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Isolation And Screening Of Thermophilic Fungi For Their Enzymes

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Abstract: Thermophilic fungi are known to present in various habitats. The enzymes secreted by the thermophilic fungi are also known for their ability to produce thermostable enzymes which are capable of improving the quality of various industrially important products. Several microorganisms are known as potential sources of enzymes but due to the thermostability thermophiles have gained much importance. More and more numbers of microorganisms are being screened for enzyme activities day by day. The new findings in these directions would allow to explore new area in the extreme industrial process. The present study was aimed to isolate the thermophilic fungi from three compost wastes sites such as Cattle dung, Goat farm waste and Poultry waste and screened the potential producers of lipases, cellulases and proteases respectively. The thermophilic fungi were isolated from about same depth from collection sites with a temperature of 45^{O}C and later screened for their thermophilic nature. Further study of enzyme activity showed the presences of lipase, cellulase and protease producing fungi present in samples collected from selected sites. The isolated fungi were screened for enzyme activity in liquid medium for 10 days at 45^{O}C. Culture filtrate obtained from the medium of production were used for the assessment of enzymatic activity. The *Rhizomucor pusillus* (1B) has shown highest lipase activity 22.147 U/ml and *Thermoascus aurantiacus* (2C) was observed with highest cellulase activity 30.056 U/ml and highest protease activity 34.059 U/ml was obtained from isolates 3C of *Thermomyces lanuginosus*. Further detailed studies with respect to these strains could be done to utilize them in industrial applications.

Index Terms **-** Thermophilic fungi, Thermostable enzymes, Lipase, Cellulase and Protease

I. INTRODUCTION

Thermozymes have gained importance due to their thermostable properties. In the industries these enzymes work in dynamic ways as thermophilic fungi serve as potential source of thermostable enzymes such as lipolytic, proteolytic, cellulolytic, lignolytic and amylolytic enzymes which are used in the industries. Numerous industries, including detergent, chemical, oil, food, brewing, pharmaceutical, leather, paper, dye and textiles ones, have discovered uses of these enzymes (Chrisnasari et al., 2018; Gulmus and Gormez, 2020). Thermophiles are found in many different parts of environment. They may be found in wood chip piles, compost sites, aquatic sediments, industrial effluents, sludge and other accumulated organic waste that offers warm, humid and aerobic conditions for their growth (Lee et al., 2014; Ahirwar et al., 2017).

When compared to the enzymes generated and extracted form mesophilic fungus, the majority of enzymes derived form thermophilic fungi frequently show a better temperature tolerance between 50 and 80^{O}C, several of these thermostable enzymes remain stable (Lee et al., 2014), any temperature below 20OC has inhibited the activity and growth of true thermophilic fungi (Maheshwari et al., 2000; Ahirwar et at., 2017).

Numerous thermophilic fungi have also been identified form extreme situations such as high salinity, high water pressure, oxygen deprivation and aridity. According to research, a large number of thermophilic fungi organisms were mostly identified from composts, the high temperatures, aerobic conditions and humidity levels found in composts are what cause these microorganisms to be so common in compost sites. Furthermore, composts provide nutrients that support the growth of microorganisms. (Lee et al.., 2014).

Many organic materials are broken down into smaller organic molecules by metabolic activities of fungus present on composts. The capacity of thermophilic fungi to release a variety of enzymes that may break down composts allows for entire processes of metabolism (Raut et. Al., 2008). The thermophilic enzymes evaluated in this study are proteases, cellulases and lipases. These are largely required in food industries to reduce time, energy and cost of operation (Raveendran et al., 2018).

Proteases are being utilized in brewing, meat tenderization as well as milk coagulation process since they catalyze peptide bonds in protein through hydrolysis (Patel et al., 2013). Cellulases work on cellulose and hydrolyze β-1, 4 links available in carbohydrates to eliminate glucose subunits. Classes of cellulases include endo –(1,4)-β-d -glucanases). (EC.3.2.1.4), exo-(1,4)- β glucanase (EC.3.2.1.91) and β- glucosidases (EC.3.3.1.21) (Schulein 1988). In addition, they are also utilized in extraction of important phytochemicals such as phenolic and flavonoids from flowers, seeds and fruits (Kabir et. al., (2015), while lipases hydrolyze long chain of triglycerides. These enzymes are utilized to extend the shelf life of baked goods while also enhancing the flavor and texture of chees, butter and drinks, (Aravindan et al., 2007). The necessity to find thermophiles that can create thermophilic and thermostable enzymes that will be extremely stable and resistant to product inhabitation during these manufacturing procedures streams from the economic benefits of these enzymes. In order to prepare for future biotechnological uses, we have thus identified and screened thermophiles (fungi) that may produce thermophilic lipases, cellulases and proteases in the current work.

II. MATERIALS AND METHODS

The compost samples were collected from three composts dumping sites as cattle dung, goat farm waste and poultry waste sites from nearby village of Sakoli Dist- Bhandara (MS) India. The samples were obtained at a depth of 1 meter using a shovel and then brought to the laboratory in sterile polythene bags. From these samples about 10 gm sample was kept in incubator for three days. Further isolations were made from these samples on Emerson's YPSS agar medium.

The three fungal isolates from different sources and having the highest enzymatic activities were evaluated for their fungal morphology by observing the colony features (colour, shape, size and hyphae) by a compound microscope with a digital micro camera using a lactophenol cotton blue-stained slide. The morphological characters were observed and identified from available literature.

2.1 Quantitative enzyme production:

For enzymes production fungi were inoculated in 250 mL Erlenmeyer flask containing 100 mL of the media described below.

(i) lipase production

The basal medium for lipase production consist of 0.1% yeast extract, 0.3% peptone, 0.05% $CaCl_2.2H_2O$, 0.05% NaCl, 1% oil and , 0.02% streptomycin; , pH was adjusted at 8.0 (Ayinla *et al*., 2017).

(ii) celullase production

Cellulase production in shaking flasks was carried out using Mandels and Weber (1969) medium, supplemented with 1% CMC and 2.5% wheat bran. The medium also consist of 0.2% KH_2PO_4, 0.03% $CaCl_2 \cdot 2H_2O$, 0.03% urea, 0.03% $MgSO_4 \cdot 7H_2O$, 0.14% $(NH_4)2SO_4$, 0.025% peptone, 0.01% yeast extract, 1 mL Tween-80, 0.005% $FeSO_4 \cdot 7H_2O$, 0.0016% $MnSO_4 \cdot H_2O$, 0.0014% $ZnSO_4 \cdot 7H_2O$, and 0.002% $CoCl_2 \cdot 6H_2O$, pH 5.0 in 250 mL Erlenmeyer flask (Saroj *et al*., 2018).

(iii) protease production

Submerged fermentation medium for protease production include the following : 1% of casein, 2.5% wheat bran, 0.1% (w/v) of each of $(NH_4)_2SO_4$, $MgSO_4.7H_2O$ and NH_4NO_3, to pH 8.0 in 250 mL Erlenmeyer flasks (Macchione *et al*., 2008).

Each medium was filled aseptically with a loopful of active fungal colonies collected from plates. Inoculated media were put in a shaking bath and incubated at 45°C with a steady oscillation of 160 rpm. After ten days, the supernatants were collected by centrifugation at 5,000 rpm for 15 minutes at 40 degrees Celsius and filtered through Whatman no. 1 filter paper before determining their relative enzymatic extracellular activities (Ayinla *et al*., 2017).

2.2 Measurement of enzyme activity:

(i) Assay for lipase

The oil served as the substrate for the lipase activity measurement method developed by Yadav et al. (1993). After thoroughly mixing 5 mL of oil and 20 mL of 0.1 M phosphate buffer, the mixture was pre-incubated for 10 minutes at 37°C. After adding 1 mL of crude enzymes to initiate the reaction, the mixture was incubated at 40 degrees Celsius for 30 minutes. The injection of 15 mL of acetone-ethanol (1:1) ultimately halted the process. Three drops of phenolphthalein indicator were added, and the free fatty acids generated during the reaction were titrated with 0.05 N NaOH. A unit of lipase activity is the amount of enzyme that produces 1µmol of fatty acids per minute under test condition (Lanka and Trinkle, 2017).

(ii) Assay for cellulase

The Ghose (1987) method was utilized to carry out the carboxymethyl cellulase (CMCase) reaction. The experiment employed a reaction combination of 0.5 mL of crude enzyme and 0.5 mL of 2% substrate (CMC) mixed in 50 mM sodium citrate buffer (pH 4.8) and ran for 30 minutes at 45 degrees Celsius. Following incubation, three milliliters of DNS (3,5-dinitrosalicylic acid) reagent were added, and the mixture was cooked in boiling water for five minutes, yielding a colored reaction mixture. The absorbance was measured at 540 nm. Saroj et al. (2018) defined one unit of cellulose activity as the enzyme required to release 1 µmol of glucose per milliliter per minute from a suitable substrate under test conditions.

(iii) Assay for protease

The TAn method established by Carrie Cupp-Enyard (2008) was utilized to quantify protease activity. In this test, 5 mL of a 0.65% casein solution was incubated for 5 minutes at 37°C. Casein is utilized as a substrate. After adding 1 mL of crude enzyme solution, the reaction was cooked in a water bath for 30 minutes at 37 °C. To complete the reaction, 5 milliliters of Trichloroacetic acid (TCA) solution was added to the mixture, which was then filtered using Whatmann No. 1 filter paper. The filtrate was then treated with five milliliters of sodium carbonate and one milliliter of Follin Ciocalteus phenol reagent, which had been diluted twice, and permitted to develop blue for thirty minutes at room temperature in the dark. Tyrosine standard was used to compare absorbance at 660 nm to a reagent blank. A protease unit is the amount of enzyme required to release 1 µM of tyrosine per minute at pH 7.5 and 37°C. (Mohapatra *et al*., 2003; Chandrasekaran *et al*., 2015).

All the experiments were done in triplicates and values are expressed as mean ± SEM (n=3). Statistical analysis was performed using a one-way analysis of variance (ANOVA).

III. RESULTS AND DISCUSSION

Table 3.1: Lipase Enzyme Activity U/ml

Name of fungi	Isolate number	Enzyme activity	SEM
Rhizomucor pusillus	1A	11.346	±0.026
	1B	**22.147**	±0.017
	1C	18.963	±0.032
Thermoascus aurantiacus	2A	8.259	±0.147
	2B	9.354	±0.065
	2C	12.550	±0.131
Thermomyces lanuginosus	3A	10.987	±0.012
	3B	16.265	±0.023
	3C	11.012	±0.020

Table 3.1 displayed the name of fungi tested, isolate number and Enzyme activity and ±SEM

Table 3.2: Cellulase Enzyme Activity U/ml

Name of fungi	Isolate number	Enzyme activity	SEM
Rhizomucor pusillus	1A	23.021	±0.016
	1B	16.321	±0.020
	1C	15.059	±0.031
Thermoascus aurantiacus	2A	25.021	±0.025
	2B	28.035	±0.029
	2C	**30.056**	±0.022
Thermomyces lanuginosus	3A	15.013	±0.012
	3B	22.054	±0.021
	3C	27.058	±0.023

Table 3.2 displayed the name of fungi tested, isolate number and Enzyme activity and ±SEM

Table 3.3: Protease Enzyme Activity U/ml

Name of fungi	Isolate number	Enzyme activity	SEM
Rhizomucor pusillus	1A	8.032	±0.012
	1B	9.987	±0.025
	1C	12.358	±0.026
Thermoascus aurantiacus	2A	21.009	±0.054
	2B	15.258	±0.024
	2C	19.369	±0.026
Thermomyces lanuginosus	3A	26.026	±0.058
	3B	30.012	±0.098
	3C	**34.059**	±0.087

Table 3.3 displayed the name of fungi tested, isolate number and Enzyme activity and ±SEM

The highest Lipase enzyme activity was shown by isolate 1B of *Rhizomucor pusillus* (22.147) (Table 3.1) Ibrahim *et. al*., (2021) and Rajnith kumar *et. al.*, (2023) has also reported the highest enzyme activity and lowest was from *Themoascus aurantiacus* (2C). Highest Cellulase activity was observed from strain 2C of *Thermoascus aurantiacus* (30.056) (Table 3.2). Rajnith kumar et. al., (2023) and lowest from that of *Thermomyces lanuginosus*. Whereas, the highest Protease enzyme activity was shown by isolate 3C (Table no. 3.3) of *Thermomyces lanuginosus.* Li *et. al*., (1997) has also reported the production Proteases form this fungus. However, *Rhizomucor pusillus* has shown the lowest cellulase activity. Proteases have long been used in the food, dairy, and detergent industries and for leather processing. The need to overcome the limitation of obtaining chymosin, the milk-curdling enzyme from the stomach contents of milk-feeding calves, which is used in the industrial preparation of cheese, led to a search for substitutes (Maheshwari *et. al*., 2000).

Conclusion:

Most of the fungi isolated were potential producers of Thermostable enzymes. In the present work only three true thermophilic fungi were tested for their hydrolytic enzymes Lipase, Cellulase and Protease. All three fungi are producers of hydrolytic enzymes. The enzymes from mesophilic sources tends to denature at high temperatures. Therefore, there is need of finding sources of enzymes which are thermostable at higher temperatures and also having good activity which can be utilized for various industrial process like Dairy and Laundry etc. The evidences suggest that the thermophilic fungal enzymes work at high temperatures of more than 50^{O}C. Thus, greater research into the biochemical and molecular characterization of these isolates is

required to improve our knowledge of their metabolic functions. Detailed understanding of the catalytic and biophysical characteristics of these thermophilic enzymes is crucial for designing these isolates to tolerate harsh industrial processes or environmental conditions.

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