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ENZYME ARRAY FROM THERMOPHILIC FUNGAL ISOLATE-RSND

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ABSTRACT

The first known thermophilic fungi was *Mucor pusillus* isolated from bread over a century ago. Thermophiles are found in various geothermally heated regions of the Earth, such as hot springs like those in Yellowstone National Park and deep sea hydrothermal vents, as well as decaying plant matter, such as peat bogs and compost. Thermophilic fungi have the exceptional ability to grow at high temperatures of 50°–60°C. Thermophilic fungi offer several advantages as they are highly thermostable and also have minimum chances of fermentor contamination at elevated temperature. Thermophilic fungi are potential sources of enzymes with academic and commercial interest. For this study, soil sample was collected from Pashan Lake, Pune for the isolation of thermophilic fungi. Soil sample was inoculated on GPYE medium and incubated at 55°C for 4-5 days. Microscopic and macroscopic analysis of the isolate RSND suggested that the genus might be *Aspergillus*. Pure culture was screened for thermophilic enzymes such as lipase, pectinase and tyrosinase because of their commercial and industrial application. Pectinase, Tyrosinase and Lipase were partially purified by ammonium sulphate. SDS-PAGE result revealed the approximate molecular weights of pectinase, tyrosinase and lipase as 29.8 kDa, 138.2kDa and 31.1kDa respectively. Specific activity of pectinase, tyrosinase and lipase in purified extract was found to be 0.26U/ml, 29.3U/mg and 80U/mg respectively. Thermophilic enzymes are gaining importance in agriculture, industry and human health. Therefore, this study contributes to the knowledge of thermophilic fungi and their ability to produce thermophilic enzymes.

KEY WORDS: Thermophilic fungi, *Aspergillus sp*, Lipase, Pectinase, Tyrosinase and SDS-PAGE.

INTRODUCTION

Most of the bacteria present in the environment are still unexplored in laboratory cultivation and hence remain unavailable for their pharmaceutical, industrial and biotechnological applications. Among eukaryotes, Thermophillic fungi have exceptional ability to grow at high temperatures of 50°–60°C.[1, 2] and can survive in all sorts of extreme environments. Thirty species of thermophilic fungi are currently known, most of which originally were isolated from composts. Their prevalence in composts is explained on the basis that prolonged elevated temperatures, humid and aerobic conditions, and supply of carbohydrate and nitrogen in the mass of organic matter favours the development of thermophilic microflora.[2] Thermophiles can be categorized into moderate thermophiles (growth optimum, 50–60°C), extreme thermophiles (growth optimum, 60–80°C), and hyperthermophiles (growth optimum, 80–110°C).[5] Because of their ability to grow at high temperatures and presence of unique macromolecular properties, thermopiles possess high metabolic rate, highly stable enzymes and lower growth but higher end product yields than mesophilic species .Thermophilic microorganisms have ability to produce thermostable enzymes[3,4] that have wide applications in pharmaceuticals and industries gaining world-wide importance.Several workers have reported thermophilic microbes from diverse environmental habitats such as geothermal sites and hot springs. The aim of this study was to isolate thermophilic fungi from muddy soil samples of Pashan Lake, Pune and screen for industrially viable enzymes like pectinase, tyrosinase and lipase. Lipases (triacylglycerol acylhydrolase; E.C.3.1.1.3) have become one of the prominent industrial enzymes for their specificity in hydrolysis and interesterification. They catalyze both hydrolysis of triglycerides and the synthesis of esters from glycerol and long chain fatty acids. Lipases of microbial origin have greater industrial attraction because they are available in large quantities and can be produced with high yields.[5-7] Pectinase has commercial application in fruit juice industry for clarification purpose.[8-11] Tyrosinase catalyzes the bioconversion of L-Tyrosine to L-DOPA (3,4dihydroxyphenylalanine) which has therapeutic importance in the treatment of Parkinson's disease.[12-15]

MATERIAL AND METHODS

Soil sample was collected from Pashan Lake, Pune. Isolation of thermophilic fungi was done on Glucose Peptone Yeast Extract (GPYE) media containing $NaNO_3$-0.3g, K_2HPO_4-0.1g,

$MgSO_4.7H_2O$-0.05g, KCl-0.05g, $FeSO_4.7H_2O$-0.001, glucose-1g, peptone-0.5g, yeast extract-0.5g, agar-2g, distilled water-100ml, pH-7. 1gm of soil sample was inoculated in Glucose Peptone Yeast Extract broth and incubated for 4-5 days at 55^oC. After incubation fungal growth was used for the screening of bioactive molecule such as lipase, pectinase and tyrosinase using different composition medium**.**

Screening of lipase

Lipase activity was carried out on Modified Agar Medium containing Peptone-1g, NaCl-0.5g, $CaCl_2.H_2O$-0.01g, tween 80-1g, agar-2g in 100ml distilled water. The hydrolysis of the Tween opacity medium is associated with the lipolytic enzymes produced by fungus. A positive result is detected by precipitation around the growth.

Screening of pectinase

Pectins are polymeric substances in the fruit lamella and cell walls. Pectinases were classified according to its specificity to attack pectin, pectic acid and oligo-D-galacturonate. Pectinesterases, depolymerizing enzymes and protopectinase are the three major types of pectinases. Presently, pectinases have been used in textile industries. Filamentous fungi are well known for their production of substances with antimicrobial activities, several of which have formed the basis for the development of new clinically important antimicrobial agents. Screening of pectinase was done on Pectin agar containing -Pectin-1g, $(NH_4)_2SO_4$-0.14g, K_2HPO_4-0.2g, $MgSO_4.7H_2O$-0.02g, agar-2g in 100ml distilled water. Pectinase activity was detected by flooding the plate with 1M NaCl and by observing zone of hydrolysis around the colony.

Screening of Tyrosinase

Biotranformation is the chemical modification of chemical compounds like amino acids, toxins of xenobiotics and drugs, brought about by an organism to produce bioactive molecule. Tyrosinase is a type 3 copper proteins and is an important enzyme participating in the process of L-DOPA and finally melanin biosynthesis. Screening of tyrosinase was done on tyrosine agar media containing -Peptone-0.5g, beef extract-0.3g, L-tyrosine-0.5g, agar-2g, distilled water-100ml, pH-7. The occurrence of a distinct brown spot (L-DOPA) which gradually changes its colour to black (melanin formation) was indicative of the fact that the above isolate was tyrosinase positive.

QUANTITATIVE DETECTION OF ENZYMES

Lipase assay

1ml of culture supernatant was added to reaction mixture containing 1ml of 0.1M tris HCl buffer, 2.5ml of deionised water and 3ml of olive oil. The solution was mixed well and kept at 37°C for 30min. Both test and blank were prepared. After 30min test solution was transferred to 500ml flask, reaction was terminated by adding 3ml of 95% ethanol. Liberated fatty acid was titrated against 0.1M NaOH using phenolphthalein as an indicator. End point is appearance of a pink color. Unit lipase is defined as the amount of enzyme which releases one µmol fatty acid per min under specific assay condition**.**

Reaction mixtures for lipase activity determination.

Protocol for Lipase assay

Reagents	Test	Blank
Deionised water	2.5ml	2.5ml
Buffer	1ml	1ml
Olive oil	3ml	3ml
Mix well		
Enzyme solution	1ml	_

$$\text{Enzyme activity unit/ml} = \frac{(\text{NaOH})\ (\text{molarity of NaOH})\ \ (1000)(2)(\text{df})}{1}$$

(NaOH) = Volume (in milliliters) of Reagent E used for Test minus volume (in milliliters) of Reagent E used for Blank..

1000 = Conversion factor from milliequivalent to microequivalent

2 = Time conversion factor from 30 minutes to 1 hour (Unit Definition)

df = Dilution factor

1 = Volume (in milliliter) of enzyme used.

Pectinase

Pectinase activity assay is based on the reducing sugar released as the product of enzyme hydrolysis, to determine the reducing sugar content, DNS method was performed. Standard curve of reducing sugar was prepared by using serial concentration of galactouronic acid solution (o.1mg/ml) in distilled water. Enzyme activity determination was performed using 0.5% (w/v) pectin substrate and 0.1M phosphate buffer pH 7.5. Substrate and enzyme were incubated for 10min at 55°C before the reaction was stopped by adding DNS reagent. One

unit of enzyme activity is defined as the amount of enzyme that catalyses the release of 1µmol galactouronic acid per min under assay condition.

Reaction mixtures for pectinase activity determination.

Protocol for Pectinase assay

Reaction	Enzyme solution(µl)	Substrate(µl)	Buffer(µl)
Blank	_	_	500
Enzyme+Substrate (ES)	250	250	_
Enzyme blank (EB)	250	_	250
Substrate blank (SB)	_	250	250

$$\text{Enzyme activity} = \frac{\text{ES-EB-SB mg/ml} \times \frac{500\ \mu l}{250\ \mu l} \times \frac{1000 \mu g/ml}{t\ min} \times \text{dilution factor} \times \frac{1}{MW\ GalA}}{\text{Slope}}$$

Tyrosinase

The reaction mixture containing 0.5 M phosphate buffer, pH 6.5, 0.001 M L-tyrosine and reagent grade water was oxygenated by bubbling oxygen through a capillary tube for 4-5 minutes. Absorbance was recorded at 280 nm for 4-5 minutes to achieve temperature equilibration and to establish blank. 0.1 ml of the supernatant of the enriched culture in tyrosine broth was added to the reaction mixture and absorbance was recorded for 10-12 minutes at 280nm. Calculation of enzyme activity was done using the following formula:

Spectrophotometer was adjusted to 280 nm.

Reaction mixtures for tyrosinase activity determination.

Following solutions were pipetted into test tube in the following order.

Protocol for tyrosinase assay

Solution to be added	Amount(ml)
0.5M Phosphate buffer, pH-6.8	1ml
0.001M L-tyrosine	1ml
Distilled water	0.9ml

$$\text{Units of enzyme/ ml} = \frac{(\Delta A280nm / min\ Test - A280nm / min\ blank)(df)}{(0.001)(0.1)}$$

$$\text{Specific activity} = \frac{\text{units of enzyme/ml/min}}{\text{mg of protein/ml}}$$

Purification of enzymes

Purification of enzymes was done by ammonium sulphate precipitation method. All the enriched broths were centrifuged at 10,000rpm for 15min. Enzymes were purified from

supernatant by Ammonium Sulphate precipitation. Ammonium sulphate was added to 10ml supernatant slowly with continuous stirring on ice bath to achieve percentage precipitation. The mixture was refrigerated overnight then centrifuged at 10,000rpm for 20min at 4˚C.The precipitate was dissolved in 10ml of 0.5M Phosphate buffer pH-6.8. The suspended precipitate was used for enzyme activity as well as for detection of total protein content by Folin reagent according to the protocol of Lowry et al. using bovine serum albumin as the standard.

Dialysis

Dialysis membrane of appropriate length was pre-treated in boiling water for 60 min and stored in 0.5 M phosphate buffer (pH 6.8). The membrane was filled up with the suspended precipitate and sealed at both ends. The dialysis bag was then suspended overnight at 4˚C in a glass cylinder containing 0.5 M phosphate buffer (pH 6.8) with continuous mixing using a magnetic stirrer.

SDS-PAGE

SDS-PAGE was carried out in a 12% polyacrylamide gel using Tris-glycine buffer (pH 8.3) by the method of Laemmli. The cell free broth (crude extract) and dialysed sample was loaded on to a denaturing polyacrylamide gel and compared with protein marker. Silver staining was performed in order to visualize the protein bands. The gel results were documented in Gel Doc EZ Imager. Silver staining was performed for viewing bands.

RESULTS

Thermophilic fungi RSND isolated from soil sample taken from Pashan lake Pune, was purified and their morphological characteristics was examined and genus was identified as *Aspergillus sp*. It was further screened for potential biotechnological enzyme producers. Thermophilic fungi showed positive result for lipase, pectinase and tyrosinase. Lipase, pectinase and tyrosinase were partially purified by Ammonium sulphate precipitation. The specific activity of tyrosinase for crude and purified extract was shown to increase from 14.86U/mg to 19.93U/mg for 60%ammonium sulphate precipitation. Lipase assay was carried out by titrimetric method using olive oil as a substrate. Specific activity of crude was found to be 25.71 mg/ml and purified extract was found to be 30 U/mg in 70% ammonium sulphate precipitation. Pectinase assay was done. Specific activity of crude was found to be 0.01U/ml and purified extract was found to be 0.015U/ml in 60% ammonium sulphate precipitation.

Microscopic Examination

Morphological study of the isolated organism was done as shown in Figure1. Microscopic analysis suggested that the isolate might be *Aspergillus sp*.

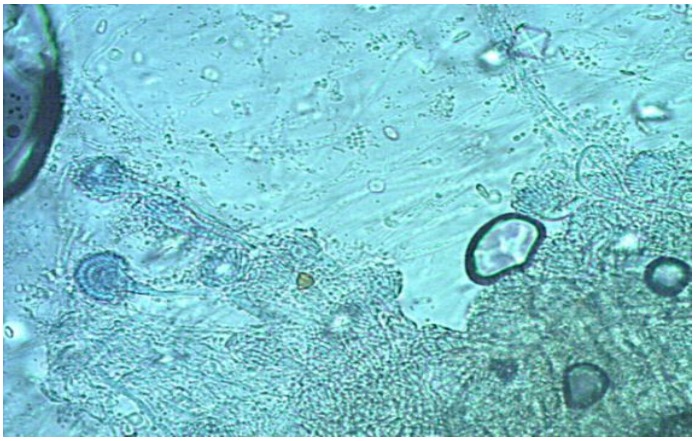
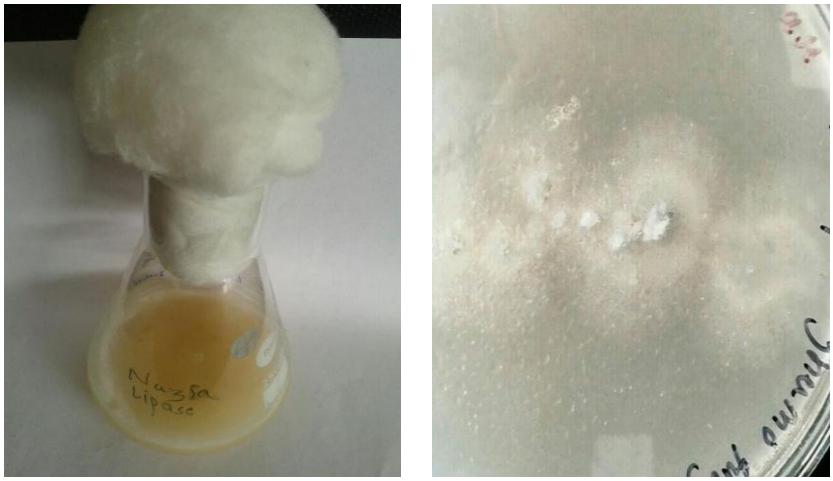


Fig .1: Microscopic view, Compound Microscope, 45X.

Enzyme Production by *Aspergillus sp*

The thermophilic fungal isolate produced lipase,pectinase and tyrosinase enzyme seen in fig 2 (a,b),fig 3 (a,b) and fig 4(a,b).

Lipase



(a) **(b)**

Fig.2 (a, b): Organism on Tween 80 broth and agar.

Pectinase

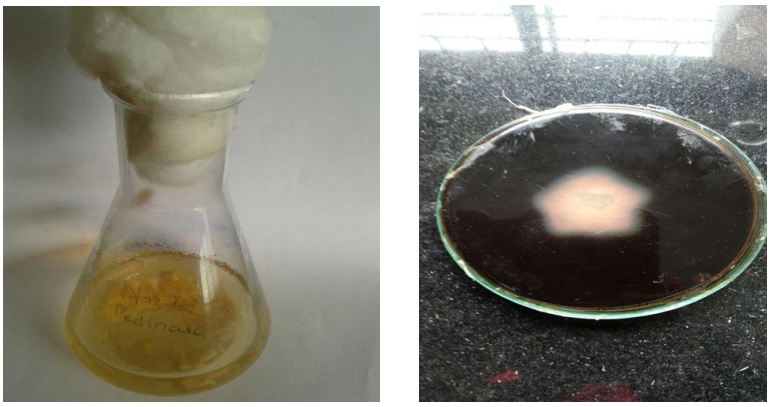


Fig.3: Organism on Pectin broth and agar.

Tyrosinase

Fig. 4: Organism on Tyrosine broth and agar.

Purification chart of enzymes

Table 1: Purification chart of crude and dialysed extract of Lipase.

Sample	Volume	Total Protein (mg/ml)	Total enzyme activity (U/ml/min)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	95	4.77	120	25.15	1	100
Dialysed extract (70% $(NH_4)_2SO_4$)	50	3.2	189.21	59.12	2.35	67.08

Table 2: Purification chart of crude and dialysed extract of Pectinase.

Sample	Volume	Total Protein (mg/ml)	Total enzyme activity (U/ml/min))	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	95	8.1	54.27	6.7	1	100
Dialysed extract (60% $(NH_4)_2SO_4$)	50	5.7	64.86	11.38	1.69	70.37

Table 3: Purification chart of crude and dialysed extract of tyrosinase.

Sample	Volume	Total Protein (mg/ml)	Total enzyme activity (U/ml)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	95	9	133	14.77	1	100
Dialysed extract (60% $(NH_4)_2SO_4$)	50	3	59.79	19.93	1.34	33.33

SDS-PAGE Results

SDS-PAGE result revealed the presence of bands and the approximate molecular weight of pectinase was determined as 29.8 kDa, tyrosinase 138.2kDa and lipase 31.1kDa respectively.

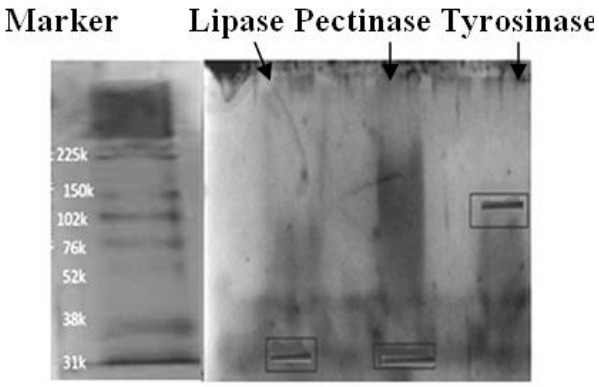


Fig.5: SDS-PAGE of Lipase, Pectinase and Tyrosinase.

DISCUSSION

Thermophilic fungi was isolated from the soil sample collected from Pashan Lake,Pune and morphological characteristics of the isolate was examined and genus might be *Aspergillus sp*.[6-8,11] It was further screened for enzymes such as lipase, pectinase and tyrosinase which provide various industrial application.[3,4] Screening of lipase was done quanlitatively by primary plate assay. Partial purification of Lipase was carried out using 70% ammonium sulphate.The molecular weight of enzyme was 31.1kDa according to SDS-PAGE.Most of the enzyme lipase reported from *Aspergillus* species have molecular weight in the range of 29-70kDa.[5-7] Screening of pectinase was done quanlitatively and quantitatively.[12] Partial purification of pectinase was carried out. SDS-PAGE was performed and the molecular weight of pectinase was determined as 29.8 kDa,[8-11] The pectinase enzyme with a molecular mass of 31 kDa has been produced by *P.chrysogenum.*[9] Similarly the tyrosinase activity of isolate was determined qualitatively and quantitatively. Tyrosinase being a bifunctional

enzyme catalyses both the orthohydroxylation of monophenols (tyrosine)- monophenolase activity or cresolase activity and subsequent oxidation of diphenols (L-DOPA) to orthoquinones (dopachrome)- diphenolase activity or catecholase activity. Quinones produced, are highly susceptible to non-enzymatic reactions, which lead to the formation of melanins and heterologous polymers.[12, 13] Partial purification of tyrosinase was carried out. SDS-PAGE results revealed the presence of a band corresponding to approximately 138.2 kDa.[14, 15]

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