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Isolation and Characterization of an Alkaline Protease Producing *Bacillus subtilis* (KHS-1) from Slaughterhouse Soil Samples

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Fifteen among 54 bacterial isolates had exhibited the prominent zone of hydrolysis on casein and skimmed milk agar media. One isolate KHS-1 had showed the highest proteolytic activity (47 mm) on both casein and skimmed milk media. Pure culture was deposited in microbial type culture collection and Gene bank (MTTC), Chandigarh, India and its MTTC No. is 10110. The optimum conditions for the production of extra cellular protease by KHS-1 were 37 ºC, pH 8.5 and 96 h of incubation period under shake flask culture at 150 rpm. The protease has the ability of dehairing of goat skin, degradation of gelatinous coating of X-ray films and degrading the blood clot proteins.

Key Words: Alkaline protease, Slaughterhouse soil sample, KHS-1, Blood clot.

INTRODUCTION

Alkaline proteases have wide industrial applications. Microbial alkaline proteases are accounting for nearly 60 % of the total world wide enzyme sales for industrial use[1-3]. Proteases are the main enzymes used in various detergent industries[4]. The proteases produced by *Bacillus subtilis* (neutrophillic), *B. licheniformis* (alakaphillic)[5] and *Bacillus subtilis* PE-11 are used in commercial detergent industry. Alkaline proteases also found potential application in the bio processing of used photographic films to recover silver and peptides[5]. Alkaline proteases secreted by both neutrophilic and alkalophilic bacilli are of particular interest due to their wide applications in laundry detergents, leather processing, protein recovery or solubilization, organic synthesis, meat tenderization and production of certain oligopeptides[6]. Hence, there is a considerable interest in the production of alkaline proteases from different sources. In the present study, isolation of an extra cellular alkaline protease producing strain *Bacillus subtilis* (KHS-1) from slaughter house soil samples of Anakapalli and optimization of enzyme production have been reported.

EXPERIMENTAL

Collection of the sample: Soil samples are collected from blood drained clay soil in the month of March, 2008 at a depth. of 0.5 cm in different slaughter houses in Anakapalli (Visakhapatnam district, Andhra Pradesh). Soil samples are transferred in to Ziploc bags with sterilized spatula. These soil samples are made different dilutions (10^{-1}-10^{-7}) with sterilized distilled water and plated (streak plate) on nutrient agar containing (g/L) peptone 10; NaCl 5; yeast extract 5; Agar 20 and incubator at 37 ºC for 24 h.

Isolation and preservation of bacterial strains: 54 Bacterial isolates were isolated individually after 24 h of incubation at 37 ºC from the nutrient agar plates. These isolates were isolated separately depending up on the morphological difference (colonies size, colonies texture and colour). All these isolates were sub cultured on nutrient slants and preserved in refrigerator at 4 ºC.

Screening for alkaline protease activity: Two screening media were used for screening of proteolytic activity, *i.e.* casein and skimmed milk agar. Casein media (pH 8.0) containing (g/L) *i.e.*, casein-5; peptone-5; NaCl-5; agar-20 and skimmed milk media (pH 8.0) containing (g/L): Skim milk agar-28; casein-5; yeast extract-2.5; peptone-1; NaCl-5; agar-20. These two media were autoclaved separately at 121 ºC/15 lbs pressure for 15 min and poured in sterilized petri plates. After solidifying pure cultured bacterial isolates were streaked on petri plates for observing proteolytic activity and these plates were incubated at 37 ºC for 24 h. After 24 h these plates were examined for proteolytic activity[7]. Colonies showing proteolytic activity were isolated in pure form by streak plate method and preserved on nutrient agar slants at 4 ºC. One isolate had shown the maximum zone of hydrolysis (47 mm), was identified as *Bacillus subtilis* by morphological and physiological characteristics according to Bergey's Manual of Determinative Bacteriology[8] and designated as KHS-1.

Assay of protease: Protease activity was assayed according to sigma quality control test procedure[9-11] protease activity was determined by using casein as a substrate. The reaction mixture (7 mL) contained 5.0 mL of tris-HCl buffer pH 8.5, 1 mL of 0.5 % casein and 1.0 mL of enzyme. After 0.5 h, of incubation at 37 ºC, the reaction mixture is terminated by addition of 0.5 mL of 10 % TCA and kept in ice for 10 min and contents are filtered through Whatman No. 50 filter paper. To 2 mL of filtrate, 5.0 mL of 0.2 M alkaline sodium carbonate and 1.0 mL of folin and ciocalteut's reagent was added and incubated at 37 ºC for 0.5 h and absorbency was measured at 660 nm in Schimedzu UV-visible spectrophotometer (Model UV 1800). One unit of protease activity was defined as mg of tyrosine liberated by 1 mg of protein at 37 ºC in 0.5 h.

Effect of incubation period on growth and enzyme production: Protease production was carried out in a production medium containing (g/L) casein-3; peptone-5; NaCl-5 at pH 8.5. The production medium was inoculated with 50 µL of the *Bacillus subtilis* (KHS-1) overnight culture (1×10^8 Cells/mL) and incubated at 37

ºC in shaking incubator at 150 rpm (Remi-Ris-24, Mumbai, India) for 96 h[12,13]. The bacterial growth and protease production was monitored at 12 h interval period by measuring the turbidity of the bacterial growth at 595 nm and protease activity at 660 nm by UV spectrophotometer. At the end of each incubation period, the whole broth was centrifuged at 10,000 g for 15 min (Plastocraft Super spin-RV/FM high speed, Mumbai, India) and the clear supernatant was used as enzyme source.

Effect of temperature on protease production: The production medium was inoculated with 50 µL of the *Bacillus subtilis* (KHS-1) overnight culture (1×10^8 Cells/mL) and incubated at different temperatures 28, 30 , 37, 40, 50, 60, 70, 80, 90 and 100 ºC in shaking incubator at 150 rpm[14,15]. (Remi-Ris-24, Mumbai, India) for 96 h to study the effect of temperature on protease production.

Effect of pH on protease production: The production medium with different pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5 and 12.0 were prepared and inoculated with 50 µL of the *Bacillus subtilis* (KHS-1) overnight culture (1×10^8 Cells/mL) and incubated at 37 ºC in shaking incubator at 150 rpm (Remi-Ris-24, Mumbai, India) for 96 h to study the effect of pH on protease production[16].

Applications of crude alkaline protease

Dehairing of goat skin: Goat skin was cut to 5 cm^2 pieces and incubated with the enzyme (8 U/mL) in 50 mM tris-HCl (pH 8) at 37 ºC. The skin was checked for removal of hair at different incubation times.

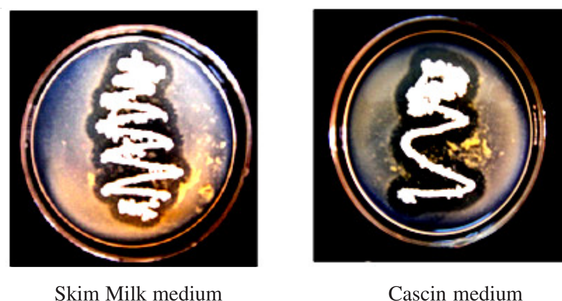
Decomposition of gelatinous coating of X-ray films: A piece of X-ray film (2 cm × 1 cm) was incubated with the enzyme (8 U/mL) and incubated at 37 ºC. The film was checked for decomposition of gelatinous coating at different incubation times.

Degradation of blood clot: The enzyme (8 U/mL) was incubated with blood clot in 50 mM tris-HCl (pH 8) at 37 ºC. Hydrolysis of blood clot was observed at different incubation times.

RESULTS AND DISCUSSION

Depending on the colony texture and colony color 54 bacterial isolates were isolated from the slaughter house soil samples. 15 among 54 bacterial isolates (MHS-1, MHS-2, MHS-3, MHS-4, KHS-1 KHS-2, KHS-3, KHS-4, RTS-1, RTS-2, RTS-3, RTS-4,9 RBS-1, RBS-2, RBS-3) had shown the zone of hydrolysis on casein and skim milk media (Table-1). As shown in Fig. 1, isolate KHS-1 had exhibited highest zone of hydrolysis on skim milk medium (47 mm) and on casein media (42 mm). Isolate KHS-1 is gram positive, rod shaped, alkaphillic, salt tolerant, spore forming bacteria. KHS-1 is moderately thermophillic and acid forming bacteria. On the basis morphological and biochemical characteristics, isolate KHS-1 was identified as *Bacillus subtilis* (Tables 2 and 3).

Effect of incubation period on growth and protease production: Since micro-organisms show considerable variation at different incubation periods, it is very essential to detect the optimum incubation period at which an organism shows the



Skim Milk medium Cascin medium

Fig. 1. Proteolytic activity on skim milk medium and casein medium by KHS-1

TABLE-1
MORPHOLOGICAL DESCRIPTION OF PROTEASE POSITIVE ISOLATES AND ZONE OF HYDROLYSIS (MM)

S. No.	Isolate name	Colony texture	Colony colour	Zone of hydrolysis (mm)
1	MCS-1	Translucent thin	Creamy, white	10
2	MCS-2	Thin colony	Yellowish	12
3	MCS-3	Thick colony	Creamy pink	25
4	MCS-4	Thick spreaded colony	White	16
5	KHS-1	Thin, spreaded	White	47
6	KHS-2	Thick spreaded	Pink	9
7	KHS-3	Thin slimy	White	14
8	KHS-4	Translucent	White	16
9	RTS-1	Thick colony	Dark yellow	18
10	RTS-2	Thick and slightly spreaded	Orange pink	15
11	RTS-3	Spreaded colony	Cream	16
12	RTS-4	Thin colony	Pink	32
13	RBS-1	Translucent and dotted colony	Creamy white	9
14	RBS-2	Thick colony	Pinkish orange	18
15	RBS-3	Thick Clustered colony	Yellowish	12

highest enzyme activity. Fig. 2 shows that 96 h of incubation period was required by *Bacillus subtilis* (KHS-1) for maximum growth and maximum enzyme production.

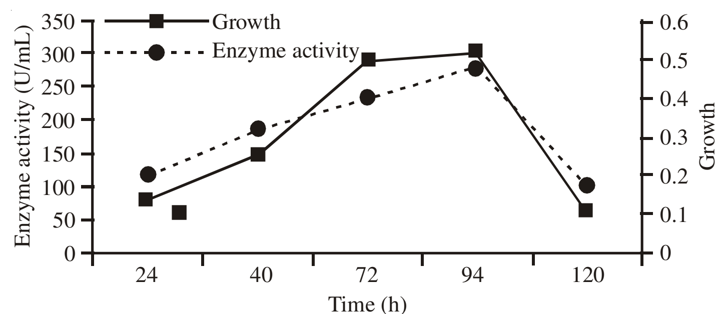


Fig. 2. Effect of incubation period on growth and protease production

TABLE-2
MORPHOLOGICAL TESTS

S. No.	Tests	Results
I	Colony Morphology	Khs-1
1	Configuration	Round
2	Margin	Entire
3	Elevation	Convex
4	Surface	Wrinkled
5	Density	Opaque
6	Pigments	Cream
II	Gram's Reaction	+
1	Shape	Rods
2	Size	Moderate
3	Arrangement	Single
III	Spore	+
1	Position	Terminal
2	Shape	Round
III	Fluorescence (UV)	–
IV	Motility	–

TABLE-3
BIOCHEMICAL TESTS

S. No.	Tests	Results
1	Growth on MacConkey agar	–
2	Indole test	–
3	Methyl red test	–
4	Voges Proskauer test	+
5	Citrate utilization	+
6	Gas production from glucose	–
7	Casein hydrolysis	+
8	Starch hydrolysis	+
9	Urea hydrolysis	–
10	Nitrate reduction	+
11	H_2S production	–
12	Cytochrome oxidase	+
13	Catalase test	+
14	Gelatin hydrolysis	+
15	Arginine dihydrolase	+
16	Lysine decarboxylase	–
17	Ornithine decarboxylse	–

+ Positive, – Negative.

Effect of temperature on protease production: The growth and enzyme activity of microorganisms is greatly influenced by incubation temperature[17]. 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 isolate KHS-1 was incubated at different temperatures. The bacterial isolates require 37 ºC for maximum production of protease. As shown in Fig. 3 *Bacillus subtilis* (KHS-1) require 37 ºC for maximum protease production.

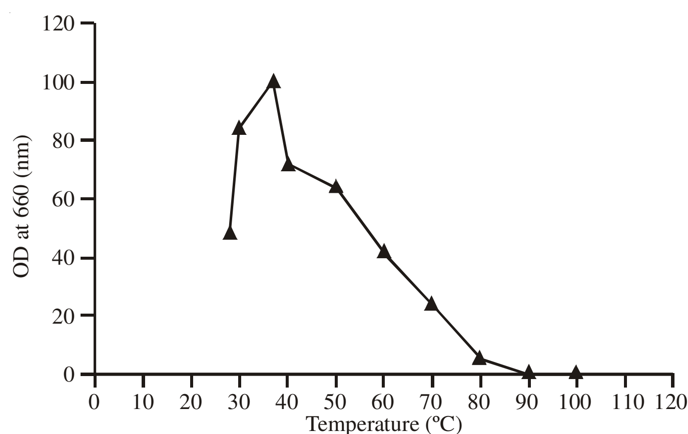


Fig. 3. Effect of temperature on protease production

Effect of pH on protease production: Microorganisms are sensitive to the change in the H^+ concentration of their environment[18]. To detect the optimum pH for protease production, *Bacillus subtilis* (KHS-1) was incubated at different pH and production of protease was recorded. Fig. 4 shows the effect of pH on protease production and was found *Bacillus subtilis* (KHS-1) exhibited maximum protease production at pH 8.5.

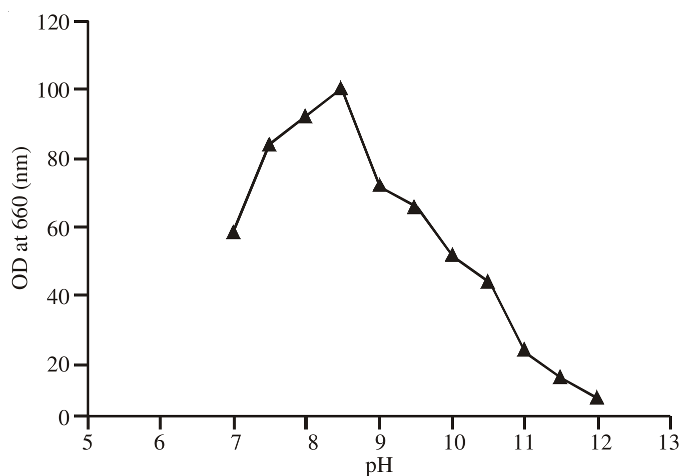


Fig. 4. Effect of pH on protease production

Applications of alkaline protease

Dehairing of goat skin: Incubation of goat skin with protease (8 U/mL) for dehairing showed that after 2-3 h incubation, hair was completely removed from the skin. (Fig. 5). The process of dehairing by proteases enhances the quality of the leather, as against traditional chemical methods. Enzymatic processes yield products of improved quality and reduce the use of hazardous and polluting chemicals.

A B

A. Control

B. Test
Goat skin after incubation with protease from
Bacillus substilis (KHS-1)

Fig. 5. Dehairing of goat skin

Decompose of gelatinous coating of X-ray films: Protease had completely decomposed the gelatinous coating on the X-ray film with an incubation of 2 h (Fig. 6). Since this protease has gelatinase activity, these results are similar to Fujiwara and co-workers[13,20]. They also reported the use of an alkaline protease to decompose the gelatinous coating of X-ray films, from which silver was recovered.

A B

A. Control

B. Test
X-ray film after treated with protease from
Bacillus substilis (KHS-1)

Fig. 6. Degradation of gelatinous coating of X-ray film

Degradation of blood clot: The protease had completely hydrolyzed the insoluble form of blood clot to soluble form after 12 h of incubation[21] (Fig. 7).

A B

A. Control

B. Test :
Hydrolysing blood cloth proteisn by protease from
Bacillus substilis (KHS-1)

Fig. 7. Hydrolyzing of blood clot proteins

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