Australian Journal of Basic and Applied Sciences, 2(3): 583-593, 2008
ISSN 1991-8178

Optimization Of The Industrial Production Of Alkaline Protease By *Bacillus licheniformis* In Different Production Scales

[1,2]El Enshasy, H., [3]Abuoul-Enein, A., [3]Helmy, S. and [4]El Azaly, Y.,

[1]CEPP, Faculty of Chemical and Natural Resources Engineering, UTM, Skudai, Johor, Malaysia.
[2]Bioprocess Development Department, GEBRI,
Mubarak City for Scientific Research, New Burg Al Arab, Alexandria, Egypt.
[3]Biochemistry Department, Faculty of Agriculture, Cairo University, Giza, Egypt.
[4]El Nasr Pharmaceutical Chemical Co., Abou-Zabal, Cairo, Egypt.

Abstract: Production of extracellular alkaline protease using an industrial strain of *Bacillus licheniformis* in different industrial media was optimized. Of different media used, the production medium composed of glucose and soybean meal as carbon and nitrogen source, respectively, in addition to inorganic phosphate, calcium chloride and magnesium chloride showed the maximal enzyme production of about 2400 U mL^{-1} after 96 h. The effect of glucose concentration on the cell growth and the enzyme production was studied in shake flask culture. The maximal enzyme production was obtained on using glucose concentration of 60 g L^{-1} in the industrial medium. Further optimization in the production process was achieved on studying the effect of aeration rate in small scale 16 L bioreactor. Whereas, the increase of airflow rate in the range between 0.5 to 2.0 v v^{-1} min showed a negative effect on cell growth, the maximal enzyme production of 4120 U mL^{-1} was obtained at airflow 1 v v^{-1} min after 84 h at agitation rate of 500 rpm. When the process was scaled up to 500 L bioreactor and cultivated at the same airflow rate, the enzyme production reached about 5100 U mL^{-1} after 80 h cultivation.

Key words: Alkaline Protease, *Bacillus licheniformis*, submerged culture, large-scale bioreactor

INTRODUCTION

Enzymes are one of the major groups of products in biotechnology business sector. The world market for enzymes grows 7.6% per year and will reach $6 billions by 2011 as predicted in the Freedonia Group Inc. business report in 2007. This is driven by continued robust growth in pharmaceutical enzyme demand, fine chemical production, bioethanol production and detergent industries. Proteolytic enzymes account for nearly 60% of the industrial enzyme market in the world. They find application in a number of biotechnological processes and used in many industries. Of different types of proteases, alkaline proteases are one of the most widely studied groups of enzymes because of their wide use in many industrial applications in food, leather, pharmaceutical and detergent industries (Kumar, C.G. and H. Takagi. 1999; Dayanandan, A., *et al.,* 2003). Moreover, they are also used for cleaning of membranes used in protein ultrafiltration (Kumar, C.G. and M.P. Tiwari. 1999). Of these different applications, the main use of alkaline protease is mainly focused on detergent industries. They are used in all types of laundry detergents and in automatic dishwashing detergents. Their function is to degrade proteinaceous stains (Maurer, K.H. 2004). Microbial proteases can be produced from bacteria, fungi and yeast using many processes like solid-state fermentation and submerged fermentation (Kumar, C.G. and H. Takagi. 1999; Anwar, A. and M. Saleemuddin. 2001; Haki, G.D. and S.K. Rakshit. 2003). However, the bacterial Serine Alkaline Protease (SAP, EC 3.4.21.62) is the most important member of alkaline proteases family for industrial application. This is an endopoptidase capable of hydrolyzing the interior peptide bonds of protein molecules. This enzyme is produced mainly by many members belonging to genus *Bacilli* especially, *B. licheniformis*; *B. horikoshii, B. sphaericus* (Mehrotra, S. *et al.,* 1999; Potumarthi, R.). The importance of alkaline protease is illustrated by the fact that the market share of this enzyme is about 20% of overall industrial enzymes business. However, beside some published reports on the production of alkaline protease by solid state fermentation (Kaur, S. *et al.,* 2001; Aijun, Z. *et al.,* 2005), the main process for the industrial production of this enzyme is carried out in large scale by submerged culture.

Corresponding Author: El Enshasy, H., CEPP, Faculty of Chemical and Natural Resources Engineering, University Technology Malaysia, UTM, 81310 Skudai, Johor, Malaysia.
E-mail: henshasy@cepp.utm.my

For industrial production of alkaline protease, technical media are usually employed that contained very high concentration of carbohydrates, proteins, and other media components. To develop an economically feasible technology, research efforts are mainly focused on: (1) improvement in the yields of alkaline proteases; and (2) optimization of the fermentation medium and production conditions in industrial scale. However, it is known that 40% of the production cost of industrial enzymes in large scale is estimated to be accounted for the cost of the growth medium. Concerning this fact, the use of cost-effective growth medium for the production of alkaline protease is especially important. For this purpose, soybean meal (*Glycine max*) was recognized as a potentially useful and cost-effective medium ingredient, because is is produced as by-product of oil industry. Soybean meal contains 40% protein and rich in other organic and inorganic components. Thus, it is a good candidate for fermentation medium formulation. In the present work, optimization of industrial medium for alkaline protease production by *B. licheniformis* was done. Moreover, the production process was scaled up in different level from shake flask up to 500-L stirred tank bioreactor.

MATERIALS AND METHODS

Strain:

The alkaline protease producer strain *Bacillus licheniforms* (DSMZ 1969) was obtained from the German culture collection and cell culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Freez dried cells were activated in nutrient broth for 3 days and stored thereafter in glycerin solution (50% v v^{-1}) at -80ºC. Cells were revived by transfer to slopes of nutrient agar (Oxoid), which were incubated at 37ºC for 3 days before use.

Inoculum Preparation:

Inoculum was prepared in nutrient broth medium using 250 ml Erlenmeyer flask with working volume of 50 mL. Incubation was done using reciprocal shaker (Haake SWB20, Germany) at 175 stroke min^{-1} and 37ºC.

Alkaline Protease Production Medium:

Fermentations were carried out using three standard pre-optimized industrial media. The first medium (MI) was composed of (g L^{-1}): glucose; 60.0; soybean meal, 20.0; Na_2HPO_4, 14.2; NaH_2PO_4, 12.0; $CaCl_2$, 0.4 and $MgCl_2$, 0.2. The second medium (MII) was composed of (g L^{-1}): dextrin, 30.0; soybean meal, 50.0; $(NH_4)_3PO_4$, 10.0; KCl, 0.3 and $MgSO_4.7H_2O$, 0.2. The third production medium (MIII) was composed of (g L^{-1}): glucose, 10.0: soluble starch, 50.0; soybean meal, 10.0; $(NH_4)_3PO_4$, 10.0 and $MgSO_4.7H_2O$, 0.2. The pH of all fermentation media was adjusted to 7.6 before sterilization.

Shake Flask Cultivations:

Cultivations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of alkaline protease production medium. The inoculated flasks were incubated on rotary shaker of 260 rpm and shaking eccentricity of 2.5 cm. All shake flask cultivations were done in temperature controlled room at 37°C.

Bioreactor cultivations

Two types of stirred tank bioreactors were used in this study. The first one was small scale 16-L stirred tank bioreactor SF-116 attached with multiple loop controller ML 4100 (New Brunswick Scientific Equipments, New Brunswick, NJ, USA) with working volume of 8 L. This bioreactor was equipped with four baffles and three Rushton six blade disc turbines with the following dimensions ($d_{i(impeller\ diameter)}$ = 80 mm; $d_{t(tank\ diameter)}$ = 215 mm, $d_i d_t^{-1}$ = 0.37). Agitation speed was adjusted to 500 rpm. and three levels of airflow rates: 0.5, 1.0 and 2.0 vvm were applied.

The pilot scale bioreactor of 500-L total volume equipped with four baffles and 3 six-blade Rushton turbine ($d_{i(impeller\ diameter)}$ = 300 mm; $d_{t(tank\ diameter)}$ = 1000 mm, $d_i d_t^{-1}$ = 0.30) was used in this study. The working volume of this bioreactor was 250 L. Antifoam Galanopon 3002 (Bussetti & Co. Gesellschaft GmbH, Wien, Austria) was used to minimize foam formation and was added intermittently during cultivation process.

Analysis:

Cell Growth Determination:

Bacterial growth was determined by optical density measurement of diluted cultures using a spectrophotometer at 600 nm and was expressed as g L^{-1} using the correlation (g CDW L^{-1}= 0.49×OD) which was calculated experimentally for this bacterial strain.

Glucose Determination:

Residual glucose was measured using an enzymatic kit (GlucGDH, Roche Diagnostics, Rotkreuz, Switzerland) based on the conversion of glucose to gluconate by glucose dehydrogenase measuring the amount of NADH formed.

Determination of Alkaline Protease and Analytical Methods:

Alkaline protease activity was determined according to the method of Fukumoto *et al.* (1971) based on the following procedure. One mL of the sample was added to 5 mL of 0.6% casein and incubated at 30°C for 10 minutes. The reaction was stopped thereafter by adding 5 mL of a TCA mixture containing 36 mL of 50% (w v^{-1}) TCA solution, 220 ml of 1 M sodium acetate solution, 330 mL of 1 M acetic acid solution in a total volume of 1000 mL. The unreacted casein was precipitated, and the resulting precipitate was filtered off using Whatman filter paper No. 1. The optical density of the filtrate was measured at 275 nm against a substrate blank. The blank was prepared by adding 5 mL of the TCA mixture to 1 mL of enzyme solution, then adding the casein and incubating at 30°C for 10 minutes. A unit of enzyme activity was defined as the enzyme quantity which liberated TCA-soluble materials equivalent for 10µg tyrosine per mL per minute.

RESULTS AND DISCUSSION

Effect of Different Industrial Media on Alkaline Protease Production by B. licheniformis:

The data in figure 1 show that through the production time course (0-96 h), the pH of cultures changed between acidic and alkaline. During the initial 24 h the pH dropped significantly in all culture media and increased thereafter for the rest of cultivation time. Cells grew exponentially during the first 72 h in all cultures studied and decreased thereafter. The maximal cell mass of about 3.5 g L^{-1} was obtained in medium No. II (which contains dextrin of 30 g L^{-1} and high soybean meal of about 50 g L^{-1}). On the other hand, the maximal value of enzyme obtained in all cultures was obtained in medium No. 1. This medium contained 60 g L^{-1} glucose and was supplemented with only 20 g L^{-1} soybean meal. The enzyme production was increased gradually in this medium with a rate of about 24 U mL^{-1} h^{-1} reaching about 2300 U ml^{-1} after 96 h. Soybean meal was usually recognized as a potentially useful and cost-effective medium ingredient, because it is largely produced as a by-product in oil extraction industry. Moreover, chemical analysis showed that it is composed of approximately 40% protein and is rich in other organic and inorganic components. Since soybean meal is an inexpensive and readily available substrate, it was used for the cost-effective production of extracellular proteases by many authors (Joo, H.S., *et al.,* 2002; Joo, H.S. and C.S. Chang. 2005; Tari, C., *et al.,* 2006). Another advantage of using soybean meal in medium is to decrease the nitrogen repression effect when using simple inorganic nitrogen sources in culture medium (Frankena, J., *et al.,* 1986). Therefore, medium I was used as the production medium in the following experiments for further medium optimization to increase enzyme production.

Effect of the Initial Glucose Concentration on Enzyme Production:

Since glucose was the most favorable carbon source for the enzyme production in the previous medium formula, it was necessary to study the effect of its concentration on the enzyme production. Different media were prepared of different glucose concentrations ranging from 0 up to 80 g L^{-1}. As shown in fig 2, the alkaline protease production was increased with the increase of glucose concentration up to 60 g L^{-1}. The maximal alkaline protease production of about 2450 U ml^{-1} was obtained after about 96 h in medium supplemented with 60 g L^{-1} glucose. Further increase in glucose concentration resulted in slight reduction in enzyme production. On the other hand, the maximal cell mass of about 3.3 g L^{-1} was obtained after 72 h in medium containing glucose in range of 50-60 g L^{-1}, other media of lower or higher glucose concentrations produced lower cell mass. As shown in the previous experiment, the pH dropped during the first 24 h and increased gradually thereafter. However, in this experiment a direct relation between the increase of glucose concentration and the decrease of pH during the early growth phase was observed. The pH of media was increased gradually after that time up to the alkaline level. However, high level of alkaline protease production was detected in all cultures under study. This indicates that, this strain is insensitive to glucose repression. In other protease producing organisms, glucose was shown to repress protease production at a concentration as low as 1% (Razak, N.A., *et al.,* 1994).

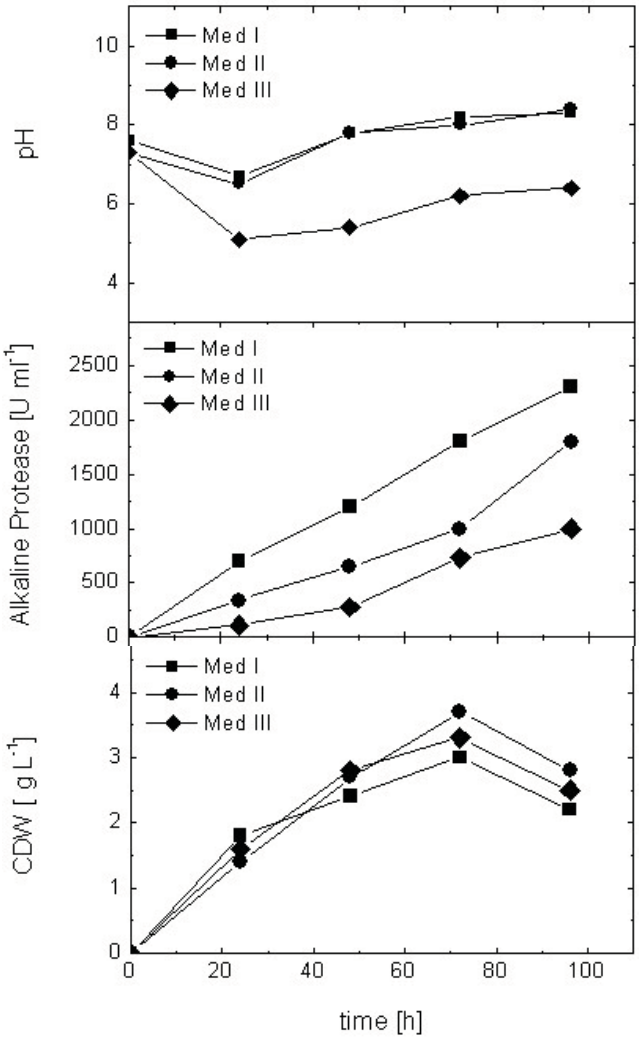

Fig. 1: Effect of different industrial media composition on cell growth, pH and alkaline protease production in shake flask cultures.

Cultivations in Laboratory Stirred Tank Bioreactor (16 L):

Mixing is very crucial for the production of alkaline protease by *B. licheniformis* (Potumarthi, R. *et al.,* 2007). Good mixing between gas-liquid phases could be achieved by means of aeration and agitation. On the other hand, agitation at higher stirring speeds may cause disruption of cells in the bioreactor by shear forces and formation of vortex which may result in poor mass transfer for both oxygen and substrate. Thus, a balance between aeration and agitation should be considered during the cultivation of aerobic microorganisms in submerged culture. Since agitation in our experiments was in a moderate agitation speed of 500 rpm, the effect of mixing and aeration was studied through the change in aeration rate. Therefore, cells were grown in batch culture at different air flow rates (0.5; 1.0 and 2.0 v v^{-1} min^{-1}) to study the effect of aeration and mixing on cell growth kinetics and enzyme production at moderate shear. Except for aeration rate variations, all other process variables were kept constant to allow the determination of the aeration influence on the overall process performance.

***Cultivation at low aeration rate (0.5 v v^{-1} min^{-1}.)*:**

Figure 3 shows the different changes of process parameters during cell growth and enzyme production. As shown, cells grew exponentially during the first 60 h reaching the maximal cell mass of about 3 g L^{-1}.

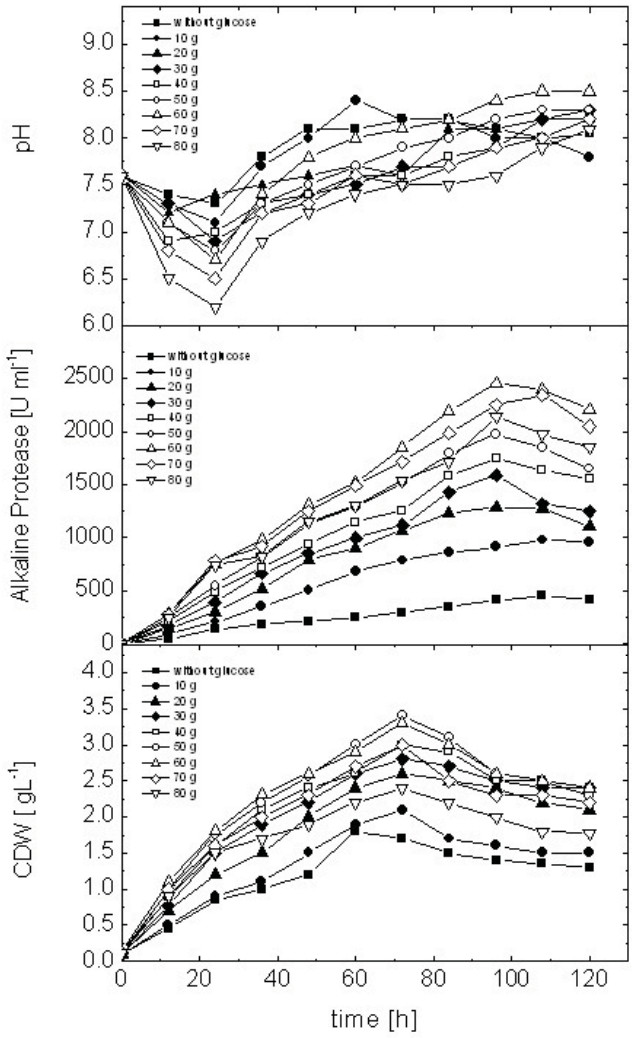

Fig. 2: Effect of different glucose concentrations on cell growth, pH and enzyme production.

During the growth phase, glucose was consumed with a rate of 1.0 g L^{-1} h, reaching complete carbon source limitation at the end of the growth phase. The production of alkaline protease started after about 12 h and increased gradually in culture medium with rate of 33.9 U mL^{-1} h^{-1}. The maximal enzyme production was about 3050 U ml^{-1} after 96 h cultivation. The pH profile of culture shows also high similarity with shake flask cultures. The pH value decreased during the first 24 h and increased gradually thereafter. The final pH value after 120 h was about 7.8.

***Cultivation at medium aeration rate (1.0 v v^{-1} min^{-1}.)*:**

In the present experiment, cells were cultivated in standard aeration rate for aerobic microorganisms in submerged culture with equi-volume of air to liquid phase. As shown in figure 4, the maximal cell dry weight reached 2.6 g L^{-1} after 72 h. The cell mass decreased after that time due to cell lysis and reached 1.6 g L^{-1} at the end of cultivation time. On the other hand, alkaline protease production reached its maximal value of 4120 U ml^{-1} after 84 h. Glucose was consumed gradually in culture during the growth phase with a rate of about 1.14 g L^{-1} h^{-1}, and no glucose was detected in culture after 60 h.

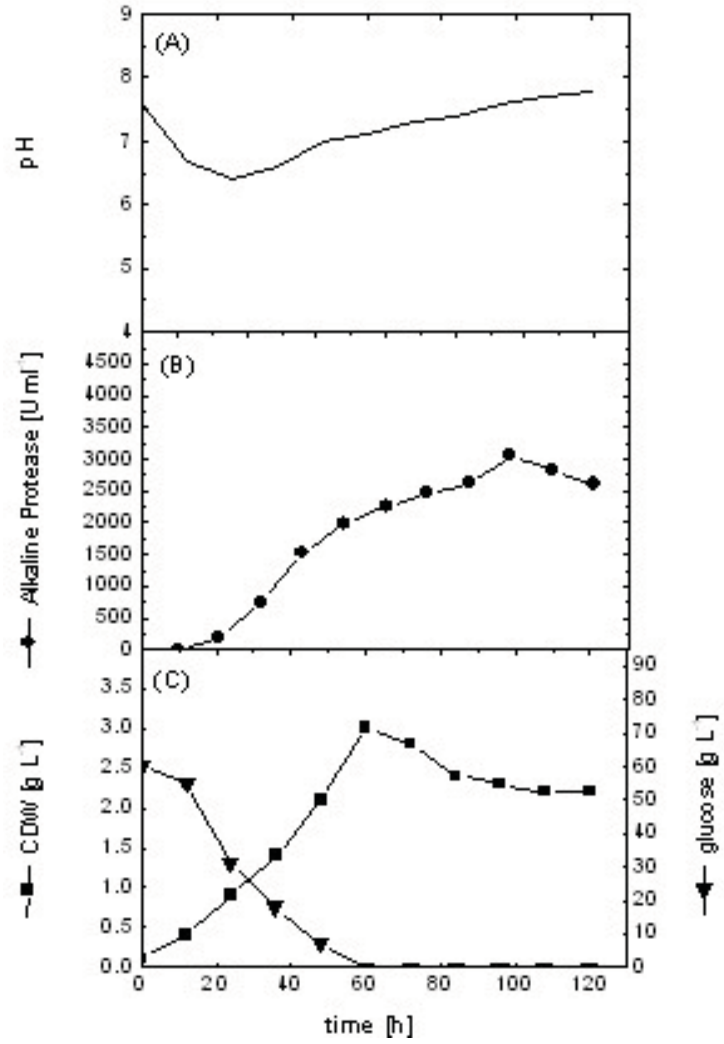

Fig. 3: Cell growth, pH and alkaline protease production during batch cultivation of *B. licheniformis* in 16 L bioreactor at aeration rate of (0.5 v v^{-1} min^{-1}.)

***Cultivation at high aeration rate (2.0 v v^{-1} min^{-1}.)*:**

The kinetics of cell growth and carbohydrate consumption as well as the change in pH during cell cultivation at high aeration rate are represented in figure 5. Cells grew exponentially during the first 60 hours and reached a maximal cell mass of 2.4 g L^{-1}. This value was less than those obtained in cultures of lower aeration rate. Cell mass was decreased thereafter with specific cell degradation rate [-μ] of about 0.014 h^{-1} which is almost 3 times and 2 times higher than the other cultures of aeration rate of 0.5 and 1.0 v $v^{-1}min^{-1}$, respectively.

The differences in cell growth and enzyme production kinetics of different cultures run under different aeration rates are summarized in table 1. As the aeration rate increase from 0.5 to 2.0 v $v^{-1}min^{-1}$, a significant reduction in both specific growth rate [μ] and the total cell mass was observed. This was due to high shear stress (namely bubble shear effect) on the cell which may reduce the cell mass as well the increase of oxidative stress on cells at high aeration rate (Jung, I.L., *et al.,* 2003; Pérez, J.A.S., *et al.,* 2006).

Concerning alkaline protease production, the maximal enzyme production rate of 49.0 U ml^{-1} h^{-1} was obtained in 1.0 v v^{-1} min^{-1} aerated culture. The increase of aeration upto 2 v v^{-1} min^{-1} resulted in decrease in production rate by about 25%. The maximal enzyme production of about 4120 U ml^{-1} was obtained

Table 1: Kinetic parameters of cell growth and alkaline protease production by *B.licheniformis* during cultivations in 16-L stirred tank bioreactor at different aeration rates.

Parameter	Aeration rate (v v^{-1} min^{-1})		
	0.5	1.0	2.0
X_{max} [g L^{-1}]	3.0	2.6	2.4
P_{max} [U mL^{-1}]	3050	4120	3560
μ [h^{-1}]	0.033	0.028	0.026
-μ [h^{-1}]	0.005	0.010	0.014
$Y_{P/X}$ [U g^{-1}]*	750	1411	1212
Q_s [g L^{-1} h^{-1}]	1.0	1.14	1.25
Q_p [U mL^{-1} h^{-1}]	33.9	49.0	37.1
- Q_p [U mL^{-1} h^{-1}]	18.3	28.1	35.4

Abbreviations: X_{max} : maximal cell dry weight; P_{max} : maximal volumetric alkaline protease produced, μ : specific cell growth rate, -μ : specific cell degradation rate, Q_p: volumetric alkaline protease production rate, $-Q_p$: volumetric alkaline protease degradation rate.
Yield Coefficient: $Y_{p/x}$: [U] Alkaline protease / [g] cell dry weight
*, This value was calculated at the time of maximal cell mass before cell entering decline phase.

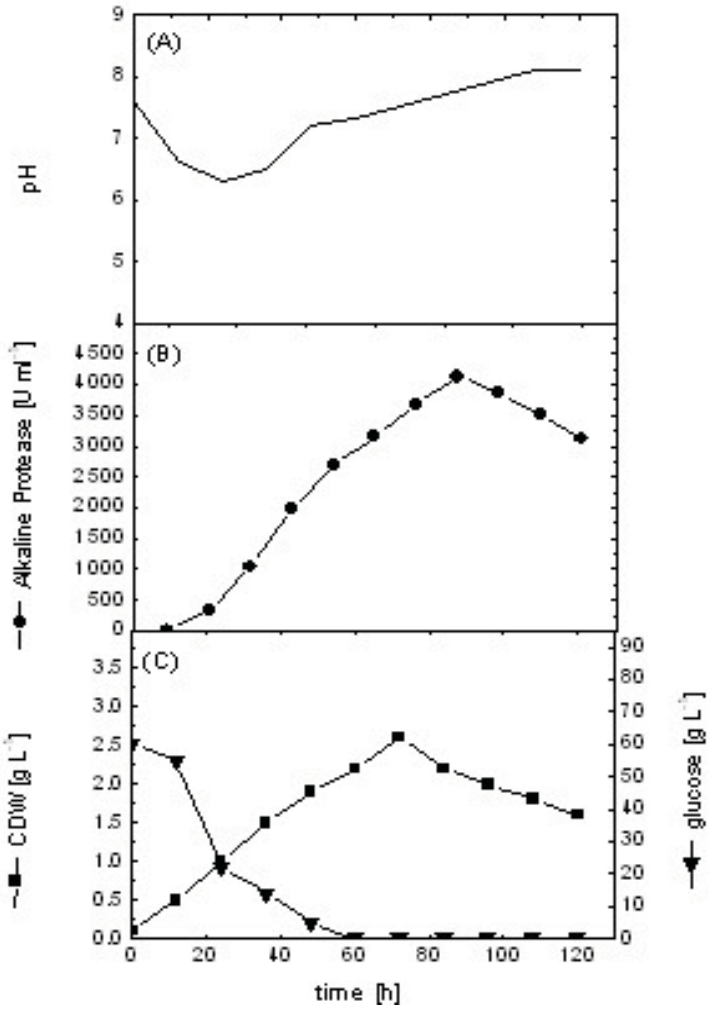

Fig. 4: Cell growth, pH and alkaline protease production during batch cultivation of *B. licheniformis* in 16 L bioreactor at aeration rate of (1.0 v v^{-1} min^{-1}).

in 1.0 v v^{-1} min^{-1} aerated culture. The enzyme production in 2.0 v v^{-1} min^{-1} aerated culture was 3560 U ml^{-1} which is higher by about 10% than 0.5 v v^{-1} min^{-1} aerated culture. This indicate that low aeration rate has more negative effect on enzyme production than high aeration rate. However, it have been reported that low aeration combined to low agitation can cause significant reduction in the protease production by highly aerobic

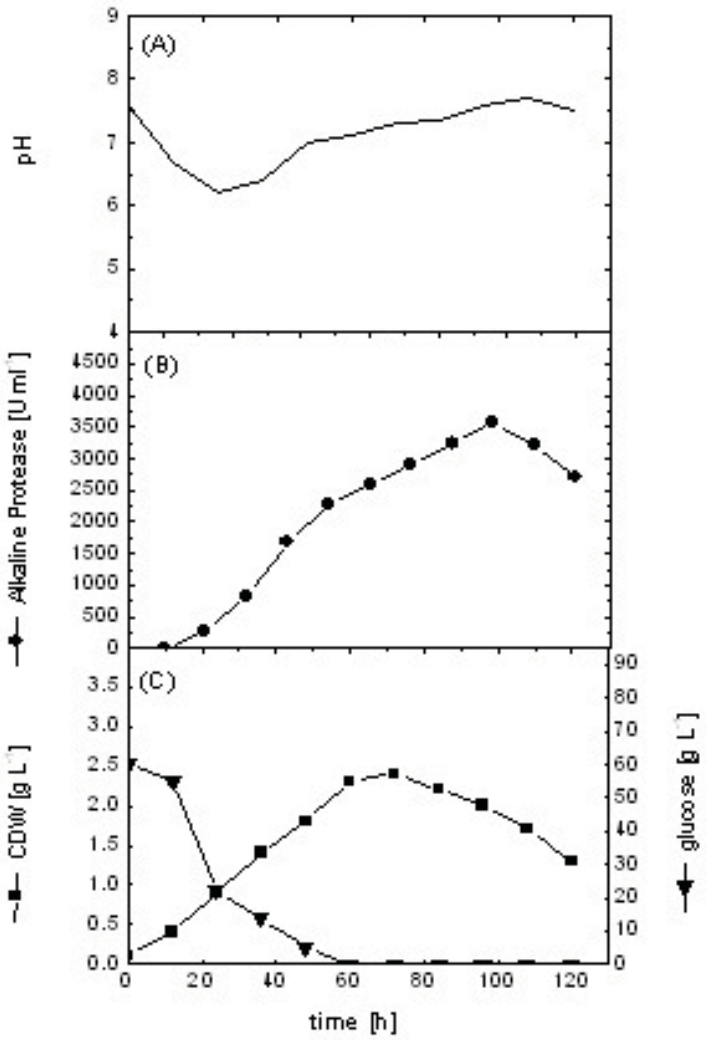

Fig. 5: Cell growth, pH and alkaline protease production during batch cultivation of *B. licheniformis* in 16 L bioreactor at aeration rate of (2.0 v v^{-1} min^{-1}).

microorganisms such as *Bacillus licheniformis* (Potumarthi, R., *et al.,* 2007; Calik, P., *et al.,* 1998; Calik, P., *et al.,* 2002). However, not only the enzyme production was also influenced by aeration rate but also the enzyme degradation as shown in figures 3-5. As the aeration rate increase, the rate of enzyme degradation [$-Q_p$] decreased significantly. The rate of enzyme degradation in 2.0 v $v^{-1}min^{-1}$ aerated culture reached about 35.4 U ml^{-1} h^{-1} which is almost the same like the production rate in the same culture and double of the rate of enzyme degradation in low aerated culture of about 0.5 v v^{-1} min^{-1}. The higher enzyme inactivation in highly aerated culture may be due to irreversible oxidation of amino acid residues of the enzyme structure (Cabiscol, E., *et al.,* 2002). In the presence of oxygen in excess, an alkoxyl radical is obtained which leads to the peptide bond cleavage (Berlett, B.S. and E.R. Stadtman. 1997). On the other hand, the increase in aeration rate resulted in significant increase in glucose consumption rate in all aeration level under study. Thus, no direct relation between glucose consumption and enzyme production was determined.

Glucose consumption rate in culture during submerged cultivations is usually regulated by the interaction between different cultivation conditions. Shear stress, which came either from agitation rate or aeration rate, is one of limiting factors for nutrient availability in culture. The glucose uptake at cellular level is driven by the proton motive force or ATP and then glucose is delivered into the cytoplasm without chemical modification and phosphorylated by glucokinase. In case of *B. licheniformis* this process is governed by two active transport

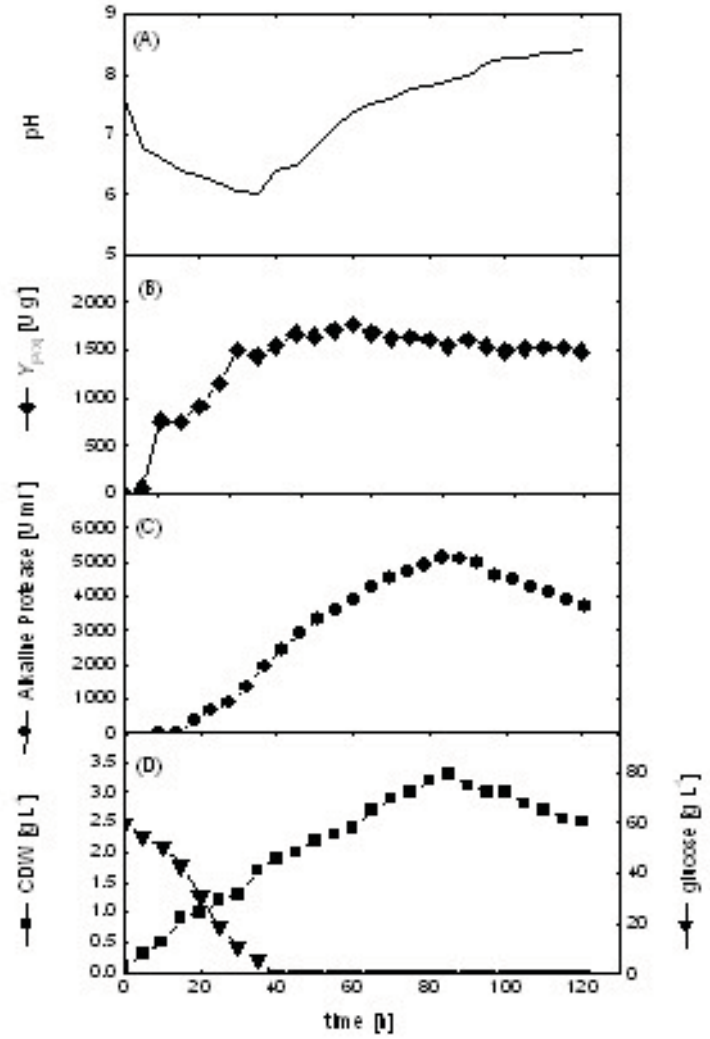

Fig. 6: Cell growth, pH and alkaline protease production during batch cultivation of *B. licheniformis* in 500 L bioreactor at aeration rate of (1.0 v v^{-1} min^{-1}).

mechanisms (Tangney, M., *et al.,* 1993). The most predominant pathway during the growth phase, where most of glucose was consumed in our study, was mainly controlled by glucose phosphotransferase system (PTS) which may be more activated by ATP (Tangney, M., *et al.,*1996). However, most of ATP is generated from aerobic respiration pathways which is more active in highly aerated cultures.

Cultivations in Pilot Plant Bioreactor (500 L STR):

Based on the data of the previous experiments in small scale bioreactors, cultivations were conducted in large scale 500 L bioreactor with a working volume of 250 L using a two-stage inoculum for seeding the bioreactor. The first-stage inoculum was developed in 500 mL Erlenmeyer flask containing 100 mL nutrient broth medium. After 24 h growth, this was transferred to second stage inoculums in 16 L bioreactor containing enzyme production medium (MI). After 12 h, the grown cells were directly transferred to 500 L bioreactor containing MI medium. The aeration rate was adjusted to 1.0 v v^{-1} min^{-1}. for the production scale bioreactor. The changes in cell growth, glucose consumption and alkaline protease production during the course of batch fermentation are shown in figure 6. As shown, like in typical other cultures of *B. licheniformis*, cells grew exponentially with specific growth rate [μ] of about 0.023 g L^{-1} h^{-1}. reaching the maximal cell mass of 3.3 g L^{-1} after 85 h. The cell concentration decreased thereafter and the rate of cell death calculated as the specific decrease in cell mass [-μ] was about 0.022 which is almost equal to the specific growth rate.

During the growth phase, enzyme production started with a rate of 64.25 U ml^{-1} h^{-1} reaching the maximal value of about 5100 U ml^{-1} after 80 h. The rate of enzyme production in large scale bioreactor was 31% higher compared to the corresponding batch culture running under the same cultivation conditions in smaller scale bioreactor. On the other hand, glucose was almost consumed after only 40 h. The rate of glucose consumption was 1.5 g L^{-1} h^{-1}. This was higher than the corresponding batch culture in 16-L bioreactor culture (Table 1) by about 30%.

Conclusion:

The composition of the industrial medium, especially glucose concentration, was shown to be important for alkaline protease production. Moreover, aeration rate was shown to be critical for alkaline protease production by *B. licheniformis*. The overall volumetric alkaline protease production process was not affected by only the enzyme production rate but also by the enzyme degradation rate. The enzyme degradation rate is highly influenced by air flow rate in culture than production rate. Thus, the aeration rate in stirred tank bioreactor seems to define the enzyme production and should be considered during the process optimization for process scaling-up.

REFERENCES

Freedonia Group Inc. World Enzymes business report. 2007. Freedonia Group Inc. OH, USA.

Kumar, C.G. and H. Takagi, 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol. Adv., 17: 561-594.

Dayanandan, A., J. Kanagaraj, L. Sounderraj, R. Govindaraju and G.S. Rajkumar, 2003. Application of an alkaline protease in leather processing: an ecofriendly approach. J. Cleaner Production, 11: 533-536.

Kumar, C.G. and M.P. Tiwari, 1999. Use of alkaline proteases for ultrafiltration membrane cleaning. Biotechnol. Tech., 13: 235-238.

Maurer, K.H., 2004. Detergent proteases. Curr. Opin Biotechnol, 15: 330-334.

Anwar, A. and M. Saleemuddin, 2001. Alkaline protease from *Spilosoma oblique*: potential applications in bio-formulations. Biotechnol. Appl. Biochem., 31: 85-89.

Haki, G.D. and S.K. Rakshit, 2003. Developments in industrially important thermostable enzymes: a review. Biores. Technol., 89: 17-34.

Mehrotra, S., P.K. Pandey, R. Gaur and N.S. Darmwal, 1999. The production of alkaline protease by a *Bacillus* species isolate. Biores. Technol., 67: 201-203.

Joo, H.S., C.G. Kumar, G.C. Park, K.T. Kim, S.R. Paik and C.S. Chang, 2002. Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. Process Biochem., 38: 155-159.

Puri, S., Q.K. Beg and R. Gupta, 2002. Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr. Microbiol., 44: 286-290.

Jasvir, S., R.M. Vohra and D.K. Sahoo, 2004. Enhanced production of alkaline proteases by *Bacillus sphaericus* using fed-batch culture. Process Biochem., 39: 1093-1101.

Potumarthi, R., S. Ch and A. Jetty, 2007. Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. Biochem. Eng. J., 34: 185-192.

Kaur, S., R.M. Vohra, K. Kapoor, Q.K. Beg and G.S. Hoondal, 2001. Enhanced production and characterization of a highly thermostable alkaline protease from *Bacillus* sp. P-2. World J. Microbiol. Biotechnol., 17: 125-129.

Uyar, F. and Z. Baysal, 2004. Production and optimization of process parameters for alkaline protease production by newly isolated *Bacillus* sp. Under solid state fermentation. Process Biochem., 39: 1893-1898.

Aijun, Z., C. Hongzhang and L. Zuohu, 2005. Air pressure pulsation solid state production of alkaline protease by *Bacillus pumilus* 1.1625. Process Biochem., 40: 1547-1551.

Fukumoto, J., T. Yamamoto and D. Tsuru, 1971. Process for producing detergent resisting alkaline protease. Canadian Patent, 910: 214.

Joo, H.S. and C.S. Chang, 2005. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. Process Biochem., 40: 1263-1270.

Tari, C., H. Genckal and F. Tokath, 2006. Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Process Biochem., 41: 659-665.

Frankena, J., G.M. Koningstein, H.W. Verseveld and A.H. Stouthamer, 1986. Effect of different limitations in chemostat cultures on growth and production of exocellular protease by *Bacillus licheniformis*. Appl. Microbiol. Biotechnol., 24: 106-112.

Razak, N.A., M.Y.A. Samad, M. Basri, W.M.Z.W. Yunus, K. Ampon and A.B. Salleh, 1994. Thermostable extracellular protease of *Bacillus stearothermophilus*: factors affecting its production. World J Microbiol. Biotechnol., 10: 260-263.

Jung, I.L., T.J. Oh and I.G. Kim, 2003. Abnormal growth of polyamine-deficient *Escherichia coli* mutant is partially caused by oxidative stress-induced damage. Arch. Biochem. Biophys., 418: 125-132.

Pérez, J.A.S., E.M.R. Porcel, J.L.C. Lopez, J.M.F. Sevilla and Y. Chisti, 2006. Shear rate in stirred tank and bubble column bioreactors. Chem. Eng. J., 124: 1-5.

Calik, P., G. Calik and T.H. Ozdamar, 1998. Oxygen transfer effects in serine alkaline protease fermentation by *Bacillus licheniformis* use of citric acid as the carbon source. Enz. Microb. Technol., 23: 451-461.

Calik, P., G. Calik and T.H. Ozdamar, 2002. Oxygen-transfer strategy and its regulation effects in serine alkaline protease production by *Bacillus licheniformis*. Biotechnol. Bioeng., 69: 301-311.

Cabiscol, E., J. Tamarit and J. Ros, 2002. Oxidative stress in bacteria and protein damage by reactive oxygen species. International Microbiol, 3: 3-8.

Berlett, B.S. and E.R. Stadtman, 1997. Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem., 272: 20313-20316.

Tangney, M., F. Priest and W. Mitchell, 1993. Two glucose transport systems in *Bacillus licheniformis*. J. Bacteriol., 175: 2137-2142.

Tangney, M., J. Tate, F. Priest and W. Mitchell, 1996. Glucose transport in stationary-phase cultures of an asporogenous strain of *Bacillus licheniformis*. Appl. Env. Microbiol., 62: 732-734.