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Protease Production by Rhizopus stolonifer through Solid State Fermentation

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ABSTRACT

Rhizopus species are known to produce various types of proteases. The isolated R.stolonifer was shown to be better producer of protease at pH 7.5 and 40 ^{0}C temperature indicating slight thermo stability. Various metal ions were shown to be effective activator of protease activity and EDTA had given 100% inhibition. Among the selected oil seed cakes ground nut oil was shown to be suitable substrate after wheat bran for the production of protease by R.stolonifer.

Key words: Protease, Ground nut oil cake, PMSF, EDTA, Mineral medium.

INTRODUCTION

Protease is essential constituents of all forms of life on earth including prokaryotes, fungi, plant and animals and is highly exploited enzymes in various industries, representing worldwide sale at about 60% of total enzyme market [1]. Extracellular enzymes are usually capable of digesting insoluble nutrient material such as cellulose, proteins and starch [2]. Proteases of fungal origin have an advantage over bacterial protease as mycelium can be easily removed by filtration. Protease produced by *Rhizopus* sp. is of greater importance due to its higher protease producing ability. Solid-state fermentation (SSF) has many advantages including superior volumetric productivity, use of inexpensive substrate, simpler downstream processing, lower energy requirement and low wastewater output [3] and the production of enzyme using different oil seed cakes is having a commercial importance and highly cost effective. This procedure of protease production with the use of low cost substrate and cost effective method allows possible production and Application of crude enzyme for various industrial processes [4]. For the production of enzymes for industrial use, isolation and characterization of new promising strain is continuous process [5].These are generally produced by solid state fermentation. Solid state fermentation has been established as a superior technique for the production of enzymes [6]. Solid state fermentation involve microbial modification of a solid, undissolved substrate in which microbial cultures are grown on moist solid with little or no free water, although capillary water may be present[7].The product can be recovered in highly concentrated form as compared to those obtained by submerged fermentations. It not only provides a natural substrate for fungal growth and fermentation, they also result in improved value of these agro-industrial residues [8]. The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of microbial population .The most important among them are

medium pH and incubation temperature. The pH of fermentation medium was reported to have substantial effect on production of proteases [9]. The purpose of this study was to isolate and produce protease from fungi. Our fungal strain *R.stolonifer* produced high protease and we report some properties of protease of this strain.

MATERIALS AND METHODS

Isolation and characterization of fungi:
The fungi was isolated on PDA plates from soil samples collected from Sri Ramakrishna Degree College surroundings, Nandyal and subjected for serial dilution. These fungi identification was using microbiological atlas [10]. This fungus were grown and transferred to PDA slants. The culture was maintained by weekly transfer on to fresh slants of Potato dextrose agar and was stored in refrigerator at 4° C for further use. This fungus was further maintained on PDA slants for further use.

Substrates and diluents:
Five oil seed cakes selected which includes sesame oil cake; ground nut oil cake; cotton seed oil cake and mustard oil cake along with wheat bran were used as substrates in this study. The composition of moistening agent (mineral medium) includes (%)) 0.5 NH_4NO_3, 0.2 KH_2PO4, 0.2 $MgSO_4$ and 0.1 NaCl in water.

Production of protease using wheat bran as substrate:
The wheat bran was used as substrate for the production and optimization studies of protease. 10gms of wheat brawn mixed with 15 ml of moistening agent after sterilization and cooed to room temperature. The slants of 5 –7 days old culture was wetted by adding 10 ml of distilled water; a homogeneous suspension was obtained by shaking for approximately 1 min. 1 ml spore suspension was used for inoculation and incubated for about 72 hrs in an incubator under room temperature. Protease activity was measured at different time intervals.

Preparation of enzyme extract:
A 250 ml of conical flasks containing 10 g of substrates with 15 ml of moistening agent were sterilized at 121^{o}C (15 1bs/inch pressure), cooled, inoculated and incubated at 30^{o}C. After incubation, 80 ml of distilled water was added to the culture flask, flask was shaken for 14 h at 200 rpm. The content of flask was filtered and filtrate was analyzed for enzymatic activity.

Protease Assay:
The Protease activity in the crude enzyme extract was assayed by using 1% casein in citrate buffer (pH 7). The reaction mixture contains1ml casein and 1ml crude enzyme extract and allowed to stand for 1hr at the room temperature. After 1hr, 5 ml TCA solution was added to stop the enzymatic reaction. After addition of the TCA, the tubes were shaken and then contents were centrifuged at 10000 rpm for 15mins for the sedimentation of the pellet. The supernatant was collected from the centrifugal tubes, and to this supernatant 5ml of NaOH solution was added and allowed to stand for another 15mins. Finally 0.5ml of FC reagent was added and the intensity of blue colour was measured at 700nm within half an hour. One unit of enzyme activity was defined as the amount of enzyme that released 1µg of tyrosine mL^{-1} of crude enzyme per hour.

Optimization of media parameters:
Production of protease from *Rhizopus stolonifer* was optimized by controlling different physico chemical parameters like carbon source, nitrogen source and other components in the medium like $MgSO_4$, KCl, KH_2PO_4, pH range and temperature for the maximum yield of enzyme. The optimization experiments were conducted uniformly by varying one compound at a time and keeping the other conditions constant. Optimization studies were carried out at 24hrs, 48hrs and 72hrs.

Optimization of Substrate concentration:
Wheat bran at different concentrations (2.5 to 12.5%) moistened with mineral medium was used for the determination of optimum concentration of substrate for the better production of protease.

Optimization of nitrogen source:
Sodium nitrate was the nitrogen source in the medium. To determine the optimum concentration of $NaNO_3$ varied concentrations of NaNO3 were taken from 3 to 7%. After incubation protease activity was studied.

Optimization of KH_2PO_4 and $MgSO_4$:
These two were also optimized by taking at different concentrations (0.1 to 0.3%) for the better production of protease.

Optimization of NaCl :
To determine the optimum concentration of NaCl, different concentrations of NaCl between 0.025% to 0.125% were taken and fermentations were carried out.

Optimization of temperature:
One of the most important factors is the incubation temperature, which is important for the production of proteases by microorganisms. For temperature optimization, different temperatures ranging from 25ºC to 45°C were taken and the activity of protease was studied.

Optimization of pH:
pH can effect growth of microorganisms either directly or indirectly by affecting the availability of nutrients or directly by action on cell surfaces. The metabolic activities of microorganisms are sensitive to pH changes and the pH of culture media has marked effect on the type and amount of enzyme produced. Changes in pH may also cause denaturation of enzyme resulting in the loss of catalytic activity [11]. In the present study, the pH range for the optimization of pH selected varies from 6 to 8.

Effect of activators and inhibitors:
Various chemicals were tested at 0.1M concentration as activators and inhibitors while assaying the protease activity. The activators used are mostly metal ions like $ZnSO_4$, $CaCl_2$ and $FeSO_4$ and the inhibitors include PMSF (phenyl methyl sulphonyl fluoride), SDS and EDTA.

RESULTS AND DISCUSSION

The protease produced from the isolated fungi, *R.stolonifer* successfully using Groundnut as substrate. 10% of the substrates has given a maximum activity of 98 IU after 72 hrs of incubation after which the production has declined.

Optimization of media parameters
Mineral media components viz. NH_4NO_3, KH_2PO_4, $MgSO_4$ and NaCl were optimized for the better production. 0.5, 0.25, 0.25 and 0.1% concentrations of the above salts had given maximum enzyme activity of 78, 66, 70 and 61 IU respectively.

Effect of inhibitors and Activators
While studying the effect of activators it was observed that $FeSO_4$ enhanced the protease activity up to 698IU followed by $ZnSO_4$ and $CaCl_2$. The selected inhibitors were found to be less effective against the protease activity (Fig-1) but the crude enzyme of *R.stolonifer* showed 90% inhibition in the presence of PMSF indicating that the enzyme is an alkaline serine protease Work on serine protease has been reported by many researchers [12, 13].

Effect of temperature and pH
To further enhance the production of protease pH and temperature were also optimized. Maximum production was obtained at 40^0C and pH 7.5. Most alkaline proteases have been reported to have optimum activity in the range pH 8-9 [14]. Protease activity at 45^0C was also reported by Shumi *et al.*2003 [15] while working with the protease of *Fusarium tumidum.* Fungal proteases are usually thermo labile and show reduced activities at high temperatures [16]. Higher temperature is found to have some adverse effects on metabolic activities of microorganism [17] and cause inhibition of the growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure [18].

Production using different oil cakes
Among the selected oil seed cakes in place of wheat bran ,ground nut oil cake was found to be effective substrate after wheat bran for the production of protease with *R.stolonifera* (Fig2). In a study by Shirish Rajmalwar and Dabholkar 2007 [19] soya bean oil gave higher production followed by sesame oil cake.

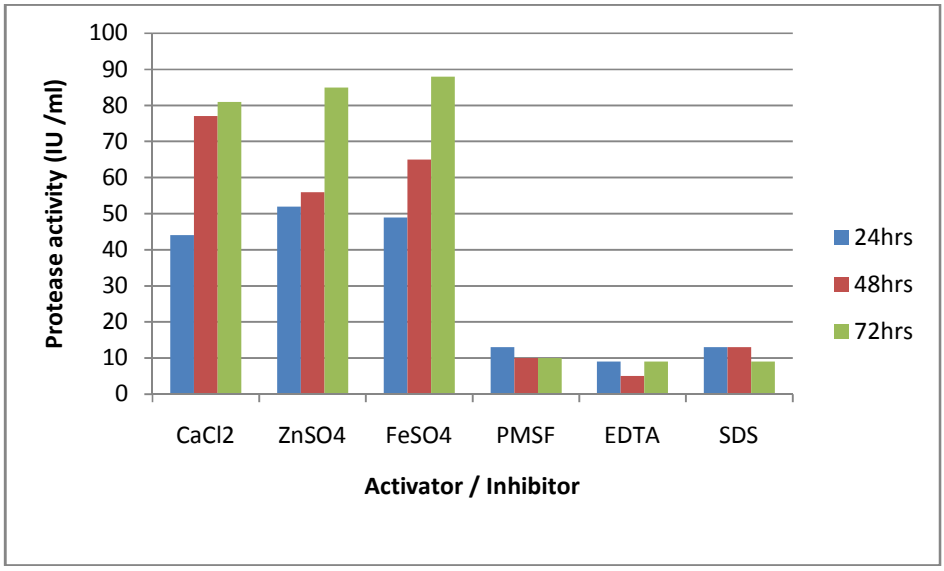

Fig-1 Effect of activators and inhibitors on enzyme activity

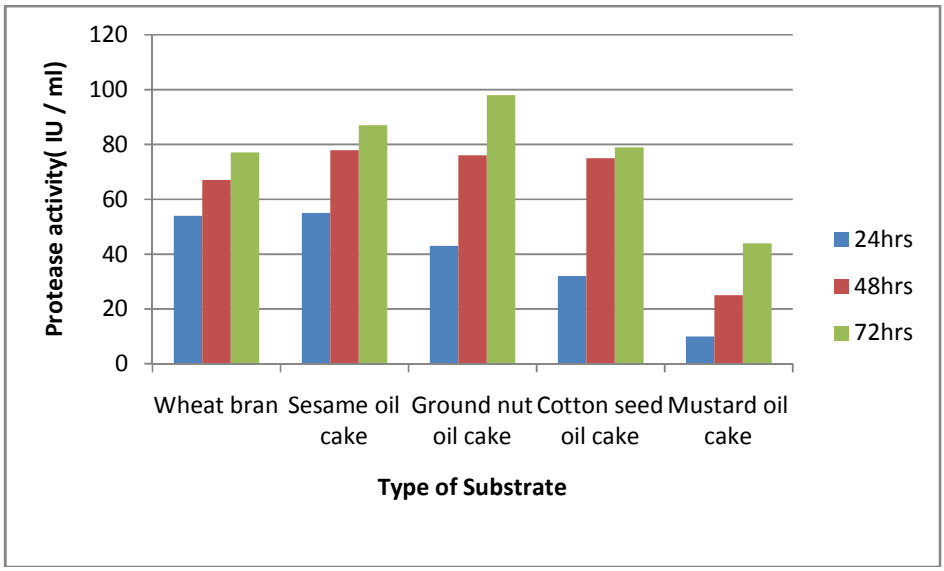

Fig-2 Effect of selected substrates on protease production

CONCLUSIONS

- The results obtained had given that the species so far subjected for enzyme production was shown to be good producer of protease.
- The Optimization studies has elevated the maximum capability of the organism for production of protease.
- Under Physico chemical conditions the organism was shown to be good producer of protease with selected cheap energy source.

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