

Full Length Research Paper

Isolation of alkaline protease from *Bacillus subtilis* AKRS3

Krishnan Ravishankar^{1,2}, Muthusamy Ashok kumar² and K. Saravanan^{3*}

¹Department of Biotechnology, Anna University of Technology, Tiruchirapally-620024, Tamil Nadu, India.

²Department of Biotechnology, Karunya University, Coimbatore-641114, Tamil Nadu, India.

³Department of Chemical Engineering, Kongu Engineering College, Perundurai-638052, Tamil Nadu, India.

Accepted 29 June, 2012

This research study was mainly focused on phenotypic, biochemical characterization, 16s rRNA sequence based species level identification of isolate and determination of the higher production of alkaline protease through optimization study (carbon, nitrogen, incubation period, temperature, pH and sodium chloride concentration), production by submerged fermentation and analytical studies (protease assay, protein and biomass estimation). The produced crude enzyme was been used for dehairing activity on goat and sheep hides. Primary screening was achieved by skim milk casein hydrolysis method. Microbiological, biochemical characterization and 16S rRNA phylogenetic analysis revealed that isolated bacterium was *Bacillus subtilis* AKRS3 with an optimum alkaline protease producing temperature, 37°C and pH 9.0. The maximum alkaline protease production was achieved at 24 h of incubation period. Among various nitrogen (organic and inorganic) sources, beef extract was found to be the best inducer for alkaline protease in the concentration of 1.5% as was reported for the maximum alkaline protease production. Effect of carbon sources for example xylose, on protease production proved high protease production than the other tested carbon sources and subsequently 2% concentration registered an optimum to enhance the protease production. The halotolerance of *B. subtilis* AKRS3 for alkaline protease production indicated that 3% of sodium chloride was optimum to yield maximum protease activity. During production, agitation rate was 250 rpm at air flow rate of 1 VVM. Maximum protease activity of 42.7556 U/ml was observed at the end of 24 h cell free supernatant of fermentation broth. Crude alkaline protease was most active at 55°C, pH 9 with casein as substrate. According to our knowledge, this study demonstrated the first report on alkaline protease producing *B. subtilis* AKRS3 isolated from fish waste. The produced enzyme could be effectively used to remove hair from goat and sheep hide indicating its potential application in leather processing industry.

Key words: *Bacillus subtilis* AKRS3, 16S rRNA sequence, alkaline protease, submerged fermentation (SmF), dehairing activity.

INTRODUCTION

Fish waste is one of the common spillage in every fish market area. This spillage incorporated with soil contains fish protein, which is considered as a good source of protein (Fukami et al., 2002). Proteolytic (proteases)

enzymes are ubiquitous, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases which account for ~60% of the total worldwide enzymes sale (Beg et al., 2003; Ellaiah et al., 2003; Nascimento and Martins,

*Corresponding author. E-mail: rumisivaesh@yahoo.com. Tel: +91-97866-05656, +91-4294-226600.

2004). Although, there are many microbial sources available for producing proteases, only a few are recognized as commercial producers. Of these, strains of *Bacillus* sp. dominate the industrial sector (Gupta et al., 2002).

The Gram-positive, aerobic, rod-shaped endospore-forming bacteria of the Genus *Bacillus* are the most widely represented organisms in the soil. Due to their ability to form spores and withstand a range of variable environmental conditions, *Bacillus* sp. adapt easily to diverse habitats (Priest, 1993). In view of their high quality protein, fish represents a potential source of industrial peptones for a wide range of applications. The fish-processing industry generates considerable quantities of byproducts as waste that includes viscera, scales, fins and bone frames. These are all often high in protein, which can be processed into useful products (fish protein concentrate, fish meal, fish silage, animal feed, etc) (Raa and Gilberg, 1982). Moreover, fish extracts readily spoil thereby proving to be an excellent source of nutrients for bacterial growth. Particularly enzymatic hydrolysate from fish waste extract (Jassim et al., 1988) and autolysate of fish viscera (Clausen et al., 1985) have been proven to be an excellent substrate for bacterial growth. The aim and objective was undertaken for isolation, identification, optimization, production and application of alkaline protease producing *Bacillus* sp. from fish waste.

MATERIALS AND METHODS

Isolation and screening

Six bacteria isolated from fish market waste soil at Ukkadam, Coimbatore (77°00'E, 11°00'N), Tamil nadu, India were studied. Samples were collected into sterile container (screw cap vials) according to microbiological procedures and shifted to the laboratory for further analysis. The collected samples were diluted in sterile saline solution (Hamidreza et al., 2007). The diluted samples were plated onto skim milk agar plates containing peptone (0.1% w/v), NaCl (0.5% w/v), agar (2.0% w/v), and skim milk (10% v/v). Plates were incubated at 37°C for 24 h. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms (Poluri et al., 2003). All the bacterial colonies were isolated and purified in nutrient agar and screened in skim milk agar plates. Among the six isolates, an alkalophilic *Bacillus subtilis* AKRS3 showed good proteolytic ability by hydrolysis of casein in skim milk agar plates.

Protease activity

The protease activity in the liquid medium was assessed first, by culturing the bacteria in an enrichment medium containing beef extract (0.3%), peptone (0.5%), NaCl (0.5%), and glucose (0.5%) at pH 7 for 24 h, and then 10% of enriched culture was inoculated in a 250 ml flask containing 45 ml basal medium containing (g/l): (NH₄)₂SO₄, 2 g; K₂HPO₄, 1 g; KH₂PO₄, 1 g; MgSO₄·7H₂O, 0.4 g; MnSO₄·H₂O, 0.01 g; FeSO₄·7H₂O, 0.01 g; yeast extract, 1 g and peptone, 10 g at pH 7.0. The culture was then incubated for two days by reciprocal shaking at 32°C. The cells were then harvested by centrifugation at 10,000xg for 15 min, and the supernatant was

used for further protease assay (Esakkiraj et al., 2007).

Protease assay

Protease activity was measured using 0.5 ml of glycine NaOH buffer (pH 10.0, 0.2 M) which was added to 0.5 ml of appropriately diluted enzyme and then incubated with 1 ml of 1% casein solution (prepared in glycine NaOH buffer, pH 10.0) 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 3000xg for 10 min, and the filtrate was used to measure protease activity based on the basis of color development. For color development, 5 ml of 0.4 M sodium carbonate solution was added to 1 ml of the filtrate and kept for 10 min. To this, 1:1 diluted Folin's Ciocalteu phenol reagent was added and kept in the dark for 30 min, and the optical density was recorded at 660 nm using UV-visible spectrometer. The amount of protease produced was measured with the help of a tyrosine standard graph. Based on the tyrosine released, the protease activity was expressed in micrograms of tyrosine released under standard assay conditions (Meyers and Ahearn, 1977).

Identification of isolate

The isolated bacterium *B. subtilis* strain AKRS3 was identified by our laboratory based on microbiological (size, configuration, margin, appearance, optical property, pigmentation, texture, Gram's reaction, cell shape, arrangements, spore staining, spore shape, sporangia bulging, motility and respiration) parameters (Harley and Prescott, 2002), physiological growth at different temperatures, pH and NaCl concentration (Esakkiraj et al., 2007; Ranilson et al., 2009) and biochemical characteristics (indole test, methyl red test, Voges Proskauer test, citrate test, nitrate reduction test, urease test, casein hydrolysis, starch hydrolysis and catalase test (Sharmin and Rahman, 2007; Elliah et al., 2003; Shah et al., 2009).

DNA isolation and purification

Pure genomic DNA was isolated following the method of Sambrook and Russel (2001). Briefly, the culture was grown overnight in 10 ml nutrient broth with shaking at 37°C. 1 ml of the culture was centrifuged at 8000xg for 10 min and the resultant pellet was suspended in 1000 µl Tris-EDTA (TE) buffer (100 mM Tris-cl pH 8.0, 10 mM EDTA pH 8.0) and centrifuged at 8000xg for 10 min. The pellet was resuspended in 400 µl of buffer A (0.25 M sucrose and 50 mM Tris-HCl pH 8.0) and vortex. Then 10 µl of freshly prepared lysozyme was added (10 mg/ml in the TE buffer), the contents were mixed and kept at 37°C for 60 min in water bath. Then freshly prepared 15 µl of 20% SDS in TE buffer added, warm to 37°C and rocked gently, which continued till the cell lysis was completed and incubated at 37°C for 10 min in water bath. After incubation 15 µl protease-K added (1 mg/ml) and mixed thoroughly. Then the mixture was allowed to incubate at 55°C for 15 min in water bath. 250 µl of Tris-saturated phenol (pH 8.0) added to the above mixture and mixed thoroughly by gentle inversion of eppendorf tube. The aqueous phase was transferred to a fresh eppendorf tube and 400 µl of chloroform was added, then the contents were centrifuged at 10000xg for 15 min. The aqueous phase was transferred to a fresh eppendorf tube and 15 µl of 3M sodium acetate and 400 µl of absolute ethanol was added and kept for incubation at 0°C for more than 30 min. Then, DNA was pelleted by centrifugation at 10000xg for 20 min. The pellet was washed with 50 µl of 70% ethanol by centrifuging at 10000xg for 3 min and the ethanol was allowed to evaporate in laminar air flow for 15 min. After ethanol evaporation, the pellet was dissolved in 20 µl

of TE buffer.

Amplification of 16S rRNA genes

The 16S rRNA genes were amplified from genomic DNA by polymerase chain reaction with two primers: 16S rRNA forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and 16S rRNA reverse primer 5'-ACG GCT ACC TTG TTA CGA CTT-3'. The cycle sequencing reaction was performed using BigDye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase (Applied Biosystems, P/N: 4337457). The sequencing reaction - mix was prepared by adding 1 µl of BigDye v3.1, 2 µl of 5x sequencing buffer and 1 µl of 50% dimethyl sulphoxide (DMSO). To 4 µl of sequencing reaction mix, 4 Pico moles of primer (2 µl) and sufficient amount of plasmid was added. The constituted reaction was denatured at 95°C for 5 min. Cycling began with denaturing at 95°C for 30 s, annealing at 52°C for 30 s and extension for 4 min at 60°C and cycle was repeated for a total 30 cycles in a MWG thermocycler. The reaction was then purified on sepheadex plate (Edge Biosystems) by centrifugation to remove unbound labelled and unlabelled nucleotides and salts. The purified reaction was loaded onto the 96 capillary ABI 3700 DNA analyzer and electrophoresis was carried out for 4 h (Annette et al., 2004). The sequence was subjected to homology search using basic local alignment search tool (BLAST) programme of the National Center for Biotechnology Information (NCBI).

Nucleotide sequence accession numbers

The partial 16S rRNA sequence derived in this study was deposited in GenBank under the accession number of HQ625025.

Optimization of protease production

Effect of incubation temperature

The effect of temperature on protease production was studied by growing each *B. subtilis* strain AKRS3 in fermentation media set at different temperatures (25, 32, 37, 40, 50 and 60°C). The inoculated substrates were incubated at different temperatures to determine the optimum fermentation temperature for alkaline protease production (Ranilson et al., 2009).

Effect of medium pH

The effect of different pHs (6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) on protease production was determined as each *B. subtilis* culture was inoculated on media with different pHs set values. The initial pH of the growth medium was adjusted with 1 M HCl or NaOH before sterilization (121°C for 15 min) (Nadeem et al., 2008).

Effect of incubation period

50 ml of selected medium was taken in each 100 ml conical flask. All flasks were autoclaved at 121°C and 15 lb pressure for 20 min. After cooling, the flasks were inoculated with equal quantity of inoculums. The flasks were incubated at 37 ± 1°C at 24, 48, 72, 96 and 120 h (Anwar et al., 2005).

Effect of nitrogen sources

The growth medium was initially supplemented with different

organic nitrogen sources that is, peptone, beef extract, meat extract, casein, gelatin and urea, each at 1% (mass per volume) and inorganic nitrogen sources, NaNO₃, NH₄Cl additionally at 1% (mass per volume). After screening, maximum protease yielding nitrogen source was further optimized by varying concentrations (0.5, 1, 1.5, 2 and 2.5%) on basal medium (Muhammad et al., 2008).

Effect of carbon sources

The effect of carbon sources on protease production was tested by using eight different carbon sources namely, glucose, lactose, fructose, sucrose, xylose, maltose, raffinose, and starch. They were tested individually at a concentration of 0.5% in the nitrogen source optimized basal medium. Then, maximum protease producing carbon source was further optimized by varying concentrations (0.5, 1, 1.5, 2 and 2.5%) on nitrogen optimized basal medium (Esakkiraj et al., 2007).

Effect of sodium chloride

Bacteria was isolated from the fish waste, hence the effect of NaCl on protease production was also tested by adding different concentrations of NaCl (1, 2, 3, 4 and 5%) in the carbon and nitrogen optimized medium (Esakkiraj et al., 2007).

Fermentor production

After optimization tests were over, yields having good value were picked and optimized in production medium containing the following (g/l): (NH₄)₂SO₄, 2 g; K₂HPO₄, 1 g; KH₂PO₄, 1 g; MgSO₄.7H₂O, 0.4 g; MnSO₄.H₂O, 0.01 g; FeSO₄.7H₂O, 0.01 g; yeast extract, 1 g; beef extract, 15 g and xylose, 20 g at pH 9, and maintained at optimized temperature value of 37°C for 24 h in bioreactor.

The pH of the medium was adjusted at optimized pH of 9.0 before sterilization. Alkaline protease production was carried out in a lab scale 5 l bioreactor (Lark-India) with 3 l working volume, fixed with 2-stage rushton type impeller of 50 mm diameter. During the fermentation time, agitation rate was at 250 rpm and aeration 1 VVM.

Estimation of protein

The protein concentration was determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951).

Estimation of biomass

Two milliliter sample was collected in a pre-weighed eppendorf tube and centrifuged at 8000 rpm for 10 min. Supernatant was discarded and the pellet was washed thrice with sterile distilled water, followed by drying the pellets at 95°C till constant weight and expressed in dry cell weight (DCW, mg/ml) (Annapurna et al., 2007).

Centrifugation of harvested broth

At the end of fermentation time, harvested broth was centrifuged at 10000 rpm for 10 min at 4°C and the clear crude enzyme supernatant was stored at -20°C for further studies of the characterization of crude protease and dehairing application studies on goat and sheep skins (Sharmin and Rahman, 2007).



Figure 1. Skimmed milk casein hydrolysis by *Bacillus subtilis* AKRS3.

Characterization of crude alkaline protease

The thermo stability of crude enzyme was measured by incubating the enzyme preparation at 25, 30, 37, 45, 55 and 60°C and pH stability was measured by incubating the enzyme at pH 4 to 11 in different buffers (0.1 M) such as $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ (6.0 to 7.5), Tris-HCl (8.0 to 9.0), Glycine-NaOH (9.0 to 13.0) and $\text{Na}_2\text{HPO}_4\text{-NaOH}$ (11.0 to 12.0) and an enzyme activity was measured (Abu-sayem et al., 2006).

Dehairing activity of crude alkaline protease

Goat and sheep skin was cut into 3 cm² pieces and incubated with 10.0 ml of crude protease for 12 h at 37°C. The skin pieces were then virtually analyzed for alkaline protease-dehairing activity (Mukherjee and Sudhir, 2009).

RESULTS AND DISCUSSION

Isolation and identification of isolate

Six microbial strains were isolated by using basic microbiological techniques of serial dilution, spread plate and quadrant streak method on nutrient agar plates. Among these, *B. subtilis* AKRS3 isolate exhibited better proteolytic ability by hydrolysis of casein in skim milk agar plate (Figure 1) compared to other isolates, which was finally selected for further studies (Anwar et al., 2005).

Caseins, gelatins and blood proteins do not enter bacterial cells. For protease production, the organism must grow under the condition that permits the solubilized proteins into the bacterial cell. To make the nutrient

proteins soluble, it needs the secretion of a protease by the cell. If this protease is temperature sensitive it cannot digest the proteins at non purposive growth temperature. It is therefore reasonable to expect the bacteria to elaborate a thermostable protease (Sharmin and Rahman, 2007).

Morphological studies revealed that the isolate *B. subtilis* AKRS3 cells were moderate size, pale yellow, circular, entire and convex. The growing cells were aerobic, rod-shaped, gram-positive, motile and produces spores that were ellipsoidal and located subterminal to terminal. The isolate was catalase negative and casein hydrolysis positive. Methyl red, Voges proskaur, indole and nitrate reduction tests also showed negative and did not utilize Simmon's citrate.

The morphological, physiological and biochemical data (Table 1) on the growth of the organism indicates that the organism used in the study might belong to the genus of *Bacillus* organisms. Though, systematic study coupled with molecular study revealed the true identity of the organism. Since the organism has industrial potentiality it was identified by 16S rRNA method. The partial 16S rRNA sequences of *B. subtilis* AKRS3 (723 bp) was compared with other strains of bacterium from the similarity matrix, calculated by number of base differences, the highest level of similarity (Table 2).

The phylogenetic tree (Figure 2) constructed using the neighbour-joining method showed that strain *B. subtilis* AKRS3 is a member of group 6 (Nielsen et al., 1994) of the genus *Bacillus*. The partial 16S rRNA sequences (723 bp) of isolated strain revealed that *B. subtilis* AKRS3 was closely related to type strains of *B. subtilis* strain STC12 (100.0%), *B. subtilis* strain PB44 (99.0%) and *B. subtilis* strain CLW-BA1-5 (99.0%) in the National Center for Biotechnology Information (NCBI) GenBank. The DNA G+C content of strain *B. subtilis* strain AKRS3 was 54.77 mol%.

Effect of incubation temperature

The growth and enzyme activity of microorganisms are greatly influenced by different incubation temperature. The growth of microorganisms can be inhibited at one temperature but it can be activated at another temperature. So, it is essential to incubate microorganisms at their optimum temperature for their successful growth. The incubation temperature is usually determined by considering the sources from which the organisms have been isolated. For this reason, to detect the optimum incubation temperature, the selected isolates were incubated at different incubation temperatures. The highest protease production temperature after incubation was analyzed and reported (Figure 3). The highest protease activity was recorded at 37°C. The bacterial isolates preferred 37°C for maximum protease production, which is in accordance with the report of Anwar et al. (2005).

Table 1. Morphological and biochemical tests for identification of *Bacillus subtilis* AKRS3.

Test	<i>Bacillus subtilis</i> strain AKRS3
Colony morphology	
Size	Moderate
Configuration	Circular
Margin	Entire
Elevation	Convex
Appearance	Shiny
Optical property	Opaque
Pigmentation	Pale yellow
Texture	Rough
Gram's Reaction	+
Cell shape	Rods
Arrangements	Single
Spores	Sporulating
Endospore	+
Position	Subterminal to terminal
Shape	Ellipsoidal
Sporangia bulging	-
Motility	+
Respiration	Aerobic
Physiological test	
Growth at temperature (°C)	
25	+
32	+
37	+
40	+
50	+
60	+
Growth at pH	
6	+
7	+
8	+
9	+
10	+
11	+
Growth at NaCl (%)	
1	+
2	+
3	+
4	+
5	+
Biochemical test	
Indole test	-
Methyl red test	-
Voges Proskauer test	-
Citrate test	-
Nitrate reduction test	-
Urease test	-
Casein hydrolysis	+

Table 1. Contd.

Starch hydrolysis	-
Catalase test	-

+, Positive; -, negative.

Table 2. Identification of bacterial strain on the basis of 16S rRNA gene sequence similarity.

Isolate (accession number)	Sequence length (bp)	Nearest phylogenetic neighbor (accession number)	Maximum identity (%)	Gap (%)	Maximum score (bit)
<i>Bacillus subtilis</i> strain AKRS3- HQ625025	723	<i>Bacillus subtilis</i> strain STC12- HM585050	100	0	1336

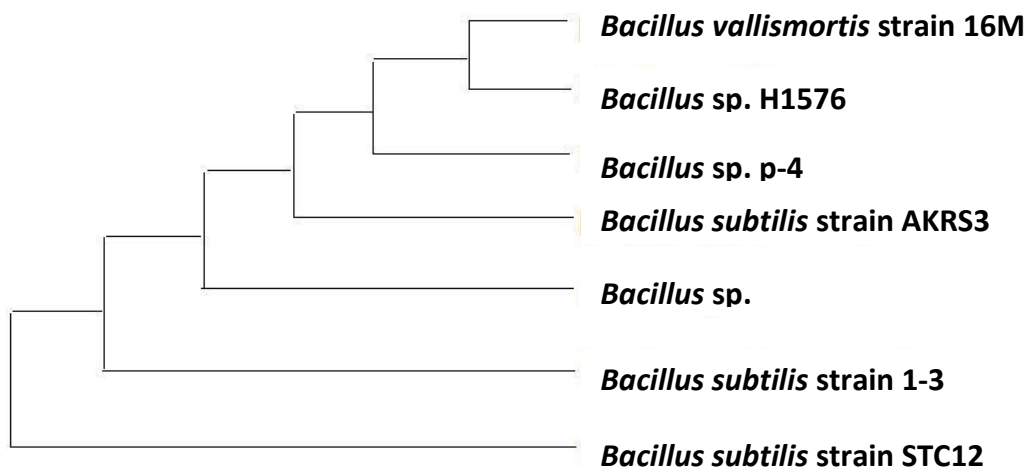


Figure 2. Phylogenetic tree of *B. subtilis* AKRS3.

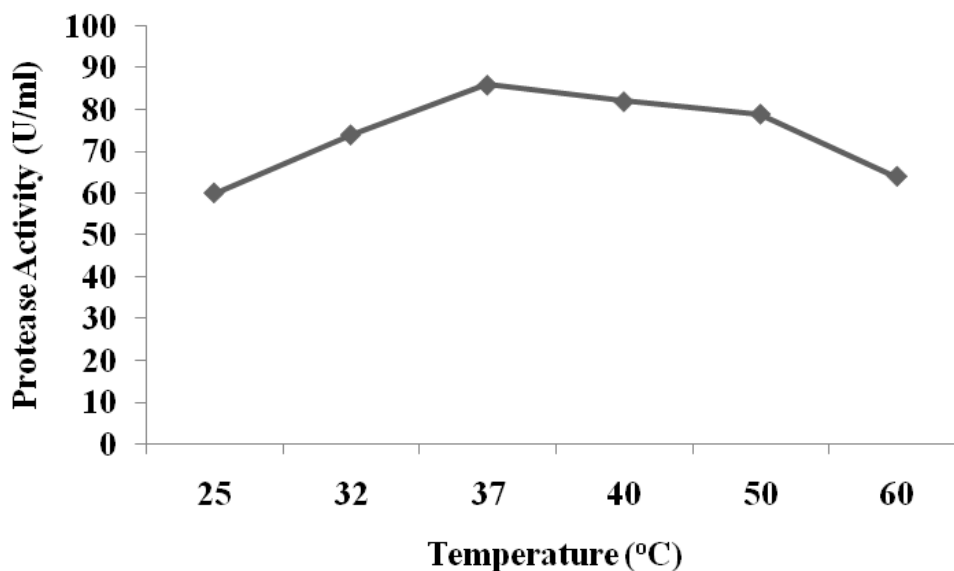


Figure 3. Effect of incubation temperature on protease production from *B. subtilis* AKRS3.

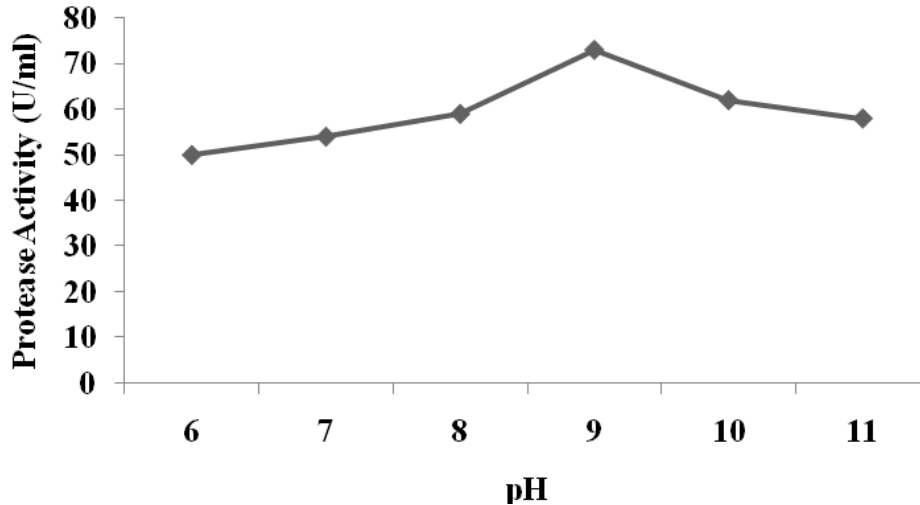


Figure 4. Effect of medium pH on protease production from *B. subtilis* AKRS3.

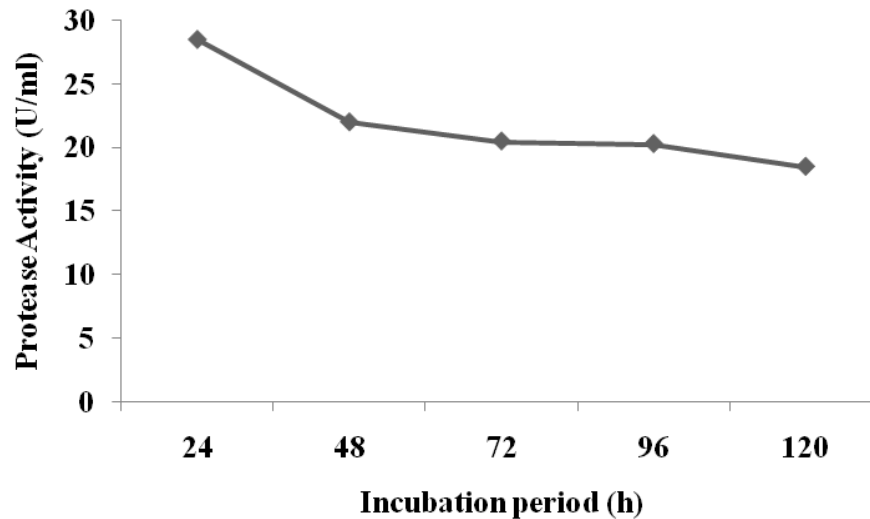


Figure 5. Effect of incubation period on protease production from *B. subtilis* AKRS3.

Effect of medium pH

Microorganisms are sensitive to the changes in the hydrogen ion concentration of their environment. Therefore, to detect the optimum medium pH, the selected organism was incubated at different pH and their production of protease was recorded. The inoculated bacteria showed a maximum production of protease at medium pH of 9.0 (Figure 4). Optimum production of protease by bacteria with the pH of 8.5 was reported by Alissara et al. (2006).

Effect of incubation period

Since microorganisms show considerable variation at

different incubation period, it is very essential to detect the optimum incubation time at which an organism shows the highest enzyme activity. When the bacterial isolate was grown in a selected media, it showed highest protease formation at 24 h of incubation time (Figure 5). Maximum production of proteases with 48 to 72 h of incubation by bacteria was reported by Andrade et al. (2003).

Effect of nitrogen sources

Complex nitrogen sources are usually needed for alkaline protease production, but the requirement for specific nitrogen sources differs from organism to organism, or even among same species isolated from different

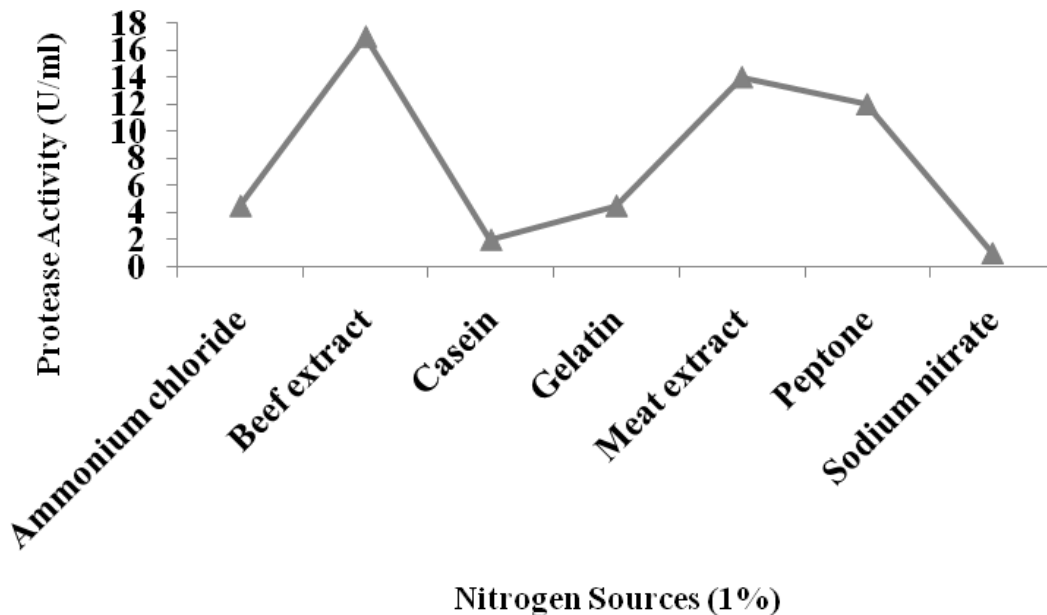


Figure 6a. Effect of nitrogen sources on protease production from *B. subtilis* AKRS3.

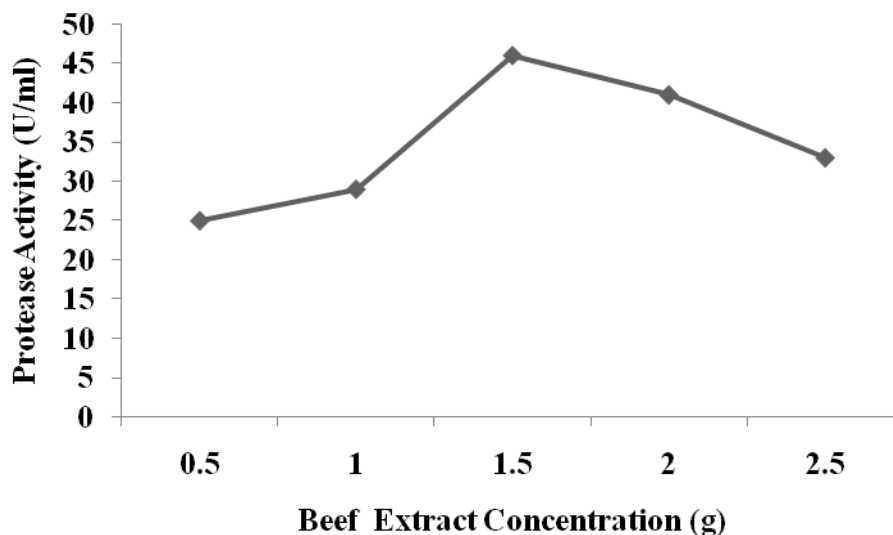


Figure 6b. Effect of different concentrations of beef extract on protease production from *B. subtilis* AKRS3.

sources. Organic and inorganic nitrogen sources at initial concentration of 1% showed significant effect on alkaline protease production. Eight different nitrogen sources were used among which beef extract basal medium added was shown to exhibit maximum yield of protease activity (Figure 6a). However, the addition of urea abruptly repressed the enzyme biosynthesis as well as growth of the inoculated bacteria. Yeast extract and casamino acids were as the most suitable sources of bacteria for alkaline protease production reported by Nadeem et al. (2008). Subsequently, beef extract was

used to optimize the different concentration level from 0.5 to 2.5 g (Figure 6b). Among the concentration used, 1.5 g reported a good alkaline protease yield.

Effect of carbon sources

In preliminary study, the effect of different carbon sources on the yield of the alkaline protease was observed in basal medium (Figure 7a). Results indicate that different carbon sources have different impact on the production of

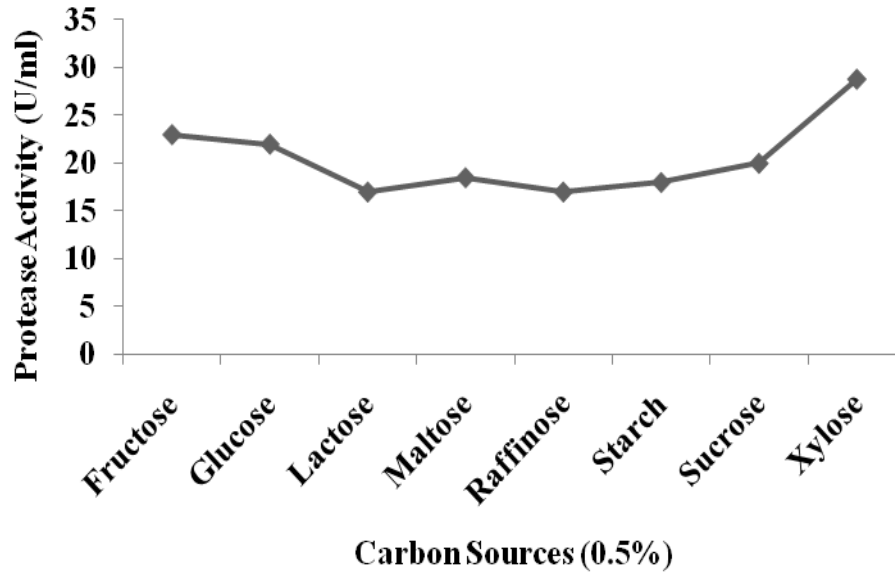


Figure 7a. Effect of carbon sources on protease production from *B. subtilis* AKRS3.

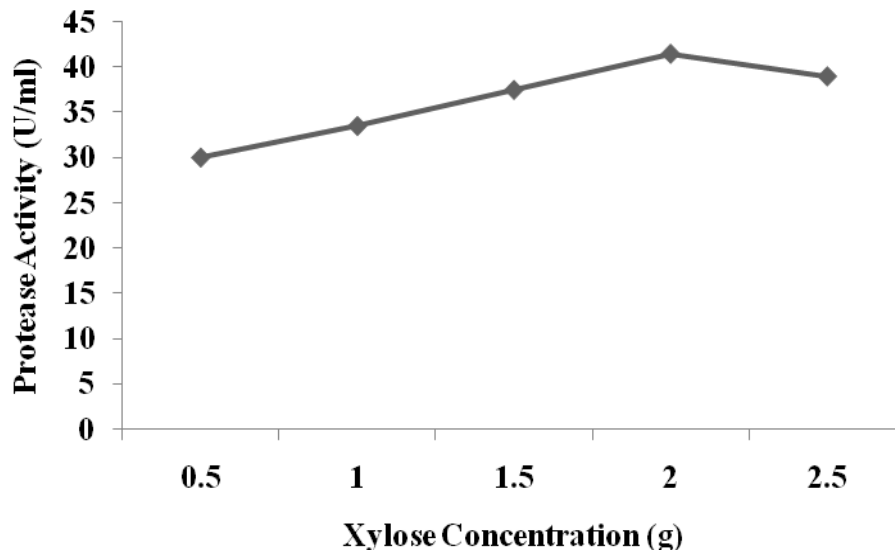


Figure 7b. Effect of different concentrations of xylose on protease production from *Bacillus subtilis* AKRS3.

alkaline protease by inoculated bacteria. It was observed that the production of alkaline protease was greatly enhanced by addition of xylose, followed by fructose. These findings indicate xylose is the best carbon source for the production of protease by isolated bacteria. Nadeem et al. (2008) reported glucose as the best carbon source of isolated *Bacillus licheniformis* N-2 for alkaline protease production. Xylose was used to optimize the different concentration level from 0.5 to 2.5 g to produce alkaline protease (Figure 7b). Among the concentration used, 2.0 g showed a maximum protease

production followed by 2.5 g.

Effect of sodium chloride

To investigate whether the concentration of NaCl affects the production of alkaline protease by inoculated bacteria, it was grown in a medium added with different concentration of NaCl (1.0 to 5.0%). A maximum protease production of NaCl concentration was recorded at 3% level (Figure 8). Esakkiraj et al. (2007) reported a

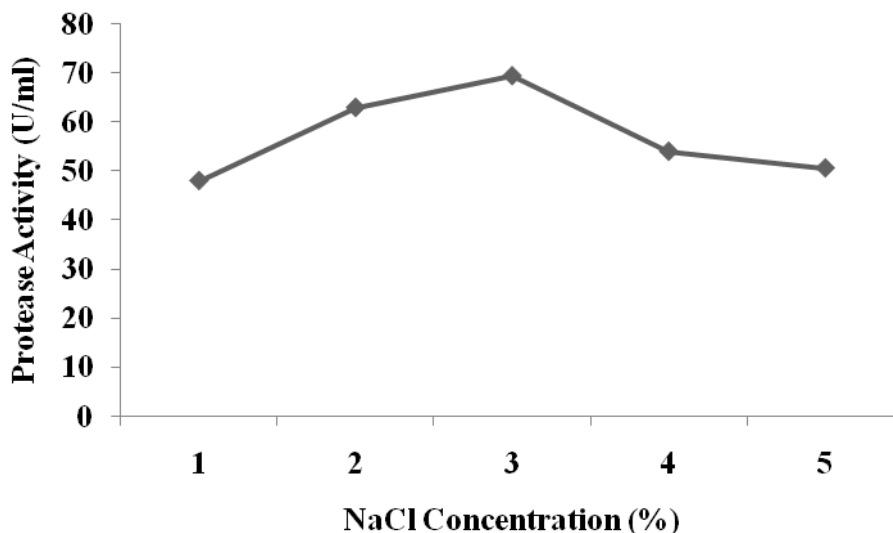


Figure 8. Effect of different concentration of sodium chloride on protease activity.

maximum alkaline protease production by *Bacillus cereus* recorded in 3% level; this result was in accordance with our observation.

Fermentor production

The amount of protease production was measured with the help of a tyrosine standard graph. Based on the tyrosine released, the protease activity was expressed in micrograms of tyrosine released under standard assay conditions (Esakkiraj et al., 2007). After 24 h fermentation with temperature at 37°C and pH of 9, excess samples were taken and analyzed by standard protease assay method. Therefore, the protease activity of final collected sample was 42.7556 U/ml.

Protein estimation

The amount of protein produced was determined by using bovine serum albumin (BSA) as the reference standard and at the end of the 24 h fermentation period, samples were drawn from the fermentor and analyzed for the protein content by Lowry's method (Annapurna et al., 2007). 32 µg/ml of protein was present at the end of fermentation broth.

Biomass estimation

Dry cell weight (DCW, mg/ml) was analysed using the bioreactor after 24 h of operation (Annapurna et al., 2007). 1.7 g/LI of dry cell weight was found in the fermentation medium. This indicates good biomass formation for alkaline protease production.

Partial characterization of crude alkaline protease

Effect of thermostability of protease

The effect of temperature on the activity of crude protease was analyzed at 25 to 60°C (Figure 9). The result on the effect of optimum temperature for protease activity was 55°C and beyond this temperature, the enzyme activity decreased. The results show bell-shaped temperature dependence with an optimal activity at 55°C. The protease had a relatively broad temperature adaptability ranging from 55 to 75°C, therefore, the protease can be classified as thermophilic (Huang et al., 2006). Esakkiraj et al. (2007) reported that their *B. cereus* indicated optimum temperature for enzyme activity at 60°C.

Effect of pH stability of protease

The effect of pH on protease activity is shown in Figure 10. The result inferred that pH 7.0 was optimum for enhancing protease activity. It registered a positive increase in enzyme activity up to pH 7.0, and a further increase in pH beyond 7.0 brought about the decline of enzyme activity. Increasing the pH beyond the optimum level (7.0 to 7.5) may interfere with the amino acid composition (Esakkiraj et al., 2007). Optimum enzyme activity of crude protease with the pH of 6.5 was reported by Shumi et al. (2004).

Dehairing activity of crude alkaline protease

The new enzyme technology would make it possible to obtain good dehairing without using sulphide and other

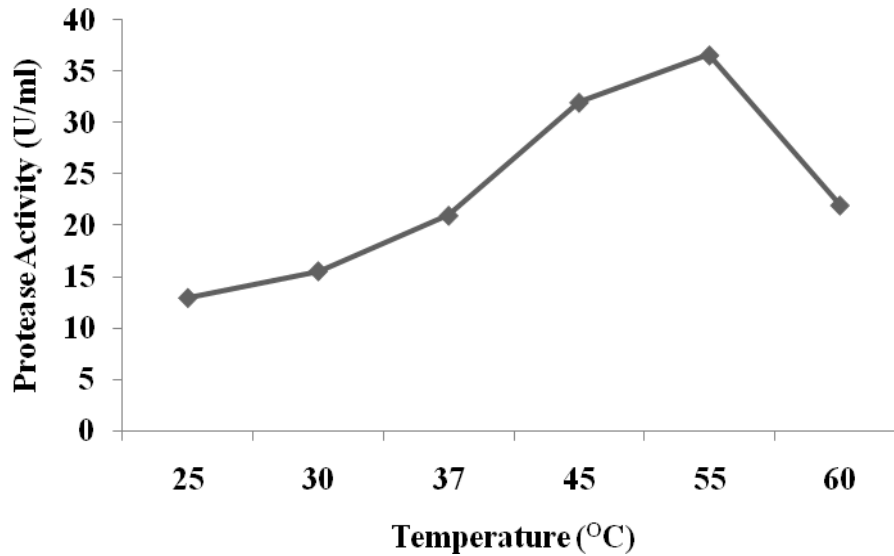


Figure 9. Determination of thermostability of protease.

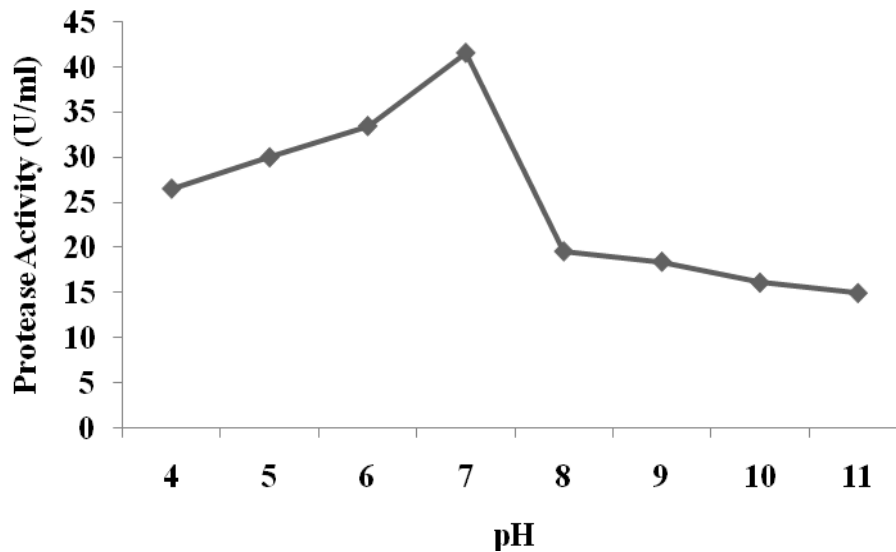


Figure 10. Determination of pH stability of protease.

chemicals. Therefore, enzymatic dehairing is an efficient method to produce quality leather without causing pollution to the environment. The action of the enzyme on raw hides and skin shows that the enzyme can remove the hair from the skin within 12 h (Figure 11). This may be due to the fact that the enzyme might have penetrated to the hair follicle and digests the filament which attaches the hair to the skin.

Today, enzymes find huge application in various industries. In tanneries, alkaline protease has been involved in soaking, dehairing and bating of skin. As leather is one of the major exportable items, it deserves quality processing by adoption techniques. The effluent

discharging system of the tanneries causes severe pollution to the water bodies. The enzymatic dehairing and bating of hides have been widely accepted as an alternative to the chemical process (Alissara et al., 2006).

Although, microbial proteases may be an alternative eco-friendly strategy to replace the use of eco-hazardous chemicals for the dehairing purpose, however, most of them were unsuitable for dehairing purpose because of the associated collagen-degrading activity. To the best of our knowledge, this is the first report on dehairing activity of purified protease from *B. subtilis*. The remarkable dehairing activity as well as less collagenase activity of Bsubap-I supported its application in leather industry

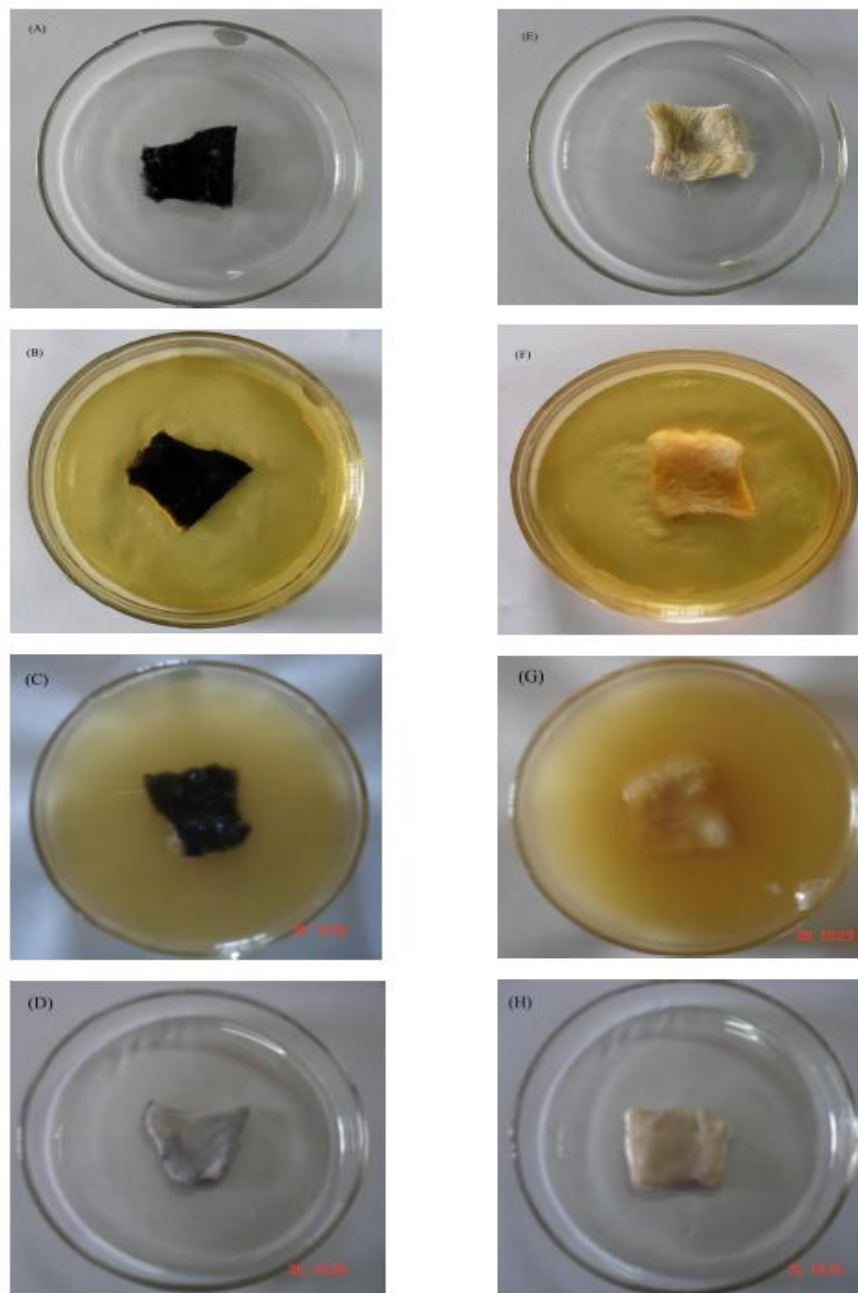


Figure 11. Application of alkaline protease on goat and sheep hides. Goat hides: A, Fresh skin; B, fresh skin with crude enzyme before incubation; C, fresh skin with crude enzyme after incubation; D, hair removed skin by enzyme. Sheep hides: E, fresh skin; F, fresh skin with crude enzyme before incubation; G, fresh skin with crude enzyme after incubation; H, hair removed skin by enzyme.

(Mukherjee and Sudhir, 2009).

Conclusion

It can be concluded that the fish waste can serve as a renewable source for the identification of alkaline protease producing *B. subtilis* strain AKRS3. The isolated

strain was subjected to phenotypic, genotypic characterization and phylogenetic analyses based on 16S rRNA gene sequences through NCBI-BLAST method. The screening of suitable medium ingredients plays an important role in the production of alkaline protease by *B. subtilis* AKRS3. In the presence of xylose and beef extract, the growth medium expressed an inducible effect on alkaline protease production. Beside

its thermostability, hydrogen ion concentration and halotolerance of alkaline protease were proven to be suitable for dehairing application on tannery industry sector.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Biotechnology (Government of India) and Tamil Nadu State Council for Science and Technology (TNSCST) an autonomous body under Government of Tamil Nadu for financial assistance for the project number BS-20 in the academic year of 2009 to 2010.

REFERENCES

- Abu-sayem SM, Alam MJ, Mozammel H (2006). Effect of temperature, pH and metal ions on the activity and stability of alkaline protease from novel *Bacillus licheniformis* MZK03. Proc. Pak. Acad. Sci. 43(4):257-262.
- Allissara R, Siriporn Y, Masaaki Y (2006). Purification and Characterization of Alkaline Protease from *Bacillus megaterium* Isolated from Thai fish sauce fermentation process. ScienceAsia 32:377-383.
- Andrade VS, Sarubbo LA, Fukushima K, Miyaji M, Nishimura K, De Campos T (2003). Production of extracellular proteases by *Mucor circinelloides* using D-glucose as carbon sources/substrate. Braz. J. Microbiol. 33:106-110.
- Annapurna J, Ravichandra P, Subhakar C (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.
- Annette SE, Megan LP, Libby AS, Sarah Q, Philip CB (2004). Bacterial diversity and ecosystem function of filamentous microbial mats from aphotic (cave) sulfidic springs dominated by chemolithoautotrophic "Epsilonproteobacteria". FEMS Microbiol. Ecol. 51:31-53.
- Anwar MN, Sharmin S, Towhid H (2005). Isolation and characterization of a protease producing bacteria *Bacillus amovivorus* and optimization of some factors of culture conditions for protease production. J. Biol. Sci 5(3):358-362.
- Beg QK, Sahai V, Gupta R (2003). Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Proc. Biochem. 39:203-207.
- Clausen E, Gildberg A, Raa J (1985). Preparation and testing of an autolysate of fish viscera as growth substrate for bacteria. Appl. Environ. Microbiol. 50:1556-1557.
- Esakkiraj PG, Immanuel S, Sowmya M, Iyapparaj P, Palavesam A (2007). Evaluations of protease producing ability of fish gut Isolate *Bacillus cereus* for Aqua Feed. Food Bioprocess Technol. Springer Sci. 2:383-390.
- Ellaiah P, Adinarayana K, Rajyalaxmi P, and Srinivasulu B (2003). Optimization of process parameters for alkaline protease production under solid state fermentation by alkalophilic *Bacillus* sp. Asian J. Microbial Biotechnol. Environ. Sci. 5:49-54.
- Fukami K, Ichijima S, Yagurumaki H, Masuzawa T, Nabeta Y, Endo K and Shimoda M (2002). Identification of Distinctive Volatile Compounds in Fish sauce. J. Agric. Food Chem. 50:5412-5416.
- Gupta R, Beg QK, Lorenz P (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 59(1):15-32.
- Hamidreza F, Mahmoud J, Naser B, Nadia M, Kianoush K (2007). Production and purification of a protease from an alkalophilic *Bacillus* sp. strain isolated from soil. Iran. J. Biotechnol. 5(2):110-113.
- Harley JP, Prescott LM (2002). Laboratory Exercises in Microbiology. 5th ed. The McGraw-Hill Companies. New York, USA. 1-116.
- Huang G, Ying T, Huo P, Jiang J (2006). Purification and Characterization of a Protease from *Thermophilic bacillus* strain HS08. Afr. J. Biotechnol. 5(24):2433-2438.
- Jassim S, Salt WG, Stretton RJ (1988). The preparation and use of media based on simple fish waste extract. Lett. Appl. Microbiol. 6:1-5.
- Lowry OH, Rosebrough NJ, Farr AL, Rondal RL (1951). Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193:265-275.
- Meyers SP, Ahearn DG (1977). Extracellular proteolysis by *Candida lipolytica*. Mycologia 69:646-51.
- Muhammad N, Javed IQ, Shahjahan B, Qurat-ul-ain S (2008). Effect of medium composition on commercially important alkaline protease production by *Bacillus licheniformis* n-2. Food Technol. Biotechnol. 46(4):388-394.
- Mukherjee AK, Sudhir KR (2009). Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04. Bioresour. Technol. 100:2642-2645.
- Nadeem A, Raj HG, Chhabra SK (2008). Effect of vitamin E supplementation with standard treatment on oxidant-antioxidant status in chronic obstructive pulmonary disease Ind. J. Med. Res. 128:705-711.
- Nascimento WCA, Martins MLL (2004). Production and properties of an extracellular protease from thermophilic *Bacillus* sp. Braz. J. Microbiol. 35:1-2.
- Nielsen P, Rainey A, Outtrup H, Priest FG, Fritze D (1994). Comparative 16S rDNA sequence analysis of some alkaliphilic bacilli and the establishment of a six rRNA group within the genus *Bacillus*. FEMS Microbiol. Lett. 117:61-66.
- Poluri E, Kunamneni A, Davuluri SA (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. AAPS Pharm. Sci. Tech. 4(4):56.
- Priest FG (1993). Systematics and ecology of *Bacillus*. In: Hoch JA and Losick R (eds.). *Bacillus subtilis* and other gram-positive bacteria: Biochem. Physiol. Mol. Gen. ASM Press, Washington DC. pp. 3-33.
- Raa J, Gilberg A (1982). Fish silage: A review. CRC Critical Rev. Food Sci. Nutr. 16:383-419.
- Ranilson SB, Talita SE, Ian PG, Amaral, Diego SB, Givanildo BO, Luiz Jr BC (2009). Fish processing waste as a source of alkaline proteases for laundry detergent. Food Chem. 112:125-130.
- Sambrook J, Russell DW (2001). Molecular cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Shah A, Qadar UI, Erum S, Samina I, Abida A (2009). Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3. Ind. J. Biotechnol. 8:286-290.
- Sharmin F, Rahman M (2007). Isolation and characterization of protease producing *Bacillus* strain FS-1. Agricultural Engineering International: the CIGR Ejournal. Manuscript FP 06 009. April. p. 9.
- Shumi W, Towhid H, Anwar MN (2004). Production of protease from *Listeria monocytogenes*. Inter. J. Agric. Biol. 06(6):1097-1100.