoprotectant which prevents the formation of ice crystal during cold storage. However, the addition of 25% glycerol may not be sufficient to stabilize the protein structure and the final concentration of 25 to 50% will be more desirable. While storing at room temperature resulted in a substantial loss in its activity after the first week. It may be due to protein degradation which commonly causes by microbial growth. This result concluded that the suitable storage temperature was 4°C and either $CaCl_2$ or $CaCl_2$ with PEG 400 could be good stabilizers for the storage of crude protease from strain C4.

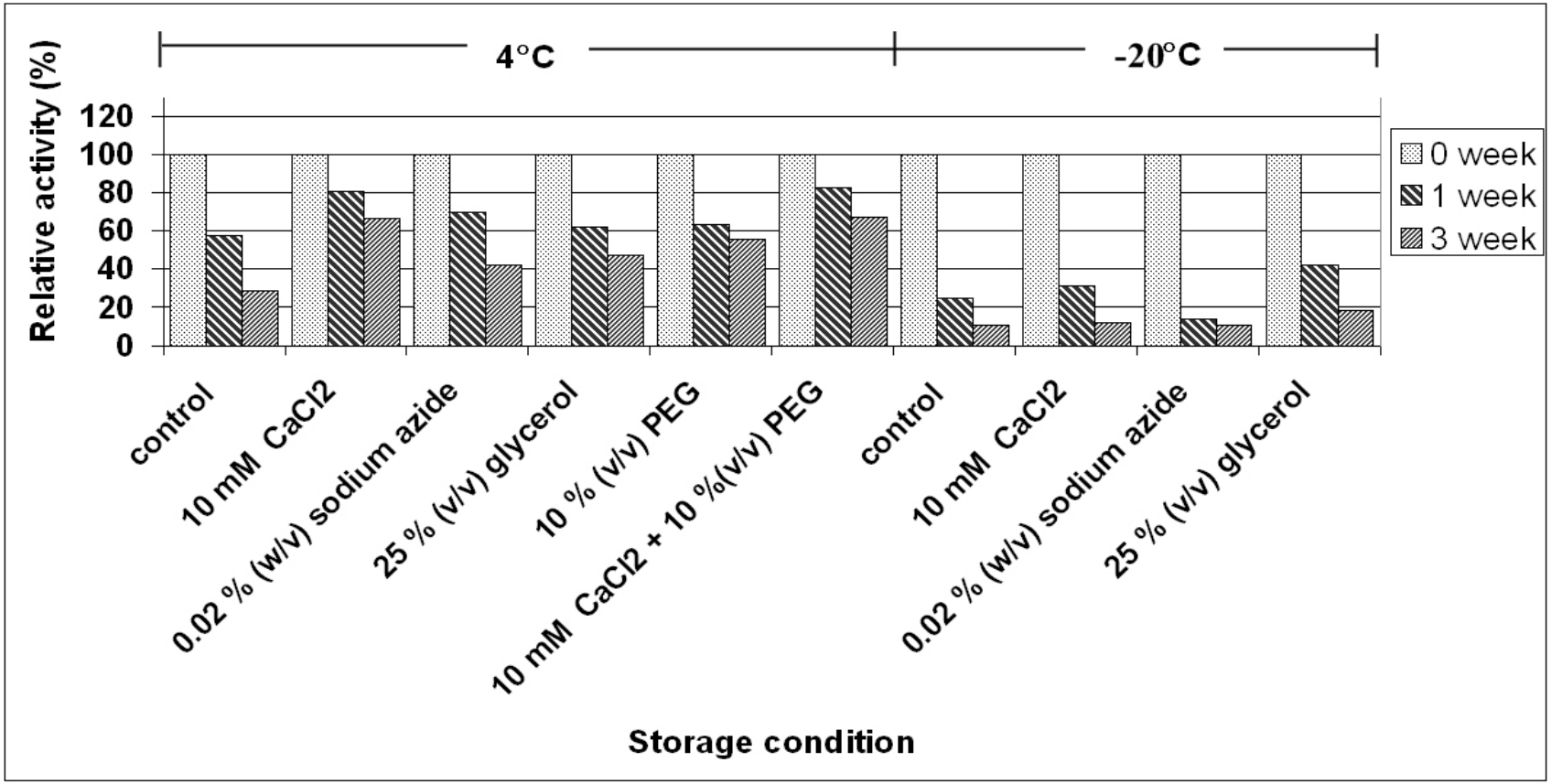


Figure 18 Relative activity of crude protease from *Bacillus* sp. C4 SS-2013 in the presence of various additives during storage for 3 weeks.

Furthermore, the effect of different concentration of $CaCl_2$ on the protease activity was also studied. The storage experiment of crude protease at 4°C with 10 mM $CaCl_2$, 10% PEG 400 or their mixture were repeated for a longer period (8 weeks) as shown in Figure 19. The residual crude protease activities were about 52%, 42% and 57%, respectively indicating that $CaCl_2$ could maintain the shelf life of the crude protease as well as the mixture of $CaCl_2$ and PEG 400 and the suitable concentration of $CaCl_2$ should be 10 mM. The lower (1 mM) or higher (100 mM) concentration of $CaCl_2$ had little effect on its stability (Figure 20). From the result of $CaCl_2$ may be explained by the strengthening of interactions inside protein molecules and by the binding of Ca^{2+} to autolysis sites. This binding is at specific sites, often external loops, which reduce the flexibility of the molecule and therefore its denaturation and autolysis (Siezen and Leunissen, 1997; Toogood *et al*., 2000). Calcium ions act as folding catalysts of many secreted protease from *Bacillus* spp. (Petit-Glatron *et al*., 1993; Harwood, 2008) especially thermolysin and subtilisin E, and it is crucially involved in stabilizing the N-terminal residues in the mature protease (Veltman *et al.*, 1998; Alexander *et al.*, 2001). Whereas protective effects of PEG were explained by the strengthening of the hydrophobic interactions inside protein molecules, inhibit protein unfolding during the freezing step of lyophilization (Taravati *et al.,* 2007) and by indirect action of polyols on water structure (Asther and Meunier, 1990; Gonzalez *et al.,* 1992).

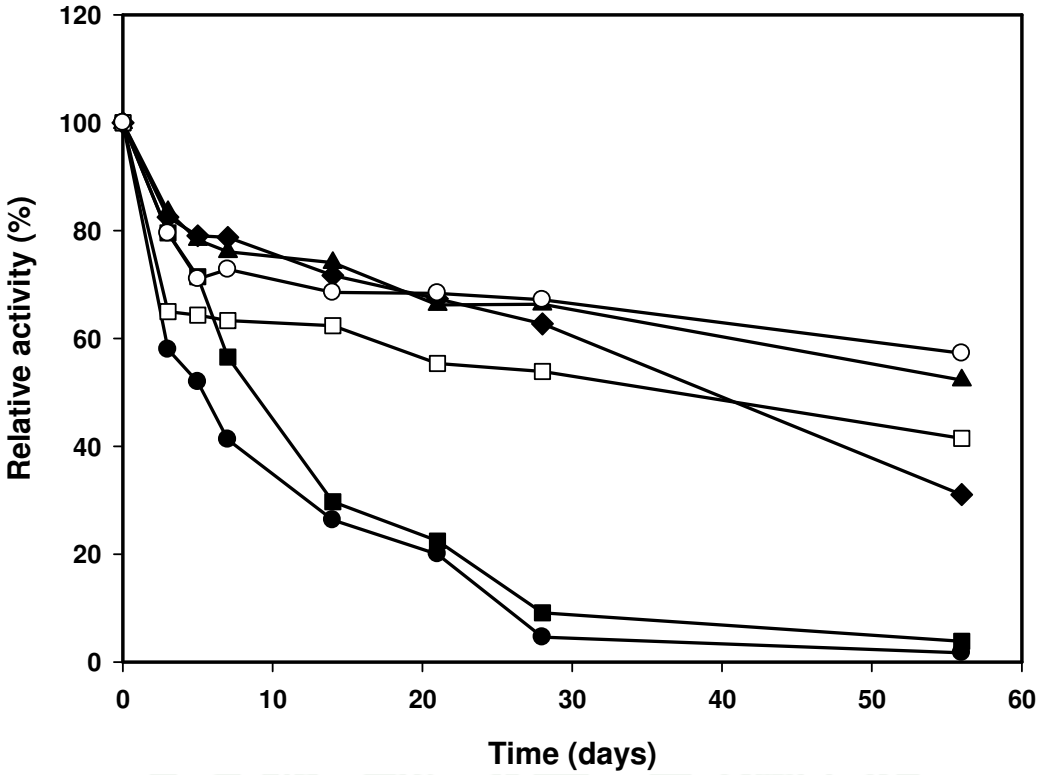


Figure 19 Relative activity of protease from *Bacillus* sp. C4 SS-2013 in the different concentration of calcium choride (●) control, (■) 1 mM, (▲) 10 mM, (◇) 100 mM (□) 10%(v/v) PEG 400, (○) 10 mM $CaCl_2$ mixed with 10%(v/v) PEG 400 during storage at 4°C for 56 days (8 week).

With a view to increase the storage stability, residual protease activity was analyzed after addition of $CaCl_2$ into crude protease and kept at 4°C. As shown in Figure 20, the proteases activity retained above 64.25 % of their initial activities up to the 8th week with the addition of 10 mM $CaCl_2$ in the crude enzyme which was about 4 times improved in the activity as compared to the control (16.41%) at 4°C. These results indicated that the addition of $CaCl_2$ in the crude enzyme can affect protease structure by directly interaction with their molecule, thus the stabilization of the protease during storage was very effective by addition of $CaCl_2$ in the crude enzyme solution which was coincident with Ma *et al.* (2006).

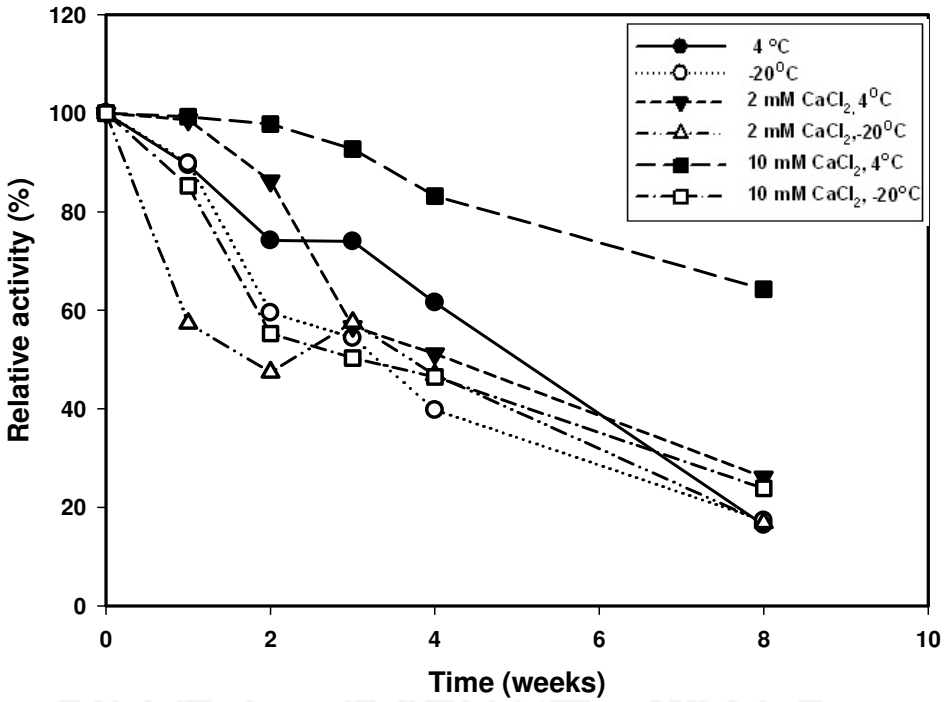


Figure 20 Comparison of the addition of calcium chloride for protease stability from *Bacillus* sp. C4 SS-2013 during storage.

Form the results it clearly appears that the suitable storage condition of crude protease from strain C4 was addition directly of 10 mM $CaCl_2$ into crude enzyme solution. Therefore the suitable storage temperature was 4°C and either $CaCl_2$ or $CaCl_2$ with PEG 400 could be good stabilizers for the storage of crude protease.

5. The effective crude protease from *Bacillus* sp. C4 SS-2013 for silk degumming.

The degumming efficiency of the crude protease obtained when growing in the optimized medium and cultivation condition was studied. The result showed a powerful silk degumming ability. The weight loss of degummed silk fiber was about 25.31±2.98, 23.70±1.41 and 17.68±1.78 % of the total dry weight of the raw silk yarn after incubation under mild conditions at pH 8 and 50^{o}C for 2 h using, 5 –fold dilution (5X; 380 U/mL), 2- fold dilution (2X; 780 U/mL) and undiluted crude protease (1,560 U/mL), respectively (Figure 21). From the visible appearance with the degumming, silk fiber became whiteness, soft and smooth surface as compared to the raw silk. It could estimate that both undiluted of crude protease and 2- fold dilution of crude protease could completely remove sericin within 2 h and better than 5- fold dilution of crude protease.

The scanning electron micrographs of the silk yarns before and after degumming are shown in Figure 22. Sericin appeared as a partial non-uniform coating on the surface of raw silk yarn (Figure 22A) which was rough with granular deposits. The degummed silk by sodium carbonate and undiluted crude protease showed similarly good results of degumming ability and uniform removal of sericin with no sign of destruction to the silk surface or fibroin (Figure 22I and 22K) The degummed silk by diluted of 2X crude protease gave fairly good results although the removal of the sericin was not very uniform (Figure 22G). While, the more diluted enzyme (5X) showed a poor sericin removal (Figure 22E).

This result ensures that the optimized medium can replace BMSM medium (previous medium) which is more expensive. The crude protease form stain C4 is considered good and suitable for silk degumming process. It degrades only sericin effectively but not fibroin because of the greater specificity of the enzyme to sericin on silk yarn. In silk degumming process, sericin is degraded into sericin peptide or hydrolyzed sericin with a molecular weight loss. Also, the decrease in the yellowness index may originate from the existence of natural yellow colorants in sericin structure

that were removed during the degumming treatment (Talebpour *et al*., 2013). In the addition, the degumming efficiency by crude protease from this strain showed similar weight loss by enzyme treatment when compared with other reports; 17.6-24% (Freddi *et al*., 2003), 21.72% (Sumana *et al*., 2013), 19.58-21.78% (More *et al*., 2013). Therefore, the crude protease from *Bacillus* sp. C4 SS-2013 showed reasonable results in terms of degumming efficiency, silk fibric properties, the cost and eco-friendly.

(A)

(B)

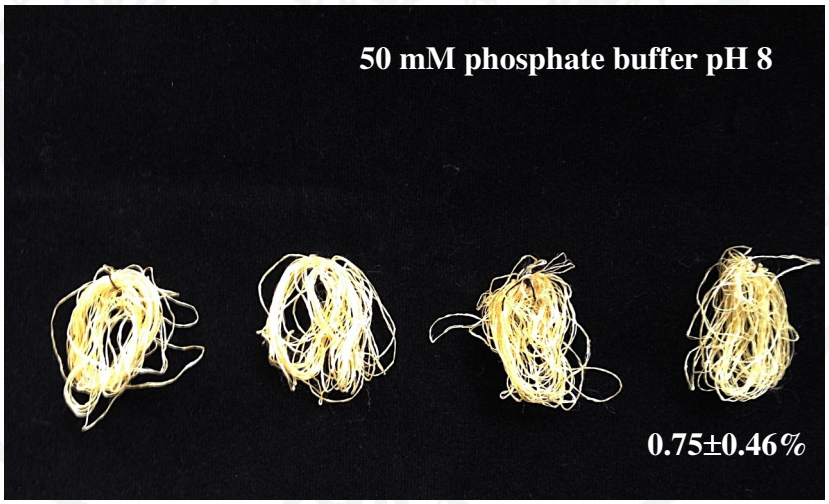


Figure 21 Silk degumming efficiency determined visible appearance of the treated silk fibers; before degumming(A), 50 mM phosphate buffer pH 8 (B), degummed 0.05% Na_2CO_3 (C), degummed with undiluted crude protease (D), degummed with diluted 2X of crude protease (E), degummed with diluted 5X of crude protease (F).

(C)

(D)

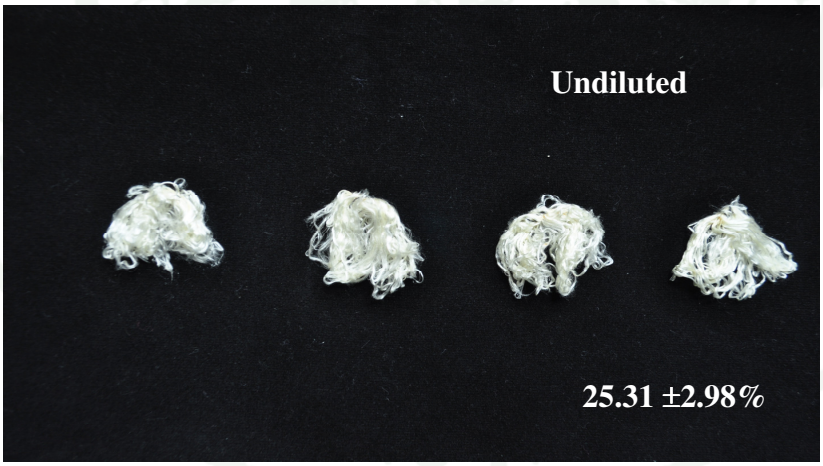
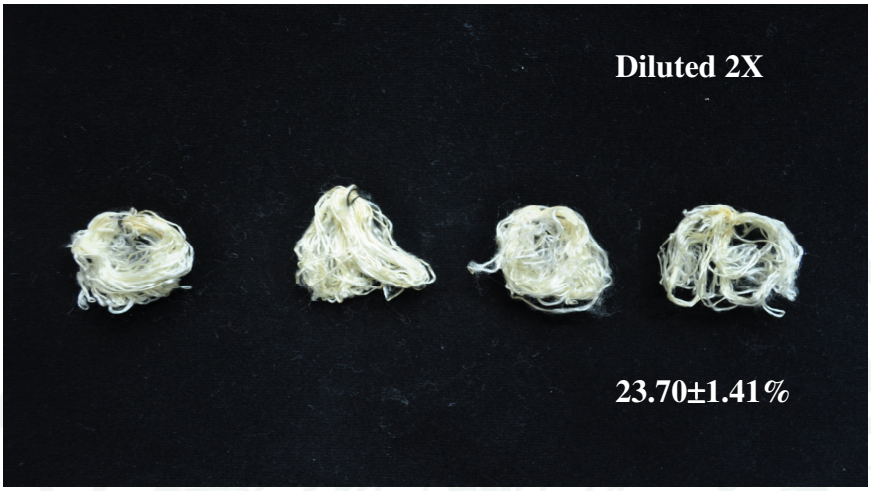


Figure 21 (Continued)

(E)



(F)

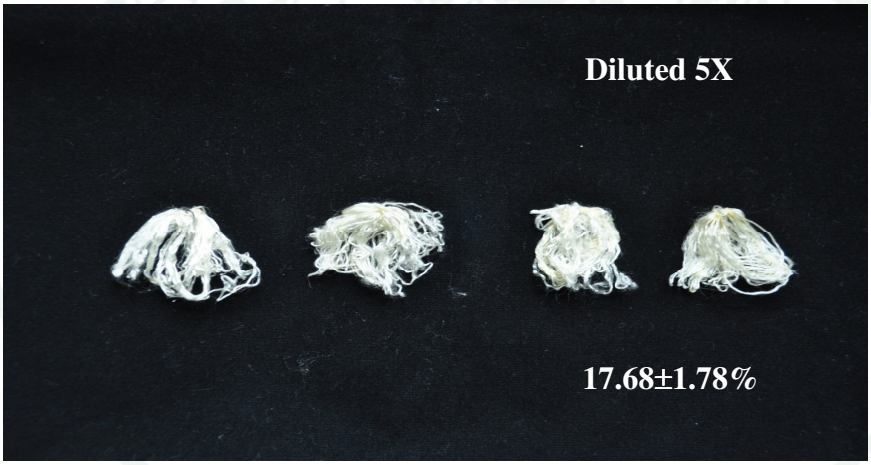


Figure 21 (Continued)

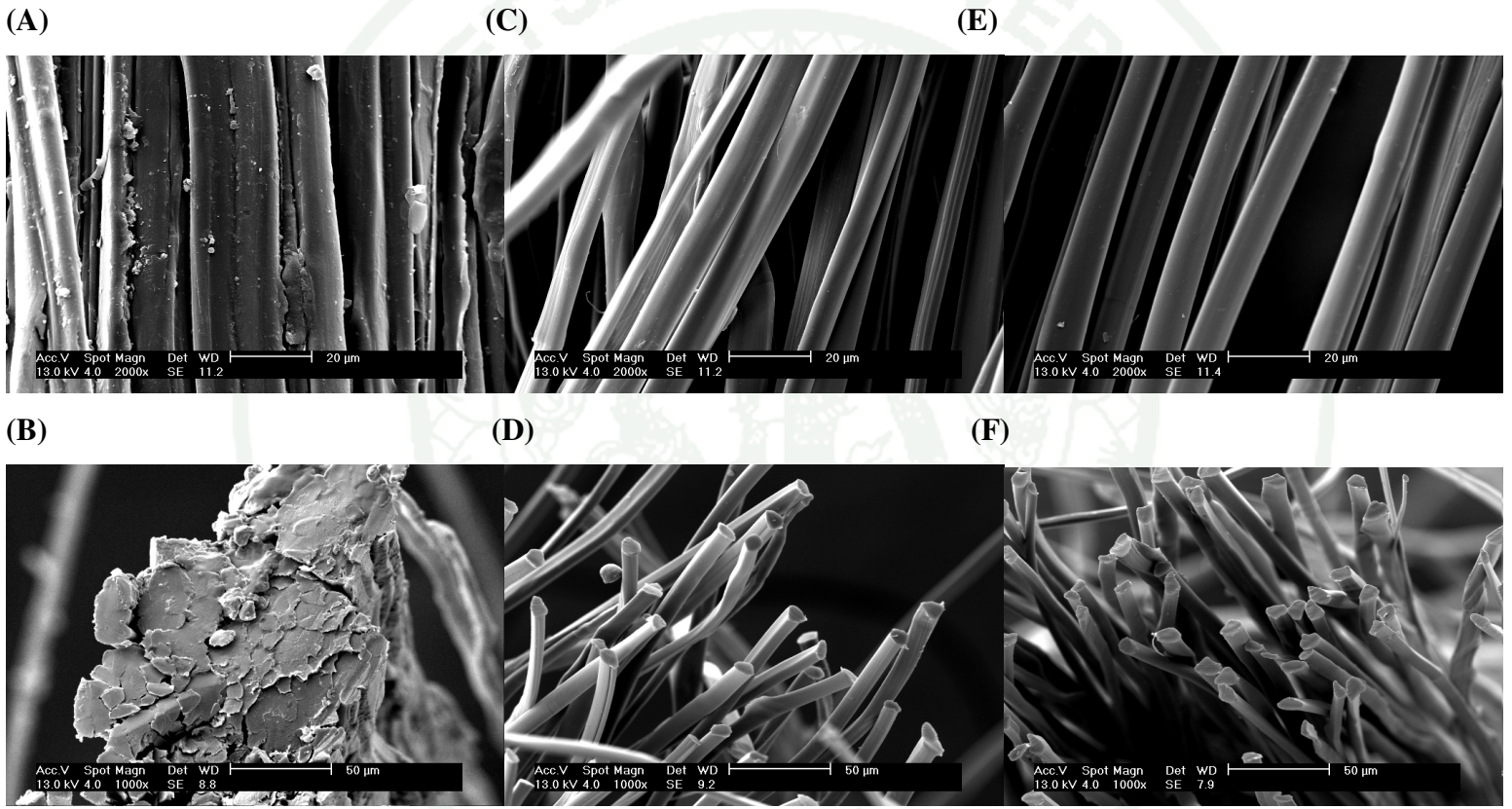
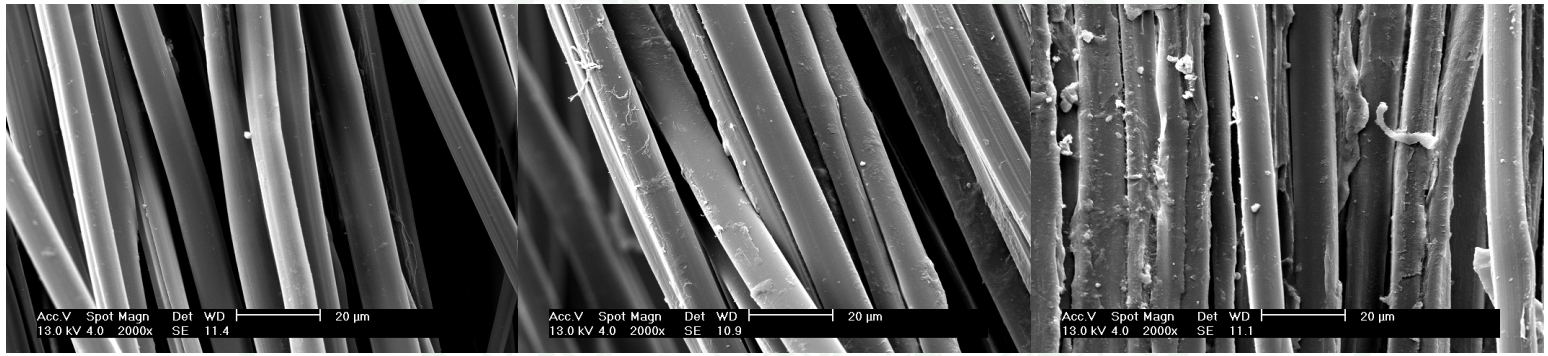


Figure 22 Comparison of fibroin (silk yarn) destruction after degumming processes by using SEM; raw silk yarn (A, B), 0.05% Na_2CO_3 (C, D), 50 mM phosphate pH 8 (E, F), undiluted crude protease (G, H), diluted 2X crude protease (I, J), diluted 5X crude protease (K, L).

(G) (I) (K)



(H) (J) (L)

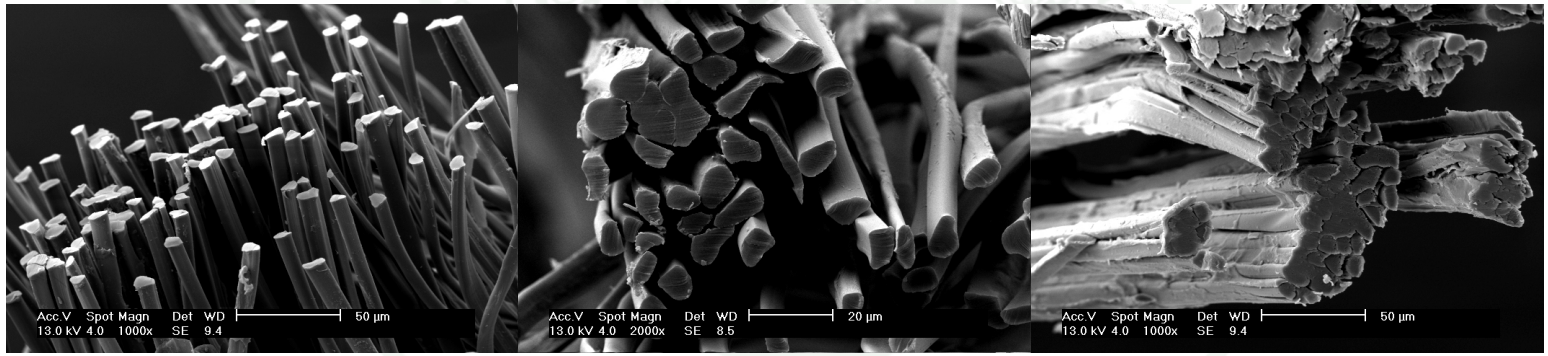


Figure 22 (Continued)

CONCLUSION AND RECOMMENDATIONS

Conclusion

In this study, the optimization of the agitation and aeration rates to improve the growth and silk degumming protease production by batch culture was performed in a fermenter using CCD and RSM methodology. Both the agitation and aeration rates significantly affected the protease production, specific protease production rate, specific growth rate and K_La. There was an increase in the protease production with the increase of agitation and aeration rate. However, increasing the agitation and aeration rates beyond these values resulted in a decrease of protease production. The similar trends were observed for the specific protease production rate, specific growth rate and K_La. The generated model was satisfied all the necessary arguments for its use. The adequacy of the model, the specific growth rate, the specific protease production rate and K_La were assessed by carrying out ANOVA. The resultant values of R^2 were 0.950, 0.919, 0.931 and 0.881, respectively. It is suggested that the model is very significant. The model was also validated by repeating the experiments under the optimized conditions, which resulted in the closeness of the predicted values and the experimental values, which indicates the good fit of the model. The optimum levels of agitation and aeration rates were accordingly determined as 400 rpm and 2 vvm, respectively, which provided the maximum enzyme activity of 1,890 U/mL. The correlation of growth, enzyme production and sporulation of *Bacillus* sp. C4 SS-2013 was also observed. The protease production was corresponded with the sporulation and the enzyme was partially associated with growth.

The unstructured models provide the good approximation of cell growth, protease production and substrate concentration kinetics profile of the batch fermentation. The data fitted the proposed mathematical model fairly well. The R^2 values were found to be 0908, 0.887 and 0.990 for Logistic model for cell growth, Luedeking- Piret model for protease production and Luedeking- Piret like model for substrate utilization, respectively. The rate of biomass and protease production started

when the cells entered the exponential phase and the maximum rate of protease was found at about 24 h of cultivation indicating the protease production was partially associated with cell growth.

The improvement of silk degumming protease production was achieved by the pH-stat fed-batch culture. The cell concentration increased up to 5.31 g/L at 42 h. A specific growth rate of 0.012 h^{-1} was obtained and remained almost constant throughout the fermentation. The protease production was improved to 4,437.34 U/mL at 45 h, resulting in a 2.35-fold increase compared to the batch culture. Recently we improved the protease production approximately 6-fold as compared to un-optimized medium (BMSM) in a shake flask (729 U/mL) and it would worthwhile to scale up to the industrial level. Moreover, the silk degumming efficiency of crude protease cultivated in the optimized medium was rechecked. It could relatively remove sericin (degum) about 25.31±2.98 % weight loss of the total raw silk which corresponded to 92.76±1.16 % sericin removal and the texture of the fiber became whiteness, soft and smooth as compared to untreated sample.

The stability of crude protease of strain C4 was also studied. Among the additives examined, the highest relative activity was obtained in the presence of 10 mM $CaCl_2$ combined with 10% (v/v) PEG 400 and kept at 4°C. The addition methods of stabilizer for storage of protease were compared, with the addition of 10 mM $CaCl_2$ in the crude enzyme that remained 64.25% of the initial activity when stored at 4°C for 8 weeks. Thus, the stabilization of the protease during storage was very effective by addition of $CaCl_2$ in the crude enzyme solution. In conclusion, the crude protease from *Bacillus* sp. C4 SS-2013 showed reasonable results in terms of degumming efficiency, silk fiber properties, the cost as well as eco-friendly and worthwhile for industrial application.

Recommendations

In this study have attempted to investigate the stabilization of silk degumming protease during storage, the suitable of the storage condition with 10 mM $CaCl_2$ at 4^oC, which hold 52% of initial activity for 8 week was observed. In order to improve stability for long term, the more suitable additive or lyophilization is recommened. Athough the optimization of agitation and aeration rate for the protease production in stirred tank bioreactor include with improvement by pH-stat fed batch culture were achieved, the exponential fed batch or multi-stage culture is interesting for studies in the future. The more suitable kinetic models should be further considered. However, some nutrient composition in optimized medium is expensive thus the cheaper and avariable nutrient should be found out for scale up to the industrial level. Finally, it is hoped that the results obtained from this study provide basic knowledge in understanding the kinetic behavior and implicated improvement silk degumming protease production in industrial scale.

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APPENDICES

Appendix A

Culture Media

1. Davis minimal medium (Davis and Mingioli (1950))

Raw silk	1.0	g
K_2HPO_4	7.0	g
KH_2PO_4	3.0	g
$MgSO_4 \cdot 7H_2O$	0.1	g
Distilled water	1.0	L

The pH was adjusted pH to 7.5 with 3 N NaOH and sterilization was performed by autoclaving at 121 at °C, 15 min.

2. Basal medium agar (BMSM agar) (Horikoshi (1991))

Glucose	10.0	g
Yeast extract	5.0	g
K_2HPO_4	1.0	g
$MgSO_4 \cdot 7H_2O$	0.2	g
Skim milk	10.0	g
Agar	15.0	g
Distilled water	1.0	L

The pH was adjusted pH to 7.5 with 3 N NaOH and sterilization was performed by autoclaving at 110 at °C, 15 min.

3. Basal medium broth (BMSM broth) (Horikoshi (1991))

Glucose	10.0	g
Yeast extract	5.0	g
K_2HPO_4	1.0	g
$MgSO_4 \cdot 7H_2O$	0.2	g
Skim milk	10.0	g
Distilled water	1.0	L

The pH was adjusted pH to 7.5 with 3 N NaOH and sterilization was performed by autoclaving at 110 at °C, 15 min.

4. Nutrient agar (NA)

Beef extract	3.0	g
Peptone	5.0	g
Agar	15.0	g
Distilled water	1.0	L

The pH was adjusted pH to 7.5 with 3 N NaOH and sterilization was performed by autoclaving at 121 at °C, 15 min.

Appendix B

Preparation of reagents and analytical method

1. Dry weight cell measurement

1.1 Dry in an oven an empty aluminum weighing pan, weigh and store them in a desiccator (W_1).

1.2 Pour out 100 mL of the culture into a graduated cylinder.

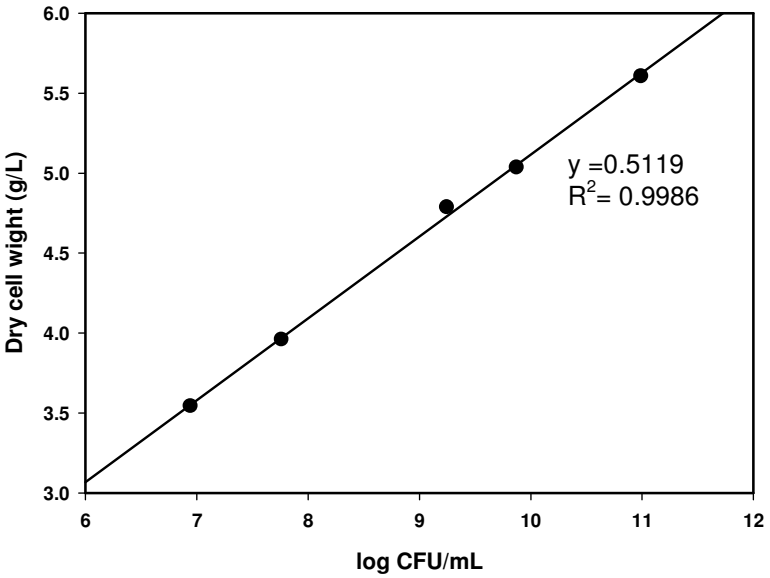
1.3 Centrifugation at 10,000 g for 5 minutes for deparate the cells from the broth.

1.4 Rinse the centrifuge tube with a few mL of water and repeat 2-3 times, pour the rinse water into the weighing pan.

1.5 Dry the cell paste in an oven set at 100ºC for 24 h, weigh and store them in a desiccator (W_2)

1.6 Calculate the difference in the weight, and express the dry weight in g/L.

$$\text{Dry weight cell (DCW) (g/L)} = \frac{(W_1 - W_2)}{100 \text{ mL}}$$



Appendix Figure B1 Standared correlation betweem dry cell weight and log CFU/mL.

2. Preparation of Phosphate buffer

Solution A: 0.2 M monobasic sodium phosphate (dissolved 31.2 g of $NaH_2PO_4 \cdot 2H_2O$ in 1,000 mL of distilled water)

Solution B: 0.2 M dibasic sodium phosphate (dissolved 53.65 g of $Na_2HPO_4 \cdot 7H_2O$ or 71.7 g of $Na_2HPO_4 \cdot 12H_2O$ in 1,000 mL of distilled water)

Working reagent was prepared by mixing solution A and B in the ratio as below.

A (mL)	B (mL)	pH	A (mL)	B (mL)	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	90.5	7.7
62.5	37.5	6.6	8.7	91.5	7.8
56.5	46.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

3. Preparation for protease activity assay

3.1 Solution

3.1.1 Substrate solution

For protease assay by using 2% (w/v) casein as substrate, was prepared by dissolving 2 g of casein in 50 mM phosphate buffer, pH 8 and adjusted volume to 100 mL with 50 mM phosphate buffer, pH 8.

3.1.2 Trichloroacetic acid (TCA) solution

10 % (w/v) Trichloroacetic acid (TCA) solution as stop solution, was prepared by dissolving 10 g of TCA adjusted volume to 100 mL with 50 mM phosphate buffer, pH 8.

3.2 Analysis

Reaction mixture containing 250 μL of enzyme solution and 250 mL of 2 % (w/v) casein in 50 mM phosphate buffer, pH 8 was incubated at 60° C for 10 min. The enzyme reaction was stopped by adding 500 μL of 10 % TCA. The reaction mixture was allowed to stand for 3 h at 4 °C and then was centrifuge at 10,000 rpm for 10 min. The supernatant collected from the centrifugation was assayed for protease activity. The protease activity was calculated according to the modified Lowry method (Lowry *et al*., 1951) using a tyrosine standard curve. One unit of protease activity was defined as 1 μg tyrosine liberated per mL under the assay conditions.

3.3 Calculation of protease activity

$$\text{Protease activity (U/mL)} = \frac{(T_1\text{-}T_2) \times D}{t \times V}$$

T_1 is concentration of tyrosine from sample (µg/mL)

T_2 is concentration of tyrosine from conrol (µg/mL)

V is total volume of assay (1 mL)

T is total volume of assay (10 mL)

4. The modified Lowry method (Lowry *et al.*, 1951)

4.1 Tyrosine standard stock solution

4.1.1 Preparation for tyrosine standard stock solution

Weight exactly 0.1000 g of tyrosine and dissolved in 0.1 M HCl and then adjusted volume to 100 mL in distilled water, the solution was kept as stock solution. The 100µg/mL of standard solution by was prepared by reaction mixture was shown as follows.

Distilled water (µL)	100	80	60	40	20	0
Stock solution of tyrosine (µL)	0	20	40	60	80	100

4.1.2 Lowry-Folin protein assay reagents

Solution A: Weight of 0.5 g of $CuSO4.5H_2O$ and 1 g of sodium citrate and dissolved in 100 mL of distilled water

Solution B: Diluted 20 g of sodium carbonate and 4 g of NaOH in 1,000 mL of distilled water

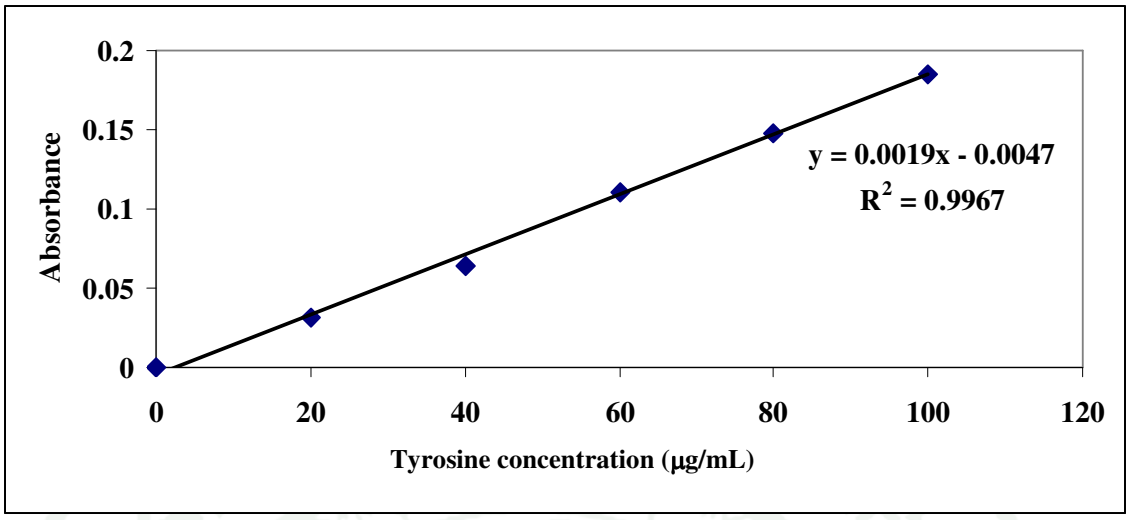
Solution C: Mixed 1 mL of Solution A with 1 mL of Solution B. (Freshly prepare before use)

Solution D: Diluted 1:1 (v/v) Folin-Ciocalteau reagent (Merck®) with distilled water

4.2 Analysis

Each assay was carried out by mixing 0.1 mL of sample solution with 2.5 mL of Solution C. The solution was mixed by vortex and incubated at room temperature for 10 min and then 0.25 mL of solution D was added and mixing. Incubation was done by standing at room temperature for 30 min and the absorbance was measured at 650 nm with visible spectrophotometer (Genesys 20; Thermospectronic) against reagent blank. The concentration of protein was determined by calculating from a standard curve prepared by using 100μg/mL tyrosine as a standared. The protein concentration was calculated as follows.

$$\text{Protein concentration (μg/mL)} = \frac{\text{Absorbance} \times \text{Dilution}}{\text{Sample (mL)}}$$



Appendix Figure B2 Standard curve of protein (Tyrosine) assay by modified Lowry method.

5. Determination of reducing sugar by 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959)

Reagents

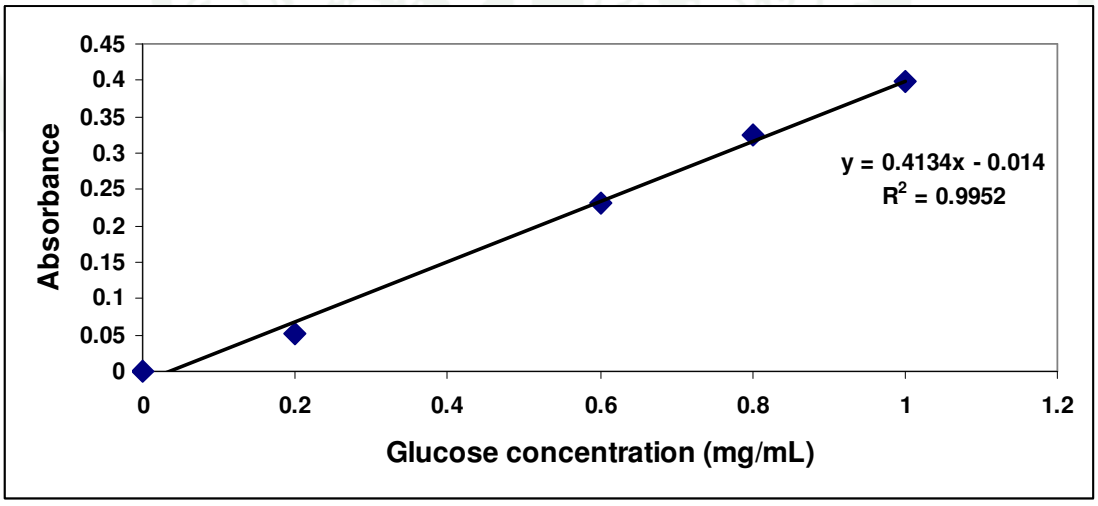
3, 5-dinitrosalicylic acid (Aldrich)	10.0	g
Na_2SO_3 (Ajax Finechem)	0.5	g
Na-K tratrate (APS Finechem)	182.0	g
NaOH (Merck)	10.0	g
Phenol (Merck)	2.0	g
Distilled water	1.0	L

5.2 DNS solution

10 g of NaOH are added into 700 mL of distilled water and mixed in order to add the 300 g of Na-K tratrate. When the solution dissolved, 10 g of 3, 5-dinitrosalicylic acid is then added and continuously stired. After that, 0.5 g of Na_2SO_3 and 2.0 g of phenol are dissolved, respectively. Finally, the volume is adjusted to 1,000 mL by distilled water and kept away from light.

5.3 Analysis

The samples 0.5 mL are mixed with 0.5 mL of DNS solution. The mixture is boiled for 10 min. And then, cooled down by immersing the sample tube into cold water immediately. 5 mL of distilled water is added. The mixture was mixed well, and measured at absorbance 540 nm. Absorbance 540 nm is converted to glucose concentration with standard curve.



Appendix Figure B3 Standard curve of glucose concentration for reducing sugar assay by 3, 5-dinitrosalicylic acid (DNS) method.

6. Determination of Total sugar using the Phenol-Sulfuric Acid Method (DuBois *et al*., 1956).

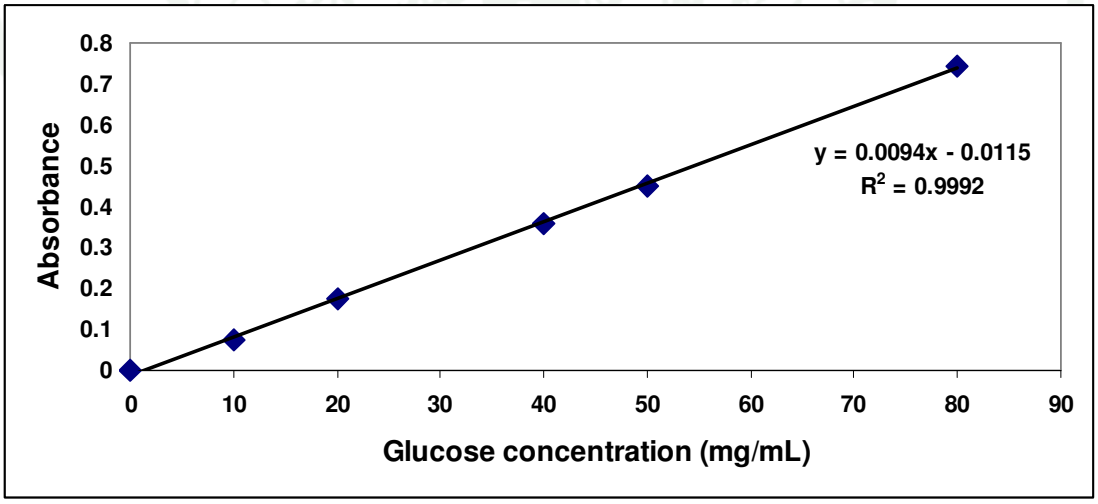
6.1 Reagents

Sulfuric acid 95-97% for analysis (EMPARTA® ACS, Merck)

Phenol GR for analysis (ACS, Reag. Ph Eur Merck)

6.2 Analysis

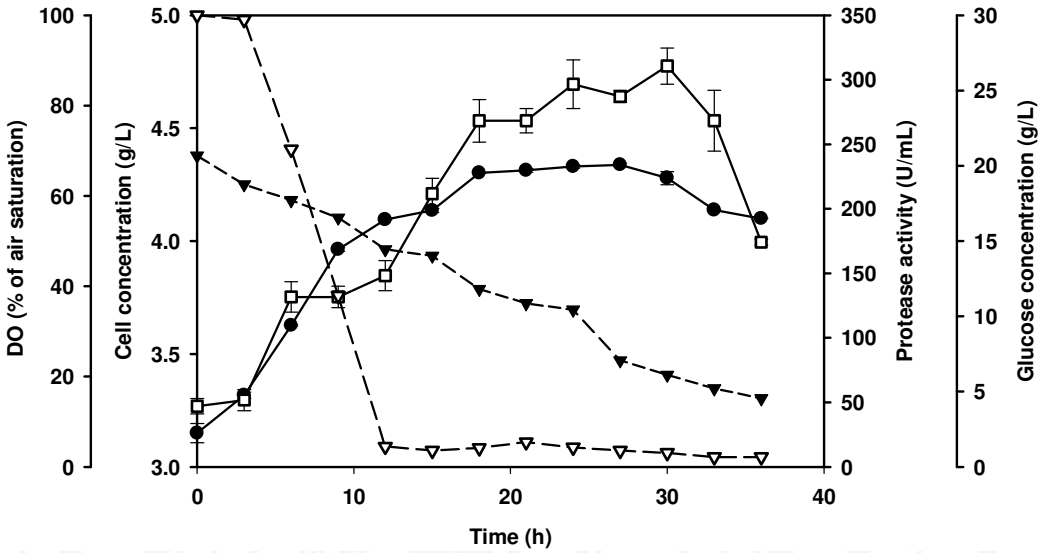
A 2 mL of a sample solution is mixed with 1 mL of 5% aqueous solution of phenol in a test tube. Subsequently, 5 mL of concentrated sulfuric acid is added rapidly to the mixture. After allowing the reaction mixture to stand for 10 min, and vortexed for 30 s and placed for 20 min in a water bath at room temperature for color development. The absorbance of the characteristic yellow- orange color is measured at 490 nm on a spectrophotometer.



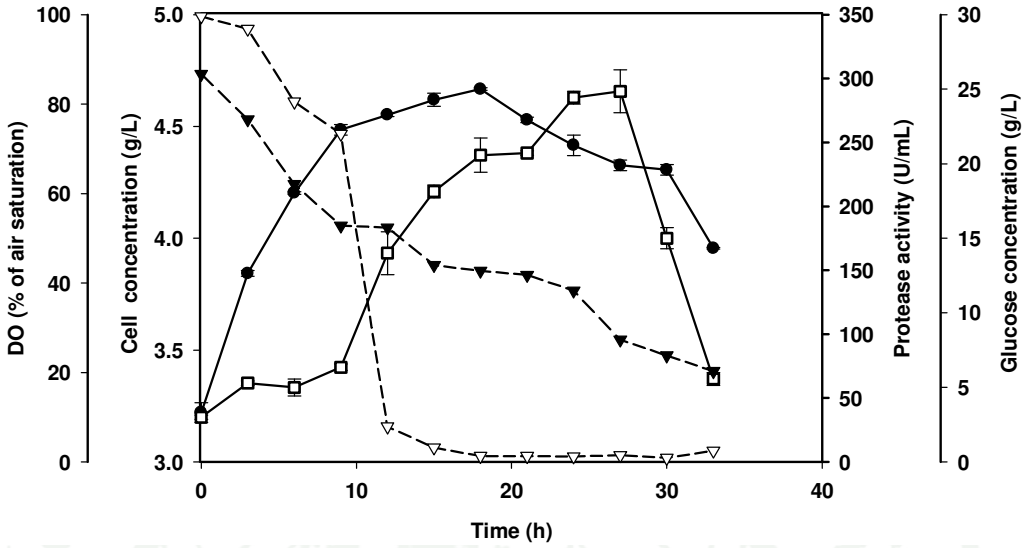
Appendix Figure B4 Standard curve of glucose concentration for total sugar assay by Phenol-Sulfuric Acid method.

Appendix C

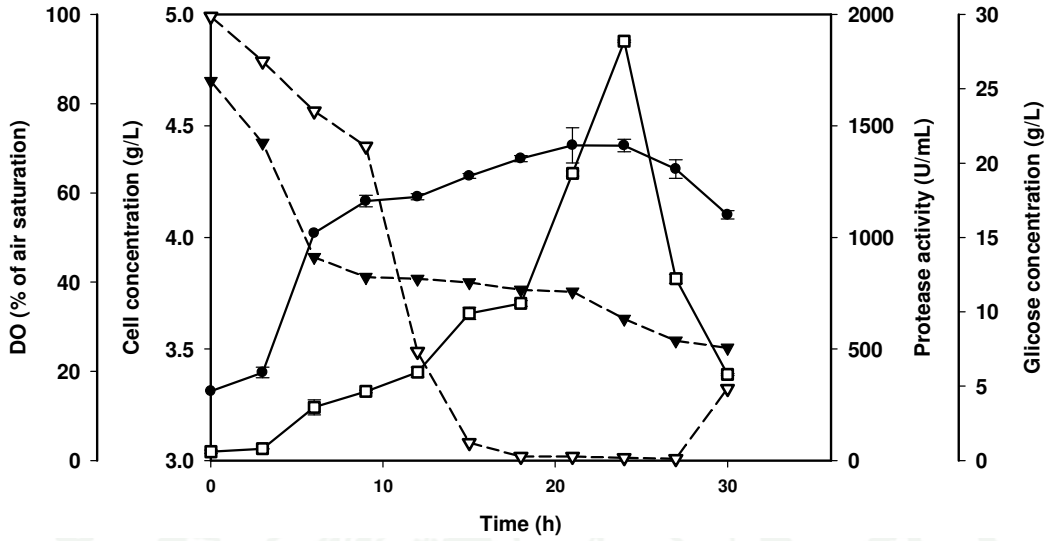
Experimental data



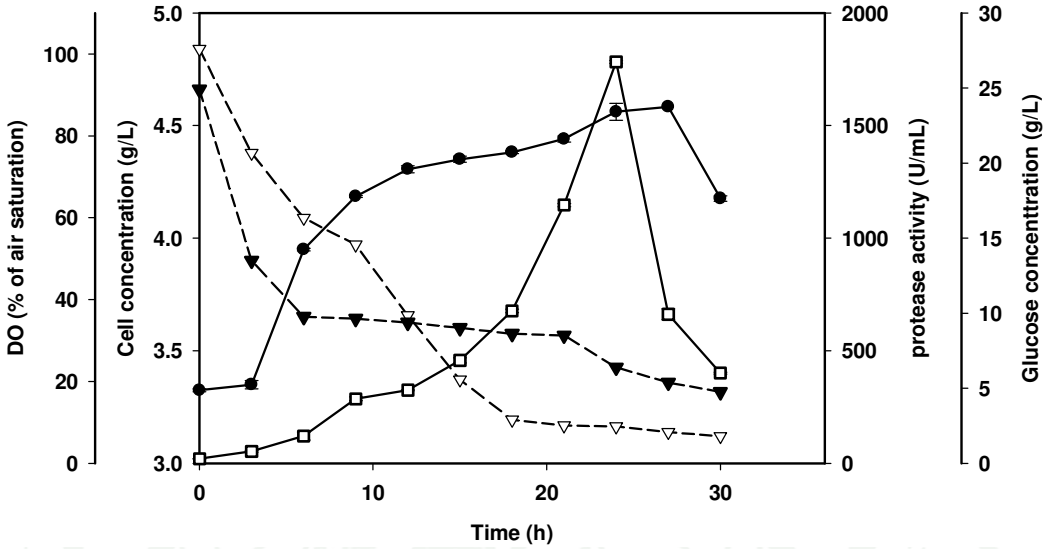
Appendix Figure C1 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 200 rpm, aeration 1 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



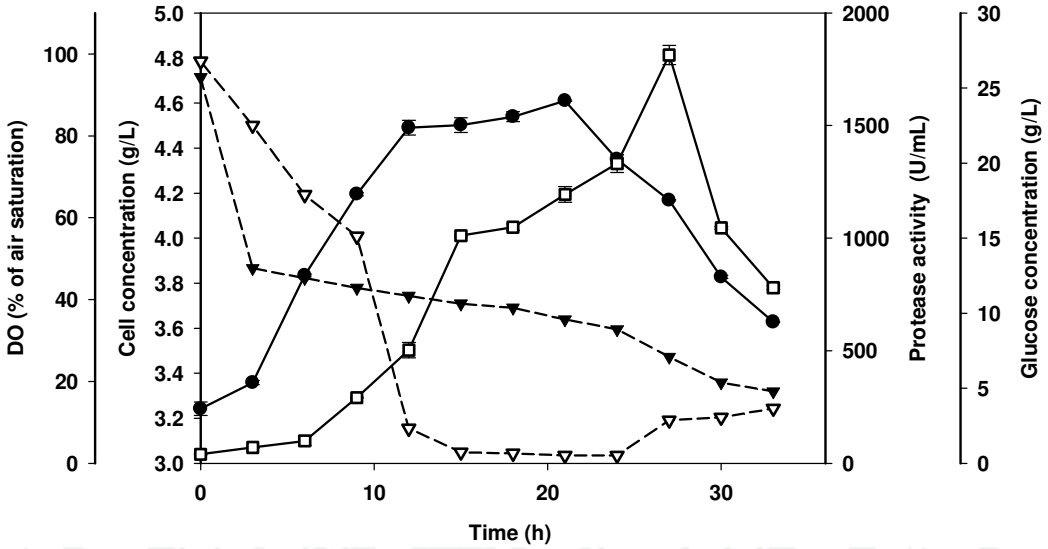
Appendix Figure C2 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 200 rpm, aeration 3 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



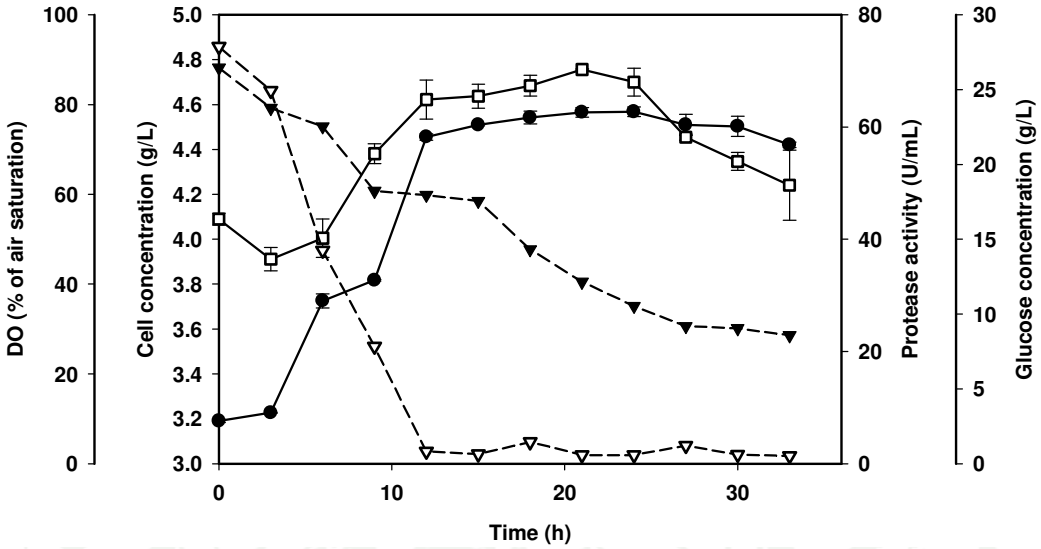
Appendix Figure C3 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 400 rpm, aeration 2 vvm; replication 1) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



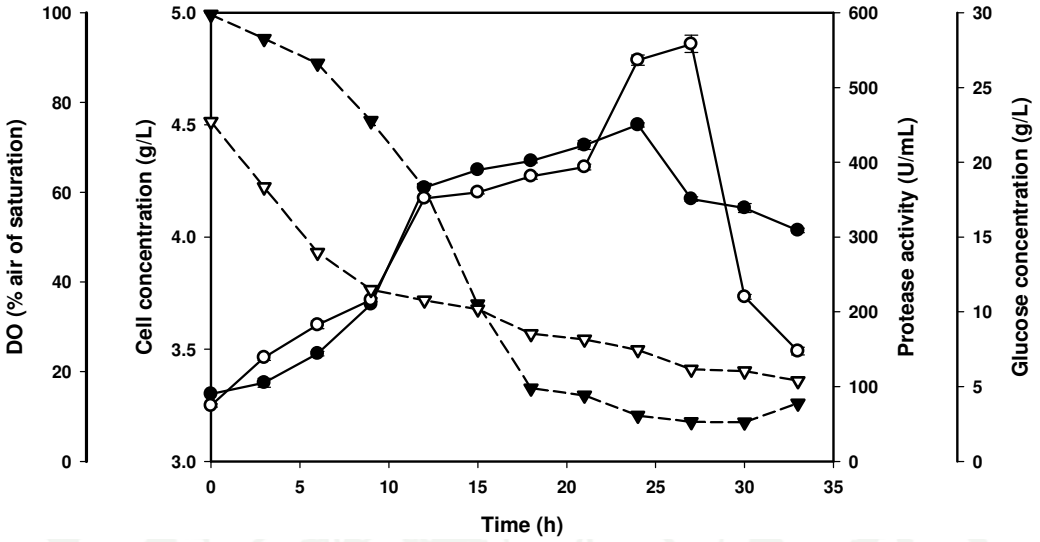
Appendix Figure C4 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 400 rpm, aeration 2 vvm; replication 2) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



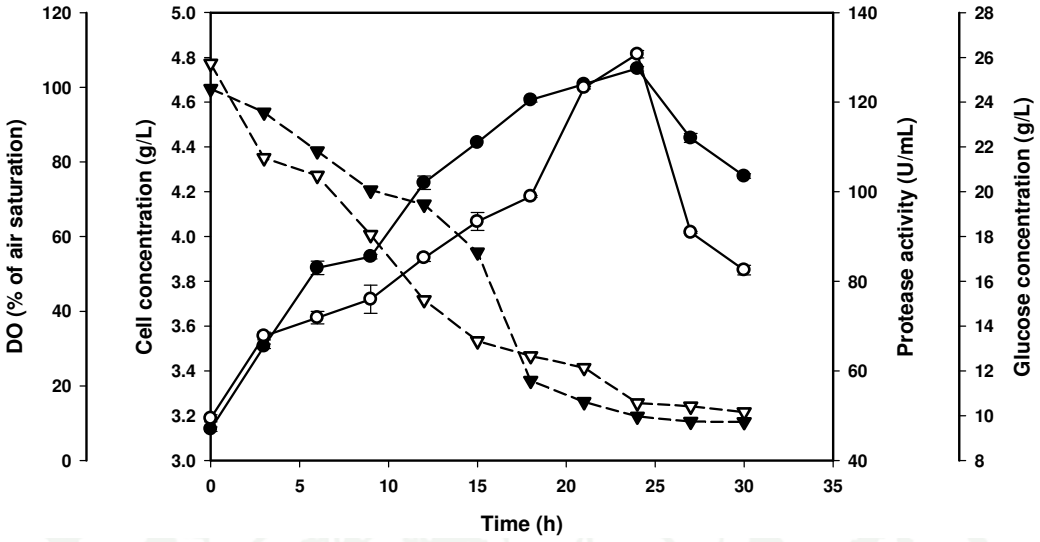
Appendix Figure C5 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 400 rpm, aeration 2 vvm; replication 3) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



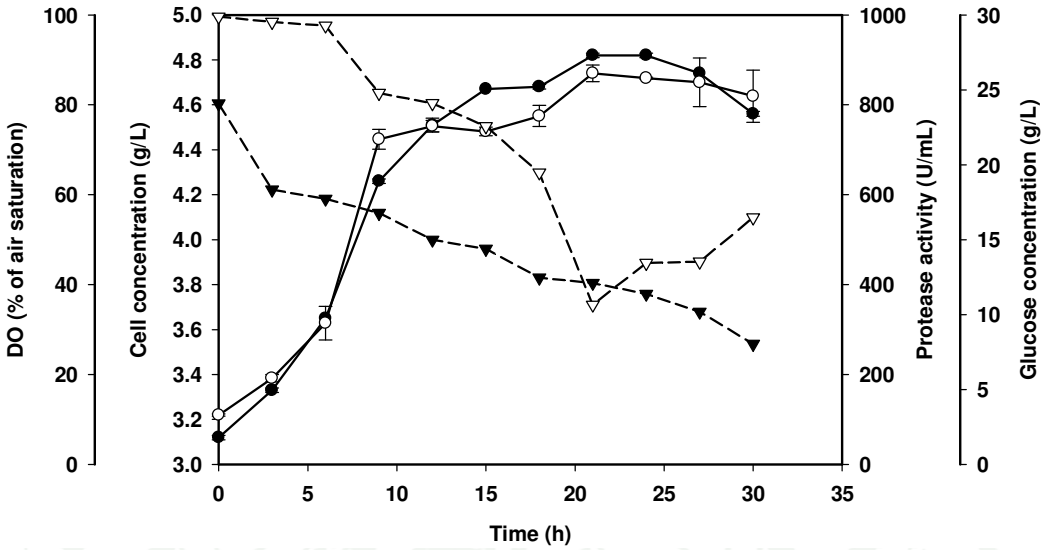
Appendix Figure C6 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 118 rpm, aeration 2 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



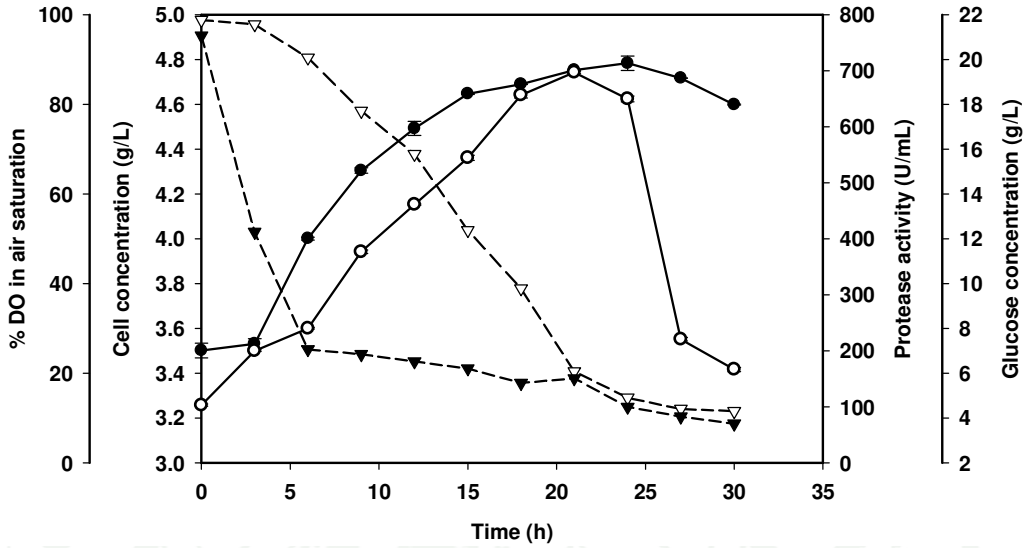
Appendix Figure C7 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 400 rpm, aeration 0.6 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



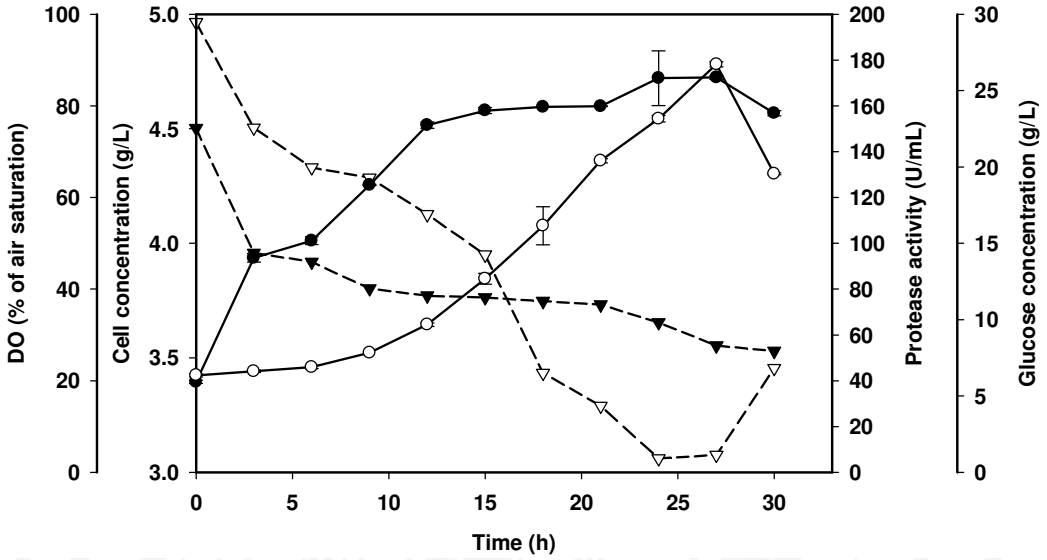
Appendix Figure C8 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 400 rpm, aeration 3.4 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



Appendix Figure C9 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 682 rpm, aeration 2 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



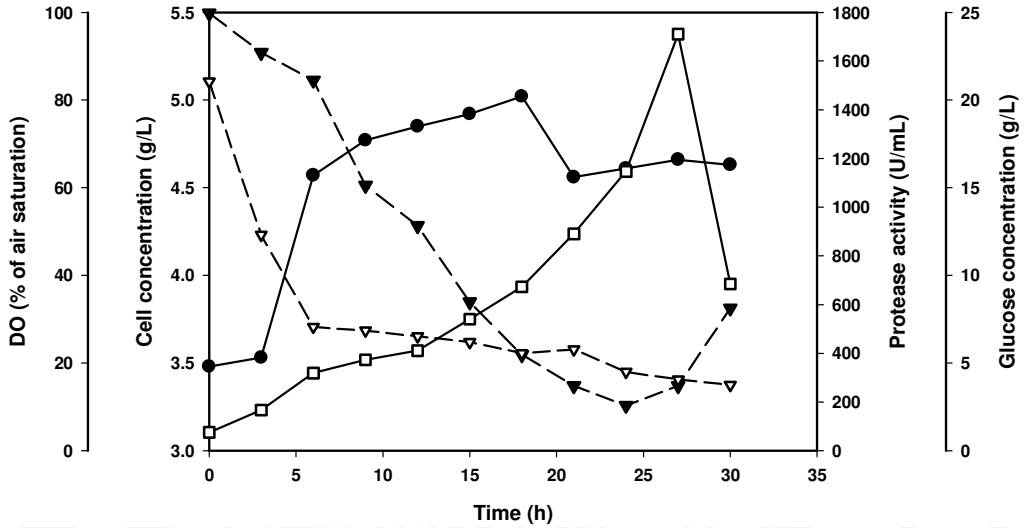
Appendix Figure C10 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 600 rpm, aeration 3 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



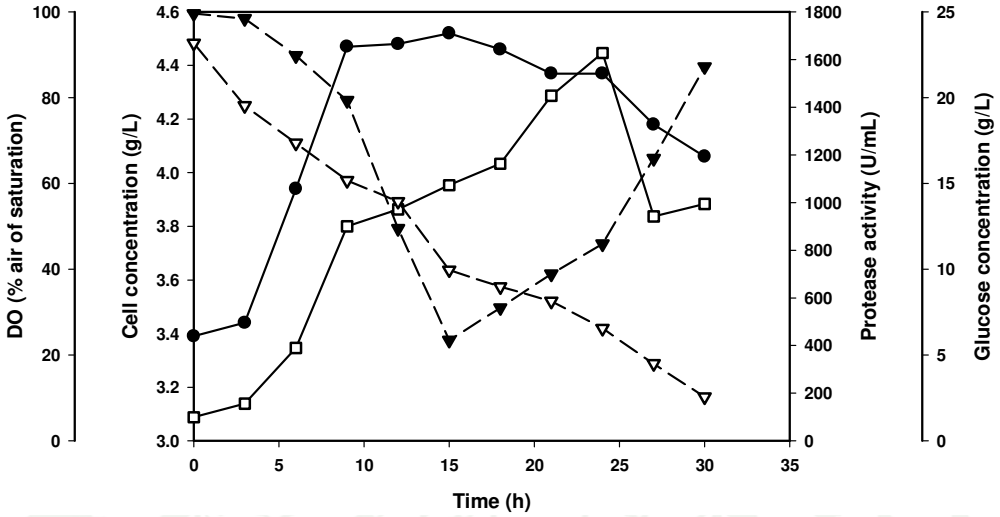
Appendix Figure C11 Time course of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 600 rpm, aeration 1 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).

Appendix Table C1 Data for validation of the experimental model.

Agitation rate (rpm)	Aeration rate (vvm)	Maximum protease activity (U/mL)		μ (h^{-1})		qp(U/g/h)	
		Predicted	Observed	Predicted	Observed	Predicted	Observed
520	2	1,702.94	1,701.18	0.073	0.120	32,242.00	20,388.00
400	2	1,831.94	1,890.00	0.084	0.094	26,015.90	27,075.00
450	2.5	1,662.39	1,627.50	0.079	0.097	29,178.90	19,510.00



Appendix Figure C12 Validation experiment of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 520 rpm, aeration 2 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).



Appendix Figure C13 Validation experiment of silk degumming protease production in stirred tank bioreactor by *Bacillus* sp. C4 SS-2013 under conditions (agitation 450 rpm, aeration 2.5 vvm) Symbols: cell growth (●), protease production (□), glucose concentration (▼) and dissolved oxygen (▽).

Appendix Table C2 ANOVA for response surface quadratic model (protease).

Source	Sum of Squares	df	Mean squares	F-value	p-value
Model	10049365.272	8	1256170.659	1075.249	0.000
X_1	5039049.042	3	1679683.014	1437.764	0.000
X_2	5490827.470	3	1830275.823	1566.667	0.000
X_1X_2	138019.953	1	138019.953	118.141	0.000
Residual	499930.219	16	31246.00		
Lack of fit	484742.83	3	161580.9433	138.3089423	>0.05
Pure error	15187.389	13	1168.261		
Cor tot					
R= 0.975; R^2 =0.950			Adjusted R^2 = 0.935		
F (5, 16) = 61.222			Std. Error of estimate = 176.76		

Appendix Table C3 ANOVA for Response Surface quadratic model (q_p).

Source	Sum of Squares	df	Mean squares	F-value	p-value
Model	2346440665.091	8	293305083.136	141.464	0.000
X_1	970959428.400	3	323653142.800	156.101	0.000
X_2	1368838985.600	3	456279661.867	220.068	0.000
X_1X_2	13739282.000	1	13739282.000	6.627	0.023
Residual	164539872	16	10283742		
Lack of fit	137586388	3	45862129		>0.05
Pure error	26953612.000	13	2073354.769		
Cor tot	2373394277.091	21			
R= 0.965; R^2 =0.931			Adjusted R^2 = 0.909		
F (5, 16) = 42.96			Std. Error of estimate = 3,206.8		

Appendix Table C4 ANOVA for response surface quadratic model (K_La).

Source	Sum of Squares	df	Mean squares	F-value	p-value
Model	142791.081	8	17848.885	117.009	0.000
X_1	83459.428	3	27819.809	182.374	0.000
X_2	43589.284	3	14529.761	95.251	0.000
X_1X_2	18202.320	1	18202.320	119.326	0.000
Residual	17225.3	16	1076.58		
Lack of fit	15242.2927	3	5080.764234		>0.05
Pure error	1983.053	13	152.543		
Cor tot	144774.134	21			
R= 0.938; R^2 =0.881			Adjusted R^2 = 0.8438		
F (5, 16) = 23.695			Std. Error of estimate = 32.811		

Appendix Table C5 ANOVA for Response Surface Quadratic Model (μ).

Source	Sum of Squares	df	Mean squares	F-value	p-value
Model	0.012	8	0.001	90.240	0.000
X_1	0.006	3	0.002	115.627	
X_2	0.006	3	0.002	130.647	
X_1X_2	0.002	1	0.002	116.521	
Residual	0.001826	16	0.000114		
Lack of fit	0.0018258	3	0.000609		
Pure error	0	13	0		
Cor tot	0.012	21			
R= 0.919; R^2 =0.845			Adjusted R^2 = 0.796		
F (5, 16) = 17.434			Std. Error of estimate = 0.01068		

Appendix Table C6 Regression analysis of response surface quadratic model.

Model	Sum of Squares	df	Mean squares	F-value	p-value
Protease					
Regression	9,564,622	5	1,912,924	61.22213	0.0000
Residual	499,930	16	31,246		
Total	10,064,600				
q_p					
Regression	2,208,850,000	5	441,771,000	42.95819	0.0000
Residual	164,540,000	16	10,283,700		
Total	2,373,390,000				
K_La					
Regression	127,548.8	5	25,509.76	23.69509	0.000001
Residual	17,225.3	16	1076.58		
Total	144,774.1				
μ					
Regression	0.009947	5	0.001989	17.434	0.000006
Residual	0.001826	16	0.000114		
Total	0.11773				

```
METHOD RK4

STARTTIME = 0
STOPTIME=30
DT = 0.02

d/dt (X) = rx
d/dt (S) = -rs
d/dt (P) = rp

init S = 23.24
init X = 3.22
init P = 48750

muemax=0.093563
Xmax = 4.94
mS = 0.012
YXSmax =0.095057
a = 179427
b = 22075


rx = muemax*(1-(X/Xmax))*X
rs = (rx/YXSmax)+ (mS*X)
rp = a*rx+b*X
```

Appendix Figure C14 Simulation by Berkeley Madonna program.

Appendix Table C7 Correation between observed data and predicted values of Logistic model.

Source	Coefficient	Std. Error	t-value	p-value
Constant	1.105	0.356	3.103	0.013
cell obv	0.768	0.0815	9.425	<0.001

cell predict = 1.105 + (0.768 * cell obv)

N = 11

R = 0.953 Rsqr = 0.908 Adj Rsqr = 0.898

Standard Error of Estimate = 0.174

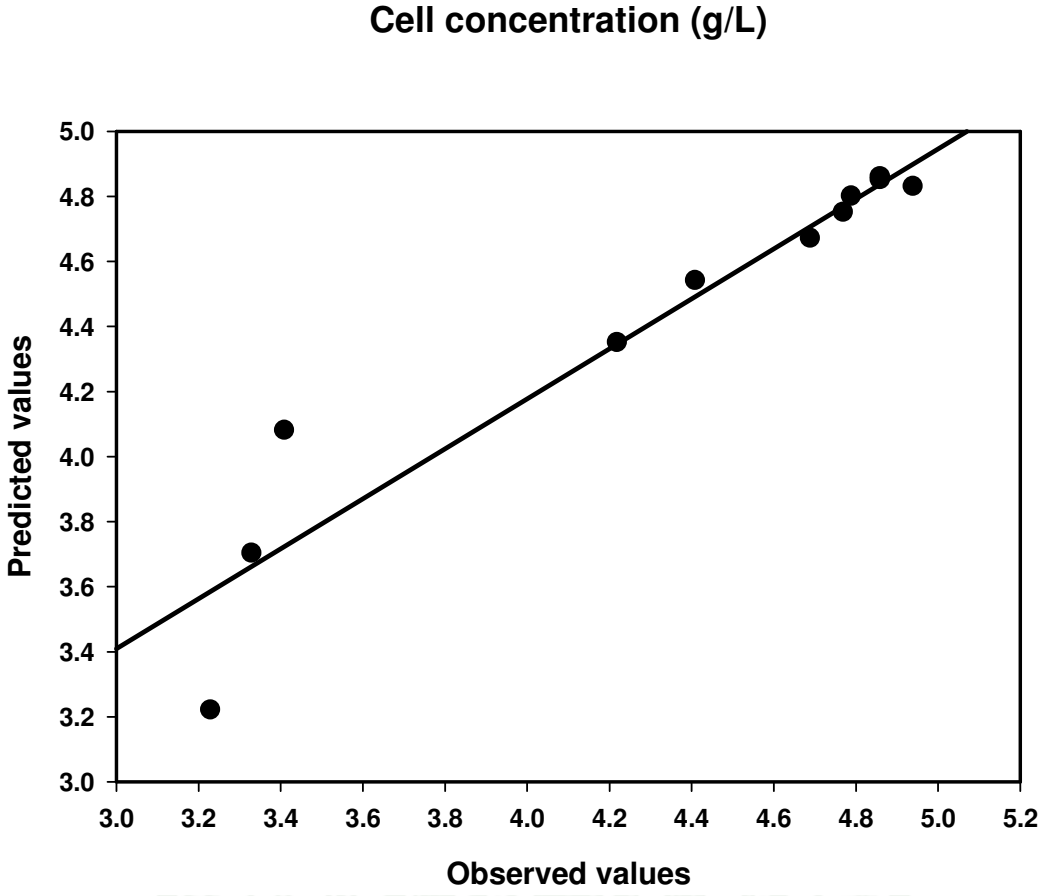
Appendix Table C8 ANOVA for Logistic model.

Source	Sum of Squares	df	MS	F-value	p-value
Regression	2.681	1	2.681	88.832	<0.001
Residual	0.272	9	0.0302		
Total	2.953	10	0.295		

Normality Test (Shapiro-Wilk) Failed (P = 0.010)

Constant Variance Test: Passed (P = 0.221)

Power of performed test with alpha = 0.050: 1.000



Appendix Figure C15 Observed data versus predicted values of cell concentration by Logistic model.

Appendix Table C9 Correation between observed data and predicted values of Luedeking- Piret model for product formation.

Source	Coefficient	Std. Error	t-value	p-value
Constant	-8080.871	152956.577	-0.0528	0.959
protease obv	1.163	0.138	8.413	<0.001

protease pre = -8080.871 + (1.163 * protease obv)

N = 11

R = 0.942 Rsqr = 0.887 Adj Rsqr = 0.875

Standard Error of Estimate = 254189.420

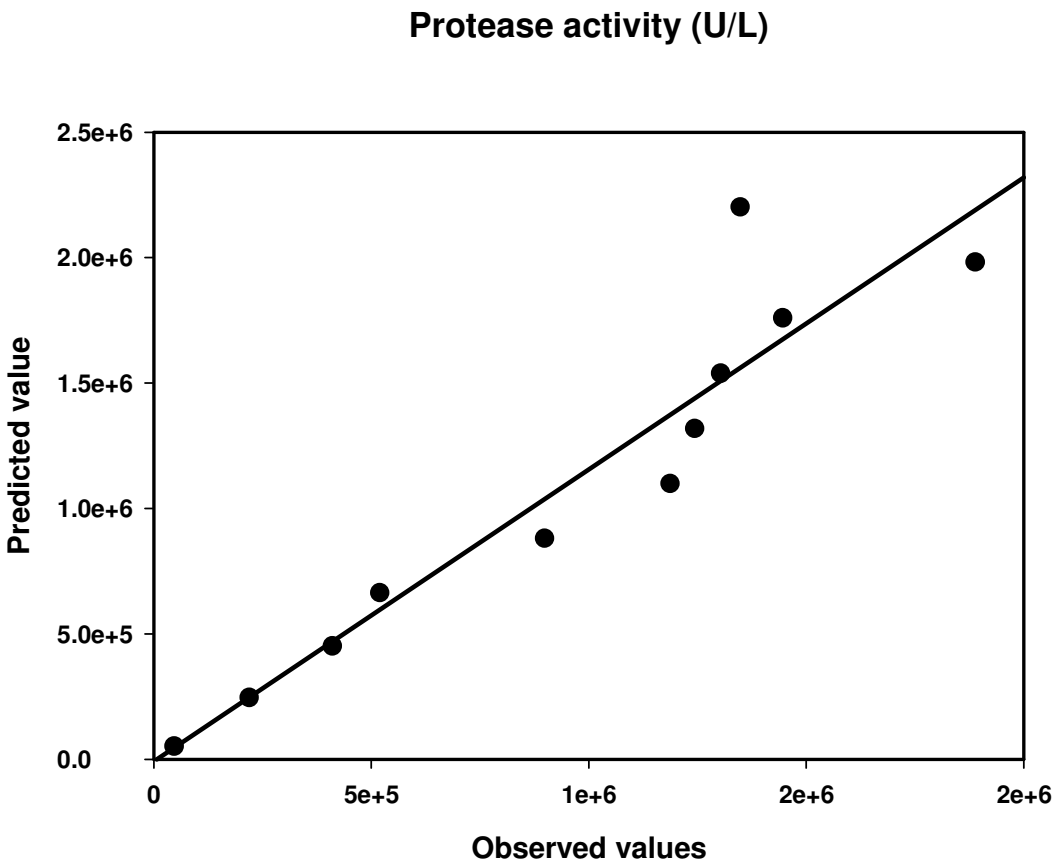
Appendix Table C10 ANOVA of Luedeking- Piret model for product formation.

Source	Sum of Squares	df	MS	F-value	p-value
Regression	4.5740E+012	1	4.574E+012	70.786	<0.001
Residual	581510349081.690	9	64612261009.077		
Total	5.155E+012	10	515515918896.364		

Normality Test (Shapiro-Wilk) Failed (P = 0.011)

Constant Variance Test: Failed (P = 0.014)

Power of performed test with alpha = 0.050: 0.999



Appendix Figure C16 Observed data versus predicted values of protease production by Luedeking- Piret model.

Appendix Table C11 Correation between observed data and predicted values of Luedeking- Piret like model for substrate consumption.

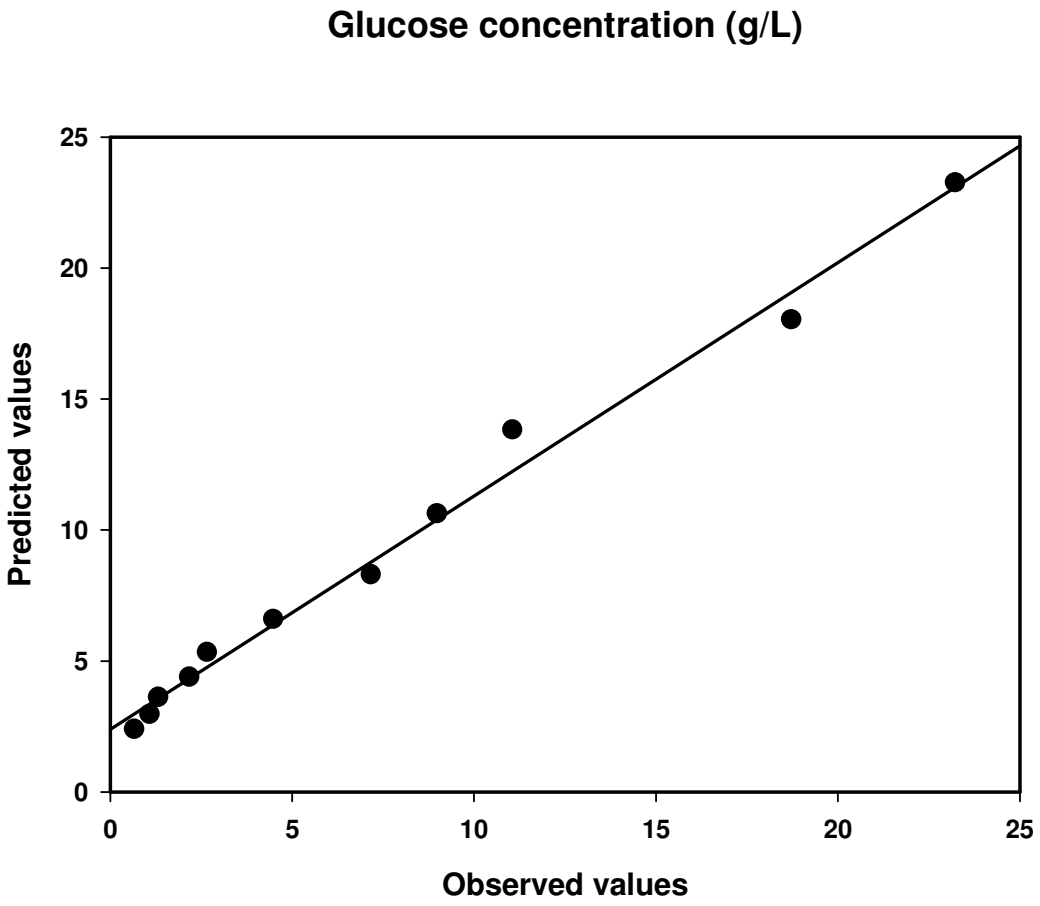
Source	Coefficient	Std. Error	t-value	p-value
Constant	2.398	0.315	7.625	<0.001
sugar obv	0.890	0.0303	29.344	<0.001

sugar pre = 2.398+ (0.890 * sugar obv)
N = 11
R = 0.995 Rsqr = 0.990 Adj Rsqr = 0.728
Standard Error of Estimate = 3.590

Appendix Table C12 ANOVA of Luedeking- Piret like model for substrate consumption.

Source	Sum of Squares	df	MS	F-value	p-value
Regression	455.795	1	455.795	861.076	<0.001
Residual	4.764	9	0.529		
Total	460.559	10	46.056		

Normality Test (Shapiro-Wilk) Passed (P = 0.452)
Constant Variance Test: Passed (P = 0.557)
Power of performed test with alpha = 0.050: 1.000



Appendix Figure C17 Observed data versus predicted values of glucose concentration by Luedeking- Piret like model.

Appendix Table C13 Stability of protease with various additives in different conditions during storage for 3 week

Time (weeks) / Replication	Storage conditions		Protease activity (U/mL)		
	Additives	Temp.	0	1	3
Control		Room temperature	1,223.16±1.99	20.34±1.69	13.24±1.12
10 mM $CaCl_2$			1,221.56±3.61	72.12±4.19	10.27±0.03
0.02%(w/v) sodium azide			1,224.56±5.06	43.89±5.57	15.97±1.26
25 % (v/v) glycerol			1,223.61±5.13	41.14±1.90	8.95±2.12
Control		4 °C	1,225.30±1.73	709.04±2.23	352.54±2.83
10 mM $CaCl_2$			1,223.21±2.89	985.02±2.79	810.62±1.99
0.02%(w/v) sodium azide			1,220.44±7.08	854.68±3.59	513.93±3.69

Appendix Table C13 (Continued)

Time (weeks) / Replication / Storage condition		Protease activity (U/mL)		
		0	1	3
Additives	Temp.			
25 % (v/v) glycerol	4 °C	1,222.99±4.51	754.08±3.31	580.49±1.04
10 % (v/v) PEG		1,228.33±2.36	905.78±38.97	792.0±32.06
10 % (v/v) PEG +10 mM $CaCl_2$		1,228.33±2.36	1041.11±36.14	977.78±6.91
Control	-20 °C	1,224.07±1.98	299.49±2.73	127.02±5.54
10 Mm $CaCl_2$		1,223.60±2.75	377.59±4.74	146.38±5.79
0.02%(w/v) sodium azide		1,224.13±1.12	172.84±1.98	134.54±3.14
25%(w/v) glycerol		1,222.30±2.70	509.89±3.87	225.61± 2.72

Appendix Table C14 Relative activity of protease with various additives in different conditions during storage for 3 week.

Storage condition	Relative activity (%)		
	0	1	3
Room temperature	100	1.66	1.08
Room temperature + 10 mM $CaCl_2$	100	5.90	0.84
Room temperature+ 0.02%(w/v) sodium azide	100	3.58	1.30
Room temperature+ 25 % (v/v) glycerol	100	3.36	0.73
4 °C	100	57.87	28.77
4 °C + 10 mM $CaCl_2$	100	80.53	66.27
4 °C + 0.02%(w/v) sodium azide	100	70.03	42.11
4 °C + 25 % (v/v) glycerol	100	61.66	47.47
4 °C + 10 % (v/v) polyethene glycol	100	63.22	55.28
4 °C + 10 mM $CaCl_2$ +10 % (v/v) polyethene glycol	100	82.67	67.05
-20 °C	100	24.47	10.38
-20 °C+ 10 mM $CaCl_2$	100	30.86	11.96
-20 °C + 0.02%(w/v) sodium azide	100	14.12	10.99
-20 °C + 25%(w/v) glycerol	100	41.72	18.46

Appendix Table C15 Comparison of the addition method of calcium chloride on protease stability during storage for 8 week.

Storage Conditions		Time of storage					
Additives	Temp.	0	1	2	3	4	8
Control	4°C	1,373.31±1.76	1,228.83±2.66	1,018.39±1.07	1,016.21±13.39	845.61±3.70	225.30±3.55
2 mM $CaCl_2$ in medium		1,473.54±1.78	735.14±1.51	730.85±2.07	672.79±1.49	554.90±1.77	417.46±2.64
10 mM $CaCl_2$ in medium		1,555.24±2.84	934.23±2.79	856.96±2.50	755.23±1.85	562.78±11.65	432.41±3.06
2 mM $CaCl_2$ in crude enzyme		1,384.32±2.80	1,365.68±2.65	1,193.57±2.16	784.96±2.09	708.21±3.47	361.63±57.79
10 mM $CaCl_2$ in crude enzyme		1,375.95±2.41	1,365.78±2.45	1,345.52±2.79	1,275.99±0.53	1,144.64±3.72	884.01±1.45
Control	-20°C	1,367.07±2.43	1,227.87±1.76	813.50±2.19	743.44±2.21	543.41±3.91	236.29±0.55
2 mM $CaCl_2$ in medium		1,477.25±1.64	1,281.27±2.96	954.20±3.12	882.97±1.56	712.11±2.33	186.63±2.79
10 mM $CaCl_2$ in medium		1,573.72±1.49	1,125.18±3.30	991.94±2.68	933.69±1.16	824.50±4.05	214.65±2.22
2 mM $CaCl_2$ in crude enzyme		1,384.12±1.83	793.16±1.64	656.35±1.80	795.72±2.79	648.48±1.59	233.48±3.71
10 mM $CaCl_2$ in crude enzyme		1,365.52±3.02	1,164.22±1.45	754.62±2.18	686.87±4.52	634.43±2.46	325.30±3.30

Appendix Table C16 Relative activity of protease with the different of calcium chloride concentration during storage for 8 week.

Storage conditions		Time of storage					
Additives	Temperature	0	1	2	3	4	8
Control	4°C	100.00	89.48	74.16	74.00	61.57	16.41
2 mM $CaCl_2$		100.00	98.65	86.22	56.70	51.16	26.12
10 mM $CaCl_2$		100.00	99.26	97.79	92.74	83.19	64.25
Control	-20°C	100.00	89.82	59.51	54.38	39.75	17.28
2 mM $CaCl_2$		100.00	57.30	47.42	57.49	46.85	16.87
10 mM $CaCl_2$		100.00	85.26	55.26	50.30	46.46	23.82

Appendix Table C17 Effect of calcium chloride on protease stability during storage for 8 week.

Time (days)	Storage conditions					
	Control	1 mM $CaCl_2$	10 mM $CaCl_2$	100 mM $CaCl_2$	10%(v/v) PEG	10%(v/v) PEG + 10 mM $CaCl_2$
0	1228.33±2.36	1228.33±2.36	1228.33±2.36	1228.33±2.36	1228.33±2.36	1228.33±2.33
3	828.67±41.48	1138.00±2.83	1193.78±10.69	1180.00±16.97	928.89±13.83	1136.78±18.38
5	742.67±20.74	1022.00±2.83	1119.56±3.46	1130.00±1.57	920.00±40.86	1015.78±11.00
7	590.00±37.71	808.89±25.14	1088.00±1.89	1126.00±51.85	905.78±38.97	1041.11±36.14
14	376.67±6.29	425.33±16.97	1058.67±49.03	1025.11±6.60	892.00±33.94	980.00±4.40
21	286.67±13.20	321.33±33.94	947.56±45.25	963.56±20.11	792.00±32.06	977.78±6.90
28	66.00±6.29	130.67±0.00	948.44±46.51	896.67±14.14	770.22±14.46	960.67±24.20
56	16.67±1.47	51.11±3.14	726.67±9.42	426.67±18.85	448.89±18.85	757.78±34.56

Appendix Table C18 Relative activity of protease with the different concentration of calcium chloride on protease stability during storage for 8 week.

Time (days)	Storage conditions					
	Control	1 mM $CaCl_2$	10 mM $CaCl_2$	100 mM $CaCl_2$	10%(v/v) PEG	10%(v/v) PEG + 10 mM $CaCl_2$
0	100.00	100.00	100.00	100.00	100.00	100.00
3	57.95	79.59	83.49	82.53	64.96	79.50
5	51.94	71.48	78.30	79.03	64.34	71.04
7	41.26	56.57	76.09	78.75	63.35	72.81
14	26.34	29.75	74.04	71.69	62.38	68.54
21	20.05	22.47	66.27	67.39	55.39	68.38
28	4.62	9.14	66.33	62.71	53.87	67.19
56	1.73	3.86	52.29	31.06	41.52	57.27

Appendix Table C19 Silk degumming protease production in stirred tank bioreactor by *Bacillus sp.* C4 SS-2013 by pH-stat fed batch culture.

Time (h)	dry weight cell (g/L)	Protease activity (U/mL)	Total sugar (g/L)	Total feed volume (L)	%DO of air saturation
0	2.83	81.18	27.35	1.31	98.6
3	3.26	155.29	24.40	1.30	92.5
6	3.65	437.65	22.35	1.28	58.1
9	4.09	562.94	20.47	1.24	50.6
11	4.47	814.12	16.05	1.20	56.9
12	4.67	877.65	32.20	1.20	57.1
15	4.46	1117.65	26.28	1.23	56.9
17	4.42	1138.24	23.97	1.28	67.8
18	4.37	1655.29	17.79	1.30	56.5
21	4.47	2421.18	17.60	1.30	48.4
23	4.70	2495.29	9.75	1.34	55.9
24	4.65	2555.29	10.30	1.34	45.3
25	4.66	2696.47	9.35	1.43	54.8
26	4.89	2770.59	11.20	1.45	58.7
36	5.07	3208.24	7.13	1.54	48.1
39	5.12	3423.53	6.85	1.61	68.1
41	5.16	3448.24	6.18	1.63	49.6
42	5.22	3516.47	2.99	1.70	58.4
43	5.31	3829.41	1.97	1.75	46.7
45	4.86	4437.24	1.51	1.81	36.6

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1. Romsomsa**,** N., P. Chim-anage, P. Sirirote and A. Jangchud. 2010. Optimization of silk degumming protease production from *Bacillus subtilis* C4 using Plackett-Burman design and response surface methodology. **ScienceAsia** 36: 118–124.

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