Short Communication

Production and purification of a protease from an alkalophilic *Bacillus* sp. 2-5 strain isolated from soil

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Abstract
This research has focused on isolation and characterization of a strain of *Bacillus* sp. from alkaline soil, which was able to produce extracellular alkaline protease at pH_s ranging from 8 to 11 and temperatures of 20 to 50ºC. Also the impact of different carbon and nitrogen sources were investigated. The yield and fold of enzyme purification was 24% and 50 times, respectively. Molecular weight of purified enzyme was measured by SDS-PAGE as 24.7 kDa. The alkaline protease produced by *Bacillus* sp. 2 - 5 showed the most caseinolytic activity (without any gelatinolytic activity) at pH>10.
Keywords: *Alkalophilic Bacillus*; Protease; Process variables; Purification.

Proteases constitute one of the most important groups of industrial enzymes and have applications in different industries such as detergent, food, feed, pharmaceutical, leather, silk and for recovery of silver from used X-ray films (Anisworth, 1994; Fujiwara, 1993). This enzyme accounts for 30% of the total world enzyme production (Horikoshi, 1996). Among bacteria, *Bacillus* spp. are specific producers of extracellular alkaline proteases (Godfrey and Reichelt, 1985). These enzymes are quite often added to laundry detergents (to facilitate the release of proteinaceous stains) (Masse and Tilburg, 1983) and in detergent preparations used in the dairy and food industries (to remove protein foulants from ultrafiltration and reverse osmosis membrane systems). Given the wide application of this enzyme, it was reported that in the year 2005, the global proteolytic enzyme demand increased dramatically to 1.0-1.2 billion dollars (Godfrey and Reichelt, 1985). Therefore, taking this demand into account and knowing the geographic richness and biodiversity of the Iranian local environment, it is assumed that there is potential for alkalophilic *Bacillus* species living in these environments. In this paper isolation and characterization of a new strain of *Bacillus* sp. from alkaline soil, and its ability to produce alkaline protease at pH_s ranging from 8 to 11 and temperature of 20 to 50ºC have been reported. Also, purification and certain properties of the alkaline protease as well as the effect of process variables such as carbon and nitrogen sources, temperature, pH and time on alkaline protease activity have been investigated.

Alkaline salty soil sample with a pH 10 was collected (surrounding regions of Yazd and Tehran, Iran), suspended in sterile saline water (100 g/l) and incubated at 80°C for 20 minutes (Hitomi, *et al*., 1994). After cooling, it was spread on specialized culture media containing (g/l): glucose 11.1, peptone 5.5, yeast extract 5.5, K_2HPO_4 11.1, $MgSO_4.7H_2O$ 0.22, and agar 16.6. The plates were incubated at 37°C for 24 h. Pure colonies were transferred to a new medium containing (g/l): peptone 5, beef extract 3 (or yeast extract 1), agar 15 to prepare stock culture. By comparing the ability of microorganisms to hydrolyze gelatin, casein and starch at two different pH_s (7 and 10), an alkaline protease producer was selected for further experimental studies. A loopful of the prepared stock culture was

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transferred into a 100-ml flask containing 9 ml of inoculum medium consisting of (g/l): peptone 5.55, beef extract 3.33, yeast extract 1.11, $MgSO_4.7H_2O$ 0.55, and adjusted to pH 10.5 with 10% (w/v) sterilized Na_2CO_3. The culture medium was incubated at 37°C with agitation at 125 rpm on a rotary shaker, for 24h. Then, 10% (v/v) of the prepared inoculum was added to culture medium containing (g/l): starch 50, yeast extract 5, casamino acid 3, K_2HPO_4 1, $MgSO_4.7H_2O$ 0.2, and adjusted to pH 10.5 (as mentioned above). After 72h of incubation in a shaker incubator (Model G-24, New Brunswick, USA) at 40°C and 125 rpm, the culture was harvested, clarified using a refrigerated centrifuge (MSE, UK) for 20 min at 10000×*g* and 4°C. Alkaline protease activity and protein content were determined in the supernatant solution. One alkaline of protease activity (APU) is defined as the amount of enzyme that produces TCA reagent soluble peptide equivalent to 1 µg of tyrosine (spectrophotometric analysis of tyrosine absorbance at 275 nm) per 1 min at 30°C and pH 10 (0.02 M borate-NaOH buffer), using Hammerstein casein (Merck, Germany) as substrate. Protein was measured by the method of Lowry *et al*. (1951), with bovine serum albumin as the standard. The absorbance of culture broth at 660 nm was measured for estimation of cell growth and biomass production (Kobayashi, *et al*., 1996). Some of the process variables which were expected to influence enzyme production during fermentation were also investigated (Fujiwara, 1993; Gessesse, *et al*., 2003; Kobayashi, 1996; Uyar and Baysal, 2004). Organic or inorganic nitrogen sources (casamino acids, yeast extract, peptone, ammonium chloride, sodium nitrate, ammonium sulfate, urea and L- glutamate) were added (5 g/l) to a basic culture medium containing (g/l): glucose 2.5, K_2HPO_4 0.1, $MgSO_4.7H_2O$ 0.02 and adjusted to pH 10.5 (as mentioned above). Also different carbon sources (glucose, starch, sodium citrate, casein and sodium acetate) were added at a concentration of 10 (g/l) to basic culture medium (containing 3 g/l casamino acids). After 48h of incubation at 37°C, with an agitation rate of 125 rpm, the media were analyzed for cell mass, total protein concentration, alkaline protease activity and final pH. The nitrogen source (yeast extract and casamino acids) and carbon source (starch) concentrations were kept constant at 5 and 3 g/l, respectively. Temperature range of 20 to 50°C with different incubation times (0 to 72 h) were investigated. Alkaline protease activity was analyzed at 6h intervals after initiation of fermentation. Cultures with different initial pH_s ranging from 6 to 14 were incubated at 37°C with shaking at 125 rpm for 36 h, in order to examine the effects of pH on enzyme production. All the purification steps were achieved at 4°C. The concentrated enzyme was applied to a CM Cellulose column (2.5 × 30 cm), after washing the column with 10mM phosphate buffer, pH 7.5. The bound enzyme was eluted using a linear gradient of KCl (0.5 M) added to the washing buffer. Fractions (2 ml each) containing alkaline protease activity were pooled (80 ml, 40 tubes) and dialyzed again. 1 ml of purified and concentrated enzyme solution was analyzed for alkaline protease activity, specific activity and protein content.

To the cell free supernatant, 125 ml solid ammonium sulphate (55% saturation) was added and cen-

Table 1. Effect of nitrogen sources on alkaline protease production by *Bacillus* sp. 2-5. in medium containing (g/l): glucose 2.5, K_2HPO_4 0.1, $MgSO_4.7H_2O$ 0.02, after 48h incubation at 37°C[1].

Nitrogen sources	Concentration (g/l)	Cell mass (660 nm)	APU[2]/ml
Casamino acid	5	0.187	878 ± 41.9
Peptone	5	0.180	1022 ± 62.1
yeast extract	5	0.710	1264 ± 63.8
L-glutamate	5	0.260	1118 ± 58.2
Urea	5	0.084	684 ± 35.2
Ammmonium chloride	5	--------	588 ± 29.3
Ammmonium sulphate	5	--------	636 ± 31.3
Sodium nitrate	5	0.111	780 ± 39.5
Casein	10	0.462	1794 ± 90.7
yeast extract + Casamino acids	5 + 3	0.264	1938 ± 96.5
yeast extract + peptone	5 + 3	0.710	1938 ± 96.9
yeast extract + L-glutamate	5 + 3	0.959	1938 ± 96.8

[1]Each value is the average of three replications.
[2]Alkaline protease unit.

Table 2. The effect of carbon source on alkaline protease production by *Bacillus* sp. 2-5. in a medium containing (g/l) casamino acids 3, yeast extract 5, after 48h incubation at 37°C.

Carbon source	Concentration (g/l)	Cell mass (660 nm)	APU[1]/ml
Starch	0.1	0.25	800
	1	0.48	1200
	2.5	0.475	1550
	5	0.495	2500
	7	0.2	1400
Glucose	1	0.629	1600
Sodium citrate	1	0.648	1504
Sodium acetate	1	0.488	1600

[1]Alkaline protease unit (APU).

Table 3. The effect of purification steps on specific activity, purification fold and percentage of purified alkaline protease recovery.

Purification steps	Volume (ml)	Protease activity (APU/ml)	Protein content (mg)	Specific activity (APU/mg)	Purification fold	Recovery (%)
Supernatant	122	237911	84.1	2829	1	100
Salting out + UF[1]	10	147857	1.8	82148	29	70
CMC[2] + UF	1	57420	0.4	143550	50	24

1Ultrafiltration
2 Carboxy methyl Cellulose

trifuged at 4°C. After resuspension of precipitated phase in phosphate buffer, it was dialyzed under vacuum (Cut off<10 kDa). The concentrated enzyme was applied to carboxy methyl cellulose (CMC) column (2.5 × 30 cm). The bound enzyme was eluted using a linear gradient of KCl (0.5 M). Fractions containing alkaline protease were pooled (80 ml, 40 tubes) and redialyzed. 1 ml of purified and concentrated enzyme was analyzed for alkaline protease activity, specific activity and protein content (Bollag and Edelstein, 1991).

Casamino acids (878 APU/ml, peptone (1022 APU/ml), yeast extract (1264 APU/ml), L-glutamate (1118 APU/ml) and urea (684 APU/ml) individually reduced protease activity (Table 1). These results are similar to those reported by Joo *et al*. (2002) who observed decreased protease activity of *Bacillus* sp. I-312 after growth on peptone. Casein addition (1 g/l) had a significant effect on biomass production and enzyme activity, although it was still less than the effect of mixed nitrogen sources. This observation is in agreement with the results of reduced alkaline protease activities of *Bacillus horikoshii, Bacillus licheniformis MIR29, Bacillus mojavensis* and *B. horikoshii 104* in the presence of casein (Beg and Gupta, 2003; Joo *et al*., 2002). But it was somewhat different from the *Bacillus* spp. I-312 (Glazer and Nikaido, 1995). It should be mentioned that application of synthetic and unpurified nitrogen sources influence yield not only as nitrogen sources but also as sources for excess carbon during protease production. Data in Table 2 shown that the highest protease production was achieved by addition of starch at a concentration of 5 g/l. Higher concentrations did not affect protease production, significantly. Similar results have been reported with respect to the influence of corn, potato starch and wheat flour as carbon sources on protease production by *Bacillus* sp. I-312 (Glazer and Nikaido, 1995). It seems that the "catabolite repression" mechanism, is the best possible explanation for the reduce of protease production in the presence of glucose (Glazer and Nikaido, 1995), therefore, it is preferable to use complex carbon sources. Glucose (1 g/l), sodium citrate and sodium acetate (1 g/l) decreased protease production yield by 34%, 43% and 20%, respectively. Addition of glucose (1 g/l) to basal media reduced alkaline protease production by *B. horikoshii* to 45% (Joo *et al*., 2002). Gessesse and colleagues (2003) also reported that protease production in *Bacillus pseudofirmus* AL-89 increased in the presence of glucose, whereas in *Nesternkonia* sp. AL-20 was suppressed. Table 3 shows the effect of purification steps on specific activ-

ity, purification fold and percent of purified alkaline protease recovery. Final purification yield and fold was obtained as 24% and 50 times, respectively (Table 3).

In this study, the alkalophilic *Bacillus* sp. 2-5 strain showed higher protease production at pH 10. Therefore, this isolate can be a potential source of alkaline protease for use as an additive in industrial applications. The objective of the future study is to find the optimal conditions for Ca-alginate gel immobilization of the new isolated bacterium and to determine the operational stability of the resulting biocatalyst for the production of alkaline protease under semi-continuous cultivation conditions.

References

Anisworth SJ (1994). Soap and detergents. *Chem Eng News*. 72:34-59.

Beg QK, Gupta R (2003). Purification and characterization of an oxidation stable thiol-dependent serine alkaline protease from *Bacillus mojavenesis*. *Enzyme Microbial Technol*., 32, 294-304.

Bollag DM, Edelstein SJ (1991). *Protein Methods*. John Wiley and Sons, New York, PP. 71-160.

Fujiwara N (1993). Production of thermopilic alkaline protease from *Bacillus sp*. B18. *J Biotechnol*. 30, 245-256.

Gessesse A, Kabul RH, Gashe BA, Mattiasson B (2003). Novel alkaline protease from alkalophilic bacteria grown on chicken feather. *Enz Microb Tech*. 32, 519-524.

Glazer AG, Nikaido H (1995). Microbial Biotechnology In: *Fundamentals of Applied Microbiology*, Freeman and Company, Washington, PP. 256-259.

Godfrey TA, Reichelt J (1985). *Industrial enzymology*: the application of enzymes in industry. The Nature Press. London.

Hitomi J, Adachi S, Hakamada Y, Takaiawa M, Yoshimatsu T, Watanabe Y, Kobayashi T, Kawai S, Ito S (1994). Alkaline protease isolation from *Bacillus sp. US patent* No. 5,296,367.

Horikoshi K (1996). Alkalophils from an industrial point of view. *FEMS Microbial Rev*. 18: 259-270.

Joo HS, Kuma CG, Park CG, Paik SR, Chang CS (2002). Optimization of the production of an extracellular alkaline protease from *Bacillus horikoshii*. *Process Biochem*. 38: 155-159.

Kalisz HK (1988). Microbial proteinases. *Adv Biochem Eng Biotechnol*. 36: 1-65.

Kobayashi T, Hakamada Y, Hitomi J (1996). Purification of alkaline proteases from Bacillus strain and their possible interrelationship. *Appl Microbiol Biotechnol*. 45: 63-71.

Lowry OH, Rosenberg NI. Farr AL. Randall RJ (1951). Protein measurement by folin phenol reagent. *J Biol Chem*. 193: 265-275.

Masse FWJL, Tilburg RV (1983). The benefit of detergent enzymes under changing washing conditions. *J Am Oil Chem Soc*. 60:1672-1675.

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