African Journal of Biotechnology Vol. 7 (10), pp. 1536-1542, 16 May, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB07.885
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Alkaline protease production on date waste by an alkalophilic *Bacillus* sp. 2-5 isolated from soil

K. Khosravi-Darani[1]*, H.R. Falahatpishe[1] and M. Jalali[2]

[1]Department of Food Technology Research, National Nutrition and Food Technology Research Institute, Shaheed Beheshti University, M.C.; P.O. Box 19395-4147, Tehran, I. R. Iran.
[2]Department of Nutrition and Biochemistry, School of Public Health, Tehran University of Medical Sciences, Tehran, I. R. Iran.

Accepted 7 March, 2008

This research focused on isolation and characterization of a new strain of *Bacillus* sp. from alkaline soil, which was able to producing extracellular alkaline protease and amylase from date waste at pH ranging from 8 to 11 and temperatures of 20 to 50°C. Purification was conducted by fractionation, concentration, and cation exchange chromatography. 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. Produced alkaline protease by *Bacillus* sp. 2 - 5 showed the most caseinolytic activity (without any gelatinolytic activity) at pH > 10. The highest protease activity was achieved in the following conditions: starch concentration (as carbon source) 0.5 g/L, yeast extract and casamino acid (as mixed nitrogen source) 0.5 and 0.3% (w/w), temperature of 45°C at pH 10.7 after 36 h. Based on the optimization studies of production and purification stages, specific and protease activity 143550 APU/mg and 57420 APU/mL were achieved.

Key words*:* Alkalophilic bacillus, alkaline protease, process variables, purification, Date waste.

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes and have applications in different industries for example in detergent, food, feed, pharmaceutical, leather, silk and for recovery of silver from used X-ray films (Anisworth, 1994; Fujiwara, 1993; Outtrup et al., 1995). This enzyme accounts for 30% of the total world enzyme production (Horikoshi, 1996). Alkaline proteases are produced by a wide range of alkalophilic microorganisms including bacteria, moulds, yeasts and also mammalian tissues have a great activity at pH around 10. The most famous of alkaline proteases are subtilisins that are produced by *Bacillus licheniformis* and other related species (Kalisz, 1988). Among bacteria, *Bacillus* sp. is a specific producer of extracellular alkaline proteases (Godfrey and Reichelt, 1985) and their enzymes are quite often added to laundry detergents to enable the release of proteinaceous soil from stains (Masse and Tilburg, 1983) and in food industries for removal of protein.

In general, most of the alkaline proteases applied for industrial purposes face some limitations, including low activity and stability towards anionic surfactants like SDS and oxidants; and their high total cost (Joo et al., 2003). Given the wide application of this enzyme, it is reported that in year 2005 the global proteolytic enzyme demand will increase dramatically to 1.0 – 1.2 billon dollars (Godfrey and Reichelt, 1985). Therefore, taking this demand into account and knowing the geographic richness and biodiversity of Iranian local environment, it is assumed that there is potential for alkalophilic *Bacillus* species living in these environments. Discovering such species, producing proteases with novel characteristics will be of great value to the enzyme industry for different applications.

In this paper, isolation and characterization of a new

*Corresponding author. E-mail: kiankh@yahoo.com. Tel: +98 21 22376426. Fax: +98 21 22360660.

Table 1. Content of filtrate of boiled date waste.

Parameter	Value
Dry substance	77.89
pH	4.77
Ash (%, w/w)	0.07
Acidity (as citric acid)	0.05
Conductivity (uS/cm)	4
Fructose (%)	37.4
Dextrose (%)	34.1
Sucrose (%)	0.08

strain of *Bacillus* sp. from alkaline soil, as one of the most important sources for separating these bacteria (Gessesse et al., 2003; Kumar et al., 1999; Mehrotra et al., 1999; Sing et al., 2001; Uyar and Baysal, 2004) and its ability for alkaline protease production at pH ranging from 8 to 11 and temperatures of 20 to 50°C is reported. Also purification and some properties of the produced alkaline protease as well as the effect of some process variables such as carbon and nitrogen sources, temperature, pH and time on alkaline protease activity was studied.

METHODS

Soil sample

An alkaline salty soil sample of pH 10 was collected from surrounding regions of Yazd, Iran. Also another sample was prepared from agricultural areas around Tehran, Iran.

Microorganism isolation and characterization

Soil was suspended in sterile saline water (100 g/L) and incubated at 80°C for 20 min (Hitomi et al., 1994). After cooling, it was spread on specialized culture media containing (g/L): glucose 11.1, peptone 5.5, yeast extract (YE) 5.5, K_2HPO_4 11.1, $MgSO_4.7H_2O$ 0.22, and agar 16.6 (18). The plates were incubated at 37°C for 24 h. For preparing stock culture, pure colonies were transferred to a new media containing (g/L): peptone 5, beef extract 3 (or yeast extract 1), agar 15. By comparison of the activities of microorganisms for hydrolysis of gelatin, casein and starch at two different pH (7 and 10) depending upon the zone of clearance on the plate, an alkaline protease producer was selected for further experimental studies.

Inoculum preparation and enzyme production

A loopful of prepared stock cultures was transferred into 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, adjusted to pH 10.5 with 10% (w/v) sterilized Na_2CO_3, which separately were added to the medium. The wastage of date was prepared from Dombaz Company of I.R. Iran as a producer of honey date. Solid waste was suspended (in water) and boiled in a 50 L tank for 30 min and filtered. Filtrate was analyzed for its sugar content. The results are summarized in Table 1.

The culture medium was incubated at 37°C and 125 rpm on a rotary shaker with agitation for 24 h. Then 10 (%v/v) of 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, adjusted to pH 10.5 with 10% (w/v) sterilized Na_2CO_3 which separately were added to the medium. After 72 h incubation in a shaker incubator (Model G-24, New Brunswick, USA) at 40°C and 125 rpm, the culture broth was clarified using a refrigerated centrifuge (High speed model 25, MSE, UK) for 20 min at 10000×g at 4°C. Alkaline protease activity and protein content were determined in supernatant solution.

Alkaline protease assay

Alkaline protease activity was determined by the method of Higahara et al. (1986). One unit of protease activity is defined as the amount of enzyme that produced TCA reagent soluble peptide equivalent to 1 µg of tyrosine (spectrophotometric at 275 nm) in 1 min at 30°C and pH 10 (0.02M borate-NaOH buffer), using Hammerstein casein (Merck, Germany) as substrate.

Protein assay

Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Cell mass estimation

The absorbance of culture broth supernatant at 660 nm was measured for estimation of cell growth and biomass production (Kobayashi et al., 1996).

Effect of nitrogen source

For evaluation of effect of nitrogen source on enzyme production in culture media, 8 different organic or inorganic nitrogen sources including casamino acid, 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 adjusted to pH 10.5 with 10% (w/v) sterilized Na_2CO_3 which separately were added to the medium. After 48 h incubation at 37°C and agitation rate of 125 rpm, the media was analyzed for cell mass, total protein concentration, alkaline protease activity and final pH.

Effect of carbon source

For evaluation of effect of carbon source on enzyme production in culture media, 5 different carbon sources including glucose, starch, sodium citrate, casein and sodium acetate were added 10 (g/L) to basic culture medium containing (g/L) casamino acid 3, yeast extract 5, K_2HPO_4 1, $MgSO_4$. $7H_2O$ 0.2, adjusted to pH 10.5 with 10% (w/v) sterilized Na_2CO_3 which separately were added to the medium. In the next step, the effect of different concentration of selected carbon source (starch) was investigated due to great impact of carbon source on enzyme production. After 48 h incubation at 37°C and agitation rate of 125 rpm the media was analyzed for cell mass, total protein concentration, alkaline protease activity and final pH.

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

Nitrogen source	Conc. (%, w/w)	Cell mass (660 nm)	Protease activity (APU/mL)
Casamino acid	5	0.187	878
Peptone	5	0.180	1022
Yeast extract	5	0.710	1264
L-glutamate	5	0.260	1118
Urea	5	0.084	684
Ammonium chloride	5	-------	588
Ammonium sulphate	5	--------	636
Sodium nitrate	5	0.111	780
Casein	10	0.462	1794
Yeast extract + casamino acid	5 + 3	0.264	1938
Yeast extract + peptone	5 + 3	0.710	1938
Yeast extract + L-glutamate	5 + 3	0.959	1938

Effect of temperature and incubation time

The nitrogen source (yeast extract and casamino acid) and carbon source (starch) were fixed at 5 and 3 g/L, respectively (obtained from previous stages). Temperature ranges of 20 to 50°C in different incubation times (0 to 72 h) were investigated. Alkaline protease activity was analyzed in 6 h interval after initiation of fermentation.

Effect of pH

Cultures with different initial pH from 6 to 14 were conducted at 37°C and 125 rpm for 36 h to examine the effects of pH on enzyme production. Alkaline protease activity was determined according to Higahara et al. method (1986).

Alkaline protease purification

All purification steps were achieved at 4°C. To the cell free culture supernatant, 125 mL solid ammonium sulphate (55% saturation) was added and centrifuged at 4°C. Precipitated phase resuspended in phosphate buffer 10 mM, pH 7.5 and dialyzed under vacuum (Cut off <10 kDa). The concentrated enzyme was applied to CMC column (2.5 × 30 cm), after washing the column with 10 mM phosphate buffer of pH 7.5. The Bound enzyme was eluted using a linear gradient of KCl (0.5 M) added to washing buffer. Fractions (2 mL each one) containing alkaline protease activity were pooled (80 mL, 40 tubes) and dialyzed again.1 mL purified and concentrated enzyme solution was analyzed for alkaline protease activity, specific activity and protein content. For protein content determination during purification (Sing et al., 2001), absorbance at 280 nm was measured against a blank (Bollag and Edelstein, 1991).

Polyacrylamide gel electrophoresis

The protein purity and the molecular weight of the purified alkaline protease were estimated by PAGE and SDS–PAGE using Laemmli's discontinuous buffer system (Rickwood and Hames, 1990). Purified enzyme solution and standard proteins (lysozyme egg white 14.3 kDa, carbonic anhydrase 29 kDa, ovalbumine 43 kDa, bovine serum albumin 68 kDa) were mixed with sample buffer (Tris-HCl, 0.0625M and pH 6.8, SDS 2%, 2-mercaptoethanol, 5%, glycerol, 10%, bromophenolblue, 0.002%) after denaturation (100°C, 3min) 100 µL were injected on 12.5% PAGE. Molecular mass markers (Sigma, St Louis, MO) were also run simultaneously. After electrophoresis, gels were fixed with 10% (w/v) trichloroacetic acid and stained with coomassie blue R-250 (Rickwood and Hames, 1990).

RESULTS AND DISCUSSION

Nine pure colonies of *Bacillus* sp. were isolated from 2 different soil samples. Bacterium isolated from alkaline soil of central areas of Iran (Yazd) exhibited prominent clear zones around the colonies on skim milk agar plates at pH 10.5 but no growth and clear zones at pH 7. Also starch hydrolysis was observed on the slant culture of bacterium on starch agar (pH 10.5). Therefore, it was categorized as a subspecies of alkalophilic bacillus. Through stepwise microbiological biochemical identification tests including catalysis, oxidase, Voges-Proskauer, acid and gas production from glucose, reduction of nitrate to nitrite as well as gelatinolytic and caseinolytic activity and hydrolysis of starch (at pH 7 to 10), the newly isolated bacterium recognized to be belong to *Bacillus* sp. 2 - 5.

Some of process variables which were expected to influence on the enzyme production during fermentation were investigated. Choice of these factors was based on literature review (Fujiwara, 1993; Gessesse et al., 2003; Kobayashi, 1996; Kumar et al., 1999; Mehrotra et al., 1999; Sing et al., 2001; Uyar and Baysal, 2004) for growing the enzyme-producing microorganism. The results of the cultures with different inorganic nitrogen sources which were conducted to examine the effects of nitrogen source and its concentration on enzyme production have been summarized in Table 2. The effect of yeast extract (0.5%) and peptone (or L- glutamate) (0.3%) on protease

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

Carbon source	Concentration (%w/v)	Cell mass (660nm)	Protease activity (APU/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

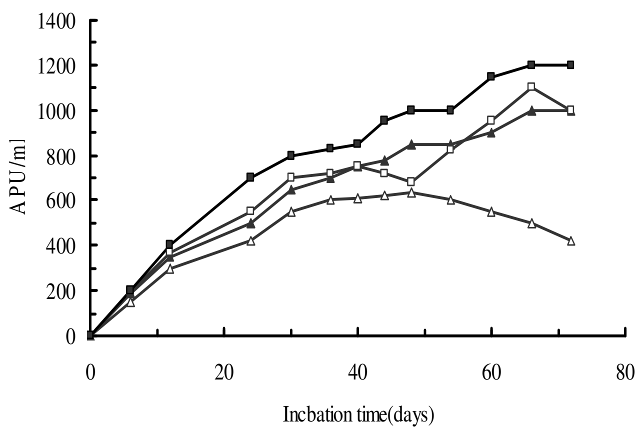

Figure 1. The effect of growth temperature and time of incubation on the alkaline protease production from Bacillus sp. 2 - 5 in a medium containing yeast extract and casamino acid (5 and 3 g/L, respectively). Symbols: (▲) 20°C, (□) 30°C and (■) 40°C, (Δ) 50°C.

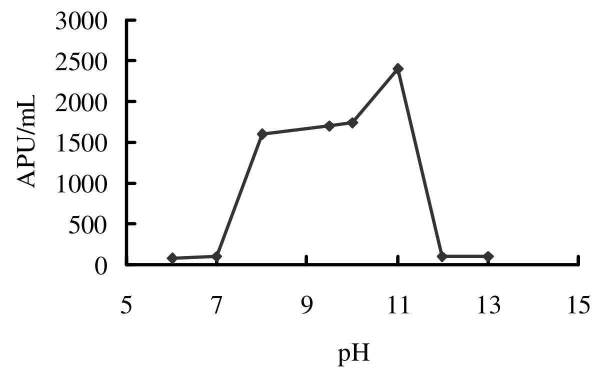

Figure 2. The effect of medium pH on alkaline protease production.

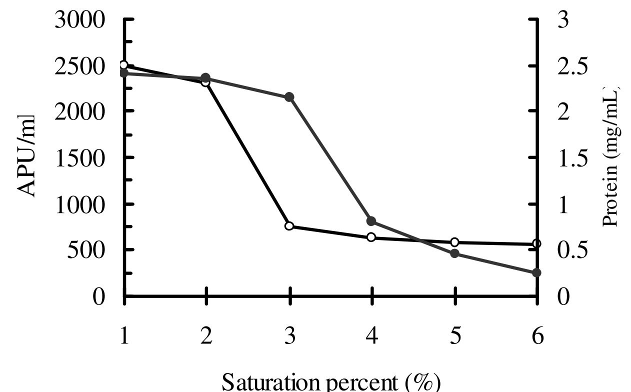

Figure 3. The effect of ammonium sulphate saturation on the protease purification yield.

production yield were investigated. Ammonium chloride and ammonium sulfate suppressed alkaline protease production significantly. Table 2 also shows usage of mixed nitrogen source i.e. yeast extract in addition to casamino acid, peptone or L-glutamate. The results of different carbon sources application on enzyme production have been summarized in Table 3. The biggest protease production was achieved by addition of starch concentration of 5% (w/v or 0.5 g/L). Higher concentrations do not affect protease production, significantly.

As Figure 1 indicates, the local optimum temperature for alkaline protease production by *Bacillus* sp. 2 - 5 was found to be 40°C and the highest protease activity (although it grows and produces alkaline protease in the range of 20 to 50°C). The results show that the bacterium cannot be assumed as a thermophile species because no growth and enzyme production were detected at temperatures above 50°C.

Figure 2 shows that production of protease increased at pH 8 to 11 and the local optimum pH was observed at pH 10.7. The enzyme production decreased at pH > 12 and pH < 8.

The effect of percentage of ammonium sulphate on precipitation of alkaline protease and other proteins was investigated. Figure 3 indicates that by using ammonium

Table 4. The effect of purification steps on specific activity, purification fold and percent of purified alkaline protease recovery.

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

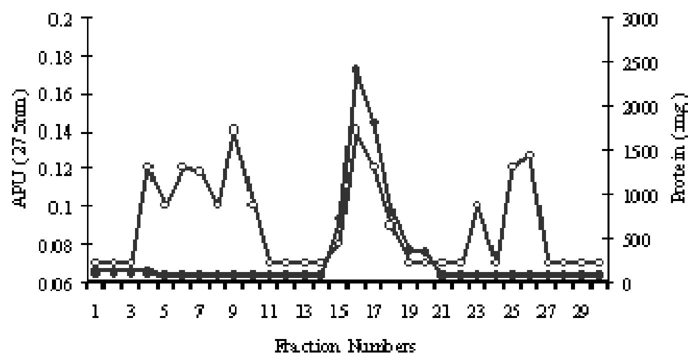

Figure 4. The elution profile on CM-Cellulose chromatography and active peak, referred to alkaline protease. Symbols: (○) protein and (●) alkaline protease.

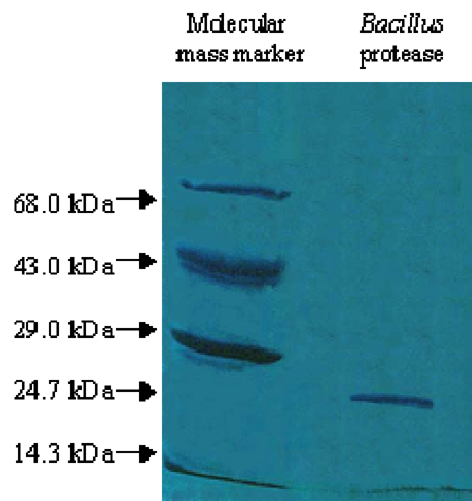

Figure 5. SDS- PAGE results of purified alkaline protease isolated from alkalophilic Bacillus sp. 2-5. Lane 1, molecular mass marker; Lane 2, Bacillus protease. Molecular mass markers are lysozyme egg white, (14.3 kDa), carbonic anhydrase, (29 kDa), ovalbumine, (43 kDa), bovine serum albumin, (68 kDa).

sulphate (up to 70% saturation) a slightly bigger fraction of alkaline protease (75%) and 24% of total protein is precipitated. The elution profile obtained on CM-Cellulose chromatography (fractions 14 - 21) revealed one active peak, referred to alkaline protease (Figure 4). Final purification yield was 24% and purification fold was 50 times (Table 4). It was sufficient to achieve homogeneity as revealed by PAGE, which showed a single band. The molecular weight of alkaline protease from *Bacillus* sp. 2 - 5 was estimated to be 24.7 kDa by SDS-PAGE (Figure 5).

Table 2 shows effects of single and mixed nitrogen source on cell concentration and enzyme activity. Although application of mixed nitrogen source results in similar enzyme activity (1938 APU/mL) but in the combination of yeast extract and casamino acid, this activity was achieved by the lower cell mass. This is due to higher productivity of enzyme production without loss of energy for biomass growth. However, casamino acid (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. These results are similar to previous report on decreased protease activity of *Bacillus* sp. I-312 by growth on peptone (Joo and Chang, 2005). Among single nitrogen sources, casein addition (1%) had significant effect on biomass production and enzyme activity, although it was still less than effect of mixed nitrogen source. This observation is in agreement to the results of reduced alkaline protease activity of *B. horikoshii, B. licheniformis* MIR29, *B. mojavensis* and *B. horikoshii* 104 in presence of casein (Beg and Gupta, 2003; Joo et al., 2002). But it was somewhat different from *Bacillus* sp. I-312 (Glazer and Nikaido, 1995). It should be mentioned that application of synthetic and unpurified nitrogen sources influences yield not only as a nitrogen source but also as a source for excess carbon during protease production (Glazer and Nikaido, 1995).

Although some of alkalophilic bacilli show gelatinolytic activity at alkaline pH (Anisworth, 1994; Masse and Tilburg, 1983; Outtrup et al., 1995), the new isolated bacterium did not show any gelatin hydrolysis (as an animal protein), so it cannot be use for recovery of silver from X-ray films (Anisworth, 1994; Fujiwara, 1993; Outtrup, 1995).

The highest protease production was achieved by addition of starch concentration of 5% (w/v or 0.5 g/L) among other carbon sources. Higher concentrations do not affect protease production, significantly. Similar

results have been reported on influence of corn, potato starches and wheat flour as carbon source on protease production by *Bacillus* sp. I-312 (Glazer and Nikaido, 1995). It seems that "catabolite repression" phenomena, is the best possible explanation for the reduction of protease production in the presence of glucose (Glazer and Nikaido, 1995); therefore it is preferable to use complex carbon sources. Glucose (1%), sodium citrate and sodium acetate (1%) decreases protease production (with yield of 34, 43 and 20% respectively) compared to starch (1%). The addition of glucose (1% w/v) to basal media reduced alkaline protease production by *B. horikoshii* to 45% (Joo et al., 2002). Gessesse also reported that in the presence of glucose in *B. psedufirmus* AL-89 culture media, an increased level of protease production was observed, whereas in *B. nesternkonia* sp. AL-20, protease production was suppressed (Gessesse et al., 2003).

A sharp decrease of protease activity was observed at temperature of 50°C after 48 to 72 h incubation (Figure 1). A possible explanation is denaturation of enzyme structure in this condition. Indeed, protease production starts simultaneously with incubation, but it reaches maximum level after initiation of sporulation (Glazer and Nikaido, 1995; Kalisz, 1988). Figure 3 indicates that ammonium sulphate (up to 70% saturation) increased the fraction of alkaline protease slightly from 75 and 24% of total protein precipitated. So, 55% saturation was selected because for maximized protease precipitation with minimum amount of impurities. In this situation, 70% of alkaline protease fraction and 4.7% of total protein were precipitated. There are some reports on ammonium sulphate (60 – 95% saturation) fractionation of enzyme solution in which the yield of precipitated enzyme has reported between 60 – 90% (Gessesse et al., 2003; Joo et al., 2003; Kumar et al., 1999; Sing et al., 2001). Since the sample buffer in chromatographic step had neutral pH, and the interested molecules had positive charge, a cation exchange resin was selected. The molecular weight of alkaline protease from *Bacillus* sp. 2 - 5 was estimated as 24.7 kDa by SDS-PAGE (Figure 5). This result is in agreement of previously reported molecular weight (19 – 29 kDa) for alkaline proteases produced by other alkalophilic bacilli (Kalisz, 1988). Based on the optimization studies of production and purification stages, specific and protease activity 143550 APU/mg and 57420 APU/mL were achieved. Rahman et al. (2006) reported that crude and purified (124.43 fold) (DEAE sephacel chro-matography) protease activities were equal to 132.29 and 16460.67 U/mg.

Conclusions

Based on the results of this bench scale study, the following conclusions can be drawn:

- The new alkalophilic strain of *Bacillus* sp. 2 - 5 shows higher protease production at pH 10. It is able to produce extracellular protease at moderately high temperatures and the enzyme may be thermostable in these temperatures.
- Date waste is a very high potential substrate for alkaline protease production without pretreatments.
- Alkaline protease produced by this newly isolated species are monomeric because its electrophoretic mobility was the same in both PAGE and SDS-PAGE. The results were similar to alkaline protease produced by *Bacillus* Ksm-k16 (Kobayashi, 1996).
- The isolate can be a potential source of alkaline protease for use as additive in industrial applications.

The objective of our 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 in the production of alkaline protease under semicontinuous 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 thermophilic 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. Enzyme Microbiol. Technol. 32: 519-524.

Glazer AG, Nikaido H (1995). Microbial Biotechnology: Fundamental 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.

Higahara H, Matsubara M, Nakai M, Okunuki K (1986). Crystalline bacterial proteinase from *Bacillus subtilis*. J. Biochem. 45: 185-194.

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. 5,296,367.

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

Joo HS, Chang CS (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.

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.

Joo HS, Kuma CG, Park CG, Paik SR, Chang CS (2003). Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. J. Appl. Microbiol. 95: 267-272.

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.

Kumar CG, Tiwari MP, Jany KD (1999). Novel alkaline serine proteases from alkalophilic *Bacillus* sp. purification and some properties. Process Biochem. 34:441-449.
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.
Mehrotra S, Pandey PK, Guar R, Darmwal NS (1999). The production of alkaline protease by a Bacillus species isolate. Bioresour. Technol. 67: 201-203.
Outtrup H, Dambamann C, Christiansen M, Aaslyng DA (1995). *Bacillus* sp. JP 395, method of making and detergent composition. US patent No. 5,466,594.
Rahman RNZA, Geok LP, Basri M, Salleh AB (2006). An organic solvent-stable alkaline protease from *Pseudomonas aeruginosa* strain K: Enzyme purification and characterization. Enzyme Microbiol. Technol. 39: 1484-1491.
Rickwood D, Hames BD (1990). Gel Electrophoresis of Proteins, a Practical Approach. Oxford University Press, Oxford, pp. 1-139.
Sing J, Batra N, Sobti C (2001). Serine alkaline protease from a newly isolated Bacillus sp. SSR1. Process Biochem. 36: 781-785.
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.