SCREENING AND PARAMETER OPTIMISATION FOR PROTEASE PRODUCTIONFROM MARINE SPONGE DERIVED FUNGI

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ABSTRACT

Assessment of *Aspergillusmelleus* strain MP3 fungi for production of protease by solid state fermentation isolated from marine sponges were thoroughly analysed. Optimization of production parameters for production of protease was done by the solid state fermentation by altering the physical conditions. Isolation, purification and characterisation of protease from this novel strain reports interesting application in textile and leather industry. This work is one of its kinds to extract protease from such novel fungi from unusual niche.

Keywords: Protease production and optimization, fungal protease, Solid state fermentation

I. INTRODUCTION

Proteolytic enzymes are abundant and ubiquitous in their occurrence, being found in almost all living organisms and essential for their cell growth and differentiation. Extracellular proteases have high commercial value and valuable industrial applications in various diverse sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery industries (Godfrey & West 1996). For these reasons, Proteases are more extensively researched upon. The proteases from microbial source represents 60% of the worldwide enzyme commercialization (Gupta et al. 2002). Its application is very broad and has been used in many fields for years, and is mainly used in food and detergent industries (Yandri et al., 2008). In recent years the potential of usingmicroorganisms as biotechnological sources of Industrially relevant enzymes has stimulated interest in the of exploitation of extracellular enzymatic activity in severalmicroorganisms (Godfrey and West, 1996; Kumar and Takagi, 1999). Protease is the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields (Pastor et al., 2001; Ward, 1985). A variety of microorganisms such as bacteria, fungi, yeast and *Actinomycetes* are known to produce these enzymes (Madan et al., 2002; Devi et al., 2008). Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Devi et al., 2008). Proteases of fungal origin have an advantage over bacterial protease as mycelliumcan be easily removed by filtration (Phadatare, 1993). The Aspergillus genus is well known for their cellulolytic, xylanolytic and proteolytic activities. Protease is an enzyme that breaks the peptide bonds of proteins (Mitchell et al., 2007). Protease breaks downpeptide bonds to produce amino acids and other smallerpeptides. Proteases work best inacidic conditions except alkaline proteases which has itsoptimal activity shown in alkaline (basic) pH (Mitchell etal., 2007). Solid-state fermentation (SSF)has many advantages including superior volumetricproductivity, use of inexpensive substrate, simplerdownstream processing, lower energy requirement and low wastewater output (Malathi and Chakraborty, 1990). Therefore, the objectives of the study were to isolate and identify protease produced from *Aspergillusmelleus* strain MP3 fungi from Marine sponge and to perform partial characterization of the enzyme production and its properties with regard to the effect of temperature and pH and to optimise the enzyme production.

II. MATERIALS AND METHODS

ISOLATION OF FUNGI AND PREPARATION OF EXTRACT

The sponge sample was washed with sterile water (distilled water: sea water; 1:1) and ground in a mortar and pestle under aseptic conditions. 1 ml of the above was mixed with 9 ml of sterile water (distilled water: seawater; 1:1) to get dilution 10^{-1} aseptically. The serial dilution was repeated till 10^{-6}. From each dilution plating was done in Sabourauds agar by

spread plate technique. The plates were then incubated at 27°C for 5 days. After 5 days, the plates were examined and the pure culture was isolated on pure agar plate. The fungal mycelia were homogenized using sea water. Then the biomass was subjected to an extraction of biologically active components which were carried out with different solvents in the order of increase polarity: hexane, ethyl acetate and methanol by soaking at ambient temperature. The residues (crude extracts) thus obtained were finally dried under rotary vacuum evaporator and screened for extra cellular enzyme production.

III. MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF ELITE FUNGI BY ITS SEQUENCING

The fungi were grown in culture in potato dextrose broth at room temperature in the dark for 48 to 72 hours. The genomic DNA was isolated and the ITS region of 5.8 sRNA was amplified using primer ITS1 TO 5' TCCGTAGGTGAACCTGCGG 3' and primer ITS5 5' TCCTCCGCTTATTGATATGC 3'[7] and sequenced using automated sequencer.

[A] ENZYME ASSAYS - SCREENING OF SUBSRATE

The fungal extract was assayed for their ability to screen for various extracellular enzymes on various substrates. Nutrient agar (half strength) medium was supplemented with gelatin, casein and skimmed milk for the assay of Protease enzyme while carboxy methyl cellulose was used for cellulose enzyme, starch was used for amylase and 1% between 20 or between 80 for lipase enzyme assay.

[B] SOLID STATE FERMENTATION

MEDIA OPTIMISATION

Agro waste namely Wheat bran, Rice bran, Bean hull, Black gram hull, Sago waste, etc were used for enzyme production in SSF.

DIALYSIS

To dialyze a sample, the tubing of required length is taken (use the gloves to handle the tubes) and both inside and outside of the tubing is rinsed with distilled water. One end of the tubing is tied securely with a thread. Now using the open end, fill the tube with distilled water. Holding the tube securely by the top, the tube is gently squeezed to check whether there is any leakage on the membrane of on the closed end. If there is no leak, the water is removed and the sample solution is filled 2/3 using a pipette or a funnel. Then the open end is also closed securely by tying with a thread (if available, dialysis clips can be used on both the sides instead of thread). Now the bag is placed in an appropriate buffer solution and dialyzed for overnight at required temperature. During this time the small molecules will be removed from the bag. the buffer can be changed, if necessary, after 3-4 hours.

Estimation of Protein

The protein was determined according to the method of Bradford (1976). This method has become a popular for estimation of proteins because the assay is simple, quick and inexpensive. To the 1 ml of culture filtrate 5 ml of CBB was added, mixed thoroughly and read at 595 nm. The protein was calculated using Bovine Serum Albumin as standard.

EFFECT OF pH ON PROTEASE ACTIVITY

The effect of pH on protease determined by assaying the enzyme activity at different pH values ranging from 5.8 to 7.8 using the buffer systems including citrate (2-6) and phosphate (7-8) buffer. The concentration of each buffer was 0.1 M. the fermentation was carried out at 37°C and the effect of enzyme production was studied.

EFFECT OF TEMPERATURE ON PROTEASE ACTIVITY

Optimal temperature was determined by incubating the reaction mixture at different temperature range between 28 - 65°C.

SDS-PAGE

SDS-Poly acrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of (Laemmli, 1970). At the end of electrophoresis, gel was removed and stained with silver staining method of Blum *et al* (1987). After staining, the gels were stored in 7% (v/v) acetic acid. Take out the gel from the glass plate carefully and transferred to a plastic tray. Pour the CBB R-250 staining solution into the gel tray and place it on the rocker for overnight. After overnight incubation remove the excess amount of stain by using destainingsolution. Transfer the gel on the Transilluminator and visualize the protein bands.

Zymogram analysis

Protease zymogram was performed by the slightly modified method of Morag et al., (1990) in polyacrylamide slab gels containing SDS and 0.1% gelatin as substrate. After electrophoresis, gels were submerged in 50 mM of Phosphate buffer (pH 7) for 15 min and incubated at 50°C for 6 hr in the same buffer. Then the gels were stained with CBB R-250 for 6 hr with mild shaking and de-stained with 1 M NaCl until protease clear band become visible. Gels were then immersed with 0.15% (v/v) acetic acid and the activity band was observed as a clear colorless area in the gel against the blue ground.

IV. APPLICATION OF PROTEASE ENZYME

DEHAIRING OF GOAT SKIN

Goat skin was cut into 5×3 cm pieces and incubated with the purified protease (5U/ml) in 0.2 M glycine NaoH buffer PH 10.537*C. The skin was checked for removal of hair at different incubation times.

DESTAINING OF BLOOD

A clean piece of white cloth (5×5) was stained with blood and allowed to dry the cloth. Then was soaked in 2% formaldehyde for 30 min and rinsed with water to remove excess formaldehyde. The cloth was incubation. After incubation times, cloth was rinsed with water wastes for 2 min and then dried The same procedure was done for the control except incubated with enzyme solution.

V. RESULTS

Isolation of fungi

In the present study,the 10^{-5} dilution of the sponge sample yielded three different isolates. The characterization and analysis was performed for Isolate1. Pure culture of Isolate1 (Fig 1a) was obtained and SEM micrograph (Fig 1b) was taken to visualize the morphological features of the fungi.

Molecular characterization and Identification of elite fungi

In the present study, the DNA was isolated from the Isolate1 and the ITS region of 5.8 s rRNA was amplified using specific primers ITS 1 and ITS4 and sequence was determined using automated sequencers. Blast search sequence similarity was found against the existing non redundant nucleotide

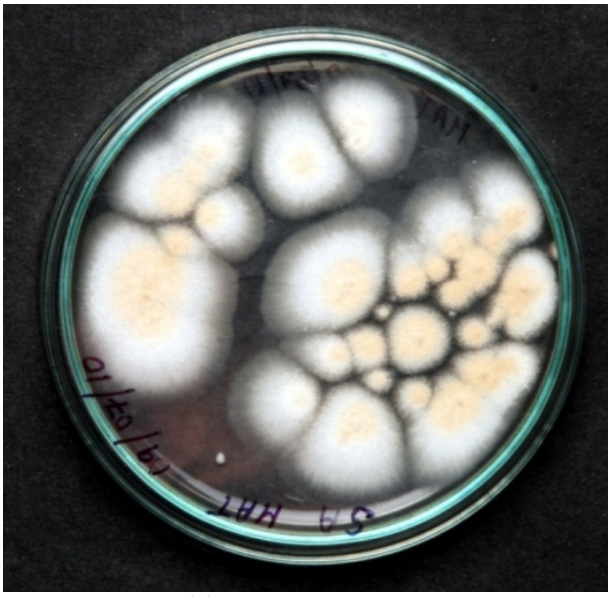

Fig. 1(a). Pure culture of Isolate1

sequence database thus, identifying the fungi as *Aspergillusmelleus*. The percentage of similarity between the fungi and database suggests it as novel strain. Thus, the novel strain was named as ***Aspergillusmelleus*** strain MP3 and made publically available in GenBank with an assigned accession number.

Enzyme Assays - Screening of Subsrate

Table 1: Screening for enzyme production by the fungi

Enzyme	Zone of Clearance (mm)
Protease	23
Amylase	–
Cellulose	–
Lipase	14

Solid State Fermentation : Media Optimisation

Table 2: Substrate optimisation for growth of fungi

Solid Substrate	Zone of Clearance (mm)
Wheat	40
Rice	27
Blackgram	26
Chennahusk	27

Effect of pH on protease activity

pH is the most important factor, which markedly influence the enzyme activity. Enzymes are affected by changes in pH. Extremely high or low pH values generally result in complete loss of activity of enzymes. It is clear from the result that the protease produced by this strain is active at pH 6.2. This property makes the enzyme suitable for leather treatment and industrial production of detergents.

Table 3: Effect of pH on enzyme production

pH	OD @650nm
5.8	1.221
6.2	1.249
7.0	1.147
7.4	1.123
7.8	1.137

Effect of Temperature on protease activity

Morimura et al. (1994) have reported that the rate of enzyme catalyzed reactions increases with temperature up to a certain limit. Above a certain temperature, enzyme activity decreases with temperature because of enzyme denaturation. Maximum protease activity was recorded at 37°C. Further increase in temperature resulted in decrease in the activity of protease. Similar observation was shown by Morimura et al. (1994) for *Aspergillususami*. It was revealed that environmental temperature not only affects growth rates of organism but also exhibit marked influence on the levels of protease production.

Table 4 : Effect of temperature on enzyme production

Temperature	OD @650 nm
28°	1.7155
37*	1.7648
55°	1.6270
67*	1.3761

VII. DISCUSSION

Marine sponge associated fungi were isolated aspectically. The isolated fungal strains were confirmed by isolating the DNA and sequencing using ITS Primer 1 and 4. The sequence obtained was subjected to BLAST to compare with library data base sequence. The BLAST result showed that the isolated fungal strains were new and the sequence were subjected to Gen Bank using the tool sequin in NCBI. The accession number were given as **HQ 449676.1**. *Aspergillusmelleus* MP3 was isolated from marine origin and completely characterised. Primary screening for the synthesis of extracellular enzymes was carried out on different substrates. Among the different enzymes screened, the fungi *Aspergillusmelleus* MP3 provided promising results for Protease enzyme on Gelatin as a substrate.

Temperature and pH are most important factors which marked by influence enzyme activity. Temperature stability studies indicate general increase in the stability of enzyme with time 5 to 50 minutes. The optimum temperature for maximum enzyme activity is found to be 37°C. The effect of pH on the enzyme activity indicates the amylase is active at pH 6.2, this suggest that the enzyme would be useful in process of wide range of pH change from slightly acidic to alkaline and vice versa.

VIII. APPLICATION OF PROTEASE

DEHAIRING OF GOAT SKIN

Incubation of the protease with skin for dehairing showed that after 2.30 - 3 hrs incubation of the enzyme (2 U/ml) with goat skin, hair was removed very easily compare to the control (Fig. 2) Since this protease can digest collagen, the process of dehairing must be controlled to avoid reducing 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.

DESTAINING OF BLOOD

In case of removing blood stain from cloth, it was seen that the protease enable to remove blood stain very easily without addition of any detergent (Fig. 3). This protease showed high capability for removing proteins and stain from cloth and also it could be used as an alkaline protease in detergent powder or solution. Its ability to act in the presence of solvents and detergents can be exploited for this purpose. Anwar and Saleemuddin (1997) reported usefulness of protease from *Spilosomaobliqua* for removal of blood stains from cotton cloth in the presence and absence

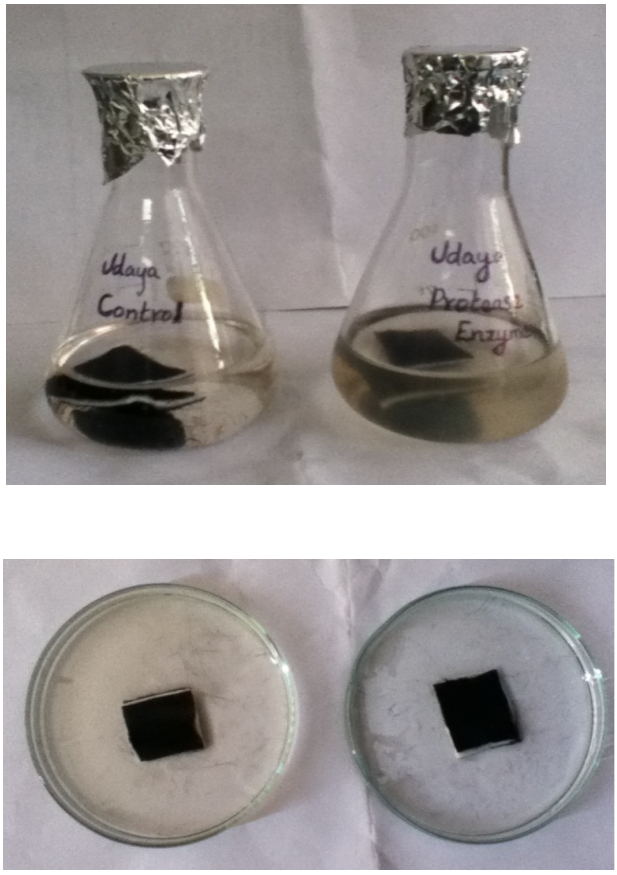

Fig. 2. Dehairing of goat skin using fungal protease enzyme

of detergents but we believe that this protease is more effective.

IX. CONCLUSION

Fungal protease was applied to show its high capability to remove stain from cloth and also it could be used as an alkaline protease in detergent powder or solution. Its ability to act in the presence of solvents and detergents can be exploited for this purpose. The protease also finds its application in leather and tanneries since it can digest collagen, the process of dehairing must be controlled to avoid reducing 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.

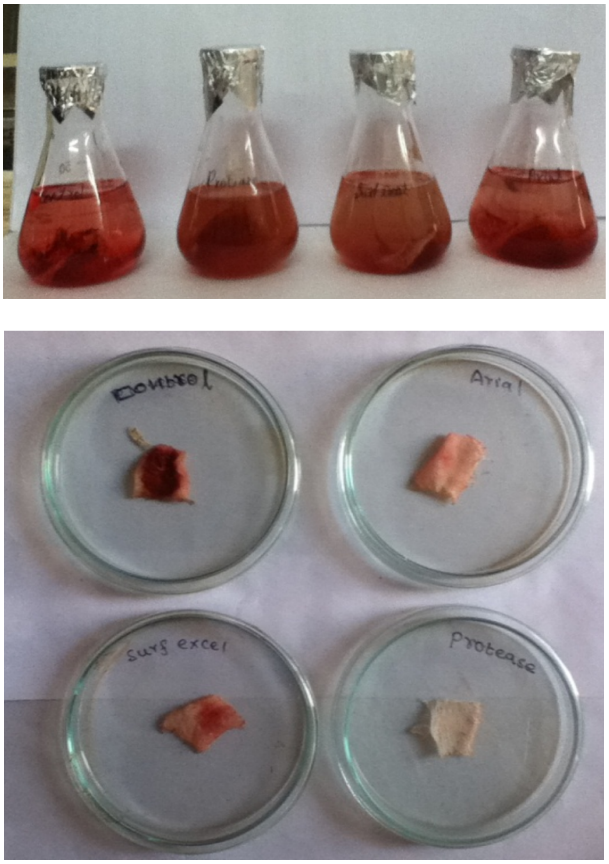

Fig. 3. Destaining of blood using detergents and protease enzyme

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