academicJournals

Vol. 10(36), pp. 1540-1547, 28 September, 2016
DOI: 10.5897/AJMR2016.8193
Article Number: B77B84E60900
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Halophile isolation to produce halophilic protease, protease production and testing crude protease as a detergent ingredient

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Received 4 July 2016, Accepted 2 September, 2016

Halophilic enzymes are potentially useful in many industries, particularly in food fermentation, pharmaceutical, textile, and leather for the treatment of saline and hypersaline wastewaters. In this study, a halophilic bacterium was isolated from saltpan environment, and was identified as *Bacillus* sp. Mk22 through biochemical test and 16S rRNA gene sequencing. During protease screening, the isolates produced 24 mm clear zone around the bacterial colony. The maximum production of proteases was due to the following conditions: 45°C, pH 8, 12% NaCl, carbon source glucose, nitrogen source skim milk powder and 42-h culture time, respectively. The protein was purified 16.5 fold, having 24.01% recovery, in DEAE-cellulose chromatography and 64 kDa molecular weight. Ca and Zn enhanced protease activity, while Hg strongly inhibited it. The protease was used to destain blood, ink, coffee and was active and stable under more than one extreme condition of high salt, pH, and temperature.

Key words: Saline environment, halophilic bacteria, protease, detergent

INTRODUCTION

Halophilic bacteria constitute a heterogeneous physiological group, including a variety of Gram-positive and negative bacteria, which grow optimally from 3 to 15% NaCl concentration, although they can also grow beyond this range (Ventosa et al., 1998). The isolation and characterization of novel industrially important enzyme from the halophilic bacteria with unique properties of salt, thermal, alkaline, and organic solvent stability may address the current demand for industrially stable enzymes in different processes (Souza, 2010). In recent years, halophilic microorganisms have been explored in different field of biotechnology (Mellado and Ventosa, 2003). Halophilic enzymes have highly negative surface charge with hydrated carboxyl groups protected by high salt concentration. Such is to avoid unfolding and maintain the solubility of protein (Joo and Kim, 2005). However, salt tolerant enzymes remain unexplored; halophilic enzyme-producing bacteria have been used for industries, particularly fish sauce or soy sauce in the form of mixed cultures (Oren, 2002).

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Different groups of enzymes are produced in halophilic bacteria particularly protease; these enzymes serve various industrial purposes (Mariana Delgado et al., 2012). Proteases are a single class of derivative enzymes that catalyze the cleavage of peptide bonds leading to total hydrolysis of proteins. They are physiologically significant for living organisms given their crucial applications in both physiological and commercial fields. Proteases are ubiquitous in various sources, such as microorganisms, plants and animals. Given that the plant and animal protease cannot meet the current global demands, microbial protease has thus gained increasing interest. Most commercial proteases are neutral and alkaline. The increase in the microbial proteases accounts for approximately 60% of the total enzyme sales in the world (Banik and Prakash, 2004). Proteolytic activity with potential industrial application has been characterized in *Halobacterium* spp. (Izotova et al., 1983). Halophilic proteases are less suitable for saline fermentation, because they require at least 12.5% (w/v) NaCl to express high activities (Ventosa et al., 1998). Protease comprises one of the most important groups of industrial enzymes with versatile applications, such as in cheese-making, meat tenderization, detergents, de-hairing, baking, waste management and silver recovery (Kumar et al., 2005)

The present study attempted to understand the enzymes used in washing detergent, as well as their efficiency in not only removing tough stains but also in being environmentally safe. Detergent industries need a new enzyme with novel properties that can further enhance the washing performance of enzyme-based detergents (Gupta et al., 1999). The stability of haloalkaliphilic *Bacillus* sp. (Gupta et al., 2005) and *Bacillus mojavensis* A21 (Haddar et al., 2009) has been reported, thus showing their strong potential in detergent industry. Laundry detergents are also popular in household dishwashing detergents and in both industrial and institutional cleaning detergents (Godfrey and West, 1996). This study primarily aimed to screen a novel/stable protease from halophiles for high industrial usage. Isolates were grown in culture media to obtain a crude enzyme for further characterization. The efficacy of the protease was tested as a detergent ingredient, which is one of the industrial applications of halophilic enzymes.

MATERIALS AND METHODS

Isolation of halophilic bacteria

Halophilic bacteria were isolated using halophilic agar (Hi-Media, Mumbai, India) composed of (g/l): Casein acid hydrolysate 10, yeast extract 5, trisodium citrate 3, potassium chloride 2, magnesium sulfate 25, sodium chloride 25, agar 20 and pH 7.2. Isolated bacteria were subjected to biochemical tests (Smibert and Krieg, 1994) and were used for screening the protease. High protease-producing bacterium was identified by 16S rRNA gene sequencing, and its morphology was examined using scanning electron microscopy (SEM).

Genomic DNA extraction

Genomic DNA extraction was conducted according to Moore (1995), using forward (AGA GTT TGA TCC TGG CTC AG) and reverse primers (ACG GCT ACC TTG TTA CGA CTT). The PCR was performed using primary heating step for 2 min at 95°C, followed by 30 cycles of denaturation for 20 sec at 95°C, annealing for 60 s at 55°C and extension for 2 min at 72°C then followed by a final extension for 7 min at 72°C. Purified DNA amplicons were sequenced using the ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems, U.S.A.). Moreover, the sequences obtained were deposited in the GenBank® (NCBI, U.S.A.). The sequences were assembled using Clustal W software version 1.82 (Thompson et al., 1994) available at http://www.ebi.ac.uk, and the identification was based on the pairwise alignment algorithms and phylogenetic tree.

Screening of extracellular protease

The protease growth medium contained (g L^{-1}) the following: NaCl 25, KCl 2, $MgSO_4$ 20, tri-sodium citrate 3, yeast extract 10, agar 20, pH 7.2 (Elevi et al., 2004). As a preparation, solid medium salts were separately autoclaved at 121°C for 15 min, cooled and then mixed with warm yeast extract-agar mixture to avoid precipitation of medium components.

Protease production by the selected isolates

The protease production medium was similar to the growth medium except that the yeast extract (10 gL^{-1}) was replaced by same quantity of skim milk. The inoculums contained 3.2- 5.3 x 10^5 CFU ml^{-1}. The production condition was 37°C, 200 rpm for 66 h, the cell growth and the enzyme activity were estimated at 6 h interval. Enzyme activity and biomass were measured at different temperatures, pH, carbon sources and nitrogen sources. The culture broth was centrifuged at 10,000 rpm for 20 min at 4°C and stored at -20°C until further analysis. The growth was monitored by measuring optical density at 660 nm (Shimadzu UV spectro-photometer [UV-1800], Japan).

Optimization of protease production at various parameters

Protease production was subjected to different culture conditions, temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C), pH (5, 6, 7, 8, 9, 10, 11 and 12), sodium chloride (1 to 24 at 3% interval), carbon sources (arabinose, glucose, maltose, raffinose, starch, sucrose and xylose), and nitrogen sources (beef extract, peptone, soybean powder, yeast extract, potassium nitrate and urea).

Purification of protease enzyme

The pre-chilled 80% acetone was gradually added to the culture supernatant with gentle stirring and was left for an hour and at 12,000 ×*g* centrifuge for 20 min, and dissolved in a minimum amount of 20 mM Tris HCl, pH 8.5 containing 50 mM NaCl and 0.5 mM $CaCl_2$ and dialyzed against the same buffer for 24 h. The

enzyme preparation was loaded on a Q-Sepharose HP column (1.6 cm × 20 cm), which had been equilibrated with the same buffer. The column was washed with equilibration buffer until no absorbance at 280 nm was detectable. The bound proteins were eluted by applying a linear gradient of 0.05 to 1 M NaCl at 1 ml/min flow rate. Active fractions (5 ml) were pooled and concentrated by ultrafiltration (Centricon, Amicon, USA) and used as the purified enzyme for further characterization. All the purification steps were performed at 4°C.

Protease activity assay

Protease activity was estimated through the modified Anson's (1938) method. The assay was performed at 37°C using 1% casein as a substrate and tyrosine in control. The substrate was prepared in 50 mM Tris-HCl buffer (pH 7.2) containing 2 M NaCl. The concentration of NaCl was set at 2 M in the assay system as casein was known to lose its original conformation at higher NaCl concentrations (Capiralla et al., 2002). Casein buffer solution (1 ml) was pre-incubated at 37°C for 5 min. The reaction was initiated by adding 1 ml of enzyme. After incubation for 10 min at 37°C, the reaction was terminated by adding 3 ml of 5% (w/v) trichloroacetic acid (TCA). For blank tubes, TCA was added prior to the enzyme. The content was centrifuged, and the absorbance of supernatant was measured at 280 nm. One unit of enzyme activity was defined as 1 µg of tyrosine released per minute. Protein was determined by Lowry et al. (1951) using bovine serum albumin as the standard. SDS-PAGE (10%) was performed to determine for molecular weight, following Laemmli et al. (1970).

Effect of different metal ions on the enzyme activity

The enzyme was pre-incubated at 37°C for 1 h in various concentrations (1, 5 and 10 mM) of different metallic salts for protease activity assay ($CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$ and $ZnCl_2$).

Effect of surfactants and detergents activity in protease

The effect of inhibitors and surfactants on enzyme activity was carried out under standard enzyme assay conditions where the assay cocktail was supplemented with phenylmethyl sulphonylfluoride (PMSF), 10 mM; EDTA, 1 mM; cysteine, 1 mM; SDS, 0.1%; Tween-80, 0.1%; Triton X-100, 0.1%. The effect was assessed by comparing with the control (A control assay was done with enzyme solution without treatment agents and the resulting activity was considered as 100%). The laundry detergents used are the following: Ariel, Tide (Procter and Gamble Ltd.), Rin, Surf excel (Hindustan Lever Ltd.), and Henko (SPIC India Ltd.), which are widely used in India. Each (7 mg/ml) detergent was examined by incubating with 1 ml of enzymes (750 U) at 40°C, 200 rpm for 1 h, and then followed by enzymatic assay. The enzyme activity in the absence of detergent was taken as 100%. The commercial detergents solutions were initially boiled to denature any pre-existing enzymes at 100°C for 60 min.

Destaining efficiency of protease

The application of enzyme used as a detergent additive was studied on pieces of white cotton cloth (5 cm × 5 cm) stained with human blood, ink and coffee. The stained cloth pieces were taken in separate flasks, and the following sets were prepared: A1, A2 and A3: Flasks with 100 ml distilled water + cloth (stained with blood, ink, and coffee); B1, B2 and B3: Flasks with 100 ml distilled water + stained cloth + 1 ml detergent (7 mg/ml); C1, C2 and C3: Flasks with 100 ml distilled water + stained cloth + 1 ml detergent (7 mg/ml) + 2 ml of crude enzyme solution.

The above flasks were incubated at 40°C for 30 min. After incubation, the cloth pieces were taken out, rinsed with water and dried. Visual examination of various pieces exhibited the effect of enzyme in removing stains. Untreated cloth pieces stained with blood, ink and coffee were taken as control (Banerjee et al., 1999).

RESULTS

In this study, a total of 278 strains was isolated in saltpan environments, which belonged to 11 genera; *Alcaligenes* sp. -5, *Bacillus* sp. -38, *Bacillus subtilis* -27, *B. pumilis* -22, *B. lichenifomis* -22, *B. halodurans* -14, *Chromo halobacter* -8, *Clostridium* sp. -14, *Escherichia coli* -24, *Enterobacter* sp. -11, *Halomonas* sp. -8, *Halobacillus* sp. -34, *Idiomarina* sp. -20, *Klebseilla* sp. -15 and *Pseudomonas* sp. -16. The 16S rDNA gene sequences of *Bacillus* Mk22 by Ashokkumar and Mayavu (2014), consist 830 nucleotides and were submitted to GenBank (National Center for Biotechnology Information, USA) with accession numbers JF794553. The *Bacillus* Mk22 is rod-shaped, catalase positive, and its growth range was from 30 to 40°C, from pH 6 to 8.5, and from 0.5 to 12% NaCl (Figure 1). The 16S rRNA gene sequence-based phylogenetic relationships of *Bacillus* sp. Mk22 (830 nucleotides) were closely related to those of genus *Bacillus* (Figure 2).

Bacillus Mk22 secreted protease with maximum activity after 7 days; clear zones (24 mm) indicated high proteolytic activity, which was similar to previously reported data (Amoozegar et al., 2008). Protease production was tested at different parameters, in which the maximum production was attained at 45°C (Figure 3), pH 8 (Figure 4), and 12% NaCl (Figure 5). The protease activity was also significantly high in the presence of glucose (Figure 6) and skim milk (Figure 7) as compared to other substrates or effector molecules (Table 1). The purification of the bacterial protease was as shown in Table 2; the molecular weight of the protease was 64 kDa, while that of halophilic protease-producing bacteria ranges between 15.5 kDa in *Bacillus mojavensis* A21 (Haddar et al., 2009) and 69 kDa (Xin et al., 2011). In addition, Ca and Mg stimulated the protease activity, but $HgCl_2$ strongly inhibited it (Table 3).

Proteases are mainly applied in industrial production of commercial detergents to enhance their washing efficiency. Hence, detergent enzymes are expected to possess ability to withstand highly harsh conditions, such as high alkaline pH and in the presence of salts, surfactants, and other detergent ingredients. Therefore, protease stability in the presence of commercial

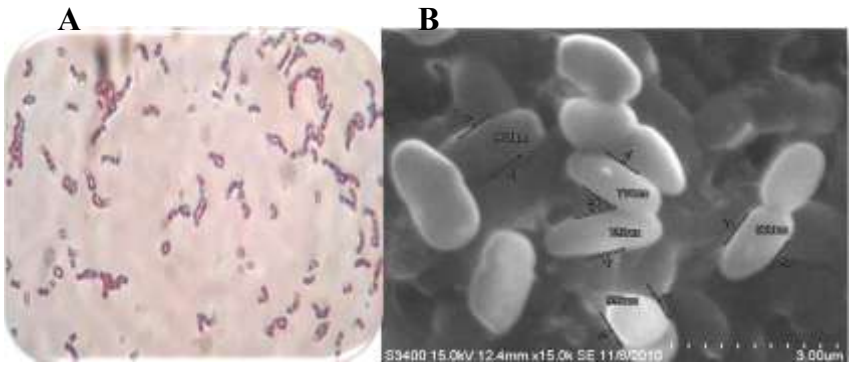

Figure 1. Morphology of *Bacillus* sp. Mk22. Microscopic (A) and scanning electron microscopic (B) view of *Bacillus* sp. Mk22 (> 31000× magnification).

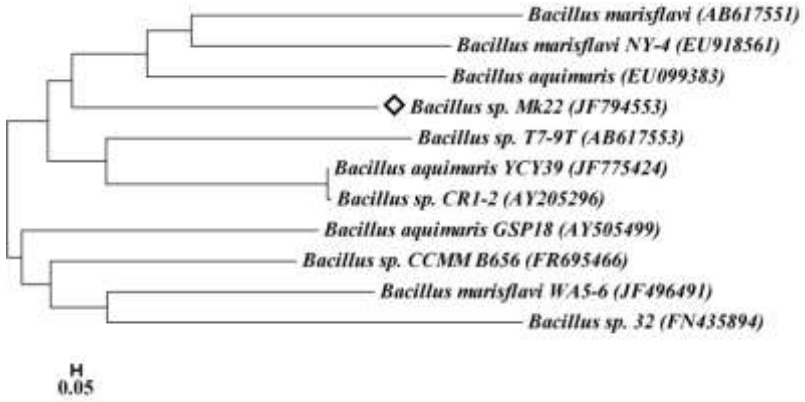

Figure 2. Phylogenetic relationship of B*acillus* sp. Mk22 and other *Bacillus* species. Genbank accession numbers are given in parentheses. Bar line 0.05 substitutions per 100 nucleotides. Tetra angle indicates the present study strain. The tree was constructed using the neighbor-joining algorithm.

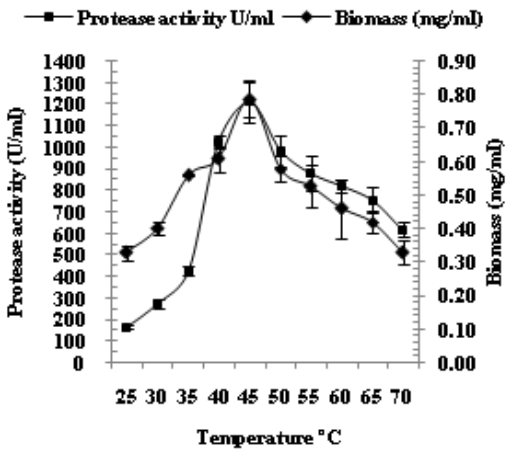

Figure 3. Protease activity and biomass at different temperatures.

detergents remains a desirable trait. The protease produced by *Bacillus* sp. Mk22 in this study significantly removed blood, ink and coffee strains at 40°C (Figure 8). The residual protease activity of *Bacillus* sp. Mk22 in the presence of various surfactants is shown in Table 4.

DISCUSSION

Halophiles have been perceived as a potential source of industrially important enzymes having exceptional stabilities. The present study intended to screen stable proteases from halophiles. In this study, protease-producing halophilic bacterium, namely, *Bacillus* sp. Mk22 was likewise isolated from saltpan and similarly halophilic bacterium *Bacillus* sp. T7-9T (AB617553) was isolated from the saltpan in South Korea (Na et al., 2011). Saltpan environment presents not only halophilic bacteria

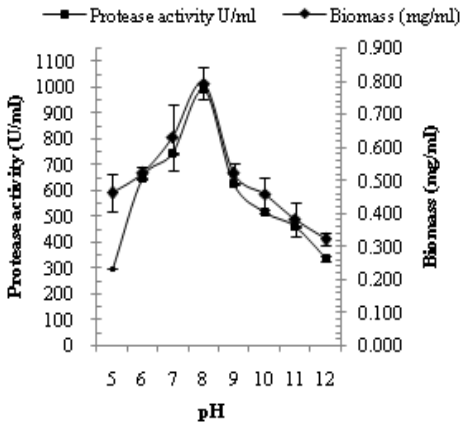

Figure 4. Protease activity and biomass at different pHs.

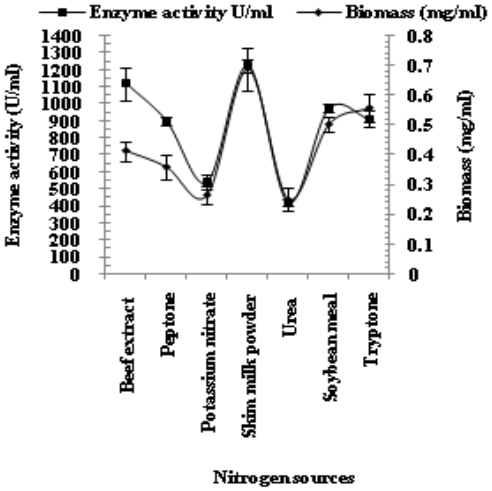

Figure 7. Protease activity and biomass with different nitrogen sources.

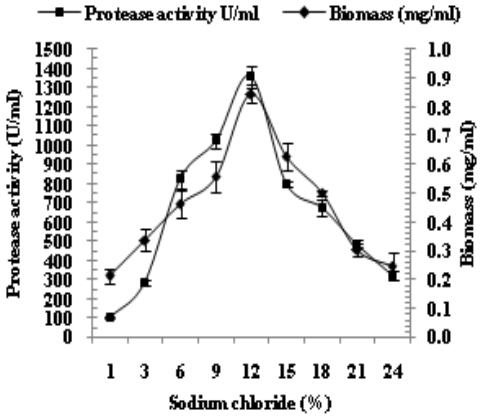

Figure 5. Protease activity and biomass at different NaCl concentrations.

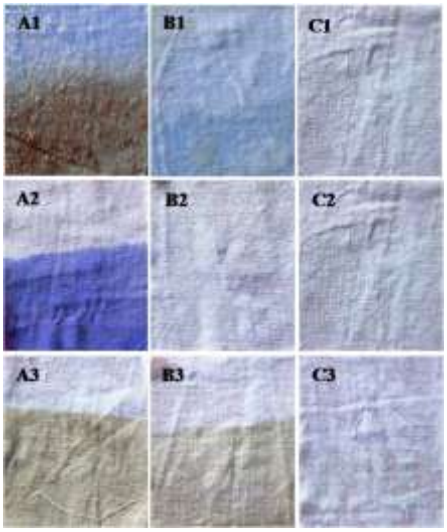

Figure 8. Supplementation of enzyme preparation in five detergents could significantly improve the cleaning performance on blood (A1, B1, C1), ink (A2, B2, C2) and coffee (A3, B3, C3).: Control (A1, A2, A3), detergents only (B1, B2, B3) and detergents + enzymes (C1, C2, C3).

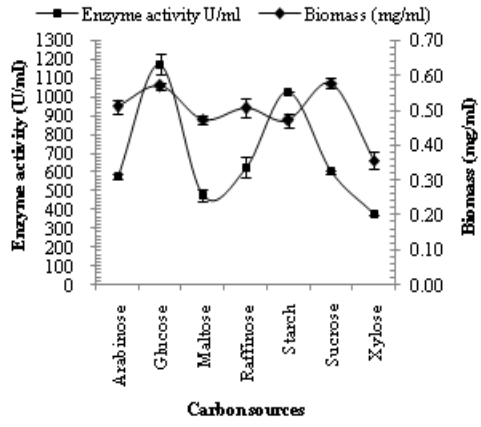

Figure 6. Protease activity and biomass with different carbon sources.

but also the non-halophilic bacteria and halophilic green microalgae, such as *Dunaliella salina* and brine shrimp *Artemia* (http://Wasterecycleinfo. com). Halophilic bacteria produce variety of enzymes particularly protease. Considerable amount of salt is used to prepare certain types of traditional fermented foods, and these salts contain halophillic bacteria producing secondary

Table 1. Conditions for maximum and minimum protease production.

Parameters	Production	Conditions	Protease activity (U/ml)
Temperature (°C)	Maximum	45	1212
	Minimum	25	165.3
pH	Maximum	8	985.7
	Minimum	5	296.3
NaCl (%)i	Maximum	12	1361
	Minimum	1	103.3
Time	Maximum	42	53
	Minimum	0	0.8
Carbon sources	Maximum	Glucose	1174
	Minimum	sucrose	371.3
Nitrogen sources	Maximum	Skim milk	1225
	Minimum	Urea	415.7

Table 2. Specific activity, recovery and purification fold of protease from *Bacillus* sp. Mk22.

Enzyme	PS	VS (ml)	TP (mg)	TA (U/ml)	SA (U/mg)	P (fold)	R (%)
Protease	CS	100	205.2	4802	23.40	0.0	100
	AMSP	25	42.60	1966	46.15	1.97	40.94
	DEAE	5	0.62	472	761.29	16.50	24.01

PS, Purification steps; CS, culture supernatant; AMSP, ammonium sulfate precipitation 80% saturation and dialysis; DEAE, DEAE cellulose chromatography; VS, volume of sample; TP, total protein; TA, total activity; SA, specific activity; P, purification; R, recovery.

Table 3. Effect of various metal ions on the activity of protease from B*acillus* sp. Mk22.

Metal	Protease activity (%)		
	1 mM	5 mM	10 mM
Control	100	100	100
$CaCl_2$	152	121	102
$CoCl_2$	93	75	96.2
$CuCl_2$	54	32	25
$FeCl_2$	104	35	26
$HgCl_2$	5	0	0
$MgCl_2$	89	107	158.3
$MnCl_2$	87	92	102
$ZnCl_2$	133	78	46.7

metabolites. Salt-rich food products are especially popular in the world, such as ‘Jeotgal’ (a traditional Korean fermented seafood), ‘fugunoko nukazuke’ (Japan’s fermented with salt puffer fish ovaries in rice bran) and ‘nam-pla’ (Thailand’s fish sauce). Although, little is still known about the microorganisms involved in

Table 4. Residual activity of protease from *Bacillus* sp. Mk22 in various surfactants.

Surfactant (concentration)	Residual activity (%)
Control	100
Phenylmethyl sulphonylfluoride (PMSF), (10 mM)	50
EDTA (1 mM)	89
Cystine (1 mM)	73
SDS (0.1%)	103
Tween-80 (0.1%)	74
Triton X-100 (0.1%)	93

the preparation of these foods, the potential of halophilic anaerobic fermentative bacteria in anaerobic treatment of saline waste waters has been reported (Kapdan and Erten, 2007).

Moderate halophilic proteases are significantly applied in biotechnology. Zavaleta and Fernandez (2007) and Moreno et al. (2007) isolated the halophilic bacteria from saltpan, and the isolated bacteria produced amylase, protease, lipase, DNase and pullulanase. Salt-tolerant proteases have high potential in the production of salt-fermented foods. However, literature on the salt-tolerant protease from halophiles, Archaebacteria and fungi remains scarce (Johnvesly and Naik, 2001). For protease production, temperature is one of the most critical parameters requiring control during the bioprocessing (Chi et al., 2007). Enzyme production is strongly influenced by temperature. The isolated bacterium *Bacillus* sp. Mk22 could tolerate high NaCl concentration and could grow on a wide range of temperature and pH. Halophilic enzyme was stable at high salt concentration and low water activity as described by Ruiz and De Castro (2007). In the present study, pH 8 produced the maximum enzyme activity, and a similar trend has been observed by various authors (Patel et al., 2006; Ganesh Kumar et al., 2008).

The 12% NaCl concentration enhanced the production, and the further increase of salt concentration significantly reduced the activity. Patel et al. (2006) have reported that maximum protease production was attained at 12% NaCl from the *Bacillus* sp. In the present study, glucose showed maximum protease production, which has been similarly reported by researchers who experimented with different type of sugars, such as lactose, maltose, sucrose, glucose and fructose (Malathi and Chakraborty, 1991). Skim milk powder produced maximum enzyme activity, while urea showed otherwise. The molecular weight of the protease investigated in this study was 64 kDa. A similar result was observed by Ravindran et al. (2011), who reported the molecular weight of protease of *B. cereus* was 66 kDa. In the present study, high protease activities were attained in Mg^{2+}, with $MnCl_2$ stimulation while $HgCl_2$ inhibited the activity. Similarly, Ca^{2+} and Mg^{2+} have been reported to increase protease activity of *B. cereus* SV1 (Manni et al., 2008). Proteases are the highest selling industrial enzymes. Their sales are projected to increase in the coming years given their applications in detergent formulations, peptide synthesis and protein processing (Chandel, 2007). In the past year, with the growing population, the demand for enzymes, particularly protease, has likewise increased.

Daily man-made and natural activities promote hypersaline environments. Therefore, halophiles and their enzymes may also be utilized in bioremediation of saline in modern day industries. The isolation and characterization of novel halophilic species that produce new enzymes may provide better opportunities. In general, majority of the commercially available enzymes are unstable in the presence of bleaching/oxidizing agents. The increased usage of these proteases as detergent additives is mainly due to the enzymes' cleaning capabilities in environmentally acceptable, non-phosphate detergents. In addition, to improve washing efficiency, the use of these enzymes allows lower water temperature and shorter activity period, often after preliminary soaking.

Conflict of interests

The authors have not declared any conflict of interests.

REFERENCES

Amoozegar MA, Ashengroph M, Malekzadeh F, Reza Razavi M, Naddaf, Kabiri M (2008). Isolation and initial characterization of the tellurite reducing moderately halophilic bacterium, *Salinicoccus* sp. strain QW6. Microbiol. Res. 163:456-465.

Anson ML (1938). The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. 22:79-89.

Ashokkumar Sekar, Mayavu Packyam. (2014). Screening, identification and antagonistic activity of halo stable *Bacillus* sp. Mk22 used as probiotic in *Penaeus monodon* Fabricius, 1798. Afr. J. Food. Sci. 8:48-53.

Banerjee VC, Saani RK, Azmi W, Soni R (1999). Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry

additive. Process. Biochem. 35: 213-219.
Banik RM, Prakash M (2004). Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiol. Res. 159: 135-140.
Capiralla H, Hiroi T, Hirokawa T, Maeda S (2002). Purification and characterization of a hydrophobic amino acid-specific endopeptidase from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates. Process Biochem. 38: 571-579.
Chandel AK (2007). Industrial enzymes in bioindustrial sector development: an Indian perspective. J. Commun. Biotechnol. 13:283-291.
Chi Z, Ma C, Wang P, Li HF (2007). Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Auerobasodium pullalans*. Bioresour. Technol. 98:534-538.
Elevi R, Assa P, Birbir M, Ogan A, Oren A (2004). Characterization of extremely halophilc archaea isolated from the Ayvalik salterns, Turkey. World J. Microbiol. Biotechnol. 20: 719-725.
Ganesh Kumar A, Swarnalatha S, Sairam B, Sekaran G (2008). Production of alkaline protease by *Pseudomonas aeruginosa* using proteinaceous solid waste generated from leather manufacturing industries. Bioresource Technology. 99: 1939-1944.
Godfrey T, West S (1996). Introduction to industrial enzymology.In: Godfrey T, West S (eds) Industrial enzymology, 2nd edn. Macmillan Press, London. pp. 1-8.
Gupta A, Roy I, Patel RK, Singh SP, Khare SK, Gupta MN (2005). One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus sp.* J. Chromatogr. A. 1075(1):103-108.
Gupta R, Gupta K, Saxena RK, Khan S (1999). Bleach-stable alkaline protease from *Bacillus* sp. Biotechnol. Lett. 21:135-138.
Haddar A, Bougatef A, Agrebi R, Sellami-Kamoun A, Nasri M (2009). A novel surfactant- stable alkaline serine protease from a newly isolated *Bacillus mojavensis* A21. Purification and characterization. Process Biochem. 44(1):29-35.
Izotova LS, Strongin AY, Chekulaeva LN, Sterkin VE, Ostoslavskaya VI, Lyublinskaya LA, Timokhina EA, Stepanov VM (1983). Purification and properties of serine protease from *Halobacterium halobium*. J Bacteriol. 155(2):826-830.
Johnvesly B, Naik GR (2001). Production of bleach stable and halo-tolerant alkaline protease by an alkalophilic *Bacillus pumilus* JB 05 isolated from cement industry effluents. J. Microbiol. Biotechnol. 11:558-563.
Joo WA, Kim CW (2005). Proteomics of *Halophilic archaea*. J. Chromatography B. 815: 237-250.
Kapdan IK, Erten B (2007). Anaerobic treatment of saline wastewater by Halanaerobium lacusrosei. Process Biochem. 42: 449-453.
Na JM, Kang MS, Kim JH, Jin YX, Je JH, Kim JB, Cho YS, Kim JH, Kim SY (2011). Distribution and identification of halophilic bacteria in solar salts produced during entire manufacturing process. Korean J. Microbiol. Biotechnol. 39:133-139.
Kumar S, Sharma NS, Saharan MR, Singh R (2005). Extracellular acid protease from *Rhizopus oryzae*: Purification and characterization. Process Biochem. 40:1701-1705.
Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of *bacteriophage* T4. Nature 227:680-685.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
Malathi S, Chekraborty R (1991). Production of alkaline protease by a new *Aspergillus flavus* isolate under solid substrate fermentation conditions for use as a depilation agent. Appl. Environ. Microbiol. 57:712-716.
Manni L, Jellouli K, Agrebi R, Bayoudh A, Nasri M (2008). Biochemical and molecular characterization of a novel calcium-dependent metalloprotease from *Bacillus cereus* SV1. Process Biochem. 43:522-530.
Mariana Delgado-Garcia, Blanca Valdivia-Urdiales, Cristobal Noe Aguilar-Gonzalez, Juan Carlos Contreras-Esquivel and Raul Rodrıguez-Herrera (2012). Halophilic hydrolases as a new tool for the biotechnological industries. J. Sci. Food Agric. 92(13): 2575-80
Mellado E, Ventosa A (2003). Biotechnological potential of moderately and extremely halophilic microorganisms. In: Barredo J.L. (Ed.), Microorganisms for Health Care, Food and Enzyme Production. Research Signpost, Kerala, 233-256. Methanogens: Ecology, Physiol, Biochem. Genet. pp. 115-120.
Moore DD (1995). Preparation and analysis of DNA. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (eds). Current. Protocols in Molecular Biology, New York: John Wiley. pp. 2-1 to 2-12.
Moreno ML, Mellado E, Garica MT, Ventosa A (2007). Diversity of extreme halophilic producing hydrolytic enzymes in hypersaline habitats. Halophiles. pp. 59-60.
Oren A (2002). Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. J. Ind. Microbiol. Biotechnol. 28: 56-63.
Patel PK, Arcangioli B, Baker SP, Bensimon A, Rhind N (2006). DNA replication origins fire stochastically in fission yeast. Mol. Biol. Cell. 17:308-316.
Ravindran B, Ganeshkumar A, Arunabhavani PS, Ganesan Sekaran (2011). Solid-state fermentation for the production of alkaline protease by *Bacillus cereus* 1173900 using proteinaceous tannery solid waste. Curr. Sci. 100(5):726-730.
Ruiz DM, De Castro RE, (2007). Effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilic archaeon *Natrialb magadii*. J. Ind. Microbiol. Biotechnol. 34: 111-115.
Smibert RM, Krieg NR, (1994). Phenotypic characterization. In: Gerhardt, P., R.G. E. Murray, W.A. Wood and N.R. Krieg (Eds.). Manual of Methods for General Bacteriology. Washington, D.C.: American Society for Microbiology. pp. 607-654.
Souza PMD (2010). Application of microbial α-amylase in industry-A review. Braz. J. Microbiol. 41(4): 850-861.
Thompson JD, Higgins DG, Gibson TJ, (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.
Ventosa A, Nieto JJ, Oren (1998). A Biology of moderately halophilic aerobic bacteria. Microbiol. Mol. Biol. Rev. 62:504 44.
Xin L, Hui-Ying Y, Xiao-Xue L, Xiao S (2011). Production and characterization of a novel extracellular metalloproteinase by a newly isolated moderate halophile, *Halobacillus* sp. LY6. Folia Microbiologica 56(4):329- 334.
Zavaleta AI, Cardenas Fernandez AM (2007). Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes isolated from Pilluana salt brine in Peru. Halophiles-2007. Congress Booklet. pp. 50-51.