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Enhanced productivity of serine alkaline protease by *Bacillus* sp. using soybean as substrate

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ABSTRACT

The growth and protease production by *Bacillus* sp. (SBP-29) was examined for poultry processing industries. The maximum protease activity was 3028 U/mL using 1.5% (w/v) of soybean meal as substrate. Soybean meal is an inexpensive and readily available, thus it can be used as the cost effective crude material for the production of an extracellular protease. Inorganic nitrogen sources proved to be less favorable, for protease production as strong catabolic repression was observed with ammonium ions. A maximum of 3208 U/mL of protease was produced in 18 h in a 10L bioreactor. The enzyme has temperature and pH optima of 60°C and 9.5 respectively. However, the temperature stability range is from 20-90 °C and pH stability range is from 6.0–12.0. The protease was completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diodopropyl fluorophosphate (DFP), with little increase (10-15%) in the production of upon addition of Ca^{++} and Mg^{++}.

Keywords: *Bacillus* sp., protease, soybean

INTRODUCTION

Proteases also known as peptidyl-peptide hydrolysis, constitute 60-65% of the global enzyme market (Banerjee *et al*., 1999; Genckel and Tari, 2006; Laxman *et al*., 2005). The applications of proteases include in the detergent, food, leather, meat tenderization industries. Proteases are also important tools in studying the structure of proteins and peptides. Besides that, they are also used in pharmaceuticals, medical diagnosis, and decomposition of gelatin on X-ray films as well as in textiles (Joo *et al*., 2002; Patel *et al*., 2005, Tari *et al*., 2006). Protease can be produced by all microorganisms, however, only microbes that produce a substantial amount of extra cellular protease have been exploited commercially. To date, the major proportion of the commercial alkaline protease are derived from *Bacillus* sp. (Joo *et al*., 2002; Manachini *et al*, 1998; Yang *et al*., 2000; Ito *et al*., 1998). The reason for this is their wide temperature and pH tolerance and stability (Genckel and Tari, 2006).

For the bulk production of commodity products like enzymes, the cost of the production media can substantially affect the overall process economics. Approximately 40% of the production cost of industrially important enzymes is estimated to derive from the cost of growth medium (Joo *et al*., 2002). Thus, the use of cost effective growth medium for the production of alkaline proteases from an alkalophilic *Bacillus* sp. is especially important, because these enzyme account for approximately 25% of the world wide enzyme consumption (Gessesse, 1997). In the current work, soybean meal (*Glycine max*) which is cheap and is commonly available was considered as a suitable ingredient in the formulation of the production medium. Soybean meal is largely produced as a byproduct during oil extraction (Gattinger *et al*., 1990). Soybean which contains 40% of protein, 17% carbohydrate, 18% oil, traces of metals, moderate amount of vitamins and amino acids supplies almost all the nutrient required for the growth of Bacilli. Besides that, soybean also contains small amount of enzymes such as protease, urease and lipoxidase.

Our preliminary experiments revealed that protease secreted by this *Bacillus* sp.(SBP-29) is suitable for use in poultry processing industry degrading the turkey and chicken feathers which otherwise contribute to several tons of waste products. However, for the commercial exploitation and for extensive industrial evaluations, it was desirable to optimize the production on large scale using inexpensive media. In this paper, we describe a low cost medium for maximum production of an extra cellular alkaline protease from *Bacillus* sp. and important biochemical properties.

MATERIALS AND METHODS

Chemicals

Casein and inhibitors of serine, cysteine and trypsin type proteases, soybean inhibitors and chelators of divalent cations (Ethylenedi aminetetra acetate (EDTA), were purchased from Sigma (St. Louis, USA). All other chemicals used were of analytical grade and were procured from local suppliers.

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Organism and growth maintenance conditions

Bacillus sp. (SBP-29), a potent protease producer was isolated from the soil by enrichment and selective screening on skim milk agar plate. The organism was cultivated at 37 ± 1 °C in a bacteriological incubator for 24 h and subsequently maintained at 4 °C in a Biological Oxygen Demand (B.O.D) incubator (Yorko, Deluxe- 10) by routine transfers after every 15 days on nutrient agar slants at pH 7.0.

Protease production medium

The modified basal medium used for protease production contained (g/L) Casamino acid (5); NH_4Cl (3); KH_2PO_4 (1.0); K_2HPO_4 (3.0); Na_2SO_4 (2.0); and $MgSO_4.7H_2O$ (0.10) with the pH of 7.0. The production medium (50 mL) was inoculated with 2.0% of inoculum with OD_{660} of 0.65. The culture was incubated at 37 ± 1 °C at 200 rpm in a incubator shaker (NBS Shaker, G-25R, Edison, USA). After the desired incubation period, the culture was centrifuged at 8000 x *g* for 20 min at 4 °C (Sorvall, RC-5C plus, Du Pont, Germany). The supernatant obtained was used as protease source.

Protease assay

The protease activity was measured as described by Meyers and Ahearn (1976) with some modifications. 0.5 mL of glycine NaOH buffer (pH 10, 0.2 M) was added to 0.5 mL of appropriately diluted enzyme and was incubated with 1 mL of 1% casein solution (prepared in glycine NaOH buffer, pH 10) for 15 min at 60 °C. The reaction was stopped by the addition of 4 mL of 5% (v/v) trichloroacetic acid. The contents were centrifuged after 1 h at 3000 x *g* for 10 min and the filtrate was used for measuring protease activity on the basis of color change. 5 mL of 0.4 M sodium carbonate solutions was added to 1 ml of the filtrate and was kept for 10 min. Folin's Ciocalteau Phenol reagent of 1:1 dilution was added and kept in dark for 30 min. The color change was determined at 660 nm. One unit of protease was equivalent to the amount of enzyme required to release 1mg/mL/min of tyrosine under standard assay conditions.

Effect of carbon and nitrogen sources

The effect of carbon and nitrogen sources for protease production were determined using different simple and complex carbon sources consisting of glucose, fructose, galactose, starch, lactose, mannose, sucrose, sorbitol, mannitol, dextrin, malt extract, wheat bran and maize starch. Organic and inorganic nitrogen sources consists casein, casamino acid, skim milk, peptone, beef extract, yeast extract, CSL, soybean meal, NH_4Cl, NH_4NO_3 and $NaNO_3$ were supplemented individually in the production medium for protease production from *Bacillus* sp. All these sources were replaced in the production medium at a concentration of 0.5% (w/v) and 2.0% (w/v) for carbon and nitrogen, respectively. Protease yield was determined after 48 h of incubation at 37 °C under shaking condition of 200 rpm.

Enzyme characterization

The effect of temperature on the stability and activity of the protease was determined by incubating the enzyme at different temperature ranging from 10-90ºC. The pH stability and activity of the enzyme was tested at different pH 6-12 using phosphate buffer pH 6-8; Tris–HCl buffer-pH 9.0 ; Glycine NaOH buffer-10.0 and phosphate buffer for pH 11.0; pH 12.0. Every reaction mixture contained the same amount of protease and the reaction was performed under the same conditions accept for the pH value or temperature.

The effect of various inhibitors and chelators on protease activity was determined by incubating with the enzyme for 30 min at room temperature and the relative activity was determined by standard assay protocols. All the inhibitors were used at 1mM final concentration unless otherwise stated. The effect of metal ions of 1mM concentration on protease activity was determined by incubating the enzyme with different metal salts at room temperature for 1 h and thereafter the relative activities were determined under standard assay conditions. The enzyme assay was carried out in the presence of Ca^{++}, Cu^{++}, Fe^{++}, Ba^{++}, Co^{++}, Cd^{++}, Mg^{++}, Mn^{++}, Zn^{++} and Hg^{++}.

Protease production in a fermentor system

Cultivation of *Bacillus* sp. for protease production was also carried out in a 10 L fermentor (Bio-flow IV, NBS, USA) with a working volume of 7.5 L. The medium was sterilized in situ at 121 °C for 20 min and was inoculated with appropriate size of the seed inoculum ($OD_{660nm} \cong 0.600$). The fermentation was carried out at 37 ± 1 °C for 24 h with controlled pH at 7.0. The impeller speed was initially adjusted to 350 rpm and compressed sterile air was sparged into the medium at constant rate of 0.5 vvm. The dissolved oxygen was not allowed to fall below a fixed set point of 25% by cascading. Samples were withdrawn periodically at an interval of 2 h and analyzed for protease production. The fermentation parameters, such as temperature, pH, dissolved oxygen and airflow rate were continuously monitored using microprocessor-controlled probes.

RESULTS AND DISCUSSION

The selected strain, SBP-29 exhibited a large zone of hydrolysis on milk agar plate and was taken up for optimization of maximum production, scale up and protein characterization. The isolate was a Gram positive, motile, rod shaped bacterium and strictly aerobic. This isolate produced 440 U/mL of protease in the initial production medium.

Effect of carbon source on the protease production

Bacilllus sp. produced maximum protease in wheat bran (1373 U/mL) followed by glucose (1100 U/mL). However, carbon sources like sorbitol, mannitol and dextrin, drastically inhibit the protease production. Further optimization of the wheat bran concentration showed that wheat bran was the effective substrate for protease production among all the carbon sources tested and the yield was enhanced by two fold by the addition of 0.5% wheat bran (1374 U/mL) when compared with a basal medium without bran (440 U/mL) (Figure1).

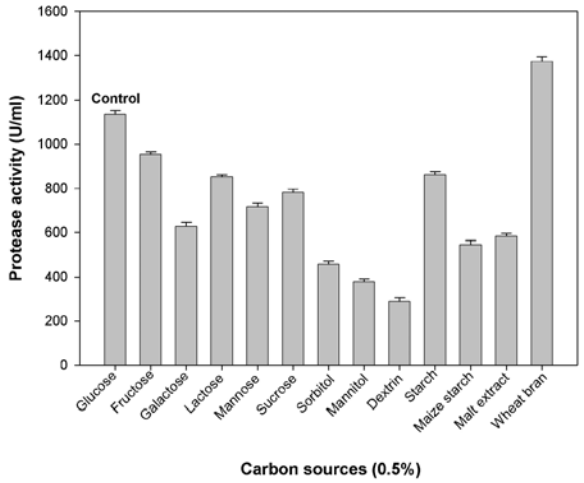

Figure 1: Effect of different carbon sources on protease production by *Bacillus* sp.

Effect of nitrogen source on the protease production

Bacillus sp. was grown in the presence of different (organic and inorganic) nitrogen sources, replacing the total nitrogen from the production medium with equivalent amount of nitrogen, in the presence of 0.5% wheat bran as carbon source. All the organic nitrogen sources used in the present study supported growth and production. Maximum production of protease (2725 U/mL) was observed with 2% soybean meal (Figure 2). This is followed by soyflour, casein, etc. Nilegaonkar *et al.* (2007) reported that the protease activity was highest with soybean meal (124.24 U/mL). Deshpande *et al.* (2004) also reported that soybean meal was used as an inducer for protease production from *Conidiobolus coronatus*. In case of inorganic nitrogen sources, except for sodium nitrate, none of the other three inorganic nitrogen source supported the growth. Our results are in accordance to the findings of Ferrero *et al.* (1996); Johnvesly and Naik (2000); Kole *et al.* (1988) who observed that inorganic nitrogen sources like ammonia are less favorable for the growth and enzyme production.

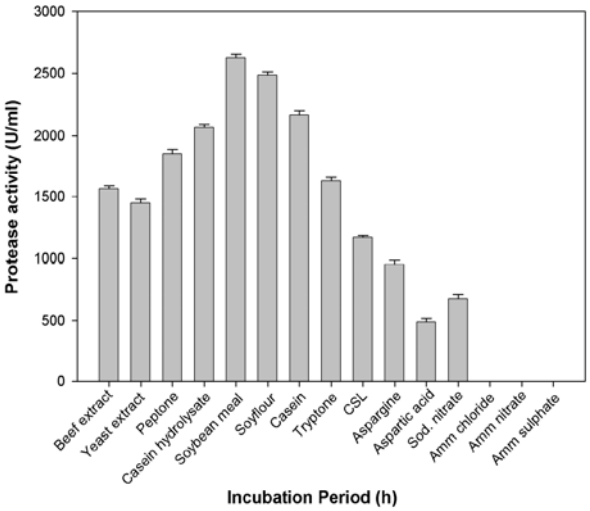

Figure 2: Effect of nitrogen source on protease production by *Bacillus* sp.

Maximum enzyme production using soybean meal

It is well documented in the literature that nitrogen is metabolized to produce primarily amino acid, nucleic acid, protein and cell wall components. These nitrogen sources have regulatory effect on the enzyme synthesis. Production of protease is highly dependent on the both carbon and nitrogen source available in the medium (Chu *et al*., 1992; Moon *et al*., 1991; Patel *et al.,* 2005). Soybean meal is a source of both carbon and nitrogen. It is an inexpensive and readily available substrate. In the present study, as soybean meal was found to be best inducer, effect of soybean meal concentration (0.5-3%) on protease production was studied. Results showed that the enzyme production reaches to 3021 U/mL with 1.5% soybean meal (Table 1). Similar finding had been reported by Joo *et al*. (2002), who observed that soybean meal at 1.5% concentration resulted in highest protease production in *Bacillus horikoshii*. Our study is further supported by the findings of Sutar *et al.* (1992); Laxman *et al.* (2005) who reported 4% and 2% soybean meal to be optimal for protease production from *Aspergillus flavus* and *Conidiobolus coronatus.*

Table 1: Effect of soybean meal concentration on protease produced by *Bacillus* sp. at 37°C and pH 7.0

Concentration of Soybean (% w/v)	Protease (U\mL)
0.5	1867 ± 11
1.0	2637 ±09
1.5	3021 ±14
2.0	2634 ±08
2.5	1905 ±10
3.0	1346 ±09

Temperature and pH stability of the enzyme

The optimum temperature of the enzyme was found to be 60 °C (Figure 3) and the enzyme tolerance is between 20-90 °C, the enzyme was 90% stable up to 55 °C after 1 h of incubation, however it showed a marked decrease over 60 °C within 30 min (Figure 4). The optimum pH of the enzyme is 9.0 (Figure 5). However, the enzyme was active up to pH 12.0. However, the activity remains 77% at pH 12 after 1 h of incubation (Figure 6). The high pH optimum is a feature of alkaline protease (Gessesse, 1997; Mehrotra *et al*., 1999; Jasvir *et al*., 1999, Joo *et al*., 2002).

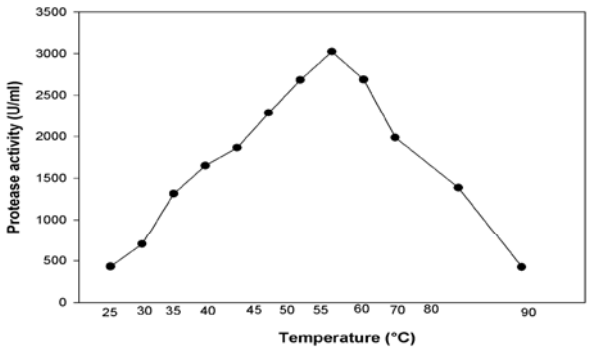

Figure 3: Temperature profile of the *Bacillus* sp. protease

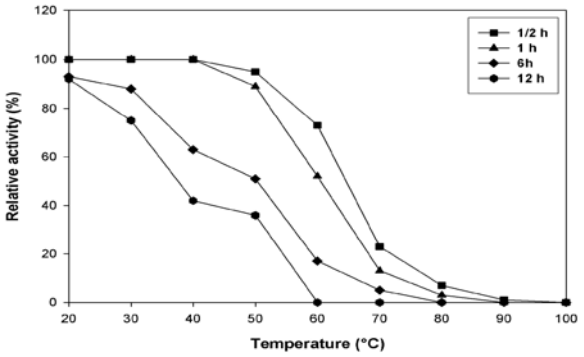

Figure 4: Temperature stability of alkaline protease produced by *Bacillus* sp.

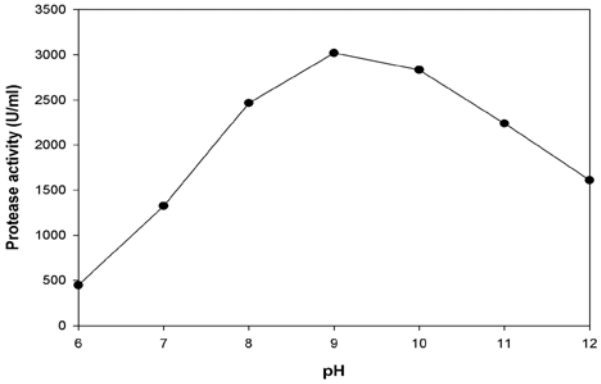

Figure 5: Optimum pH profile of the *Bacillus* sp. protease

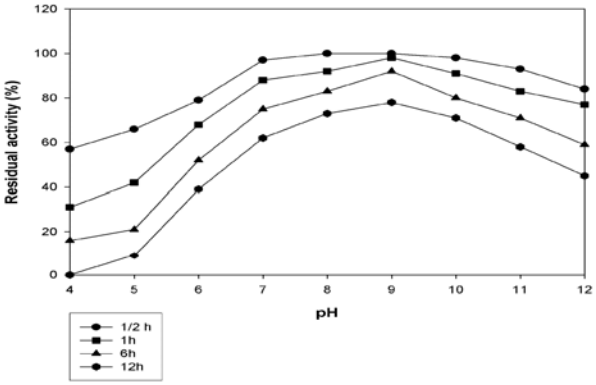

Figure 6: pH stability of the alkaline protease by *Bacillus* sp.

Effect of inhibitors on protease synthesis

The activity of the protease was completely inhibited in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), diodopropyl fluorophosphate (DFP) and Iodoacetic acid. Inhibitors like cyanamide and N-ethylmaleimide slightly inhibited the enzyme activity (Table 2). Several specific protease inhibitors such as bestatin, chymostatin and *N*-bromosuccinyl also inhibit the enzyme activity upto more than 90%. PMSF is known to strongly inhibit the serine residue at the active site causing complete loss of enzyme activity (Huang *et al.,* 2003; Tang *et al.,* 2004). This inhibition profile suggests that the extracellular protease from *Bacillus* sp. belongs to a family of serine protease. Among the metal ions tested, Ca^{++} and Mg^{++} marginally stimulated the protease activity upto 15% at 1 mM concentration (Table 3). These cations probably protect the enzyme against thermal denaturation and therefore maintain the active conformation of the enzyme at high temperature (Donaghy and Mc Kay, 1993). Ag^{+} and Co^{++} inhibit the protease activity. Similar inhibitor and metal ion studies have been reported by Joo *et al.* (2002); Beg *et al.* (2003); Tang *et al.* (2004).

Scale up of protease production in 10L bioreactor

Scale up studies were carried out in a 10 L bioreactor with a working volume of 7.5 L. Protease production started early within 4 h, however, there was no significant increase in the production up to 8 h. This is because, at this time, the organism was in the exponential phase. A significant fall (below 25%) in the dissolved oxygen was observed which was automatically maintained by an increase in the agitation from 300 up to 600 rpm. Similar has been observed by Laxman *et al.* (2005) who reported that 500 rpm was suitable for the growth and protease activity. The exponential phase was followed by the stationary phase wherein maximum protease activity was observed resulting in 3208 U/mL of protease in 18 h (Figure 7). Thereafter, a decline in the protease

Table 2: Effect of inhibitors on protease activity

Inhibitors (1mM)	Relative activity (%)
Control	100
β-mercaptoethanol	113
Antipain	60
Bromoacetic acid	82
Ethylene diamine tetra acetate	64
Dithiothrietol (DTT)	77
Cyanamide	90
Phenyl methyl sulphonyl Fluoride (PMSF)	No activity
N-ethylmaleimide	95
N-Bromosuccinyl	09
diodopropyl fluorophasphate (DFP)	No activity
Iodoacetic acid	02
Bestatin	11
Chymostatin	05

Table 3: Effect of metal ions on protease activity

Metal ions (1mM)	Relative activity (%)
Control	100
Ca^{++}	116
Mg^{++}	110
Pb^{++}	90
Ba^{++}	85
Mn^{++}	78
Zn^{++}	75
Cu^{++}	69
Fe^{++}	48
Co^{++}	33
Ag^{++}	16
Hg^{++}	9
EDTA	85

Note: Control: Protease activity 3021 (U/mL)

production was observed in the bioreactor. Moon *et al.* (1991); Chu *et al.* (1992); Gupta *et al.* (2002) have also reported a similar cessation in protease production once a maximum amount of the enzyme is produced during the run. Although, there are several theories such as autoproteolysis (Jang *et al*., 2001) and protease degradation by some proteolytic activity on the cell surface on nitrogen starved cells in the fermentation broth have been put forward (Chu *et al*., 1992; Beg *et al*., 2003), however, the exact mechanism is yet to be clearly known. During the fermentation, the aeration rate also influences the mixing of media components and thus affects the nutrient availability to microorganisms (Moon *et al*., 1991; Chu *et al*., 1992*).* In the present model, the high agitation rate (>200 rpm) favored maximum production and a decrease in the agitation rate drastically lowered the total protease yield. Protease production at high agitation rate up to 360–600 rpm has been reported by Moon *et al.* (1991) with *B. firmus* and by Jang *et al.* (2001) while working with *Bacillus* sp B21-2. Similar observation has been found in the present investigation, where when the agitation rates increases from 350 to 700 rpm the organism grows at a fast rate achieving maximum protease (3978 U/mL) in a minimal time 18 h in a bioreactor.

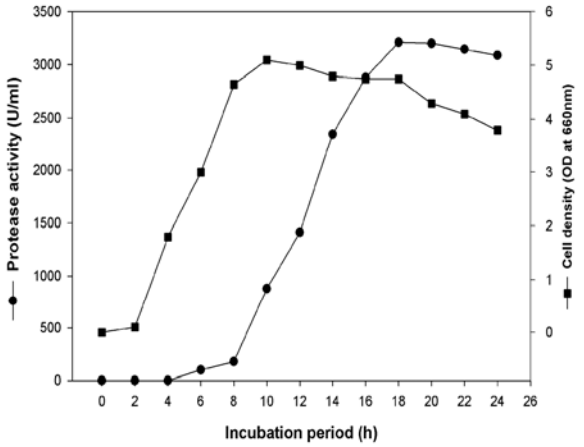

Figure 7: Fermentation profile of the protease produced by *Bacillus* sp.

CONCLUSIONS

Bacillus sp. (SBP-29) produces high levels (3208 U/mL) of an extracellular alkaline serine protease which has a pH optima of 9.0 and temperature optima of 60 °C. Based on optimization studies, it was ascertained that soybean meal is the most effective substrate for protease production. Moreover, soybean meal is an inexpensive nitrogen source as is produced as a byproduct during oil extraction. From the present investigation, it is envisaged that this isolate can be potential source of alkaline protease for use as additive in industrial application such as poultry processing industry. This protease has a good stability at high alkaline pH value and broad heat stability thereby permitting its wide biotechnological application potentials to be exploited in many industries as an environmentally benign product.

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REFERENCES

Banerjee, C.V., Sani, R.K., Azmi, W. and Soni, R. (1999). Thermostable alkaline protease from *Bacillius*

brevis and is characterized as a laundry determine additive. *Process Biochemistry* **35: 213 – 219.**

Beg, Q.K., Sahai, V. and Gupta, R. (2003). Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Process Biochemistry* **38: 1 – 7.**

Chu, I.M., Lee, C. and Li, T.S. (1992). Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. *Enzyme and Microbiol Technology* **14: 755 – 761.**

Donaghy, J.A. and Mc Kay, A.M. (1993). Production and properties of an alkaline protease by *Aureobasidium pullulans*. *Journal of Applied Bacteriology* **74: 662 – 666.**

Ferrero, M.A., Cartro, G.R., Abate, C.M., Baigori, M.D. and Sineriz, F. (1996). Thermostable alkaline protease of *Bacillus licheniformis* MIR 29: isolation, production and characterization. *Applied Microbial Biotechnology* **327 – 322.**

Gattinger, L.D., Duvnjak, Z. and Khani, A.W. (1990). The use of canola meal as a substrate for xylanse production by *Trichoderma reesei. Applied Microbial Biotechnology* **33: 21 – 25.**

Genckal, H. and Tari, C. (2006). Alkaline protease production from alkalophilic *acillus* sp. isolated from natural habitats. *Enzyme and Microbial Technology* 39**: 703 – 710.**

Gessesse, A. (1997). The use of nugmeal as low-cost substrate for the production of alkaline protease by the alkaliphilic *Bacillus* sp. AR-009 and some properties of the enzyme. *Bioresource Technology 62***: 59 – 61.**

Gupta, R., Beg, Q. K., Khan, S. and Chauhan, B. (2002). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbial Biotechnology* **60: 381 – 395.**

Ito, S., Kobayashi, T., Ara, K., Ozaki, K., Kawai, S. and Hatada, Y. (1998). Alkaline detergent enzymes from alkaliphiles: enzymatic protiens, genetics, and structures *Extremophiles* **2: 185 – 190.**

Jang, J.W., Jun, H. K., Kim, E. K., Jang, W.H., Kang, J. H. and Yoo, O. J. (2001). Enhanced thermal stability of an alkaline protease, prP, isolated from a *Pseudomonas* sp. by mutation at an autoproteolysis site, Ser-331. *Biotechnology and Applied Biochemistry* **34: 81 – 84.**

Jasvir, S., Gill, N., Devashahayam,G. and Sahoo. D.K. (1999). Studies on alkaline protease produced by *Bacillus* sp. NG 312. *Applied and Environmental Microbiology* **6: 57 – 63.**

Johnvesly, B. and Naik, G.R. (2000). Studies on production of thermostable alkaline protese from thermophilic and alkalophilic *Bacillus* sp. JB-99 in a chemically defined medium. *Process Biochem*istry **37: 39 – 144.**

Joo, H.S., Kumar, C.G., Park, G.C., Kim, K.M., Paik, S.R. and Chang, C.S. (2002). Otimization of the production of an extracellular alkaline protease from *Bacillus horikoshii. Process Biochem*istry **38:155 – 159.**

Kole, M.M., Draper, I. and Gerson, D.F. (1988). Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. *Journal of Chemical Technology and Biotechnology* **41**: **197– 206**.

Laxman, R.S., Sonawane, A.P., More, S.V., Rao, B.S. Rele, M.V. Jogdand, V.V., Deshpande, V.V. and Rao, M.B. (2005). Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus* . *Process Biochemistry* **40: 3152 – 3158.**

Manachini, P.L. and Fortina, M.G. (1998). Production in a sea –water of thermostable alkaline proteases by a halotolerant strain of *Bacillus licheniformis. Biotechnology letters* **20: 565 – 568.**

Mehrotra, S., Pandey, P.K., Gaur, R. and Dharmwal, N.S. (1999). The production of alkaline protease by *Bacillus* species isolate. *Bioresource Technology 67***: 201 – 203.**

Meyers, S.P. and Ahearn, D.G. (1977). Extracelllular proteolysis by *Candida lipolytica*. *Mycologia* **69: 646-651.**

Moon, S.H. and Parulekar, S.J. (1991). Parametric study of protein production in batch and fed batch cultures of *Bacillus firmus*. *Biotechnology and Bioengeneering* **37: 467 – 483.**

Patel, R., Dodia, M. and Singh S.P. (2005). Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization. *Process Biochemistry* **40: 3569 – 3575.**

Sutar, I.I., Srinivasan, M.C. and Vartak, H.G. (1992). Production of alkaline proteinase from *Conidiobolus coronatus* and its use to resolve DL- phenylanine and DL- phenyl glycine. *World Journal Microbiology &. Biotechnology* **8: 254 – 258.**

Tang, X.M., Lakay, F.M., Shen, W., Shao, Wei, L., Fang, H.F., Prior, B.A., Wang, X.Z. and Zhuge, J. (2004). Purification and characterization of an alkaline protease used in tannery industry from *Bacillus licheniformis*. *Biotechnology Letters* **26: 1421 – 1424**.

Tari, C., Genckal, H. and Tokatali, F. (2006). Optimization of a growth medium using a statistical approach for the production of alkaline protease from a newly isolated *Bacillus* sp. L 21. *Process Biochemistry* **41: 659 – 665.**

Yang, J.K. Shih, I.L., Tzeng, Y.M. and Wang, S.L. (2000). Production and purification of proteases from a *Bacillus subtilis* that can deproteinize crustaceans wastes. *Enzyme and Microbial Technology* **26: 406 – 413.**