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PRODUCTION OF PROTEASE UNDER SOLID STATE FERMENTATION USING DIFFERENT LOW COST SUBSTRATES

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Abstract

Bacterial proteases constitute an important group of industrial enzymes having applications in detergent, leather, food and pharmaceutical industries. A bacterial strain isolated from forest soil, presumably belonging to the genus *Bacillus* is an efficient source of proteolytic enzymes. The submerged fermentation studies revealed the capacity of the isolate for growth related production of extracellular proteolytic enzymes. Solid state fermentation using five different substrates such as wheat bran, rice bran, ground nut meal, black gram husk and green gram husk has been attempted for enzyme production. Wheat bran was found to be the most suitable substrate for protease production having an activity of 372.96 units after two weeks of growth. This was found to be 3.3 times more than submerged fermentation. The physiological conditions of fermentation such as inoculum size and moisture level play a vital role in protease production. Their influence in enzyme production was next studied in wheat bran medium. Maximum protease production was observed at the inoculum level of 5% and moisture level of 1:1.5.

Keywords: Proteases, Production, Activity

Introduction

Wastes from food industries can often represent a serious contamination problem. Utilization of various agro-industrial wastes by microorganisms to produce enzymes of commercial significance is economically and environmentally attractive (Celina *et al*., 1995). A number of microbial enzymes are produced on large scale and used in commercial operations. Of these proteases, amylases, xylanases, glucoamylases, lipases, cellulases and gelatinases are the most important ones. In addition, a large number of enzymes are produced on much smaller scales and are mainly used for research purposes.

Proteases are an important group of extracellular enzymes produced by microorganisms. They represent a large group of enzymes, ubiquitous in nature and found in a wide variety of microorganisms. The molecules of proteases are relatively small in size and compact spherical structures that catalyse the peptide bond cleavage in proteins. Based on the mode of action they are mainly classified into two major classes known as peptidases and proteinases. Peptidases cleave the peptide bonds of amino acids from C or N termini and degrade protein. Proteinases hydrolyse the internal peptide bonds and cause degradation.

Protease have prominent commercial importance. Microbial protease represents about 60% of all the industrial enzyme's sales in the world due to their enormous applications in various industrial sectors. They have applications in various industries including detergent, food, pharmaceutical, silk and leather (Gupta *et al*., 2000). They have been used for meat tenderization and in some medical applications (Gajju*et al*., 1996). In modern diary and food industryalkaline enzymes are widely used to synthesize automatic dish washing detergents used to clean the ultra filtration (UF) and reverse osmosis (RO) membranes. These membranes are

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used in various activities such as concentration, fractionation, clarification and/or sterilization of different food materials including milk, whey, egg white, fruit juices, wine and other beverages (Divakar *et al*., 2006). In recent years, usage of thermostable alkaline enzyme has been increased in a wide range of other biotechnological applications such as silver recovery from used x ray films (Masse, 1983), in feeds (Dhar and Sreenivasulu, 1986) and in peptide synthesis (Lin, 1996).

Fermentations are mainly of two types depending on the state of medium used. They are submerged fermentation and solid state fermentation. In submerged fermentation technique liquid medium is used. The solid state fermentation is the growth of organism on moist substrates in the absence of free flowing water. The substrate provides a rich and complex source of nutrients, which may or may not need to be supplemented. solid state fermentation using agro-biotech wastes as substrate for the biosynthesis of various enzymes possess advantages in productivity, cost effectiveness,labor time and medium components along with environmental advantages like less effluent production and waste minimization (Pandey *et al*., 2000).

The ideal solid substrate is the one that provides maximum nutrients to the microorganism for its optimal growth and metabolic function. There are ample reports on the production of protease using a wide variety of agro-food wastes. Wheat bran, rice bran, ground nut meal, black gram husk and green gram husk can act as a potent source of protein (Pandey *et al*., 2000). The present work was undertaken with the objective of finding the most appropriate substrate for the production of protease under solid state fermentation. Attempt was also made to study the effect of few physical parameters on enzyme production.

Materials and methods

Bacterial strain

The bacterial strain used in the present investigation was isolated from forest soil samples. It is a gram positive rod-shaped bacterium producing endospores and has been tentatively identified as *Bacillus* sp. The procedure of isolation and screening has been reported elsewhere. The proteolytic activity of the strain was identified by the clear zone produced in a protein rich culture medium.

Maintenance of culture

The culture was revived using a medium composed of nutrient agar (3.0 g/L), glucose (5.0g/L), NaCl (5.0 g/L), casein (3.0 g/L) and agar (20g/L) and maintained in petriplates by streak plate method. Subculturing was performed continuously to purify the culture. Slant cultures were prepared using single colonies present on the plates. After 48 hours of incubation, properly grown slants were maintained at 4^0C.

Submerged fermentation

Submerged fermentation was carried out by using the liquid medium having peptone (10g/L), NaCl (0.1g/L), $CaCl_2.H_2O$ (0.5g/L), KH_2PO_4 (0.2g/L) and $MgSO_4.7H_2O$ (0.5g/L). The fermentation was performed in triplicate in 250mL Erlenmeyer flasks containing 50mL of the above described medium. 5.0% inoculum was used and the initial pH of the medium was maintained at 7.0 using 1% sterile Na_2CO_3. The flasks were kept in a shaker at about 120rpm for a period of 96 hours. Samples were taken in a regular interval of 24h and were assayed for protease activity, growth and change in pH.

Protease assay

The enzyme assay was carried out according to the procedure of Tsuchida *et al*. (1986) with the following modifications. Casein (2.0%) at pH 7.0 was used as the substrate The culture supernatant was centrifuged at 10000 rpm for 15 minutes at 4^0C served as the source of enzyme. 0.5mL of substrate was incubated for 10 minutes at a temperature of 40^0C. To this solution, 0.5mL of enzyme was added. After 10 minutes

of incubation of reaction mixture, the activity was arrested by adding 1mL of TCA (trichloro acetic acid). The mixture was centrifuged at 2000 rpm for two minutes. To 1mL of 10 times diluted supernatant, 5.0 mL of 0.44M Na_2CO_3 was added and incubated for a period of ten minutes. 0.5mL of two-fold diluted Folinciocalteu reagent was added to this. After 20-25 minutes the colour developed was read at 660nm against a blank. Tyrosine prepared in the same procedure served as the standard.

One unit of enzyme activity can be equivalent to microgram of tyrosine released per mL per minute under the above mentioned reaction conditions.

Growth

Growth of the bacteria in different samples was determined by reading the samples at 600nm in a spectrophotometer against water blank.

pH of the samples

pH of the samples was observed before centrifugation using an electronic pH meter.

Solid state fermentation

Fermentation study was carried out in 5 different agro industrial wastes such as wheat bran, rice bran, groundnut meal, green gram husk and black gram husk.

Preinoculum preparation

A loop full of strain from the slant culture was carefully transferred to the 50mL standard medium and kept in a shaker for 24 h.

Different substrates selected for the present study

In SSF, the solid substrate not only serves as an anchorage but also supplies nutrients to the organism. The ideal solid substrate is the one that provides maximum nutrients to the microorganisms for its optimal growth. Five various substrates were used for the fermentation study. Commercial grade of wheat bran, rice bran and groundnut meal were procured from the local market. The particle sizes were homogenized by sieving. The green gram husk and back gram husk were obtained by removing the husk after soaking the seeds and then dried.

Fermentation conditions

10.0g of the five different substrates were taken in 250ml Erlenmeyer flasks and were well mixed with the salt solution containing $MgSO_4.7H_2O$ (0.5g), KH_2PO_4(0.5g) and $FeSO_4$ - 0.01g (g/L). The initial substrate moisture ratio was maintained as 1:1. Experiments were done in triplicates and incubated for a period of three weeks and samples were taken for analysis every 7^{th}, 14^{th} and 21^{st} days of incubation.

Extraction of enzyme

Flasks were removed at regular intervals of one week for three weeks. Contents were extracted after one hour shaking of the substrate with 40mL of 0.1N phosphate buffer at pH 7.0. The contents were filtered through cheese cloth. The filtrate was centrifuged at 10,000rpm for 15 minutes at 4^0C and the supernatant was used as the enzyme.

Effect of inoculum size on enzyme production

Various inoculum levels such as 4%, 5%, 10%, 15% and 20% were tried to study their effect on enzyme production. Standard liquid medium prepared was incubated for 24h and used as preinoculum. The samples were removed after 14 days of incubation and the protease activity was estimated as mentioned earlier.

Effect of moisture level

Effect of moisture level was studied on protease production at 1:1, 1:1.5 and 1:2 ratios. Samples were analysed after two weeks of incubation.

Results and Discussion

Proteases are a major group of enzymes that hydrolyse proteins by the addition of water across peptide bonds and catalyse peptide synthesis in organic solvents with lower water content (Beg *et al*., 2003). They are one of the most important group of enzyme used in pharmaceuticals and food industry for peptide synthesis. These are used in leather industry for dehairing and as an additive of detergent formulation in detergent industry (Joe *et al*., 2004). Bacterial

proteases are commonly used for a particular process due to their enzyme characteristics. The most important of which are pH stability, temperature stability and substrate specificity. Bacterial proteases are superior to other proteases because of their activity in narrow range of pH. Thus, controlling of activity can be easily performed.

The production of protease was attempted in submerged as well as solid state fermentation in the present study. A bacterial strain isolated from forest soil samples were used for the present investigation (Fig 1).

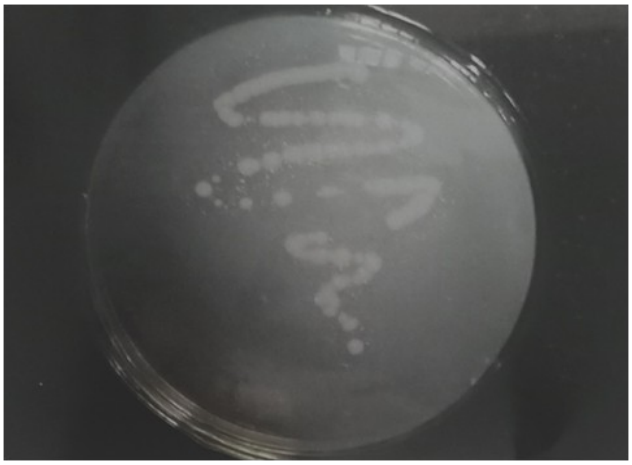

Figure 1. Bacterial isolate used in the present study

Submerged fermentation

Submerged fermentation was carried out to understand the production pattern of protease from the bacterium. The culture was grown in an initial pH of 7 and inoculum size was 5%. Samples were taken at 24,48,72,96 h of incubation to estimate protease activity, growth and pH change as described in the materials and methods. Maximum enzyme activity could be observed after 24 hours of growth. The growth of the culture also reached a peak at 24 hours where after it showed a decline both in enzyme activity as well as in growth (figure 2 and figure 3).

Protease production started from an early logarithmic growth phase that increased with increasing growth rate reaching a peak at which growth ceased as evidenced by the decrease in absorbance after 24 hours. There are several reports regarding growth associated production of proteases (Mehrothra*et al*., 1999, Chu *et al*., 1992). Ward (1983) has stated that *Bacillus* sp. usually produce more alkaline protease during the late exponential phase.

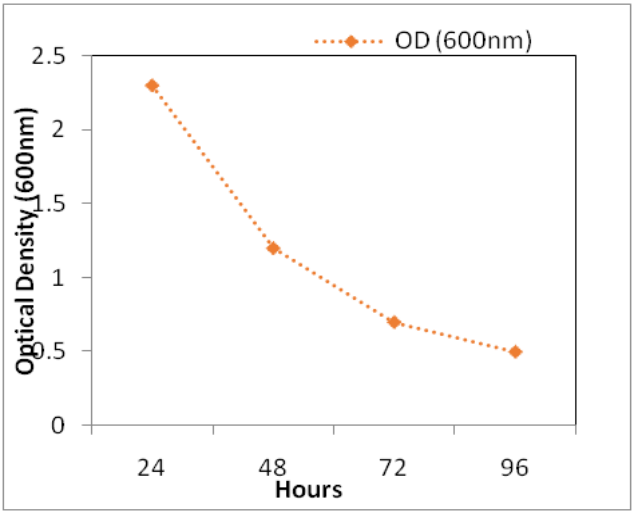

Figure 2. Growth of the bacterium during submerged fermentation

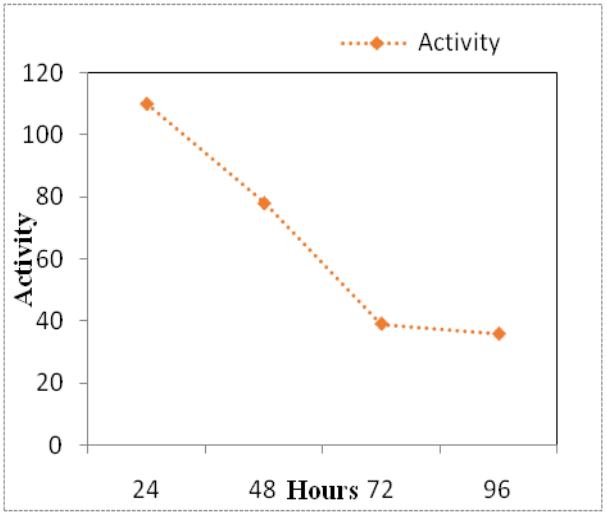

Figure 3. Protease production from the bacterium during submerged fermentation

There was decline in enzyme activity after the active growth phase. The decrease in enzyme activity might have caused by the cessation of enzyme synthesis after active growth together with the deactivation of existing enzyme.

Denaturation, degradation by other proteases and autolysis are the three major mechanisms that are involved in the deactivation of proteases (Moon and Parulekar, 1991). Regarding the medium pH, it shifted from 7.2 to 8.3 after 24 hours of growth and thereafter it maintained approximately around the value 8.5. The enzyme retaining the activity around this pH is suggestive of its alkaline nature.

Solid state fermentation

Microorganisms are generally grown in two conditions, in liquid state (SmF) and in solid state (SSF). SSF is advantageous over SmF in low waste water output, simplicity, high reproducibility and simple fermentation media requirement. In the present study, although fairly good amount of activity was obtained under submerged fermentation, the cost of production is a concern in SmF. So attempt was made to study the production of protease under SSF using different substrates which are easily available and often treated as waste material.

Effect of different substrates on enzyme production

The selection of an ideal agro industrial waste for enzyme production in a solid state fermentation depends on several factors mainly related with cost and availability of substrate material and thus may involve screening of several agro industrial residues (Pandey *et al*., 2000). There are several reports regarding the use of different low cost substrates for the production of different microbial enzymes are available. The five low cost substrates used for the current study are wheat bran, rice bran, ground nut meal, green gram husk and black gram husk.

Production in wheat bran

Wheat bran has been reported to be used as a substrate for protease production in different organisms. In the fungus *Rhizopus oryzae* this has been reported for protease production. (Aikat and Bhattacharyya, 2000). Neutral protease production has been carried out using wheat bran under solid and submerged fermentation (Couri *et al*., 2000; Sandhya *et al*., 2005). Satyanarayana (1994) reported a protease activity of 3.5 U/dry bacterial strain using wheat bran in *Bacillus* sp.

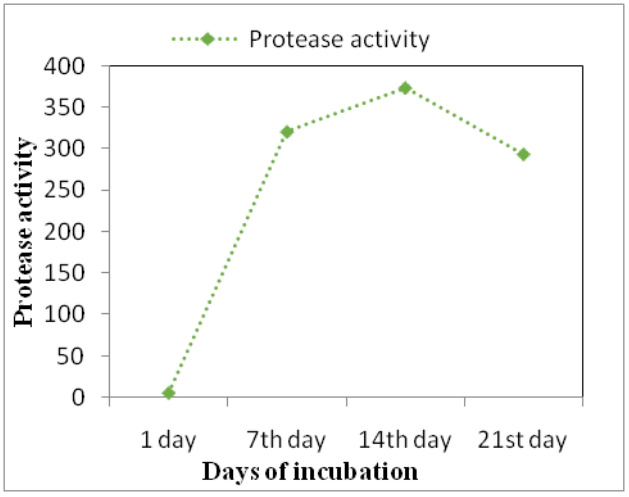

Figure 4. Production of protease under SSF using wheat bran as the substrate

In the present study using wheat bran as the substrate, maximum activity could be obtained after two weeks of growth (372.96 units) of which 86% could be observed after one week of growth and 78.6% after three weeks of growth (Fig 4). Increase in protease production after first week clearly suggests the role of the enzyme as a primary metabolite being produced for the utilization of the nutrients present in the solid substrate. The decrease in later days in the most of the substrates may be due to the inactivation of existing enzymes by other proteases (Paranthaman *et al*., 2009).

Production in rice bran

Maximum enzyme activity has been observed after two weeks of growth (253 units) in rice bran medium of which 68.4% could be observed after one week of growth and 92.5% after three weeks of growth (Fig 5).

Production in groundnut meal

When groundnut meal was used as the substrate, maximum activity could be obtained after two weeks of growth (186.48 units) of which 14.5% could be observed after one week of growth and 50% after three weeks of growth (Fig 6).

Production in green gram husk

Green gram husk when used as the substrate, maximum activity was found as 93.04 units after three weeks of growth of which 42.85% could be observed after one week of growth and 42% after three weeks of growth (Fig 7). Prakasham*et al*. (2006) used green gram husk for protease production by SSF in *Bacillus* species.

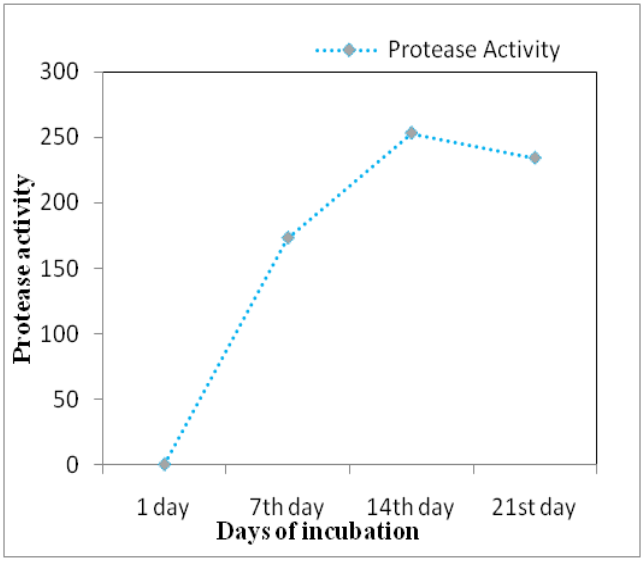

Figure 5. Production of protease under SSF using rice bran as the substrate

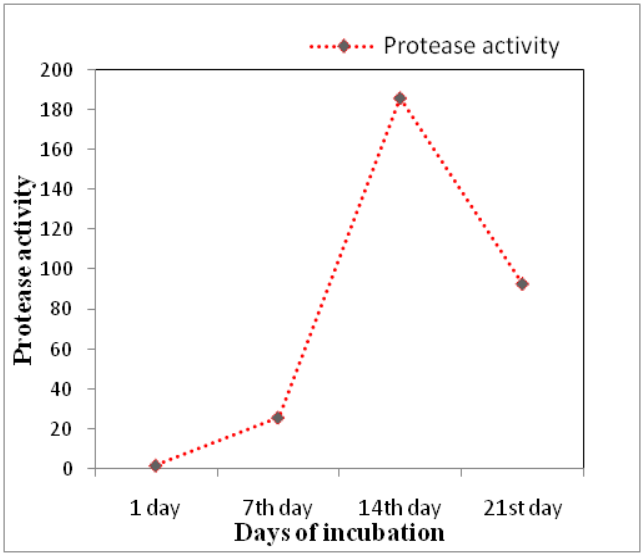

Figure 6. Production of protease under SSF using ground nut meal as the substrate

Production in black gram husk

Protease production was negligible when black gram husk was used as the substrate. A very low activity was observed after one week of growth, which failed to retain in the later stages of growth.

The selection of a suitable substrate for SSF mainly depends upon the cost and availability of the substrate material (Pandey *et al*., 2000). Five different substrates that can be easily procured locally were selected for the current study. Profile of the highest activities of different substrates is present below (Fig 8). The production pattern varied with the type of substrates used. This could be attributed to solid material's dual role, supply of nutrients to the microbial culture and anchorage for the growing cells.

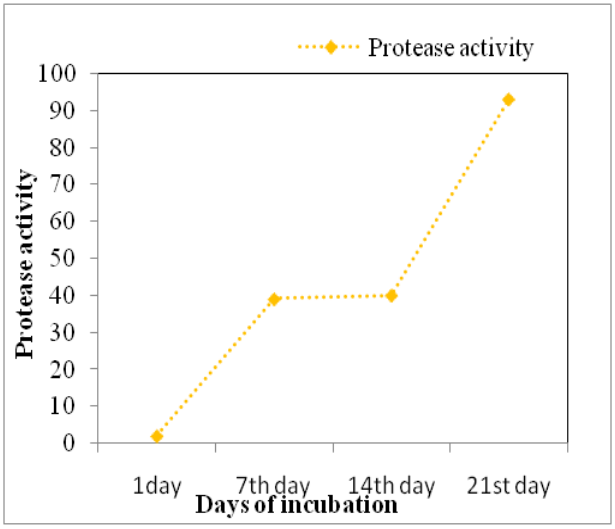

Figure 7. Production of protease under SSF using green gram husk as the substrate

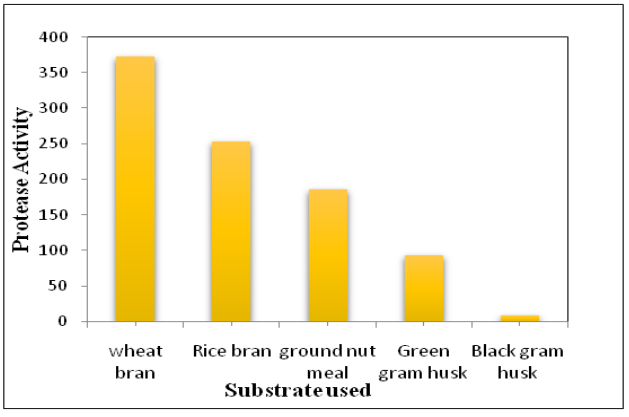

Figure 8. Comparison of protease production in five different solid media

The graph clearly indicates that the highest activity could be observed with wheat bran as the substrate followed by rice bran and ground nut meal. Only 67.9% of activity using wheat bran has been observed in rice bran. Compared to the production in submerged fermentation (110 units), wheat bran gave nearly 3.3 times more production (Fig 9). Considering this wheat bran medium was then selected for further studies.

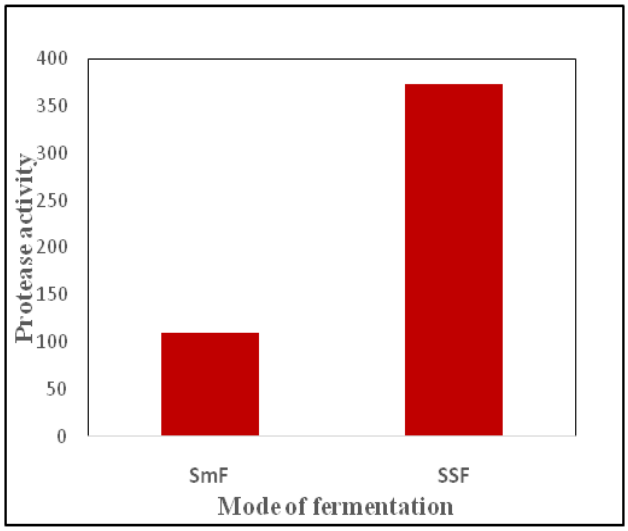

Figure 9. Comparison of protease production by SmF and SSF

Effect of inoculum size

Inoculum size played an important role in the protease production. The inoculum size studied were 4%, 5%, 10%, 15% and 20%. The maximum protease production was observed with 5% inoculum when we used wheat bran as substrate (Fig 10). Further increase in inoculum size decreases the protease production. Prakasham *et al*. (2006) obtained maximum protease production on green gram husk with 3% inoculum level.

Effect of moisture level

Increase in moisture level is believed to reduce the porosity of the wheat bran, thus limiting oxygen transfer, while lower moisture content causes reduction in the solubility of nutrients of the substrate, lower degree of swelling. Hence an optimum level of moisture is required for maximum enzyme productivity. Divakar *et al*. (2004) observed high enzyme titer when the initial moisture level was 20% (W/V) in comparison with that at low or high moisture levels. Ikasari and Mitchell (1994) while studying protease production on *Rhizopus oryzae* attained maximum protease production with initial moisture content of 47%. At the highest initial moisture content (60%) growth occurred only on the substrate surface, and the protease yield was very low. This may be due to the filling of interparticle spaces within the substrate mass with water, which limits the diffusion of O_2.

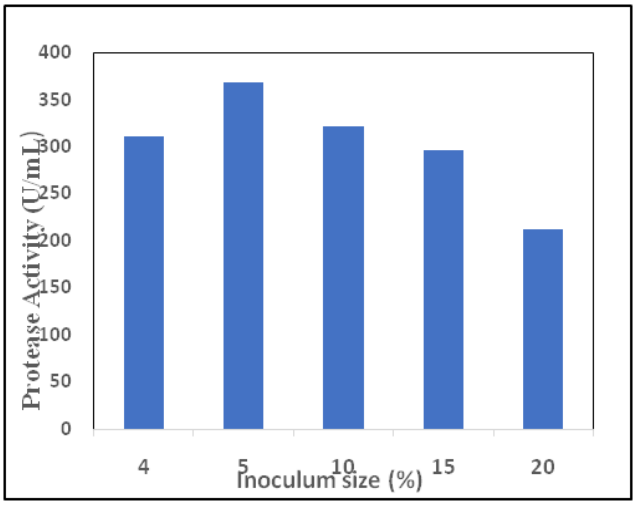

Figure 10. Effect of inoculum size on protease production

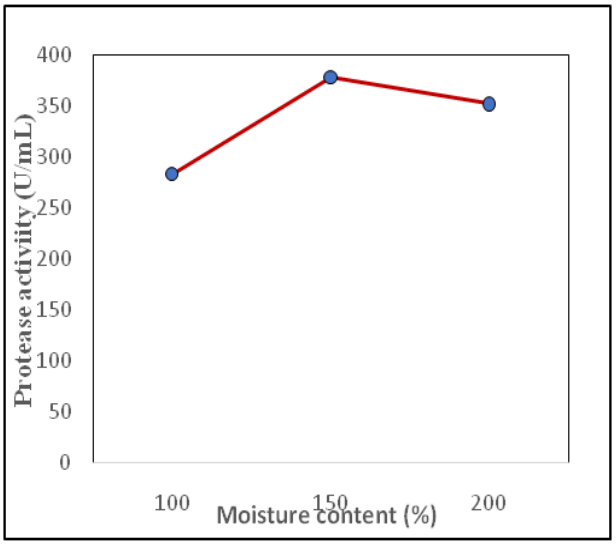

Figure 11. Effect of moisture content on protease production

In the present study highest activity could be obtained when the moisture level was in the ratio 1:1.5 (Fig 11). Wang *et al*. (1974)

obtained similar results for the growth of *R. oligosporus* on a wheat bran medium. Poor growth and low protease yields occurred at 35% moisture level. At 63% moisture, growth was more rapid than at 50% moisture, but protease yield was found lower.

Conclusion

Proteases are an important group of enzymes that occupies a large share of the commodity enzyme market. They have a wide range of applications in detergent industry, leather processing and food industry. Protease production is influenced in a very complex manner by various factors. Different strains of bacterium may behave differently and optimization of parameters for maximum protease production should be performed for each strain.

Submerged fermentation studies were carried out to characterize the protease production pattern. The pH changes of the medium, growth of the culture and protease production were observed. Since the medium components of submerged fermentation study are high cost chemicals, we opted the solid state fermentation study using five different agro industrial wastes like wheat bran, rice bran, ground nut meal, green gram husk and black gram husk. The SSF process was observed to be less sensitive to contamination than submerged fermentation process. In contrast to a general belief that SSF technique is not suitable for bacterial and other cultivation because of their requirement for higher water activity, the enzyme titers produced in SSF were higher than those in submerged fermentation.

In SSF, the selection of a suitable solid substrate for fermentation process is a critical factor. All the substrates used in the study supported the growth and enzyme formation, while wheat bran proved superior to other substrates followed by rice bran. 5% inoculum size and 1:1.5 moisture levels were optimum for enzyme production. The maximum enzyme production was observed at the end of second week of incubation. When these factors are optimized an increase in protease production was observed. These results indicates that this protease possess good characteristics for industrial application and further studies can be carried out both in respect to fermentation level and further increase in production.

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