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Research Article

MOLECULAR CHARACTERISATION OF ALKALINE PROTEASE PRODUCING *BACILLUS SUBTILIS* FROM SOIL

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ABSTRACT

Alkaline proteases are one of the most important enzymes in the biological world. Here, a comparison between poultry waste contaminated and detergent contaminated soil were carried out to detect the most potent alkaline protease producer. Soil samples were serially diluted and plated on skim milk agar to yield 4 potent isolates. Protease activity was compared using protease assay and the best isolate selected was B2 isolate from detergent contaminated soil. It showed a higher protease production of 300.666 U/ml/min after 24 hrs incubation. The selected isolate was identified as *Bacillus subtilis* using standard identification parameters such as gram staining and biochemical tests followed by molecular analysis. The 16S rDNA of the selected organism showed 99% similarity in the BLAST search with *Bacillus subtilis*.

Keywords: Enzymes, Alkaline protease, *Bacillus subtilis,* Protease assay.

INTRODUCTION

Alkaline proteases are obtained from many microbial sources like bacteria, fungi and certain yeasts. Of all the microbial sources, bacterial proteases are of special interest due to their various applications in industries such as detergent, textile, leather, food and feed industry. An important source of bacterial alkaline proteases is *Bacillus* species, which has been studied more. Some fungal species also produce alkaline proteases of industrial use, of which *Aspergillus* species has been thoroughly studied. Alkaline proteases from *Aureobasidium pullulans*, *Yarrowia lipolytica*, *Issatchenkia orientalis* and *Cryptococcus aureus* with optimum pH of 9-10 and optimum temp of 45-50 ^{0} C have been reported because of their good bioactive peptide production abilities (Li et al.,2009). Some halophilic strains have also been screened for the secretion of alkaline proteases. Extracellular alkaline proteases isolated from halophilic bacteria with very good pH and thermostability, organic solvent stability and compatibility in detergents have been also reported (Makhija et al., 2006).Alkaline protease from *Halobacterium halobium* S9 was reported to have the potential in debittering of protein hydrolysates (Capiralla et al., 2002).

Alkaline proteases from a variety of sources have been characterized by various workers in order to use them for specific applications. For example alkaline proteases with broad pH range activity, high thermostability and bleach stability can be used in detergent and leather industry. The alkaline protease isolated from *Pseudomonas aeruginosa* is found to be active at a broad pH range of 6–11 and a temperature range of 25–65 ^{0}C (Patil &Chaudhari , 2009).The molecular mass for most of the alkaline proteases is in the range 30-45 KDa and bacterial alkaline proteases which shows molecular weight below or above this particular range have been rarely reported.

Leather processing is one of the major industries related to everyday life. Enzymatic unhairing with the help of proteolytic enzymes is of great commercial importance. They contributed more than 40% of the world's commercially produced enzymes. Approximately 50% of the enzymes made are used for industrial

processes (Pepper et al., 1963). Further, proteolytic enzymes are more efficient in enzymatic dehairing when compared to amylolytic enzymes (Puvankrishanan, 2003). The enzymes can loosen the hair, without destroying the fibrous collagen of dermis (Arunachalam & Saritha , 2009). A metalloprotease produced from *Pseudomonas aeruginosa* MTCC 7926 retrieved from solvent-contaminated area is found to be beneficial for dehairing of animal skin, anti- staphylococcal activity and of X-ray film processing (Patil & Chaudhari, 2009). A metallotype protease produced from *Salinivibrio* sp. strain AF-2004 exhibiting broad pH ranges (5.0– 10.0), moderate thermoactivity and halo tolerance is suggested for its high commercial value, as it is a thermophilic and halophilic alkaline protease (Amoozegar et al., 2007).

The current paper aims the comparative screening and identification of alkaline protease from two different contaminated soils viz, with poultry and detergent wastes. The best isolate was selected based on protease assay and genotypically identified.

MATERIALS AND METHODS

Isolation and screening of Alkaline protease producing bacteria from soil samples:

Soil samples from poultry waste contaminated and detergent contaminated area were collected and serially diluted upto 10^{-6} and plated onto skim milk agar containing 0.5 gm of peptone, 0.25 gm of yeast extract, 0.5 gm of NaCl and 2.5 gm of agar. Plates were inverted and then incubated for 24 hours at 37^0C. The colonies showed clear zone of skimmed milk hydrolysis were identified as potent producers of protease (Khan et al., 2013).

Alkaline protease assay

The extent of protease activity was measured by using casein as a substrate. The extent of tyrosine liberated as a result of protease action was estimated spectrophotometrically at 660 nm after reaction with Folin ciocalteau reagent as per the Sigma protocol. The amount of tyrosine liberated was quantitatively estimated by comparing with absorbance values of known tyrosine concentrations used in the construction of a standard curve. One unit of protease activity were expressed in terms of units and was defined as the amount of enzyme needed to liberate one micromoles of Tyrosine equivalents released from casein per minute. Briefly, the culture supernatants were treated with casein substrate, incubated at 37^0C for 10 min, treated with NaCO3, reacted with F-C reagent and the absorbance was analysed using a spectrophotometer along with suitable controls.

Protease activity is directly proportional to the amount of tyrosine released and the enzyme activity was calculated as per equation given below.

$$\text{Protease activity} = \frac{\text{Mole of tyrosine released} * \text{Total volume of assay}}{\text{Time} * \text{Volume of enzyme} * \text{Volume used in cuvette}}$$

Morphological and molecular characterization of the isolate

Phenotypic characterization of the isolate was done based on its morphological and biochemical characteristics as per Bergey's Mannual of Systematic Bacteriology. Molecular identity of the isolate was further verified by 16S rDNA typing using universal 16 S typing forward primer: 5'-GAGTTTGATCCTGGCTCAG-3' and reverse primer: 5'-GAATTACC GCGGCGGCTG-3'. DNA was isolated as per the standard phenol chloroform method and the 16S rDNA PCR amplicon was verified by agarsose gel electrophoresis. The amplicon was purified and sequenced at SciGenome for further analysis. The nucleotide sequence was checked for similarity with known sequences in the NCBI database using BLAST programme.

RESULTS AND DISCUSSION

Isolation and screening of alkaline protease producing bacteria

Alkaline protease producing bacteria were isolated from poultry waste contaminated area and detergent contaminated area. Four different isolates A1, A2, B1 and B2 were initially selected as alkaline protease producing microbes as per the halo obtained on skimmed milk agar as shown in fig 1. Zone of clearance was observed in both samples. A clear zone of skim milk hydrolysis gave an indication of protease producing organism as in (Khan et al., 2013). Alkaline proteases have the ability to degrade proteins in a wide range of pH ranging from 9-11 and temperature range of 50^0C-70^0C.They have a wide range of applications in detergent industry, feather degradation, food industry etc.

The microbial isolates were further quantitatively screened for protease activity to identify the most potent protease producer (table 1). The study revealed that the microbial isolate B2 of the detergent contaminated soil showed pronounced protease activity of 300.666 U/ml/min after 24 hrs incubation as illustrated in fig 2. The alkaline protease assay was done by using casein as a substrate to find out the amount of tyrosine liberated from the isolated organism and got a higher amount of protease activity in detergent contaminated soil sample. Hence this particular organism was used for further process. One unit of protease activity was defined as the amount of enzyme that liberated 1 mg of amino acid equivalent to tyrosine in a minute under the standard assay conditions . Similar procedure was carried out in the work of (Riffel & Brandelli,2006) where azokeratin and azocaesin as substrates.

Morphological and molecular characterization of bacteria

The protease assay helped to select the most potent producer of protease enzyme and that particular species was used for further procedures. The isolate B2 revealed to be a gram positive rod and showed various biochemical properties characteristic of *Bacillus* sp as enlisted in table 2. Molecular typing of the isolate with 16S rDNA PCR yielded a 1.5 kb PCR amplicon as shown in fig 3. The BLAST analysis revealed that B2 showed showed 99% similarity with *Bacillus subtilis*.

Bacillus subtilis (natto) is the major microbial member in the ongoing production of the soya-based traditional natto fermentation, and some *Bacillus* species are in the Food and Drug Administration's GRAS (generally regarded as safe) list. The selected strains have a higher capacity to produce and secrete large quantities (20–25 g/L) of extracellular enzymes. With the recent studies of the genome of *B. subtilis* 168 and of some related strains, *Bacillus* species are supposed to become the preferred hosts for the production of many new and improved products (Schallmey et al., 2004).So *Bacillus* species especially *Bacillus subtilis* the most potent candidate of this particular work can be regarded as the best producer of alkaline proteases for industrial and research purposes due to its special features and easy availability.

Most of the studies related to alkaline proteases indicate the efficiency and role of *Bacillus subtilis* in production of proteases and its ability to withstand higher temperatures. Even though they are present in the upper layer of soil and have the ability to withstand temperatures it is very rare to cause diseases to humans. It has the capacity to form spores during unfavorable conditions and remains alive for decades. The ability to withstand high temperatures is very much helpful to use it in protease production in industrial levels. Best growth and reproductive capacities enable the use of this particular species in future researches also. Immobilized alkaline protease isolated from *Bacillus subtilis* having therapeutic properties has been studied for making soft gel-based medicinal formulas, ointment compositions, gauze, non-woven tissues and new bandage materials . Good recovery of silver from X-ray films have been reported by alkaline proteases derived from *Bacillus subtilis, Conidiobolus coronatus, Streptomyces avermectinu*s (Singhal et al., 2012).

Table 1: Quantitative estimation of tyrosine released by isolates as a result of protease activity

Bacterial isolate	Absorbance at 660 nm	Equivalents of Tyrosine released
Poultry waste contaminated area		
Bacteria A1	0.09	12
Bacteria A2	0.05	6
Detergent contaminated area		
Bacteria B1	0.02	2
Bacteria B2	0.16	22

Table 2: Tentative identification of the isolate B2 as per phenotypic characteristics

Name of test	Observation
Gram staining	+
Spore staining	+
Starch hydrolysis	+
Indole test	_
Methyl red test	_
Voges-Proskauer	+
Citrate utilization test	+
Oxidase test	+
Catalase test	_
Motility test	+
Tentative identification	*Bacillus* sp

Fig. 1: Screening of protease isolates on skimmed milk agar

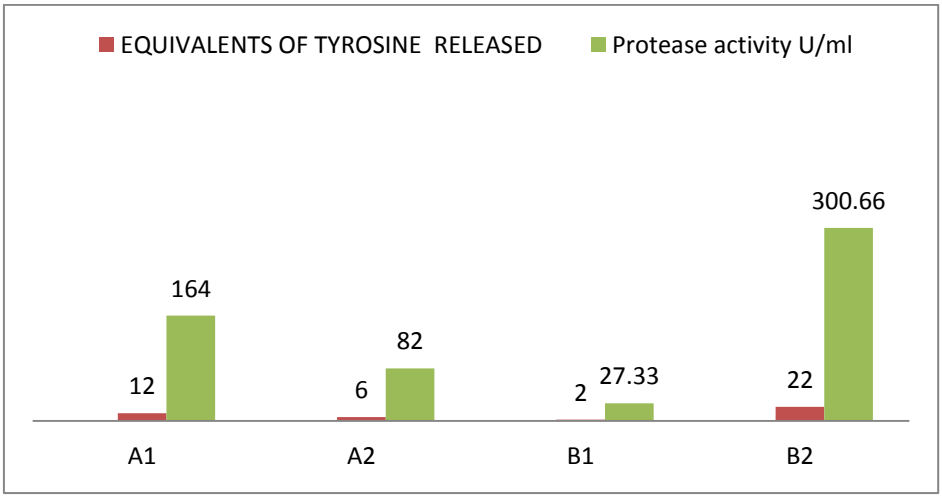

Fig. 2: Comparative analysis of tyrosine yield and Protease activity of isolates

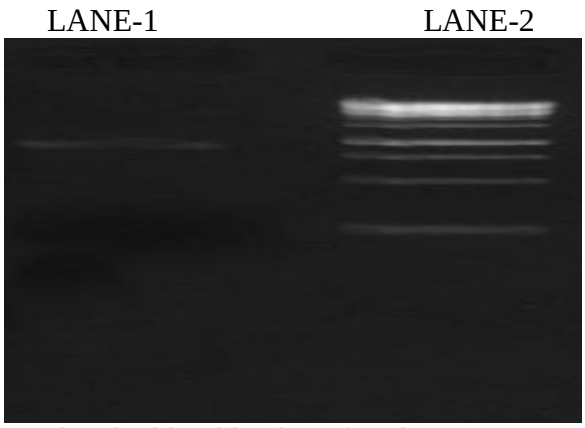

Fig. 3: Molecular identification of B2 by 16S rDNA typing
(LANE 1- 1.5 kb PCR amplicon, LANE 2- 1kb ladder)

CONCLUSION

Bacterial alkaline proteases are important participants of detergent industry, feather degradation etc. Investigations are ongoing for the invention of new varieties. Any experiment which is related to alkaline protease production gains an importance in the scientific world. Existing knowledge on bacterial alkaline proteases is increasing its depth day by day due to the innovative findings on the same .Here in this work the bacteria which produced the best quantity of alkaline protease was identified as *Bacillus subtilis* and early investigations have already proved the ability of this particular species in alkaline protease production . As this particular strain has its own unique characters and it has the ability to withstand high temperatures and stressful conditions they are suitable candidates in experiment and research purposes. Researches also proved that it is a non-pathogenic organism too. So these findings on alkaline protease producing *Bacillus subtilis* are definitely helpful for the industrial purposes and it definitely show its own importance in the scientific world.

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