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RESEARCH ARTICLE

PROTEIN PROFILING OF PROTEASE ENZYME OF BACILLUS SPP.,ISOLATED FROM SOIL SAMPLES

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ABSTRACT

Enzymes are proteins present in the cells of living organisms and are capable of speeding up chemical reactions. These enzymes are in great demand and are mostly commercialised by many industries. Microbial enzymes are preferred to those from both plant and animal sources as they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable, this is mainly because the microorganisms have a short life span and the production rate is higher. The estimated value of the worldwide sales of industrial enzymes is $1 million a year, with proteases accounting for about 60 % of it, thus the present study aims at identifying the microorganisms producing protease enzyme isolated from different sources mainly the *Bacillus spp.* In the present study the *Bacillis spp*., were screened for the production of protease and the proteins were purified by ammonium precipitation and the molecular weights of the protein was determined by SDS-PAGE so that, the method can be used for identifying the different proteins produced by the microorganisms. A total of 33 isolates were obtained from 6 different samples out of which 9 isolates were found to be belonging to Bacillus spp., and among these only 5 isolates produced protease. The SDS-PAGE gel with 50% ammonium sulphate precipitation of the samples showed multiple banding patterns with varied molecular weight. Isolate 2 showed six prominent bands with varied molecular weights of 21KDa, 22KDa, 25KDa, 42KDa, 68KDa and 110KDa.

INTRODUCTION

Proteases are enzymes that hydrolyze proteins to short peptides or free amino acids. Proteases constitute one of the most important groups of industrial enzymes, as it accounts for at least a quarter of the total global enzyme production (Layman PL 1986). About 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria sources (Ferrero *et al*., 1996).

Bacteria secrete proteases to hydrolyse the peptide bonds in proteins and therefore break the proteins down into their constituent monomers. Microorganism proteases are particularly important in recycling of proteins as they play a major role in carbon and nitrogen cycles, and such activity tends to be regulated by nutritional signals in these organisms (Sims, G.K. 2006). The nutritional regulation of protease activity in most of the species present in soil has been observed as the overall microbial community level as proteins are broken down in response to carbon, nitrogen, or sulfur limitation.

Bacillus strains have proved to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures (Prakasham RS *et al*., 2005).

Alkaline protease have been produced commercially from bacillus for applications in detergent industry (Ferrero *et al*., 1996; Manachini and Fortina, 1998; Johnvesly *et al*., 2002; Fu *et al*., 2003). Thermophiles such as *Bacillus stearothermophilus* (Boonyanas *et al*., 2000), *Thermus aquaticus* (Gabriela *et al*., 2003), *Bacillus licheniformis* (Ferrero *et al*., 1996), *Bacillus pumilus* (Kumar, 2002), and *Thermoanaerobacter yonseiensis* (Hyenung *et al*., 2002) have produced thermostable proteases.

MATERIALS AND METHODOLOGY

Isolation of microorganisms

The microorganisms were isolated from different sources like soil, vegetable and fruit waste. The bacteria were isolated by serially diluting the sample and plating them on Nutrient agar plates. The inoculated plates were incubated at 37^0C for 24-48 hours for the growth of the microorganisms. The microorganisms were identified based on their Gram's staining and standard microbiological technique. The bacillus spp., were selected for the further investigation as these microorganisms have been proved to produce protease enzymes.

Fermentation process: The bacillus spp., were screened for the presence of proteolytic activity by determining the zone of inhibition around the colonies. The microorganisms showing positive and higher zone of inhibition were selected for the fermentation. The fermentation was carried out by inoculating 48 hours old culture into media containing addition casein. The fermentation process was carried out in optimised condition for 48-72 hours in 37^0C.

Purification of the enzyme

Ammonium sulphate precipitation

The proteins were precipitated from the fermentation media by centrifuging the broth at 10000rpm for 25 minutes. The protein

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purification was carried out at -4ºC as proteins are sensitive to temperature and may get degraded. The precipitation of the proteins was carried out according to the standard chart. The sample was then brought to 50% and 60% (w/v) saturation with solid ammonium sulphate by keeping it overnight at -4ºC. The precipitate was collected by centrifugation in an ultracentrifuge at 10,000 rpm for 20 minutes. The obtained pellet was dissolved in 1ml of 0.1M Tris Hcl buffer for further purification through dialysis.

Dialysis

The resultant ammonium sulphate precipitate was introduced to a special plastic bag called the dialysis tube. Dialysis was carried out overnight by placing the tubes in the buffer on a magnetic stirrer to remove all the traces of the ammonium sulphate. The buffer was replaced at a regular interval of 4 hours.

SDS-PAGE

One dimensional Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried**.** Concentration of 12% separating gel and 5% stacking gel were prepared according to standard protocol. 35µl of each crude sample were mixed with 30µl of sample loading buffer in an Eppendorf tube. The Samples with loading buffer were then heated in the dry bath at 95˚C for 5 minutes for denaturation. The denatured protein samples were centrifuged briefly at 5000rpm for 5 minutes to precipitate out the debris. 40µl of each sample and 20µl of standard marker were loaded into the gel wells. The samples were run at 120V with 25mA in 1X running buffer according to standard protocol. After dye reaches the bottom of gel, it took it out from the electrophoresis apparatus and the gel was fixed with 10% TCA for 10 minutes at -4^{0}C. The staining was carried out with 0.25% CBB-250 for 10 hours. The excess of the dye was removed by de staining the gel for 4 hours.

RESULTS

Isolation of microorganisms

A total of 33 isolates were obtained from 6 different samples. 20 isolates had Gram positive Bacilli, 9 isolates were Gram negative bacilli and 4 isolates were Gram positive cocci. The Gram positive bacilli were selected for the further study and biochemical tests were performed and the isolates which showed similar biochemical characterisation to *Bacillus spp*., as Bergey's Manual were chosen and screened for the production of protease enzyme. Nine isolates showed to be Bacillus spp., out of which 5 isolates showed that they were capable of producing protease.

Purification of the intracellular enzyme and SDS-PAGE

The five isolates showing protease enzyme production were used in the fermentation process and the proteins were precipitated by ammonium sulphate precipitation. 50% and 60% ammonium precipitation was done. SDS-PAGE was carried out for the samples along with a protein ladder to separate the proteins based on their molecular weight. The molecular weight from the samples were identified by comparing with a standard protein ladder bands.

50% ammonium sulphate precipitation of the samples showed multiple banding patterns with varied molecular weight. Isolate 2 showed six prominent bands with molecular weights of 21KDa, 22KDa, 25KDa, 42KDa, 68KDa and 110KDa. 3 isolates showed bands with molecular weight nearing to 42KDa

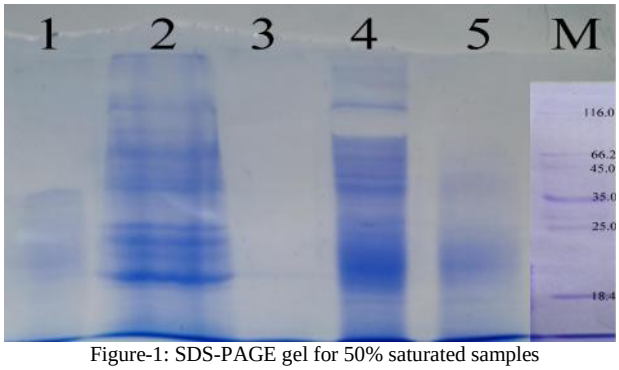

Figure-1: SDS-PAGE gel for 50% saturated samples

60% ammonium sulphate precipitation of the samples showed few and faint bands with varied molecular weight.

DISCUSSION

Hirushi, S *et al*., 1976 and Mantsala, P *et al*., 1980, studied that serine proteases are produced within *Bacillus* species other than variety of extracellular and intracellular proteases.

Wellingta Cristina Almeida do Nascimento *et al*., 2004 produced protease by thermophilic *Bacillus* sp strain SMIA-2 in liquid cultures containing trisodium citrate reached a maximum in 9h, with levels of 1.93U/mg protein.

The effect of nitrogen source in production of protease by different microorganisms was carried out and was found that tryptone, peptone and yeast extract had a impact on protease production (Beg, Q.K *et al*., 2003; Johnvesly, B *et al*., 2001; Nunes, A.S *et al*., 2001). Rahman *et al.*1994 and Manachini *et al.*1998 studied the effects of Mn2+ on the activity of proteases. Horikoshi, K *et al*., 1990 studied the effect of temperature on bacillus protease and found the optimum temperature to be 60^{0}C.

Jen-Kuo Yang *et al*., 2000, isolated and identified as a strain of *Bacillus subtilis* Y-108, optimized conditions for protease and found when the culture was shaken at 30°C for 3 days in 100 ml of medium (phosphate buffer adjusted to pH 6.0) containing 7% shrimp and crab shell powder (SCSP), 0.1% K2HPO4, 0.05% MgSO4, 1.0% arabinose, 1.5% NaNO3, and 1.5% CaCl2. Under optimised conditions, the protease activity was high as 20.2 U/ml. The protease was purified in a three-step procedure involving ammonium sulfate precipitation, DEAE-Sepharose CL-6B ionic exchange chromatography, and Sephacryl S-200 gel permeation chromatography. The enzyme was shown to have a relative molecular weight of 44 kDa by SDS polyacrylamide gel electrophoresis.

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