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Characterization of a thermo tolerant lipase from *Pichia anamola*

Prakash Tiwari* and Mukesh K. Upadhyay

School of Life Sciences, Jaipur National University, Jagatpura, Jaipur, India

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ABSTRACT

A yeast strain of Pichia anamola was characterized for optimal production of liapse.The organism shown maximum enzyme production on 5th day of incubation. The optimum temperature and pH for the enzyme activity was 45°C and 7.0 respectively. The enzyme retained 85 % activity at 55°C. Few metal ions like Ca^{+2} and Mg^{+2}enhanced the activity whereas Fe^{+3}, $Hg^{+2}$and Ag^{+} declined the enzyme action. The enzyme was characterized by some inhibitors and solvents; amongst inhibitors, β – mercapto ethanol, SDS and urea had shown maximum inhibition where as amongst the solvents phenol and chloroform strongly repressed the activity.

Keywords: *Pichia anamola*, lipase, enzyme activity, production, relative activity.

INTRODUCTION

Lipases (EC 3.1.1.3) catalyze the hydrolysis of acyl glycerols tofatty acids, di-acyl glycerols, mono-acyl glycerols andglycerol and widely occur in bacteria [1,2,3], yeasts and fungi [4,5]. Among microbes, fungi including yeasts are extensively recognized as the best lipase sources and are used preferably for industrial applications because fungal enzymes are usually excreted extracellularly, facilitating extraction from the fermentation media. A large number of unicellular as well as filamentous fungi have been studied for lipase production [5,6]. *Aspergillus*, *Rhizopus* and some strains of yeast are well known lipase producer and suitable for use in many industrial applications [7,8]. Studies on conditions for the production of extra- cellular lipases by fungi show variations among different strains but the requirement of lipid carbon source is crucial for enzyme production. Hence, the technique of solid state fermentation (SSF) is exploited at industrial scale and involves the growth and metabolism of microorganisms on moist solids. This technique has many advantages *viz*. economy of space needed for fermentation, simplicity of fermentation media, less energy demand, lower capital and recurring expenditure [9,10]. Although, almost all literature on SSF refers to fungal systems but there are very few reports on lipase production in SSF by yeast to date [11,12]. Lipases active at highly acidic pH have not been reported so far from microbial sources. Therefore, the present study is standardized for lipase production by a selected yeast stain *Pichia anamola* and characterization of the enzyme with regard to thermo stability, pH stability and optimum temperature and pH conditions for reaction.

MATERIALS AND METHODS

Isolation of fungi from soil

A high lipolytic yeast *Pichia anamola* was isolated from oil rich samples collected from different niches of Rajasthan. The pure culture of yeast was maintained on GYPA medium at 28±1 °C whereas one set of culture was maintained at 4 °C in refrigerator for further use.

Enzyme assay

Crude enzyme extract was prepared from the culture supernatant which was centrifuged at 10,000 rpm for 15 min at 4˚C. Lipase activity was measured spectrophotometrically using p- nitrophenyl acetate (pNPA) as a substrate at 45°C in 100 mM phosphate buffer of pH 7.0[13]. The substrate for this reaction was composed of solution A and B. Solution A contained 40 mg of p-nitro phenyl actetate dissolved in 12 ml of isopropanol, solution B contained 0.1 g of gum arabic and 0.4 ml of triton X-100 dissolved in 90 ml of distilled water. The substrate solution was prepared by adding 1 ml of solution A and 19 ml of solution B. The assay mixture contained 1 ml of the substrate, 0.5 ml of buffer, 0.1 ml of enzyme and final volume was made up to 3 ml with distilled water. The enzyme activity was stopped by adding 0.2 ml isopropanol and liberation of *p*-nitrophenol at 45 °C was detected in spectrophotometer at 400 nm. One enzyme unit was defined as 1 µ mol of p-nitrophenol enzymatically released from the substrate per minute [1].

Effect of various parameters on lipase activity

Effect of incubation period

Enzyme production was observed from 1st to 10th days of incubation and the activity was determined by similar protocol as described above. 0.1 ml of crude enzyme extract added in substrate solution, incubated at 28 °C for 30 minutes and lipase activity was measured spectrophotometrically against control.

Effect of temperature

To ascertain the optimum temperature for the enzyme activity, the assay mixture was incubated in the temperature range of 5°C to 75°C (5 °C, 15 °C, 25 °C, 35 °C, 45 °C, 55 °C, 65 °C and 75 °C) for 30 minutes and assayed for the lipase activity.

Effect of pH on lipase activity and stability

The titrimetric assay of lipase produced by the test strain was performed according to the method of Kamimura *et al.* [14]. Lipase activity was measured with 0.05 M NaOH using emulsified olive oil as the substrate. One ml of culture supernatant, treated as crude enzyme was added to 5 ml of oil emulsion containing 25 % (v/v) olive oil, 75 % (v/v) gum arabic and 2 ml of 0.5 M phosphate buffer at pH 7.0. The assay was carried out at 45°C during 30 min incubation. After this time interval the reaction was stopped by addition of 15 ml of acetone/ ethanol (1:1 v/v) and the amount of fatty acids was then titrated. 1 ml of the titration volume is equal to 2.5 units of lipase.

Effect of metal ions on lipase activity

For determining the effect of different metal ions on lipase activity, the enzyme was incubated with different metal ions (concentration 50 µ g/ml each) *viz.* Mn^{+2} ($MnCl_2$), Cu^{+2} ($CuSO_4$), Zn^{+2} ($ZnSO_4$), Ba^{+2} ($BaCl_2.5H_2O$), Fe^{+2}($FeCl_3$), Hg^{+2} ($HgCl_2$), Ca^{+2} ($CaCl_2.6H_2O$), Co^{+2} ($CoCl_2.6H_2O$), Mg^{+2} ($MgCl_2.6H_2O$), NH_4^+ (NH_4NO_3 anhy.) and Ag^+ ($AgNO_3$) for 30 min at 45 °C under standard assay conditions.

Effect of inhibitors on lipase activity

Different inhibitors *viz*; Urea, H_2O_2, sodium nitrite, sodium hypochlorite, sodium bisulphate, EDTA, DTT, SDS, β- mercapto ethanol, DMF and PMSF were used at concentration of 50 µ g and activity was determined incubating the assay mixture at 45 °C for 30 min (pH 7.0) under standard assay conditions.

Effect of solvents on lipase activity

The effect of different solvents on lipase activity was determined by incubating the assay mixture with acetone, aniline, benzene, chloroform, ether, iso propanol, ethanol, glycerol, phenol, titran X- 100 and toluene (concentration 50 µl/ml each) individually for 30 min at 45 °C under standard assay conditions.

RESULTS AND DISCUSSION

From the Table-1, it is revealed that the production of enzyme increased during cell growth of *Pichia anamola* and reached maximum (236.2 U/ml) on 5th day of incubation in batch culture, after that the production was decreased with increase in incubation period. Gumienna *et al.* [16] reported similar observation while studying the lipase production from *Candida bombicola* ATCC 22214. In contrary to this Kim *et al.* [4] reported optimum production from *Pichia lynferdii* on 4 days of incubation. The optimum temperature for lipase production by this organism was determined and tested at the range of 20 to 65 ˚C (Table -2). It was observed that 35 ˚C was the optimum incubation temperature. Imandi and Garapati [17] reported maximum yield of enzyme incubating the organism at 30°C while studying the lipase production from *Yarrowia lipolytica* NCIM 3589.

Table 1: Effect of incubation period on lipase production by *Pichia anamola*

Incubation Period (Days)	1	2	3	4	5	6	7	8	9	10
Lipase activity (U/ml)	29.7	62.3	139.8	167.5	236.2	221.1	192.5	157.5	129.3	124.1

Effect of pH and temperature

The pH plays crucial role for the detection of enzymatic activity. The study of the effect of pH on enzymatic activity provides valuable clues regarding the type and identity of amino acids present in the enzyme. Enzymes are affected by changes in pH and show maximum activity at a specified pH. Extremely high or low pH values generally result in complete loss of activity for most of enzyme. In this study, highest activity at pH 7.0 was recorded (Table-3) and as the pH increased beyond 7.0, the activity was dropped. Korbekandi *et al*. [18] and Kim *et al*. [4] both reported optimum pH 7 while working on *Candida rugosa* and *Pichia lynferdii* respectively.

The crude enzyme shown maximum activity at 45°C and retained 85 % of its activity at 55°C (Table- 4). This shows that the lipase produced by this organism is thermo tolerant. Therefore, this enzyme can be exploited for detergent and food processing industries. Similar observations were recorded by Deive *et al*. [19] and Jatta *et al*. [20] while working on *Kluyveromyces marxianus* and *Candida albicans* respectively.

Table 2: Effect of temperature on lipase production by *Pichia anamola*

Temperature (°C)	20	28	35	45	55	65
Lipase activity (U/ml)	204.5	278.2	318.6	224.2	208.3	168.3

Table 3: Effect of pH on lipase activity of *Pichia anamola*

Ph	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	9.0	10	11	12
Lipase activity (U/ml)	73.5	89.6	160.5	193.8	217.5	250.7	277.3	304.8	289.2	227.6	179.6	153.5	117.2	79.6

Table 4: Effect of temperature on lipase activity of *Pichia anamola*

Temperature (°C)	5	15	25	35	45	55	65	75
Lipase activity (U/ml)	86.5	124.1	184.7	274.4	307.5	262.1	148.3	72.3

Effect of various metal ions, inhibitors and solvents

Besides coenzymes, certain enzymes require a metal ion for their full activity. As reported from studies on this isolate at the concentration of 50 µ g/ml of some metal ions could affect the enzyme activity. Ba^{+2}, Ca^{+2} and Mg^{+2} enhanced the activity of enzyme. Whereas metal ions Fe^{+3}, Mn^{+2}, Zn^{+2}, Cu^{+2} and Hg^{+2} showed above 44-60 % relative activity (Table- 5). Hg^{+2} showed the strongest inhibitory effect (44.1 % relative activity). Kakugawa *et al*. [21] characterizing lipase of *Kurtzmanomyces* sp. reported 19 % and 46 % relative activity with Fe^{+2} and Al^{+2} using metal ion concentration of 50 µg/ml.

Table 5: Effect of different metal ions on lipase activity of *Pichia anamola*

Metal ions	Control	Mn^{2+}	Cu^{2+}	Zn^{2+}	Ba^{2+}	Fe^{2+}	Hg^{2+}	Ca^{2+}	Co^{2+}	Mg^{2+}	NH_4^{+}	Ag^{+}
Relative activity (%)	100	74.5	66.9	73.9	111.7	58.5	44.1	121.6	91.8	121.9	88.2	32.8

A total of eleven inhibitors were studied with crude enzyme extract at the concentration of 50 individually in enzyme assay. β-mercapto ethanol (0.7 %) strongly inhibited the enzyme activity followed by SDS (19.5 %), other inhibitors showed 20-60% relative enzyme activity (Table- 6). In contrary to this Balaji and Ebenezer [10] reported that SDS strongly reduces the enzyme activity while working on extracellular lipase produced from *Colletotrichum gloeosporiodes*.

Amongst 11 solvents, phenol and choloform (concentration 50µ l/ml each) showed greater inhibitory effect but acetone, iso- propanol and ethanol partially inhibited the enzyme activity (Table-7). The enhancement of lipase activity via these solvents have many applications as in oil based fuel manufacturing [22], plastic and chemical manufacturing, biodegradation, pulp and paper industries, dairy and food industries [11]. Devie *et al*. [19] reported that lipase produced by *Kluyveromyces marxianus* shown 70 % of its residual activity after 2 days in solvent combination of *n*-hexane and cyclohexane (80 % v/v). Kakugawa *et al*. [21] reported stimulation of *Kurtzmanomyces* sp. lipase activity in the presence of 50 % isobutanol, xylene, benzene and toluene however they reported dramatically reduced enzyme activity in the presence of methanol, chloroformand dimethylsulfoxide (DMSO).

Table 6: Effect of different inhibitors on lipase activity of *Pichia anamola*

Inhibitors	Control	A	B	C	D	E	F	G	H	I	J	K
Relative activity (%)	100	29.2	49.5	48.4	52.7	44.5	49.6	57.3	19.5	0.7	37.9	17.6

Whereas; A= Urea; B=H_2O_2; C= Sodium nitrite; D=Sodium hypochlorite; E=Sodium bisulphate;F=EDTA; G=DTT; H=SDS; I= β -mercapto ethanol; J=DMF and K= PMSF

Table 7: Effect of different solvents on lipase activity of *Pichia anamola*

Solvents	Control	A	B	C	D	E	F	G	H	I	J	K
Relative activity (%)	100	79.6	72.8	74.5	41.8	69.8	74.8	71.8	54.7	00.0	54.5	77.1

Whereas, A = Acetone; B= Aniline; C= Benzene; D= Chloroform; E= Ether; F= Iso propanol; G= Ethanol; H= Glycerol; I = Phenol; J= Titran X - 100 and K= Toluene

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