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Purification, Optimization and Production of Alkaline Protease from *Penicillium* Isolates Through Solid State Fermentation

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Penicillium **isolates were isolated from leather industry effluents and screened for alkaline protease enzyme production. This study reports the production of alkaline protease by *Penicillium* isolates using agricultural wastes in a chemically defined basal medium under solid-state fermentation. A comparative study was carried out on the production of alkaline protease using rice ban, wheat bran as substrates in solid state fermentation by *Penicillium* isolates. Among the tested substrates wheat bran produced the highest activity as 3.9 U/ml/min while rice bran produced alkaline protease as 3.1 U/ml/min by *Penicillium* isolate-2 under solid state fermentation. The cultural conditions were optimized for higher yield of alkaline protease enzyme. Extracellular alkaline protease was purified from *Penicillium* isolate in a two-step procedure involving ammonium sulphate precipitation and Sephadex G-100 column chromatography. The molecular mass of the enzyme was determined to be 30kDa by SDS-PAGE. The utilization of agro industrial waste not only fulfills the requirement as a substrate for the production of several value added products, but also reduce the pollution.**

Key words: *Penicillium*, Alkaline protease, Tannery waste, Solid state fermentation, Optimization and Purification.

Protease enzyme breaks down proteins. It conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases precisely act on peptide bonds formed by specific amino acids to hydrolyze them (Muhammad Ayaz Shaikh., 2010). Alkaline proteases have numerous applications of daily life of people such as food, complementary of beasts and poultries, confectionary, bakery, leathering, oil manufacturing industries, alcohol production industries, beer production. Alkaline proteases are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine centre (serine protease) or are of metallo-type (metalloprotease); and they are the most important group of enzymes exploited commercially (Gupta *et al.*, 2002b). Especially, alkaline proteases of microbial origin, which dominate the worldwide enzyme market, possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures(Agrawal *et al.*, 2004, Gupta *et al.*, 2002a). Fungal alkaline proteases are also used in food protein modification. A variety of microorganisms such as bacteria, fungi, yeast and *Actinomycetes* are known to produce protease enzyme (Madan *et al.*, 2002; Devi *et al.*, 2008). Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing

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proteases, as several species of these genera are generally regarded as safe (Devi *et al*., 2008). Production of alkaline protease enzyme using agro biotech substrates under solid state fermentation provides several advantages in productivity, cost-effectiveness, time and medium components in addition to environmental advantages like less effluent production, waste minimization (Pandey, 2000; Scool, 2003). The industrial demand of proteolytic enzymes, with novel properties continues to stimulate the search of new enzyme sources for different applications. Recently, large portions of commercial alkaline protease are available from the *Bacillus* species (Kazan, 2005; Miyaji, 2006) although the potential use of several fungal sources is now being increasingly realized (Phadatare, 1993; Tunga, 2003). The present study reports the purification, optimization and production of alkaline protease from *Penicillium* isolates through solid state fermentation.

MATERIALS AND METHODS

Collection of sample

The protein rich tannery effluent samples were collected from leather industry at Common Effluent Treatment Plant, Varadayapalam and transported to laboratory under sterile conditions.

Isolation and Screening of alkaline proteolytic fungi

1ml of effluent samples was taken and was serially diluted up to 10^{-3} dilution. 0.1 ml aliquots of 10^{-1}, 10^{-2}, 10^{-3} dilutions was spread onto the potato dextrose agar medium and incubated at room temperature for 5-6 days and observed at frequent intervals for the development of colonies. For the screening of alkaline proteolytic fungi, skim milk agar medium (pH -8) was sterilized and poured into petri plates. The fungal spores were inoculated and the plates were incubated at room temperature for 3-5 days. After incubation, the plates were flooded with an aqueous solution of Coo-massive Brilliant Blue (0.1% CBB) and shaken at 50 rpm for 15 min.

Production of alkaline protease through solid state fermentation

Inoculum preparation

The potent isolates were maintained on Potato dextrose agar slants. To these slants, 3 ml of sterile distilled water and Tween-20 were added to release the spores and this spore suspension was used as inoculum for alkaline protease production.

Preparation of fermentation medium

Production media

Rice bran and Wheat bran procured from local market was used as solid substrate. Solid state fermentation was carried out in 250 ml Erlenmeyer flask containing 5 g of wheat bran moisture to 60% with salt solution containing (g/l) $NaNO_3$ - 3, KH_2PO_4 - 1, KCl - 0.5, $FeSO_4.7H_2O$ - 0.01, Glucose - 30, pH-8 (Ellaiah *et al*., 2002). Spores of the selected fungus were harvested from seven day old slant cultures by suspension in sterile distilled water containing 0.01% Tween-80 (Agrawal *et al*., 2005). The spore suspension diluted to desire count (5 x 107 spores /ml) served as an inoculum. Inoculum was added to 5g rice bran and 5g wheat bran in a 250 ml conical flask and was moisture to 60% water content with salt solution and incubated at room temperature for 7 days.

Alkaline protease Enzyme assay

The protease activity was assayed by the method of Lovrien *et al* (1985). Three ml of reaction mixture containing 0.5% casein in 2.95ml of 0.1M Tris Hcl buffer pH-8, add 0.1ml of each enzyme was incubated at 40°C. After 30 min, the reaction was stopped by adding 3 ml of cold 10% TCA. After one hour, each of the culture filtrate was centrifuged at 8000 rpm for 5 min, to remove the precipitate and absorbance of the supernatant was read spectro-photo-metrically at 540 nm. One unit of enzyme was defined as the amount of the enzyme that liberated 1 µg of tyrosine from substrate (casein) per minute under assay conditions.

Extraction of the enzyme

To the fermentation medium, 75 ml of sterilized distilled water was added to the conical flasks and kept on rotatory shaker for about one hour to obtain uniform suspension. Then filtered through Whatmann No.1 filter paper and the filtrate were collected and used as an enzyme extract.

Optimization of Alkaline protease production by solid state production

Alkaline protease production by solid state fermentation was carried out by Rice bran, Wheat bran as solid substrates. The enzyme production was optimized with supplementation of following parameters like carbon sources, nitrogen sources, pH and temperature.

Enzyme purification and determination of molecular weight

The organism was grown for 48 hours as described previously. The cells were separated by centrifugation (10, 000 rpm, 20 minutes) and the supernatant was collected. The crude enzyme from *Penicillium* sp. was purified using ammonium sulfate at 60% saturation. The precipitates were collected by centrifugation at 12,000 rpm for 20 minutes at 4°C and re-suspended in 0.1M Tris-HCl buffer, pH 8.0 and dialyzed against the same buffer in dialysis bag at 4°C. Dialysis is a very simple technique used extensively to separate macromolecules from smaller molecules. The concentrated sample was applied to a Sephadex G-100 gel filtration column (90 × 12 cm) and equilibrated with the same buffer. The fractions possessing protease activity were pooled and concentrated.The molecular mass of the purified enzyme of *Penicillium* sp. was determined using 12% SDS –PAGE gels (Laemmli 1970) at constant voltage for 3 h.

RESULTS

Isolation and screening for proteolytic fungi

Penicillium isolates were isolated from leather industry effluents using potato dextrose casein agar plates. The cultures showing larger zones of casein hydrolysis on the plates were picked up and transferred to PDA slants (Fig. 1 -2). The culture was maintained by weekly transfer onto fresh slants of potato dextrose agar (PDA) and was stored in refrigerator at 4°C.

Production of alkaline protease through solid state fermentation

The alkaline protease enzyme produced by solid state fermentation using rice bran and wheat bran (Fig.4 and 5). *Penicillium* isolate-2 showed the highest activity of the protease enzyme (3.9 U/ml/min) with wheat bran, where as the same isolate showed the less enzyme activity with rice bran (3.1 U/ml/min) *Penicillium* isolate-1 showed the highest activity of the protease enzyme (3.4 U/ml/min) with wheat bran, where as the same isolate showed the less enzyme activity with rice bran (2.8 U/ml/min) and the results were graphically represented in the figure 3.

Optimization of Alkaline protease production by solid state fermentation

Influence of supplementation of different carbon sources including sucrose, glucose, lactose and starch media for alkaline protease production by *Penicillium* isolates were examined and listed in figure 4. Sucrose and lactose did not accelerate alkaline protease production. The alkaline protease activity of *Penicillium* isolate-2 in medium supplemented with glucose showed maximum activity (3.9/U/ml/min) than *Penicillium* isolate-1 (3.6/U/ml/min). The best carbon source for alkaline protease production by *Penicillium* sp. was glucose. Nitrogen source is an important amendant that effects enzyme production. In the present study four nitrogen sources were taken and

Fig. 1. Casein hydrolysis by *Penicillium* isolate-1

Fig.2. Casein hydrolysis by *Penicillium* isolate-2

assessed for alkaline protease production. Yeast extract, peptone, casein, sodium nitrate were incorporated in the medium. *Penicillium* isolate-2 had maximum alkaline protease activity with Casein (4.5/U/ml/min) compared to peptone (3.9/U/ml/min) and yeast extract (2.8/U/ml/min). The best nitrogen source for alkaline protease production by *Penicillium* sp. was casein (Fig. 5).The pH of the medium was adjusted to different levels ((8, 9 and 10)). *Penicillium* sp. showed maximum alkaline protease production at pH - 8, although significant levels of alkaline protease could be recorded at

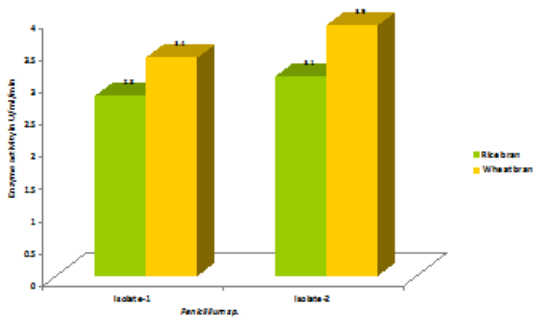

Fig. 3. Solid state fermentation of alkaline protease by using substrates

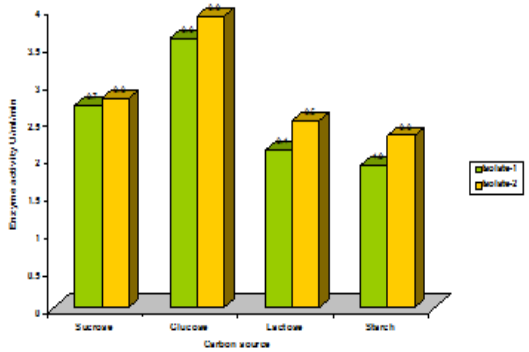

Fig. 4. Effect of carbon sources on alkaline protease production

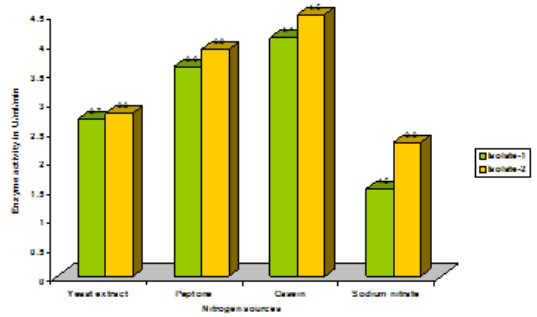

Fig. 5. Effect of nitrogen sources on alkaline protease production

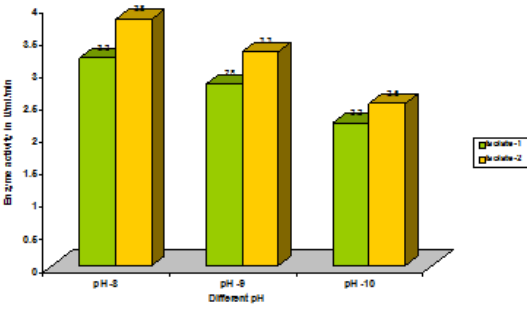

Fig. 6. Effect of pH on alkaline protease production

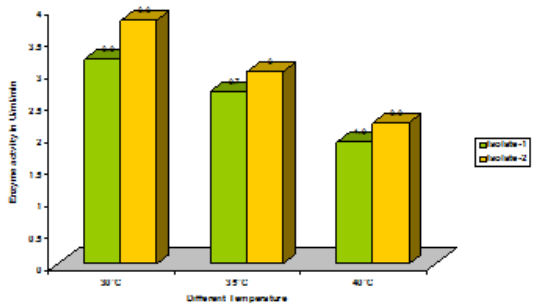

Fig. 7. Effect of temperature on alkaline protease production

Determination of molecular weight

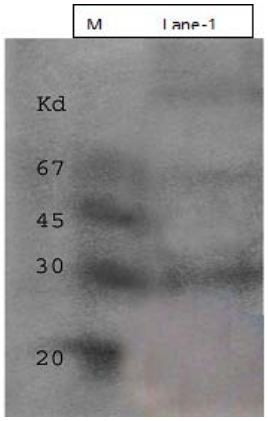

Fig. 8. SDS-PAGE of the alkaline protease from *Penicillium* isolate. Lane M, molecular mass markers; lane 1, purified alkaline protease

other pH. The *Penicillium* sp.were able to grow in the pH range 8.0 to 10.0 and produce alkaline protease in the alkaline range (Fig. 6). The medium was incubated at various temperatures (30, 35 and 40 °C). *Penicillium* sp. showed maximum alkaline protease production at 30°C. Significant range of alkaline protease was produced at temperature ranging from 30 - 40°C. (Fig. 7) Optimum temperature for the production of alkaline protease by *Penicillum* species was at 30°C.

Enzyme purification and determination of molecular weight

Molecular weight of the partially purified alkaline protease from *Penicillum* sp. was found to be 30 kDa on SDS-PAGE (Fig. 8).

DISCUSSION

The media optimization is an important aspect to be considered in the development of fermentation technology to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. The alkaline protease enzyme produced by *Penicillium* under solid state fermentation using rice bran and wheat bran. Wheat bran supported the maximum production of protease enzyme however rice bran also had comparable results. Studies have revealed that productivity is sometimes better under solid state fermentation conditions than under submerged fermentation condition(Wang, 1974).The optimization of alkaline protease activity with different parameters like carbon, nitrogen, pH and temperature were observed. Influence of supplementation of different carbon sources including glucose, sucrose, lactose and starch media for alkaline protease production by *Penicillium* sp. was examined and showed in figures, among carbon sources tested glucose served as the best carbon source. Among the tested nitrogen sources, casein exhibited highest activity than other nitrogen sources. Among physical parameters**,** pH of the growth medium plays an important role by inducing changes in enzyme secretion. *Penicillium* sp. showed maximum alkaline protease production at pH – 8. Fungal proteases are active over a wide pH range (pH 4.0 to 11.0), but they have a poor heat tolerance than the bacterial enzymes. These results were correlated with those recorded by Hussain *et al* (2006) who reported optimum pH of 8 for protease production by *A.flavus*. *Penicillium* sp. showed maximum alkaline protease production at 30°C. The results were supported by the earlier studies of the different species of *Penicillium* including *P. citrinum*; *P. perpurogerum* and *P. funculosum* gave highest yield of protease when incubated at 30°C (Sharma *et al.,* 1980). Haq *et al*. (2004) have also reported that maximum production of protease by *Penicillium griseoroseum* was obtained at an incubation temperature of 30°C and the enzyme production was reduced when the incubation temperature was increased above 30°C. Molecular mass of the partially purified alkaline protease from *Penicillum* sp was found to be 30 kDa on SDS-PAGE while other workers have reported molecular mass ~48 kDa for alkaline protease from *Aspergillus* sp (Boer, 2000; Hossain., 2006). Moreover Joshi (2010) found that the molecular mass of serine protease from *B. firmus* Tap5 was 34kDa. The different molecular weight of the alkaline proteases were obtained from *Aspergillus* species such as 33 kDa from *A. fumigatus* (Monod, 1991), 35 kDa from *A. clavatus* (Tremacoldi, C.R, 2007), 37 kDa from *A. terreus* (Chakrabarti, S.K .,2000). We have attempted to combine the economic advantages of an SSF system with the capacity of the *Penicillium* isolated in our laboratory to grow very rapidly and produce prolific amounts of alkaline proteases such that the final enzyme product is available at a commercial price attractive to tanners. The potential use of protease enzymes in leather processing eliminates the pollution causing chemicals such as sodium, lime and solvents.

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