**International Journal of Emerging Engineering Research and Technology**
**Volume 2, Issue 4, July 2014, PP 327-332**
**ISSN 2349-4395 (Print) & ISSN 2349-4409 (Online)**

# Potentiality of Soil Fungi to Produce Protease through Solid Substrate Fermentation Technique

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**Abstract:** *Proteases are a group of industrially important enzymes and have application in leather industry, food industry, pharmaceutical ,detergent industry and bioremediation process. In the present study soil fungi were screened for the production of proteases. The enzyme activity of the isolates was determined. Rhizopus sp showed maximum activity which was then cultured on wheat bran through SSF technique by standardizing various growth parameters. The Rhizopus sp showed maximum enzyme activity at pH 7 on 4$^{th}$ day of incubation at 30$^0$C.*

**Keywords**: *Soil fungi, Solid Substrate Fermentation, Protease activity, Rhizopus sp*

## 1. INTRODUCTION

Microorganisms vary greatly from one to another in their ability to produce enzymes. Commercial microbial enzyme production utilizes mainly various fungi, bacteria and yeasts. Fungal enzymes are gaining importance in agriculture and industry as they are often stable at high temperature and extreme pH than the enzymes derived from plants and animals (Waiter and Morgan 2007). Protease is the single class of protein degrading enzymes, which occupy pivotal position with respect to their applications in both physiological and commercial fields. Protease represent one of the largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation process (Walsh, 2002).

Solid Substrate Fermentation (SSF) technique holds tremendous potential for the production of enzymes. This system offers numerous advantages over submerged fermentation system, including high productivity relatively higher concentration of the products, less effluent generation requirements for simple fermentation equipments, etc. A large number of microorganisms including bacteria, yeast, and fungal culture are employed for enzyme production in SSF systems.

Agro industrial residues are generally considered the best substrate for the SSF process. Some of the substrate that have been used included sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, saw dust, tea waste, casava waste, steamed rice (Demain and Solomon, 1996). In fungal and bacterial SSF, moisture levels, incubation temperature and incubation period are an important parameter. Optimum temperature for protease production by most of the culture has been reported in mesophilic range (Sandhya et.al, 2004). According to Benazir et.al.,(2011), *Aspergillus* sp showed maximum enzyme activity on solid substrate fermentation when compared to submerged fermentation on 6$^{th}$ day of incubation at pH 7 and temperature 40$^0$C.

Protease enzymes are gaining more importance in industries due to their wide application. The present work was conducted to screen the protease producing fungi from the soil and to check their enzyme activity. The isolates were then cultured by SSF technique by optimizing different physical parameters and assayed for enzyme activity.

## 2. MATERIALS AND METHODS

### 2.1. Screening of Protease Producing Fungi

Soil samples collected in clean polythene bags from the forest areas were mixed to form composite samples and brought to the laboratory. Samples were serially diluted up to $10^{-6}$ dilutions and inoculated on Skimmed milk agar media to isolate protease producing fungi (Aneja, 2003**)**.

### 2.2. Identification And Maintenance of the Isolates

The fungal isolates showing clear zone of hydrolysis around growth were identified based on their morphological characteristics and microscopic observation with the help of a manual by Nagamani et.al. 2006(Aneja, 2003)

### 2.3. Production and Extraction of Protease Enzyme

Production of protease from the isolates was carried out using production medium containing (g/l) Peptone-10, Dextrose 40, pH 6.5. 1ml of the spore inoculum was inoculated to 100ml of production medium, incubated at $28^0$c , in a rotary shaker for 4 days. Centrifuged at 10,000 rpm for 10minutes at $0^0$c and the supernatant was used as crude enzyme source (Modified method of Anitha et. al., 2010).

### 2.4. Protease Enzyme Assay

Protease activity was determined by a procedure based on Madhanraj et.al., 2009, using casein as a standard.2ml of casein was added to sterile test tube.0.5ml of crude enzyme was added, incubated at $50^0$C for 30 minutes. The reaction was stopped by adding 2.5ml of 10%TCA.Blank was maintained by 2ml of 1% casein and 2.5ml of 10%TCA. After incubation 0.5ml of water was added. Filtered through Whatmann No.1 filter paper .0.5ml of the filtrate was taken .5ml of 0.5mM sodium carbonate and 0.5ml of 2 fold diluted FC reagent was added. Incubated at room temperature for 30minutes.Absorbance was measured at 660nm using spectrophotometer. The amount of protease activity was determined using standard graph prepared from Tyrosine. From the standard graph enzyme units were calculated (Madhanraj et.al., 2009).

### 2.5. Total Protein Assay

Total protein of the cell free filtrate was determined by the method of Lowry et. Al using Bovine Serum Albumin as a standar (Sadasivam and Manickan, 1991)

### 2.6. Optimization of Growth Parameters for the Maximum Enzyme Production through SSF Technique

The isolate showing maximum enzyme activity was cultured through SSF technique using wheat bran as substrate. 5g of wheat bran was taken.The moisture content was adjusted to 65% with different pH of 6.0, 6.5, 7.0, 7.5, 8.0 using 0.1 M Phosphate buffer. Sterilized using autoclave at $121^0$C for 20minutes at 15 Lbs pressure.1ml of the spore suspension was inoculated and Incubated at various temperatures i.e $20^0$C,$30^0$C ,$40^0$C $50^0$Cand $60^0$C respectively. The samples were withdrawn at an interval of 2, 4, 6, 8 and 10 days (Modified method of Madhanraj et.al., 2009).

### 2.7. Extraction and Estimation of Enzyme

The samples were centrifuged at 15,000 rpm for 15minnutes. Supernatant was used as enzyme source. The enzyme activity was determined using Tyrosine standard graph. (Sadasivam and Manickan, 1991)

## 3. RESULTS AND DISCUSSION

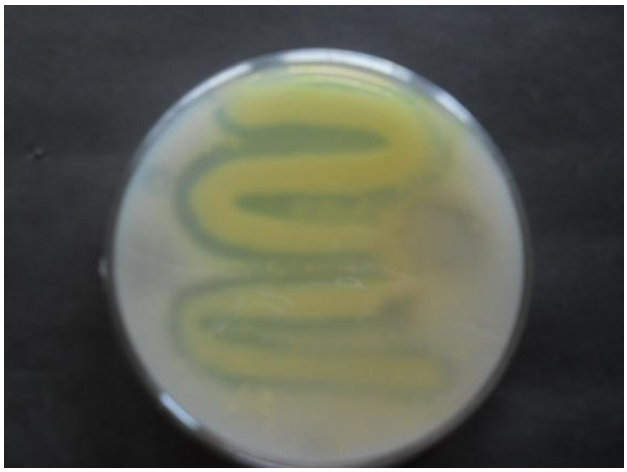

**Plate1.** *Isolate showing clear zone of hydrolysis on skimmed milk agar media.*

In the present study protease producing fungi were isolated from soil. These isolates were screened for protease production by casein hydrolysis. About 7 isolates showed a very clear zone of hydrolysis around the growth**(Plate:1).** They were identified as species of *Aspergillus, Penicillium, Cldosporium, Gleocladium , Rhizopus, Mucor and Alternaia* ***respectively*** based on their morphological characters and microscopic observation.

The diameter of the clear zone of hydrolysis is listed in Table 1. *Aspergillus* sp showed clear zone of 2cm diameter followed by *Rhizopus* sp. According to Benazir et. al., (2011), *Aspergillus niger* produced zone of hydrolysis with diameter of 3cm in casein medium.

**Table1.** *Diameter of clear zone on skimmed milk agar media*

| **Isolates** | **Diameter (cm)** |
|---|---|
| *Aspergillus* sp | 2 |
| *Penicillium* sp | 0.8 |
| *Cladosporium* sp | 0.5 |
| *Gleocldium* sp | 0.3 |
| *Rhizopus* sp | 1.2 |
| *Mucor* sp | 0.1 |
| *Alternaria* sp | 0.4 |

According to Walsh (2002), *Aspergillus niger, Aspergillus oryzae,*

*Penicillium roqueforti, Rhizopus oryzae* etc are the largest protease producing microoraganisms.

### 3.1. Protease Activity of the Isolates

The protease enzyme activity was estimated by Caseinolytic method, using Tyrosine as a standard (Fig:1).

In the present study protease producing fungi were isolated from soil. These isolates were screened for protease production by casein hydrolysis. About 7 isolates showed a very clear zone of hydrolysis around the growth**(Plate:1).** They were identified as species of *Aspergillus, Penicillium, Cldosporium, Gleocladium , Rhizopus, Mucor and Alternaia* ***respectively*** based on their morphological characters and microscopic observation.

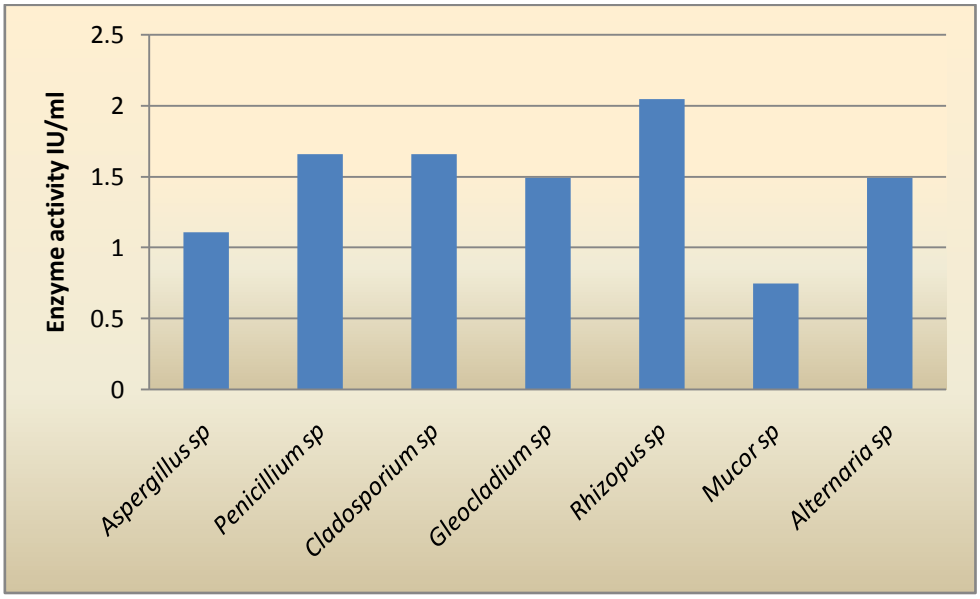


**Fig1.** *Protease activity by caseinolytic method*

According to Sumantha et.al., (2006), *Rhizopus microsporus* showed the maximum enzyme activity. According to Walsh (2002), *Rhizopus oryzae* is also the best producer of the protease enzyme.

### 3.2. SSF of Rhizopus for Protease Production

*Rhizopus* sp showing the higher enzyme activity was selected for optimisation of growth parameter for maximum production of protease by SSF technique using wheat bran as the substrate. The media

was adjusted to pH (6.0, 6.5, 7.0, 7.5, 8.0), incubation period (2, 4, 6,8,10 days) and temperature ($20^0$C, $30^0$C, $40^0$C, $50^0$Cand $60^0$ ) and 65% moisture.

The samples were analysed for protease activity. The *Rhizopus* sp showed maximum enzyme activity of 1.380 IU / ml on $4^{th}$ day of incubation at $30^0$C and pH 7.

### 3.3. Effect of Incubation Period on Protease Production

The present study showed that the production of protease was maximum on $4^{th}$ day of incubation **( Fig 2)**. A gradual decrease in enzyme activity was observed with increasing incubation time. According to Sumantha et al. (2006), *Rhizopus microsporus* showed maximum enzyme activity at 72h of incubation. *Aspergillus niger* showed maximum yield on $7^{th}$ day of incubation (Benzir et.al., 2011).

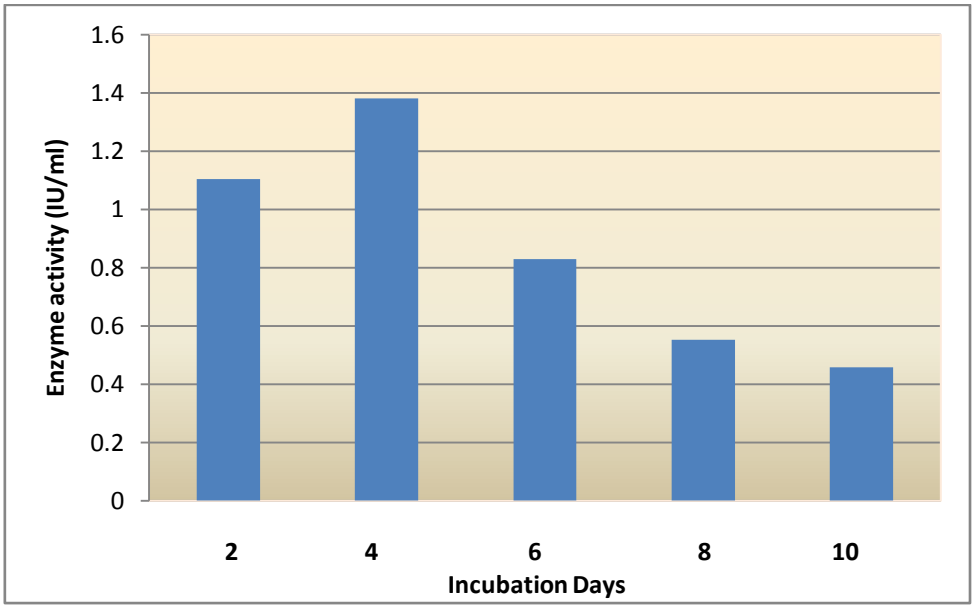


**Fig2.** *Protease activity at different incubation period at pH 7 and incubation temperature of $30^0$c*

### 3.4. Effect of Incubation Temperature on Protease Production

The temperature of incubation was found to play an important role in the production of enzyme. When the inoculated substrates were incubated at different temperatures incubation at $30^0$C was best suited for enzyme production **( Fig 3)**. However *Rhizopus* sp showed a progressive decline in enzyme production at $40^0$c and beyond. According to Sandhya et.al., (2004), the optimum temperature for protease production by most of the culture is in mesophilic range. According to Sumantha et.al.,(2006) fermentation temperature of $30^o$C is the optimum temperature for the maximum enzyme production of *Rhizopus* sp. Species of *Aspergillus* showed maximum enzyme activity at $40^0$-$45^0$ (Devi et.al.,2008 and Benazir et.al.,2011).

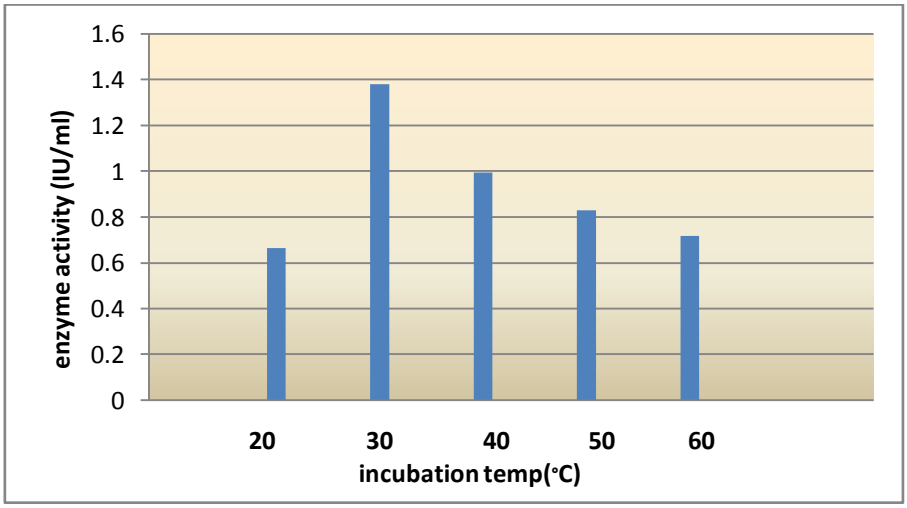


**Fig3.** *Protease activity at different incubation temperature at pH 7 on $4^{th}$ day of incubation*

### 3.5. Effect of PH on Protease Production

Sarkar *et al* have reported that maximum protease production was obtained when pH of the culture media was maintained at 6.0. According to Sumantha *et al* (2006), *Rhizopus microsporous* exhibited highest enzyme activity at pH 7 when provided with different pH of phosphate buffer. The present study showed maximum protease activity at pH 7. The enzyme activity decreased with increase in pH **( Fig4).** It was observed that enzyme activity was comparatively higher at alkaline pH.

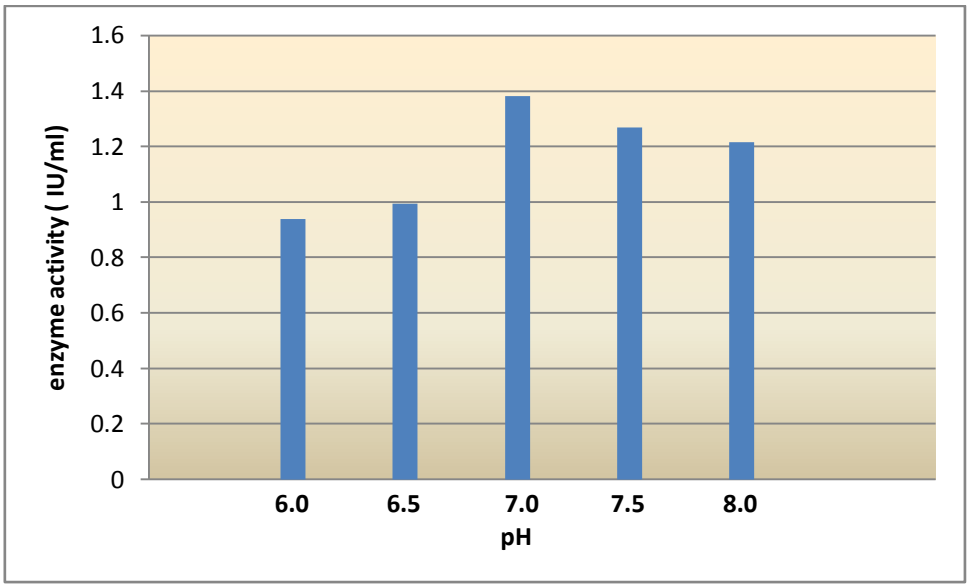


**Fig4.** *Protease activity at different pH on 4th day of incubation at incubation temperature 30$^0$c*

## 4. CONCLUSION

From the present study it can be concluded that soil harbours a diverse group of fungi which can be used as a source of industrially important protease enzyme. The *Rhizopus* sp which showed maximum protease activity can be grown on specific media or waste substrate such as wheat bran for maximal enzyme production. The growth parameters optimized to pH 7, incubation temperature of 30$^0$C and 4 days of incubation period is found ideal for protease enzyme production using wheat bran as a substrate. Thus *Rhizopus* sp can be used for industrial protease production and also for degradation of protein rich waste substrates.

## ACKNOWLEDGEMENT

The author is very grateful to Mangalore University, for providing necessary facilities and to everyone who has given their support.

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