Efficacy of *Aspergillus* sp in the production of protease enzyme with different substrates.

ABSTRACT

In the present study, the soil samples were collected from marine environment of Arichalmunai, Dhanushkodi, Ramnad District, Tamilnadu ,India. Fungal species were isolated by plating method, in 50% sea water containing potato dextrose agar medium .Totally 16 fungal species were isolated and identified from the soil sample. The production of protease from *Aspergillus niger , A.flavus and A. terreus* by liquid state fermentation. The production of protease enzyme was optimized by using fermentation medium containing different substrates. The maximum protease production was observed on wheat bran, containing medium. The protease production was maximum in temperature 35°C were recorded. Wheat bran produced the maximum level protease. The optimization work also carried out. This study revealed that coastal environment provides impressive density of fungi in the East Coast of India and are unexplored for microbial resources can be use full in industry.

Key words ; Marine soil, Optimization, Substrates, *Aspergillus* sp, Protease enzyme.

1.INTRODUCTION

Protease is one of most important classes of industrial enzymes and accounts for about 60% of commercial enzymes in the world [1]. They find application in a number of biotechnological processes, viz, in food processing and pharmaceuticals, leather industry, detergent industry, etc. [2 & 3].Two third (2/3) of the industrial produced protease are from microbial sources [4].

A variety of microorganisms such as bacteria, fungi, yeast and Actinomycetes are known to produce these enzymes [5 & 6]. Most of the genera *Aspergillus, Pencillium* and *Rhizopus* are especially useful for producing protease, as several species of these genera are generally regarded as safe [7 & 6] . *Aspergillus clavatus* ES1 has been recently identified as producer of an extracellular bleaching stable alkaline protease [8]. Protease enzymes play an important in both physiological and commercial fields [9 & 10]. Proteases are the enzymes which hydrolyse the peptide bonds through which amino acids are linked together in the polypeptide chain [11].Proteases represent about 60% of the total world wide sales of the enzyme[12] . Proteases are classified into six groups based on their catalytic activity and the presence of amino acid residues at the active site: aspartate, cysteine, glutamate, metallo, serine and threonine [13]. These enzymes are widely found in plants, animals as well as in microorganisms[14] Proteases now known for their many physiological commercial applications and also perform various other functions too. They are widely used in biological processes, in regulation of metabolism and

digestion of dietary proteins to allow absorption of amino acids [15] . These enzymes carried out various other processes involving maturation of prohormones, blood coagulation, apoptosis, immune function, and the recycling of cellular proteins [11]. Proteases also have various industrial applications as they can be widely used as a biochemical reagent and also used for manufacturing of various products.

Proteases are utilised in various food processing applications. In cheese making processing, chymosin performs hydrolyzation of the specific peptide bond (the Phe-15-Met 106 bond) and generate park-k-casein and macro peptides [16]. They also find their application in pharmaceutical and leather tanning industries [17]. Beside this, they are also used in preparing soy sauce, other hydrolyzates and for meat tenderization [18]. Due to rapidly increasing demand of these enzymes in various industrial applications, manufactures are always looking for various strain improvement techniques to improve the production, stability and specificity of proteases [14]. Therefore , the present study is focused on to isolate and identify protease producing fungi from wheat bran, to perform partial characterization of the enzyme production and its properties with regard in relation to the pH, temperature, incubation period, carbon sources and nitrogen souces in order to maximize the importation of enzyme.

2.MATERIALS AND METHODS

2.1 Sample collection

The marine sediment soil sample were collected from Arichalmunai, Dhanushkodi, Ramnadu Dt, Tamilnadu, India.

2.2 Isolation

One gram of marine sediment soil sample was diluted serially in distilled water Potato Dextrose Agar medium (PDA) was prepared and sterilized in an autoclave at 121°C for 15 minutes. The medium was incorporated with streptomycin sulphate solution (1:1) and poured into the petriplates. After solidification 0.1mL of serially diluted soil sample were inoculated into the medium. The inoculum was spread uniformly and kept undisrupted in dust free chamber at room temperature for a period of 3-5 days. The fungal colonies were counted. The pure cultures were maintained in the conventional potato dextrose agar medium.

2.3 Identification and photomicrography

Morphological features of fungi were photographed using Nikon microscope. All the fungi were identified with the standard manual [19].

2.4 Estimation of Protien

The total protein content was estimated by the method [20] . To 0.1 mL of sample, 4.5 mL of alkaline copper reagent was added. The mixture was shaken well and allowed to stand for 20 min. Then, 0.5 and incubated at 37ºC, for 15 min. Standards containing BSA at concentrations from 20 µg to 100 µg were treated similarly. The blue color developed was read at 620 nm in a spectrophotometer. The total protein content was expressed in mg/g of sample.

2.5 Production of enzymes *Aspergillus niger A.flavus* and *A.terreus* by using carbon and nitrogen sources.

Carbon Sources. The effect of various carbon source such as starch, glucose, maltose, lactose, and fructose at the concentration of 1 to 5% was examined in the production medium.

Nitrogen Sources. Various nitrogen sources like yeast extract, peptone, urea, and ammonium sulphate were examined for their effect on enzyme production by replacing 0.5% peptone in the production medium. The available carbon and nitrogen substrates such as Wheat bran, Rice bran and Coir pith were used in the present study.

2.6 Optimization of Protease enzymes production

Modified Reese's Medium (glucose 0.25 g, casein 0.5 g, yeast extract 0.05 g, K_2HPO_4 0.20 g) for protease production. The effect of the following parameters on protease production by the *A.niger A.flavus* and *A.terreus*. Each established parameter was kept constant in the subsequent study of the next parameter. Enzyme assay was carried out after 72 h incubation period in each parameter (Table-2).

2.7 Effect of pH

The pH values of 4.0, 6.0, and 8.0, in different flasks using 1 N HCl and 1N NaOH and sterilized. The cultures are inoculated and incubated at particular temperature on protease production using the method [21].

2.8 Effect of temperature

The effect of temperature on activity of protease produced by *A. niger*, *A. flavus* and *A. terreus* was studied by taking various temperatures ranges like 25, 30 and 35°C. The optimization media was inoculated with the test samples at different temperatures and the protease assay was done after 24 h [22].

2.9 Effect of incubation period

The effect of incubation period on protease production was determined by incubating for different time intervals of 24, 48 and 72 hours. The protease activity was carried out by the method [23].

3. Effect of carbon sources

The effect of different carbon sources such as rice bran and wheat bran on protease production was investigated. The Carbon source present in the production medium was replaced with 10.0 g of each of the carbon source under study. Furtherrmore, for carbon source optimization, different concentrations of the best carbon source (25, 50 and 75 mg/g) were used for the protease production. This was carried out using the method. [24]

3.1 Effect of nitrogen sources

The influence of nitrogen sources coir pith was determined for protease production, to optimize the nitrogen source, different concentrations of (25,50 and 75 mg/g) was used to produce protease [24].

3.2 Effect of Phosphorous and potassium

A.niger, *A.flavus*, and *A.terreus* were grown in Erlenmeyer flasks (250 mL) containing 50 mL of liquid medium phosphorous were added individually to the basal medium in a 0.3% ratio.The flasks were sterilized at 121°C for 20 min and inoculated with two mycelia disc (10 mm) cut from 7-day fungal cultures grown on potato dextrose agar medium .The inoculated flasks were incubated at 30°C for 6 days when protease activity was determined.

4. RESULTS AND DISCUSSION

The fungi provides a fascinating and almost endless source of biological diversity which is a rich source for exploitation .Costal region of India are dispersed in tropical as well as subtropical condition. Marine fungi play an important role in nutrient regeneration cycle as decomposers of head and decaying organic matter in the estuaries. The Indian costal line provide niches and habitats for many marine organisms and very little is known about the fungi associated with them till recently.

In the present study totally 16 fungal species were isolated from marine soil sample of Arichalmunai, Dhanushkodi, Ramand (Dt) Tamilnadu, India. The previous report has reported [7] the (*T.viride)* produced chitinolytic enzymes have been detected and purified.In our study concentration of protein in the enzyme samples were determined with reference to standard Bovine Serum Albumin. [25]. Four different previously isolated *Aspergillus* strains were screened quantitatively and qualitatively for extracellular protease production. Among them, *A. niger* was capable to release high quantity of protease (33.0 U mg^{-1}) into the fermentation medium. [26]The quantification of amylase and cellulase activities from *A. niger* ASP2 was measured in the supernatant by the DNS method. The result demonstrated the ability of *A. niger* ASP2 to produce amylase and cellulase enzymes with activities of 8.37±0.09 and 1.76±0.09 U/mL,

respectively. The three substrate were optimized with *A. niger, A.flavus* and *A.terreus* for the production of protease. The maximum production of protease was observed in *A.niger* optimized with wheat bran at 35°C. (Fig-1) (Table -1).

The influence of pH on the activity of protease was determined at various pH ranging from 4,6 and 8. The optimum production of protease was achieved by *A.niger* at pH 8 (0. 90 IU/mL). Variation observed in earlier report of *Aspergillus brasiliensis* strain BCW2 at pH 9 (2304 IU/mL) [25]. Purification and characterization of protease from *A.niger* would be to a large extent help in various industrial, biotechnological and environmental aspects. (Fig-1) (Table -1).

In the present investigation, the influence of temperature on the activity of protease was determined at 35ºC . Similar kind of result was recorded [22].The optimum enzyme activity of purified protease was at 35 °C 2.51 IU/Ml (Fig-1) (Table -2). Reports of [26] had similar observations as temperature of 35 °C.

The present study focussed the Incubation period for protease production by *A. niger* was optimum at 48 h with protease 0.38 IU/mL (Fig -1) (Table-2).Deviation in the incubation period was recorded at 120 h by [27]. The yield of protease was (95.2IU/Ml) . Further increase in the incubation time reduced the enzyme activity.

The present study of organic carbon substrates such as rice bran, and wheat bran were evaluated for their effect on protease production was high at 1.66 IU/mL in wheat bran and followed by Rice bran 1.05 IU/mL were recorded (Fig-1) (Table-2). Vast difference in the yield was recored by [25] when organic carbon substrates such as rice bran, rice husk, corn cob, sorghum, yam peel, orange peel and wheat bran were used as supplement. For their effect on protease production by *A. brasiliensis* BCW2. Citrus peel supported optimal protease production with protease activity of 1604 U/mL and at 10% concentration. The least amount of protease produced was by sorghum (260 U/mL) .

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Nitrogen sources were tested in this study for protease production by *A. niger*. Maximum protease production was observed in coir pith with the activity of 0.56 IU/mL at 50 mg/g concentration (Fig-1) (Table-2). Contrary to the present study, six different nitrogen sources were tested for protease production by *Aspergillus brasiliensis* BCW2. Maximum protease production was observed with yeast extract of 1515.99 U/mL at 2% concentration .[25]

In this study the Phosphorus for protease production by *A. niger* was optimum at 75 mg/g with protease activities of 0.99 IU/mL and followed by poor protease production was recorded at 25 mg/g is 0.10 IU/mL. The effect of Potassium on protease production by *A.niger* was optimum at 75 mg /g , 0.98 IU/ mL followed by low protease production in 25 mg/g is 0.29 IU/ML. (Fig-1)(Table-2). This result is in tandem with the earlier reports [27].

5. CONCLUSION

The present study focused on optimization and characterization of protease enzyme produced by *A. niger*, *A.flavus*, and *A.terreus*. Production of Protease by *A.niger* was optimized at pH 8.0, temperature of 35 °C, and 75 mg/g as carbon source, The nitrogen source also 75 mg/g were recorded the incubation period of 72 h. This study provides the cost effective technology for the enzymes protease using lignocelluosic residues as substrate.

CONSENT

It is not applicable.

ETHICAL APPROACH

It is not applicable.

COMPETING INTERESTS

Authours have declared that no competing interests exist.

Table1: Effect of micro fungi for the production of protease enzyme with different substates

S. No	Substrates (g)	Quantity (µl/mL)		
		A.niger	***A.flavus***	***A.terreus***
1.	Wheat bran	16.1 ± 8.66	0.96 ± 0.00	10.6 ± 5.33
2.	Rice bran	3.34 ± 6.67	1.85 ± 3.33	8.24 ± 0.00
3.	Coir pith	9.60 ± 0.00	3.20 ± 0.00	13.0 ± 9.20

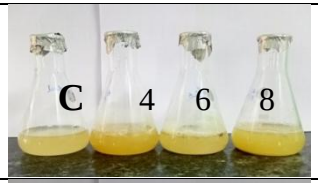
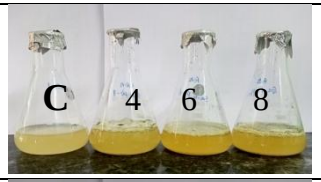
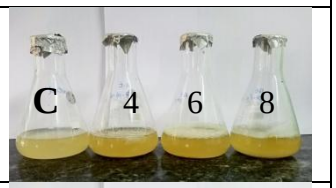
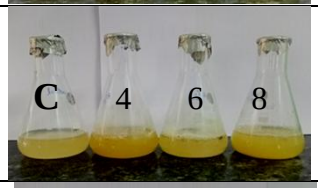
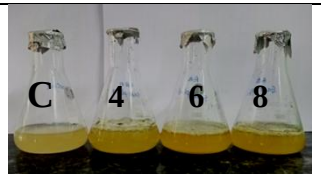
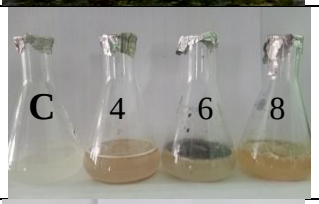
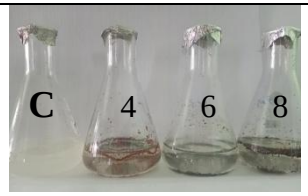
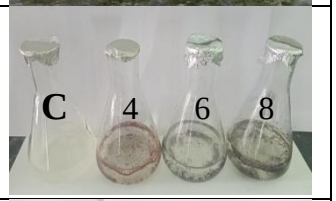
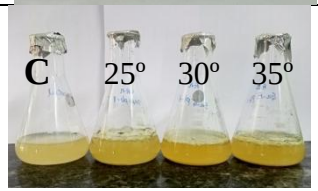
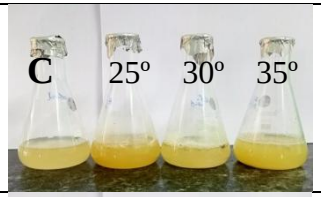
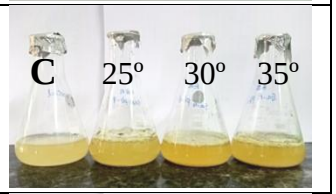
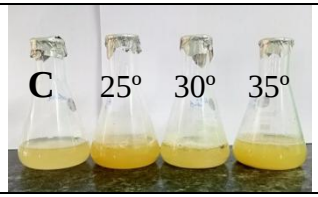
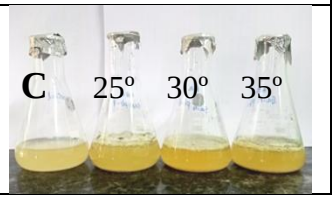
The values are expressed in SD ± SE

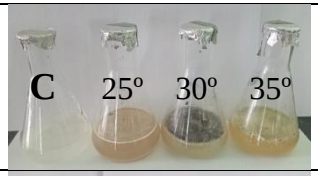
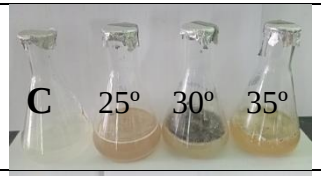
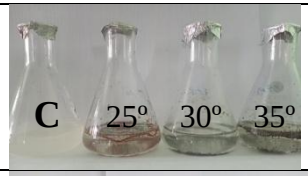
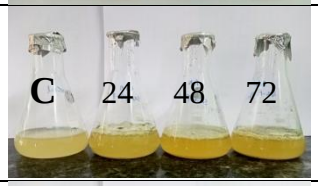
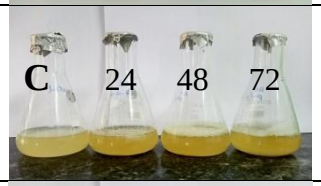
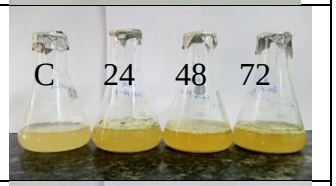
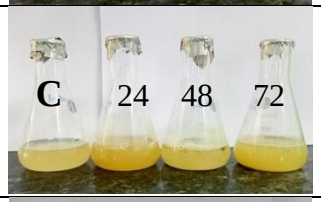
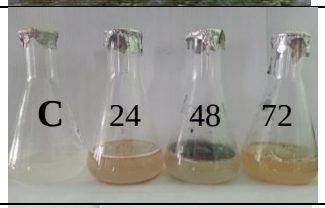
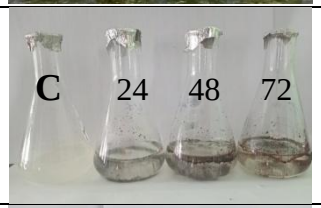
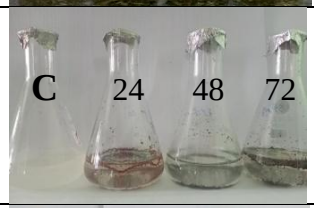
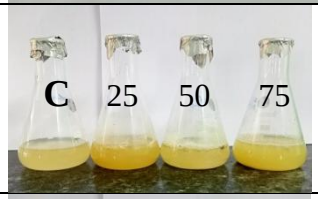
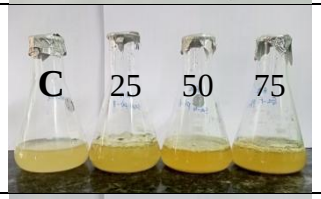
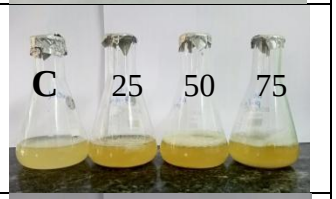
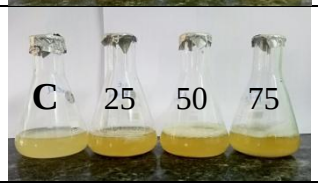
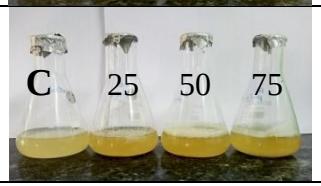
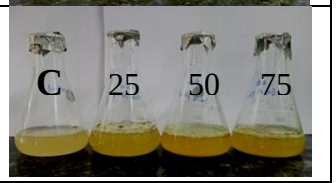
Table :2 Production and optimization of Protease enzyme from potential fungi

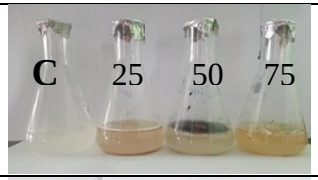
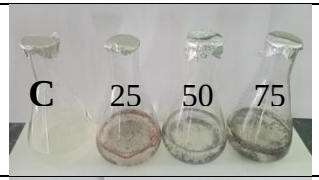
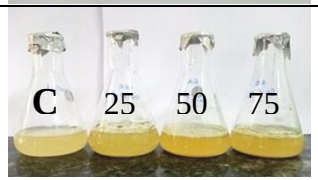
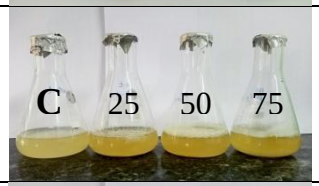
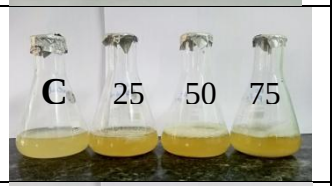
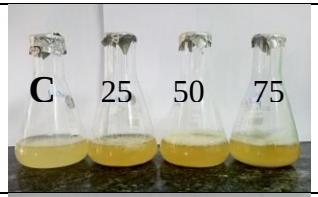
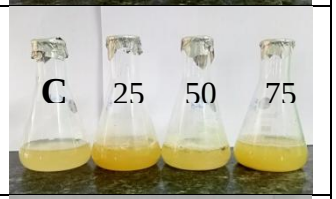
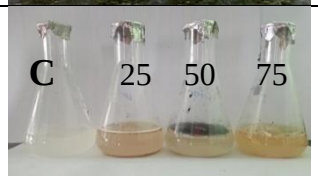
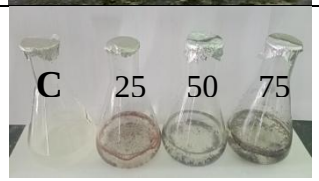
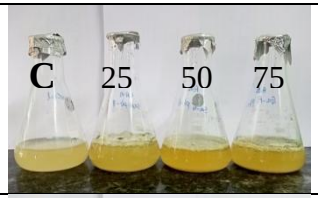
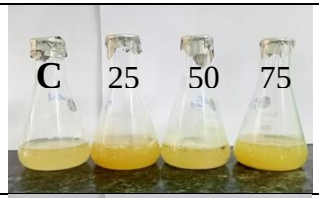
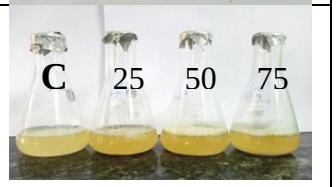
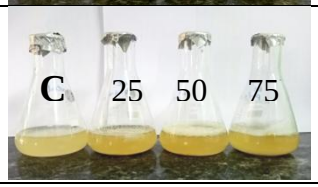
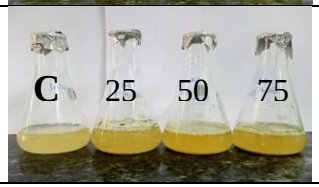
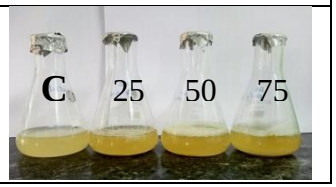
S.NO	Parameters	Inference	Quantity (IU/mL) (Wheat bran)			Quantity (IU/mL) (Rice bran)			Quantity (IU/mL) (Coir pith)		
			A.niger	***A.flavus***	***A.terreus***	***A.niger***	***A.flavus***	***A.terreus***	***A.niger***	***A.flavus***	***A.terreus***
1	P^H	4	0.38±0.00	0.15±0.03	0.26±0.00	0.45±0.00	0.58±0.00	0.22±0.02	0.17±0.03	0.18±0.03	0.35±0.03
		6	0.51±0.03	0.43±0.00	0.39±0.02	0.80±0.00	1.04±0.03	0.30±0.07	0.34±0.00	0.49±0.03	0.55±0.04
		8	0.90±0.08	0.62±0.05	0.66±0.03	1.99±0.05	1.20±0.00	0.41±0.02	0.50±0.03	0.68±0.03	0.63±0.04
2	Temperature(^{o}C)	25^o	0.59±0.05	0.22±0.00	0.24±0.07	0.60±0.03	0.19±0.03	0.31±0.01	0.10±0.00	0.26±0.05	0.20±0.00
		30^o	1.55±0.00	0.32±0.02	0.40±0.01	1.36±0.02	0.38±0.00	0.51±0.08	0.27±0.01	0.30±0.00	0.39±0.01
		35^o	2.51±0.09	0.67±0.06	0.76±0.03	1.49±0.05	0.69±0.02	0.71±0.08	0.56±0.00	0.45±0.00	0.48±0.05
3	Incubation period(hours)	24	0.03±0.03	0.01±0.02	0.01±0.03	0.01±0.00	0.02±0.06	0.01±0.03	0.01±0.08	0.01±0.03	0.01±0.00
		48	0.38±0.06	0.01±0.01	0.01±0.00	0.01±0.05	0.01±0.03	0.01±0.02	0.05±0.00	0.01±0.03	0.01±0.00
		72	0.03±0.03	0.01±0.03	0.01±0.03	0.01±0.00	0.01±0.05	0.01±0.00	0.01±0.07	0.00±0.00	0.00±0.00
4	Nutrient content (Carbon) (mg/g)	25	0.63±0.04	0.65±0.03	0.20±0.09	0.59±0.02	0.29±0.05	0.20±0.02	0.19±0.05	0.29±0.05	0.26±0.02
		50	1.20±0.02	0.80±0.00	0.59±0.00	0.96±0.05	0.54±0.06	0.42±0.09	0.25±0.09	0.52±0.03	0.30±0.20
		75	1.66±0.00	1.00±0.00	1.00±0.02	1.05±0.06	0.68±0.09	0.70±0.07	0.37±0.08	0.60±0.06	0.48±0.05
5	Nitrogen (mg/g)	25	0.22±0.00	0.33±0.02	0.15±0.01	0.49±0.02	0.22±0.00	0.37±0.06	0.10±0.03	0.23±0.09	0.18±0.02
		50	0.56±0.02	0.40±0.02	0.44±0.04	0.68±0.05	0.43±0.06	0.52±0.05	0.29±0.01	0.40±0.05	0.25±0.03
		75	0.50±0.02	0.60±0.00	0.67±0.02	0.87±0.02	0.50±0.00	0.98±0.00	0.86±0.02	0.53±0.06	0.47±0.06
6	Phosphorus (mg/g)	25	0.10±0.00	0.25±0.02	0.15±0.02	0.34±0.00	0.36±0.00	0.27±0.03	0.22±0.01	0.26±0.00	0.19±0.06
		50	0.52±0.03	0.62±0.03	0.35±0.02	0.55±0.04	0.50±0.02	0.46±0.06	0.30±0.00	0.46±0.03	0.30±0.00
		75	0.99±0.00	0.80±0.05	0.77±0.06	0.63±0.06	0.63±0.02	0.60±0.05	0.47±0.02	0.59±0.05	0.55±0.03
7	Potassium (mg/g)	25	0.29±0.00	0.10±0.23	0.29±0.02	0.29±0.00	0.40±0.03	0.16±0.09	0.20±0.01	0.13±0.06	0.28±0.06
		50	0.50±0.08	0.40±0.02	0.49±0.02	0.52±0.06	0.56±0.06	0.59±0.05	0.43±0.00	0.29±0.06	0.36±0.00
		75	0.98±0.09	0.60±0.00	0.78±0.06	0.90±0.00	0.66±0.00	0.79±0.00	0.59±0.05	0.46±0.00	0.46±0.00

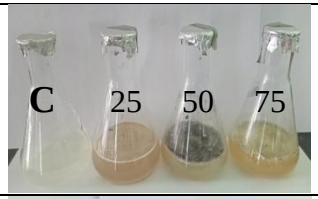
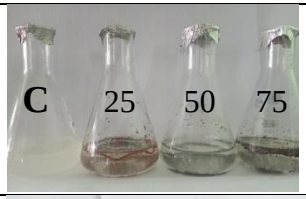
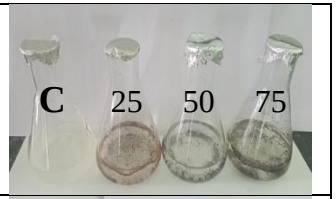
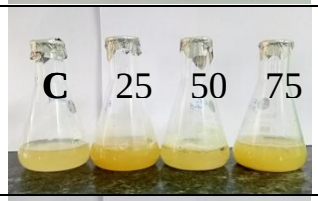
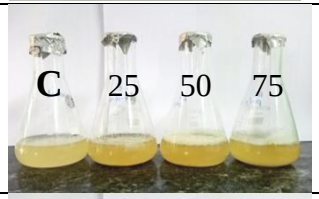
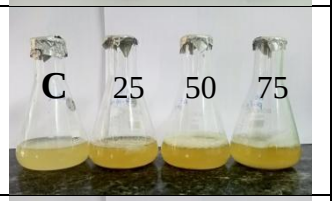
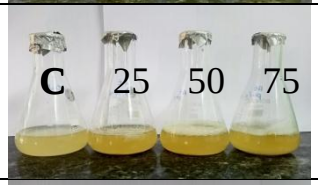
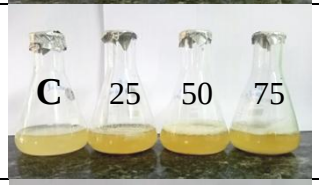
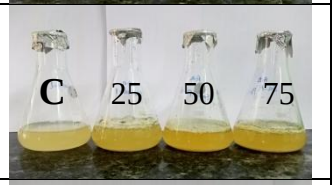
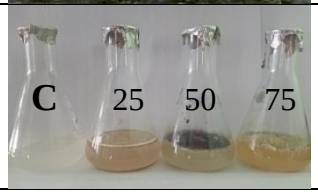
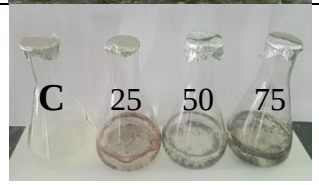
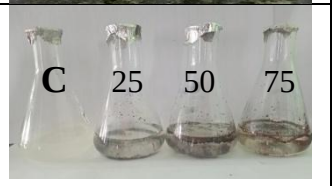
Standard deviation ± Standard error

Fig 1 : Production and optimization of protease enzyme from potential fungi

S.NO	Parameters	Substracte	Quantity (IU/mL)		
			A.niger	*A.flavus*	*A.terreus*
1	P^H	Wheat Bran			
		Rice Bran			
		Coir Pith			
2	Temperature(°C)	Wheat Bran			
		Rice Bran			

		Coir Pith			
3	Incubation period (days)	Wheat Bran			
		Rice Bran			
		Coir Pith			
4	Nutrient content (Carbon) (mg/g)	Wheat Bran			
		Rice Bran			

		Coir Pith			
5	Nitrogen (mg/g)	Wheat Bran			
		Rice Bran			
		Coir Pith			
6	Phosphorus (mg/g)	Wheat Bran			
		Rice Bran			

		Coir Pith			
7	Potassium (mg/g)	Wheat Bran			
		Rice Bran			
		Coir Pith			

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