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Protease characteristics of bacteriocin producing *Lysinibacilli*, isolated from fruits and vegetable waste

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Abstract:
This study describes the physical stability and optimization of nutrient components for an extracellular protease produced by *Bacillus* strains isolated from fruits and vegetable waste, Lucknow, India. The isolated proteases could hydrolyze various native proteinaceous substrates such as bovine serum albumin, casein, skim milk, but not the gelatin. The strain JX416854 and isolate 10 yielded maximum protease (831; 703 U/ml) under optimized conditions: Nutrient, Casein broth; pH 7.0; shaking condition 37 °C for 36 h. Crude protease exhibited activity over a wide range of pH (6.0–10.0) and found to be stable at (10–70 °C), pH stable at 7-9.0. The significant protease activity was observed with divalent cations Ca^{2+} and Mg^{2+} and EDTA. Further, significant blood destaining properties and stabilities with detergents were also observed. Thus, the significant potency and stability of these enzymes indicated their industrial importance and could be an alternative protease for various industrial applications.

Key words: Protease, *Lysinibacillus*, 16S r RNA gene, Phylogenetic analysis.

Background:
Bacillus produces linage of extracellular enzymes including proteases. Protease occupies a significant part of world enzyme production by contributing nearly 60% share of the total world enzyme market. *Bacillus* protease contributing 35 % sale of total enzymes **[1, 2].** They are generally classified into four groups; serine proteases- presence of serine group, Thiol/cysteine protease- Thiol group/cystein group, acidic proteases and metalloproteases. Proteases having diversity in industrial application like pharmaceuticals, food, detergents, leather and textile **[3].** A number of proteases are produced not only from plant, but also from bacteria and fungi. There is still a need to search a novel protease of desirable physical and chemical stability that can be used in various medical and chemical applications. Proteases from. *A niger* and *A. oryzae* are commercial produced and used as enzyme therapy **[4, 5]** like to improve blood circulation, to prevent abnormal blood clotting, to reduce pain and inflammation associated with Phlebitis, to alleviate the pain, inflammation, and discomfort of varicose veins; to minimize muscle pain that occurs after exercise, to minimize the inflammation and pain associated with Osteoarthritis and Rheumatoid Arthritis, to alleviate the symptoms of Sinusitis and to alleviate (reverse) Edema **[4, 5].** It's also helpful in studying of kinetic and protein or peptide structure. Various researches have been shown that production of protease and activity influences by various physicochemical parameters like nutritional and cultural cultural characteristics. Hence, to obtain maximum yield, it is necessary to optimize the media components and the cultural characteristics like pH, temperature, incubation time, inoculum size, agitation and others **[6]** suitable for each strain. Alkaline proteases from microbial sources are most commonly used in various industries. The commercial superiority of alkaline proteases is due to their suitability for use in the field of detergent industry, where they are required in large quantities. It is desirable to develop a non-phosphate based product with

decreased detergency which can be achieved by incorporation of enzymes to remove the proteinaceous stains. Moreover, liquid laundry detergents are becoming increasingly popular among consumers in developed countries. Furthermore, to improve washing performance of liquid detergent, enzymes are to be incorporated to remove the proteinaceous materials. An ideal detergent protease must be cost effective, stable, and compatible to detergents, active at a high pH (8-12) and in a wide range of temperature. Although various *Bacillus* ideal proteases have been reported and significantly used in various industrial applications, but *Lysinibacillus* protease characteristics and its production optimization were not much reported. So, in this study, we have evaluated protease characteristics and optimization of production parameters to enhance protease production from *Lysinibacillus*, which can use in various industrial applications for the human welfare.

Methodology:

Isolation and screening for protease production

Isolation was done on MRS agar, in search of bacteriocin producing isolates and protease screening was the result of their biochemical characterization. So, to elaborate the industrial potential of *Lysinibacillus* its protease spectrum was characterized. For this, collected samples were serially diluted in sterilized normal saline and seeded on to MRS agar plates which were incubated at 37 ^{0}C for 24 h. Grown colonies were separated by streak plate method. Primary screening for protease was done by streaking the isolates on casein agar plate and secondary screening was done from cell free supernatant according to **[7].** Different media used in this study were- Nutrient broth**;** Peptone: 5g/l, Beef extract: 3g/l, NaCl : 5g/l, Skim milk broth; Skim milk : 100g/l, Peptone 1.0 g/l, NaCl: 5g/l, LB broth; Tryptone: 10g/l, NaCl: 5g/l, Yeast extract: 5g/l, Glucose: 1g/l, Glucose broth**;** Glucose: 1.o g/l, Peptone: 10.0 g/l, Yeast extract: 0.2 g/l, $CaCl_2$: 0.1 g/l, K2HPO4: 0.5g/l, $MgSO_4$: 0.1 g/l. MRS broth; contained-proteose peptone: 10.0g/l, Beef extract:10.0g/l, Yeast extract: 5.0 g/l, Dextrose: 20.0g/l, Poly sorbate-80: 1.0 g/l, Ammonium citrate: 2.0g/l, Sodium acetate: 5.0g/l, magnesium Sulphate: 0.10 g/l, Maganese Sulphate: 0.05 g/l, Di potassium phosphate: 2.0 g/l.

Enzyme assay

Protease activity (U/ml) was determined according to Anson and Folin **[8]** by using casein as substrate solution and TCA as reaction blocking reagent and total protein conc was estimated by Bradford's method **[9].** One unit of enzyme activity (U/ml) was defined as the amount of the enzyme that liberates 1 µg of tyrosine per minute per milliliter under the standard assay conditions.

Calculation

(i) Standard Curve**:** ΔA_{660} Standard = A_{660}nm Standard - A_{660}nm Standard Blank

Plot the A_{660}nm Standard Vs. µ moles of Tyrosine.

(ii) Sample Determination*:*

A_{660}nm Sample = A_{660}nm (Test) - A_{660}nm (Sample Blank)

Determine the µ moles of Tyrosine equivalents liberated using the Standard curve.

Units/ml enzyme= (µ mole Tyrosine equivalents released) (11) / (1)(10) (2)

Where: 11 = Total volume (in milliliters) of assay; 10 = Time of assay (in minutes) as per the Unit Definition ***c;*** 1 = Volume of enzyme (in milliliter) of enzyme used; 2 = Volume (in milliliters) used in Colorimetric Determination

Accession number and phylogenetic analysis

Being potential inhibitor of bacterial and fungal pathogens, isolate 6 was characterized by 16 S rRNA and isolate 10 was used for the comparative study of both the proteases isolated from the same sample. The amplified gene sequence was submitted to NCBI (JX416854). The Sequence was retrieved to constract the phylogenetic tree (FigTree 1.4.0) using the Neighbor –Joining method **[10].**

Optimization of fermentation conditions

Physicochemical factors like media, carbon and nitrogen source, pH, Temp and salt concentration. Influences protease productions which were optimized by one parameter at a time approach, using submerged fermentation system carried out at constant time, rpm and seeded volume. The selected isolate was inoculated in four different media (LB, NB, GP and casein broth was assayed for protease activity. The enzyme activity and protein conc. were estimated as described previously. The optimized media used for further characterization of other parameters. Similarly the effects of carbon (citric acid, sucrose, fructose, glucose, lactose and starch), nitrogen (tryptone, casein, yeast extract, peptone, ammonium nitrate, ammonium chloride and corn steep liquor) were evaluated. The tests were conducted in triplicates and average reading was recorded **[11].**

Optimization of culture conditions

For enhanced protease production, different culture variables such as pH (3.0–12.0), temperature (15, 25, 37, 45 and 55 °C), salt concentration (0.25-2 % NaCl) and inoculums size (0.5–2.5 %) were optimized.

Characterization of protease activity

Effects of pH and Temperature

Pre incubated CFS at specified conditions, was used to evaluate the protease activity at different sets of experiment needed. The enzyme activity was screened at various pH and temperature viz. 3.0–11.0 with an interval of 0.5 units using 0.1 M of citrate buffer. (pH 3.0–6.0), phosphate buffer (pH 6.0–8.0), Tris–HCl (pH 7.5–9.0) and carbonate buffer (pH 9.0–11.0). The pH stability was determined by pre-incubation of crude enzyme for 30 min at room temperature with appropriate buffers (pH 3.0–11.0), and the activity was measured under standard assay conditions. Similarly, optimum temp for the protease activity (10-50 ^{0}C) and stability (20-90 ^{0}C) was determined by calculating the activity as described previously **[11].**

Effect of substrates

The proteolytic activity of the crude protease was determined 1% w/v various proteinaceous substrates such as BSA, casein, skim milk, and gelatin under the standard assay conditions.

Effect of protease inhibitors

The effect of various chemicals like (w/v)—phenylmethanesulphonyl fluoride (PMSF), dithiothreitol (DTT); surfactants (1% v/v) Tween 80, Triton X-100; detergents

(w/v)—SDS; chelators (w/v)—ethylenediaminetetraacetic acid (1mM EDTA) and various metal ions (1% w/v) (Mg2+, Fe3+, Zn2+ and Ca2+) at different concentrations on protease activity was determined. The enzyme was pre-incubated in these chemicals for 30 min at room temperature, and the residual activity was measured under standard conditions.

Wash Performance Assay and stability with detergents

Wash efficiency was tested with three pieces of muslin cloth. They were stained with goat blood, dried at 60 °C and fixed with 1 % (v/v) formaldehyde. The stain fixed fabrics were immersed in the crude enzyme for 10 min at room temperature and examined for stain removal. The same procedure was followed for the control without the enzyme **[11].** A negative control without any treatment was maintained.

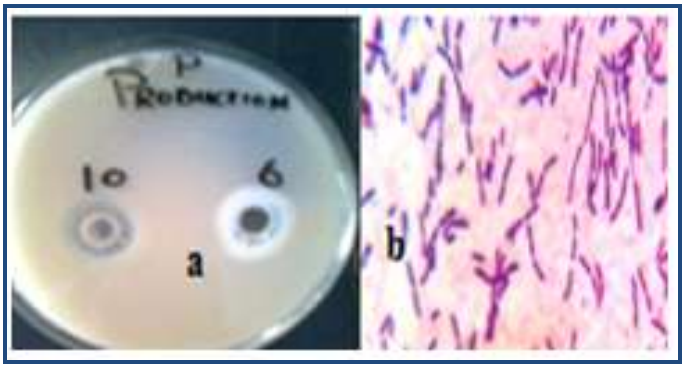

Figure 1: a) Screening of protease on casein agar plates, **b)** gram staining showing gram positive bacilli

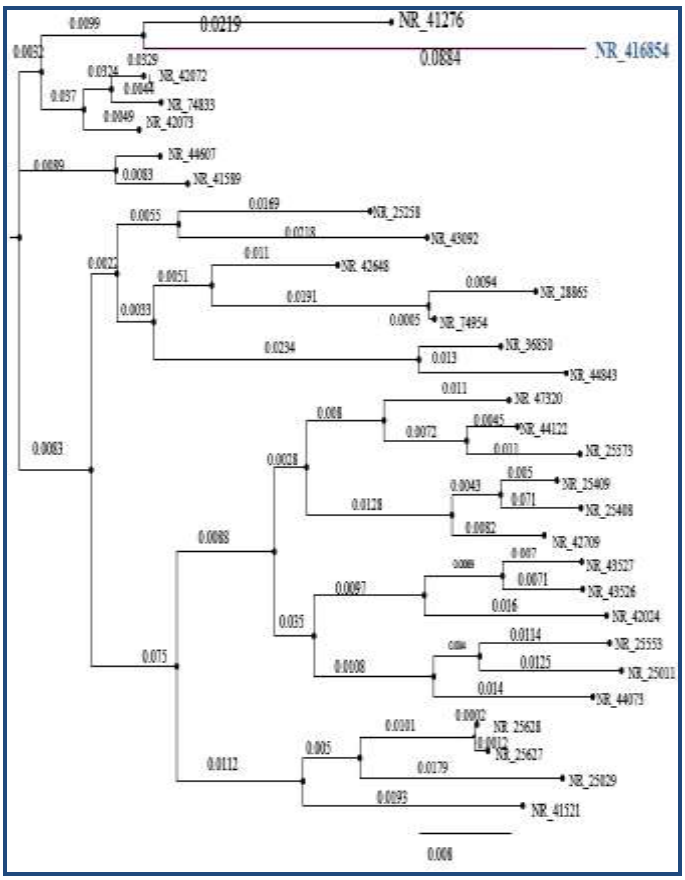

Figure 2: Phylogenetic position of isolate JX416854 is highlighted. The tree was constructed by Neighbor-Joining method and visualized by FigTree 1.4.0 by using 30 species, with 1000 replicon at the scale of 0.008. The highlighted position of isolate representing a separate sp among the bacilli.

Results:

Isolation, identification and screening of isolate

Protease producing isolate was selected from bacteriocin producing isolates during the course of their biochemical characterization. Among them two isolates form clear zone on a casein agar plate and recorded to be protease positive **(Figure 1).** Both isolates were gram positive rods and negative with gelatinase, amylase, nitrate reductase, urease but positive with catalase and protease. Isolate 06 was 16S rRNA based molecularly found to be as *Lysinibacillus*. It is closest to *Lysinibacillus* (96% similarity) and lies on separate parenthesis on radius of fermicutes on the phylogenetic tree constructed by neighbor- joining method using FigTree 1.4.0 (**Figure 2).**

Optimization of media and nutritional components

Four different media (LB, NB, GP and CB were tested for protease production. It was observed that both strains showed highest protease activity (889U/ml; 742U/ml) with CB followed by nutrient broth, glucose broth and LB broth but strain 6 Showed greater activities as compared to isolate 10. The used carbon components were found significant as glucose 763; 725U/ml, fructose 720; 697U/ml, sucrose 640;580 U/ml, citric acid 580;550U/ml, lactose 530; 515 U/ ml and starch 520; 500 U/ml Similarly, nitrogen sources casein and peptone found to be significant in protease production (**Figure 3).**

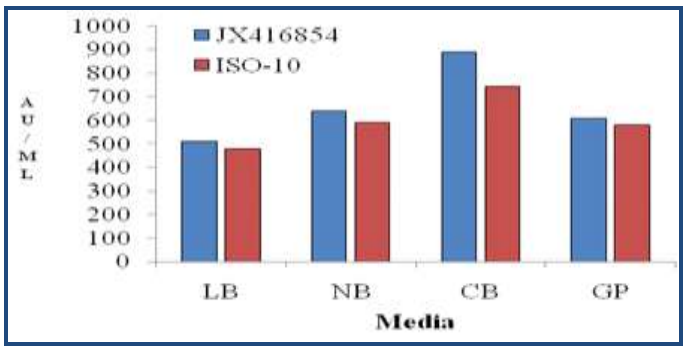

Figure 3: Showing effect different media on protease production LB; Luria-Bertani broth, NB; nutrient broth, CB; casein broth and GP; glucose broth

Effect of culture conditions on Protease Production

Different pH, Temp, incubation time, shaking conditions and inoculums volumes for both the strains were optimized. The activity enhanced gradually from pH 6.0 and reached the utmost production at pH 8.0 (921; 821 U/ml) while no activity was observed with pH ranging 3.0–5.0 and activity decreased at pH 9-11.0. The isolate produced protease at temperature 15, 25, 37, 45 and 55 °C with maximum production at 37 °C (970; 960 U/ml). Further increased protease production was observed with shaking @ 120 rpm for the 36 h followed by 48 h, 30 h and 25 h. Prolonged incubation and static condition showed the reduced protease production. We did not observe any significant effect of NaCl conc. on protease production.

Effect of pH on activity of protease

Different buffer systems of pH (3-11) were used to evaluate the effect pH on protease activity. The enzymes were found to be

stable at a pH range of 7.0–11.0 and retained maximum activities up to pH 9.0. The enzymes reduced residual activity nearly 30% between pH 6.0 and pH 11.0 (**Figure 4B).**

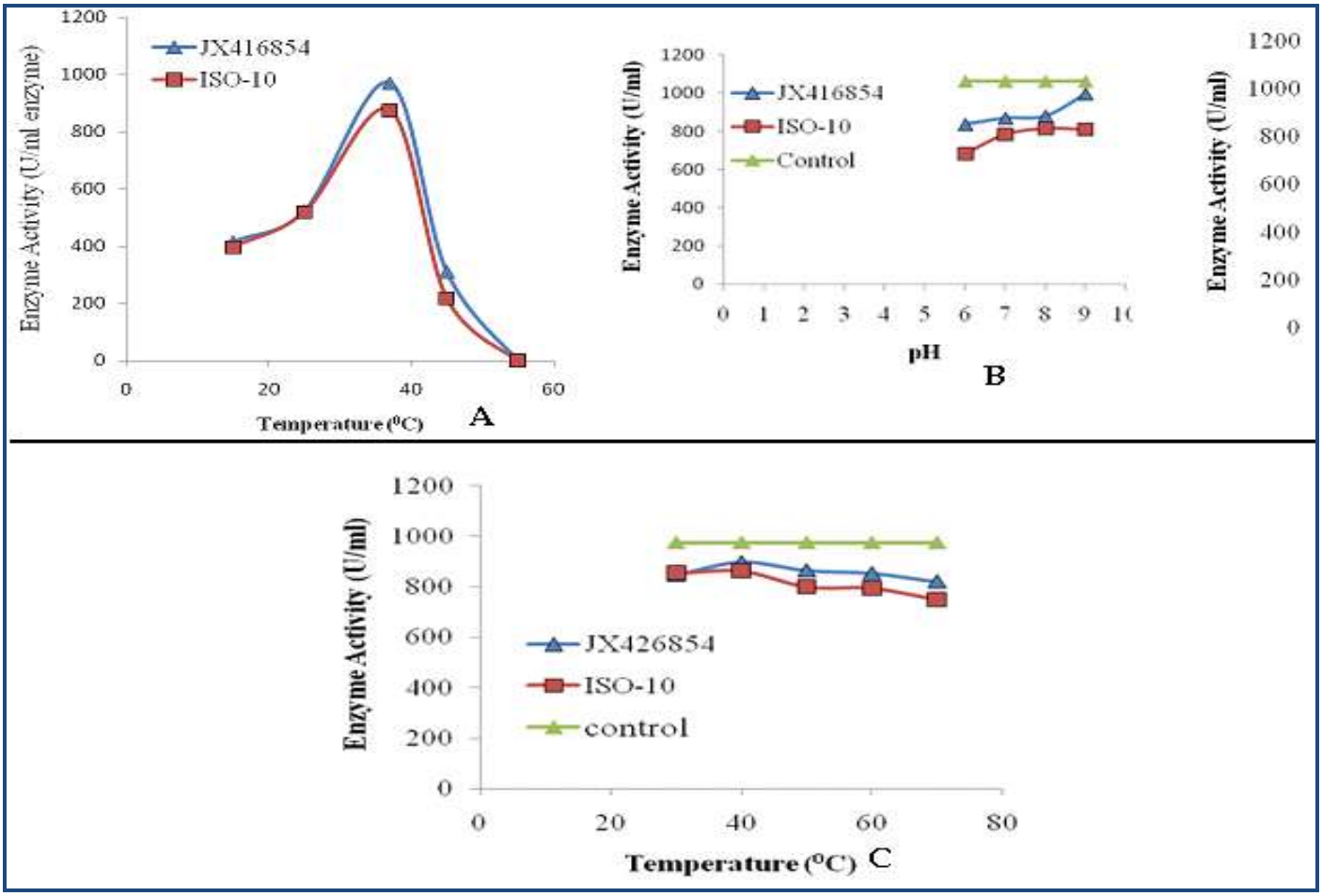

Figure 4: A) Showing effect different media on protease production LB; Luria-Bertani broth, NB; nutrient broth, CB; casein broth and GP; glucose broth; **B)** Showing effect Temperature on protease production; Optimum production of protease was achieved at the 370 C; Figure 6: Effect of pH on activities of enzymes; **C)** Effect of Heat on stability of enzymes.

Effect of heat

Heat stabilities of the enzymes were estimated at various temperatures from 40 to 120 °C. The enzyme exhibited optimum residual activity at 40 °C (898; 903 U/ml) followed by 50 °C (865; 854 U/ml), 60°C (854; 843 U/ml) and at 70 °C (7821; 811 °C) the protease enzymes nearly lost 20% their activities. Both the enzymes found to be retained nearly 80 % of its original activity in the range of 30–70 °C. At higher temp (70-120 °C) protease activity was not recorded **(Figure 4C).**

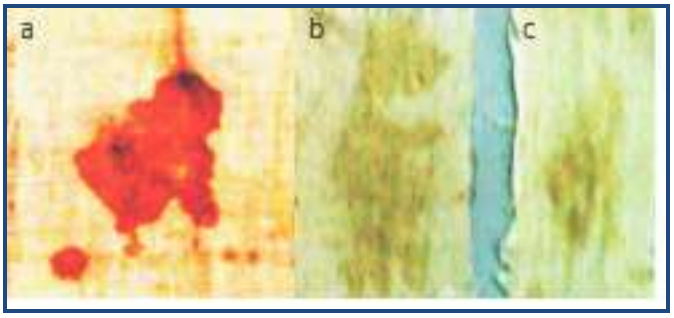

Figure 5: Washing effect of enzyme Jx416854 **a**; control, **b)** washing with detergent without enzyme showed detergent itself is not sufficient to remove the blood stain **c**) The blood destaining test showed that the enzyme has the efficiency to remove blood stain almost completely from the muslin cloth piece without the use of any of the detergents, also additives washing activity of enzyme (Decreased area and sensitivity of blood clot in fig C) with detergent further signified the stability of enzyme in harsh washing conditions.

Effect of substrates

The hydrolytic activity of the crude protease was observed to be more than 90% with the tested pertinacious substances BSA, casein, skim milk while no activity was recorded with gelatin.

Effect of Chemicals

The chemical stabilities of both the enzymes are summarized in the **Table 1 (see supplementary material)**. 0.25M protease inhibitor, PMSF significantly inhibited the enzyme activity to 35 % and 20% with while enzymes retained about 78 % of its total activity upon treatment with DTT. Surfactants like Triton X-100, T-80, SDS and metal ions like Ca^{2+}, Mg^{2+} and EDTA significant enzyme activities nearly 95 % were retained while 85 % reduction in activity was observed with Zn ions. Destaining of blood on muslin cloth was observed without the addition of detergents, taken from local market (ghadi

detergent). Further quick and easy destaining of blood was observed when used with detergent that indicated it's stability with detergent (**Figure 5)**.

Discussion:

In the present study, protease producing *Lysinibacillus* was isolated from fruits and vegetable waste, Lucknow India. Many *Bacilli* proteases were significantly reported as an additive of many formulations in many industries **[2].** Substrate consumption in production is an important criteria for a product to be cost effective. Optimization of carbon and nitrogen sources in the media plays a vital role to determine the cost of the substrate and enzyme production **[5].** So, It is necessary to formulate the media with cost effective components and to optimize the culture conditions for enhancing protease production. Many *bacillus* proteases optimized and stability conditions have been reported by various researchers **[12, 13, 14].** The nitrogenous sources like CB, corn steep liquor have been reported to be a cost effective nitrogenous component in various protease production. Similarly, glucose, fructose and sucrose are also reported to be used as suitable carbohydrates for enhancing protease production **[15].** There is gradually increased in protease production in the pH range of 6-11 but optimum production was achieved at 37 ^{0}C with the pH 8. This finding indicated the alkalophillic nature of the proteases. Many alkalophillic proteases have been described **[16, 17].** The enzyme showed maximum activity at pH 9.0 which was supported by the finding of **[16, 17].** Generally, the commercial proteases from various *bacilli* have maximum activity in the alkaline pH range of 8.0–12.0 **[18].** This stability at higher pH indicated that it can be used as an additive of detergents. Protease activity with bivalent ion was found to be not affected; this signifies the protection from denaturation and helping the enzyme to maintain its native conformation for the activity. Washed performance assay of enzyme showed that it helped to remove the blood stain from muslin cloth with the addition of any detergent. This blood destaining application and stability at higher pH and indicated its importance and might be used an additive in detergent industries.

The cost effective optimized media components like Casein broth and NB broth with the glucose as carbohydrates and peptone or corn steep liquor as nitrogenous sources were analyzed for the proteases of this study and the further characterization showed that these protease was found to be stable at pH 9 and temperature 70 °C. it was observed from the conducted study that both enzymes were found to be stable at higher pH and temp, indicated that these enzyme could be belong to serine (inhibited by PMSF) alkaline protease family. Its significant blood destaining activity with tested surfactant further showed that it could be used as a stain remover in detergent industry.

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References:

[1] Kalisz HM, *Adv Biochem Eng Biotechnol*. 1998 **36**: 1 [PMID: 3142224]
[2] Outtrup H & Boyce COL, In C.T Fogarty & K. Kelly (Eds.), 1990 227 London: Elsevier
[3] Kumar CG & Takagi H, *Biotechnol Adv*. 1999 **17**: 561 [PMID: 14538129]
[4] Patel R *et al*. *Process Biochem*. 2005 **40**: 3569
[5] Laxman RS *et al*. *Process Biochem*. 2005 **40**: 3152
[6] Hinman RL, *Chemtech*. 1994 **24**: 45
[7] Hadj-Ali *et al*. *Enzyme Microbes Tech*. 2007 **40**: 515
[8] Anson ML, *J Gen Physiol*. 1938 **22**: 79
[9] Bradford MM, *Anal Biochem*. 1976 **72**: 248 [PMID: 942051]
[10] Saitou N & Nei M, *Mol Biol Evol*. 1987 **4**: 406 [PMID: 3447015]
[11] Jayakumar R *et al*. *Appl Biochem Biotechnol*. 2012 **168**: 1849 [PMID: 23076563]
[12] Najafi MF *et al*. *Electron J Biotechnol*. 2005 **8**: 79
[13] Haddar A *et al*. *Bioresour Technol*. 2009 **100**: 3366 [PMID: 19269812]
[14] Maurer KH, *Curr Opin Biotechnol*. 2004 **15**: 330 [PMID: 15296930]
[15] Boominadhan *et al*. *J Bot Res Int*. 2009 **2**: 83
[16] Sivasubramanian S *et al*. *Chemosphere*. 2008 **70**: 1015 [PMID: 17983644]
[17] Ghorbel B *et al*. *Enzyme Microbial Technol*. 2003 **32**: 513
[18] Rao MB *et al*. *Microbiol Mol Biol Rev*. 1998 **62:** 597 [PMID: 9729602]

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Supplementary material:

Table 1: Effect of various chemicals on enzymes activities

Enzyme activity (U/ml)		
Chemicals	JX416854	Isolate-10
Control	1022 ± 1.52	1005 ± 1.84
PMSF	665 ± 2.34	805 ± 1.73
DTT	795.6 ± 2.5	780.2 ± 1.5
Triton-X	970.90 ± 3.76	944.70 ± 2.63
T-80	965.40 ± 3.54	938.95 ± 3.5
SDS	986.50 ± 1.83	946.90 ± 1.78
$BaCl_2$	955.32 ± 2.43	942.78 ± 2.5
$CaCl_2$	952.20 ± 1.5	944.25 ± 1.5
EDTA	975.67 ± 1	953.45 ± 1.5
$ZnSO_4$	123.30 ± 2.32	117.75 ± 3.21