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Biosynthesis of lipases through solid substrate fermentation technique using *Penicillium* sp. isolated from pickle (MBL) 40

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Abstract

Lipases are important enzymes because of their various applications in different industries. Fungal lipases are used in many industries like Vitamins, amino acids fermentation, organic salts. Lipases have their own pH different from acids. The present study is concerned with production of lipase from locally isolated *Penicillium* sp. from pickle. Lipase showed its maximum activity (98.7±0.42U/ml) with teramera at 10%(v/w) inoculums after 72h of fermentation at the temperature of 30°C by using tap water as moistening agent when pH was maintained at 9 with 10g of substrate and 1% ammonium sulphate used as nitrogen source

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Introduction

Lipase is important enzymes because of their various applications in different industries (Banjamin and Panday, 1998). Fungal lipases are used in many industries like Vitamins, amino acids fermentation, organic salts. Lipases have their own pH different from acids (Nadia *et al*., 2010). Fungi which are preferably used for lipase production are *Penicillium* sp *Rhizopus* sp *Aspergillus* sp *Fusarium* sp. (Gracheva *et al*., 1980, Rajoka *et al*., 2006). *Penicillium* sp is used at industrial level for production of liapase and other enzymatic process (Hillenga *et al.,* 1995). The cold and pH tolerant isolates of *Penicillium* spp has been reported to produce cold active lipases (Pandey *et al.,* 2016*)*. Both solid state fermentation technique and submerged fermentation technique have been used for lipase production but solid state fermentation is better than submerged fermentation (lwashita, 2002). Lipases have special contribution in waste management as they have biodegradable properties with no harmful residues.

They are also used in glycerol's synthesis and splitting of fats. Lipase has low energy requirements and can produce high quality products (Haq *et al*., 2001). Pakistan imports lipase from other countries to fulfill its commercial needs and it requires large amount of money. Although researchers are working but the production of lipase is not increased in Pakistan as required. In this work, we have produced lipase from locally isolated *Penicillium* sp which will open new ways of progressing fermentation in biotechnological industries.

Materials and methods

Micro-organism

A lipase producing fungal strain of *Penicillium* sp. was obtained from Laboratory of Mycology and Biotechnology, Department of Botany, Government College University Faisalabad. The culture was maintained on the slants of potato dextrose agar (PDA) and was stored at 4°C and revived once every fortnight. In the present studies spores from 4-6 days old culture were used.

Solid state fermentation technique

10g of each substrate was taken in 250 ml flasks, cotton plugged, and moistened each with 10 ml of distilled water and sterilized them. The flasks were inoculated with 1.0 ml of fungal inoculum under sterilized conditions which were incubated at 30 °C for 72h. All the experiments were carried out in triplicates.

Enzyme assay

After 72 h of incubation 100 ml of phosphate buffer solution was added in each fermented flask, and then were shaken for 1h at 200 rpm. The contents of the flask were filtered by Whattman filter no.1 paper and the filtrate thus obtained was used for lipase estimation. 1ml of filtrate was taken in a flask and 10ml of 10% olive oil into 10% gum acacia, 2ml of 0.6% $CaCl_2$, 5 ml of 1MPhophate buffer was added in it and then was shaken for 1h at 200 rpm. 20 ml of alcohol: acetone (1:1) mixture was added to the reaction mixture to stop the ongoing reaction between assay substrate and enzyme. Then Lipase activity was determined by titration method. Titration of the liberated fatty acids was carried out against 0.1N NaOH using phenolphthalein as an indicator. The end point was light pink color. A lipase unit is defined as an amount of enzyme which can release one micromole fatty acid under specific assay condition (Kundu and Pal, 1970). The extra cellular lipase activity is expressed as unit per ml (U/ml).

The units were calculated by following formula.

$$Lipase\ \ Activity\ \ = \frac{\Delta V \times N}{V_{(Sample)}} \times \frac{1000}{60}$$

$\Delta V = V_2 - V_1$

V_1= Volume of NaOH used against control flask.

V_2= Volume of NaOH used against experimental flask.

N= Normality of NaOH.

$V_{(Sample)}$ =Volume of enzyme extract.

Specific Activity= $\frac{\text{Lipase Activity}}{\text{Total Protein (mg/ml)}}$

The calculations of production of lipase units are made by using following formula

$\Delta V = V2 - V1$

$U/ml = \frac{\Delta V \times N \times 1000}{1ml \times 60}$

The protein contents were measured by the method of Bradford (1976) using Bovine serum Albumin solution (BSA) as standard.

Optimization of cultural conditions

Firstly, oily substrate such as almond meal, Soybean meal, *Brassica* meal; Coconut meal, til meal, teramera and khaskhas meal were screened for optimizing production of lipase. Various quantities of substrate including 5g, 10g, 15g, 20g, 25g and 30g were used for maximum production of lipase. The effect of different inoculum size including 5%, 10%, 15% 20%, 25%, 30% (v\w) were screened for the maximum production of lipase. The effect of different temperature was studied. Moreover the different time period was optimized for production of lipase. Then the effect of different pH (3, 5, 7, 9, and 11) was checked for maximum production of lipase. Similarly the different moistening agents were used for example tap water, distilled water, acetate buffer and phosphate buffer. Different carbon and nitrogen sources were also checked for maximum production of lipase.

Statistical analysis

The data collected were subjected to ANOVA for the analysis of variance by using Costat software, where the difference between means was significant ($p \leq 0.05$), the mean values were compared using the LSD (least significant difference).

Results and discussions

In the present studies biosynthesis of lipases was conducted by *Penicillium* sp. *Penicillium* sp. has been reported for the production of various industrially important enzymes as in case of lipase, it hydrolyzes the triacylglycerols (Kondo *et al.*, 1997).

Table 1. Selection of substrate for the production of extracellular lipase by *Penicillium* sp. throughsolid substrate fermentation technique**.**

SL	Quantity of substrates (gm)	Lipase activity (U\mL)	Proteins (mg\mL)	Specific activity (U/mg)
1	Teramera	$77.26 \pm 2.00.73^{a}$	0.44 ± 0.11^{e}	33.03
2	Til meal	52.34 ± 0.6^{b}	0.96 ± 0.03^{a}	12.65
3	Coconut meal	39.6 ± 0.58^{c}	0.5 ± 0.05^{d}	18.64
4	Brassica meal	31.51 ± 0.50^{d}	0.3 ± 0.03^{f}	33.03
5	Khaskhash	20.49 ± 0.94^{e}	0.83 ± 0.05^{b}	11.57
6	Almond meal	11.14 ± 0.6^{f}	0.72 ± 0.07^{c}	12.1433
	LSD	1.3	0.11	

Every value use in triplicates.

The production of secondary metabolites like lipase from *Penicillium* sp. can be increased or decreased by altering eco-cultural conditions i.e. effect of different temperatures, change in pH level, different quantities of substrates and moistening level. Effect of pH and temperature are more important (Pommerville, 2001).

Table 2. Effect of quantity of substrate on production of extracellular lipase by *Penicillium* sp. through solid substrate fermentation technique.

SL	Quantity of substrate(g)	Lipase Activity (U\mL)	Proteins (mg\mL)	Specific activity (U/mg)
1	5	49.5 ± 0.7^{d}	0.5 ± 0.04^{de}	22.08
2	10	76.96 ± 0.8^{a}	$0.4 \pm 0.1e$	38.23
3	15	67.56 ± 0.58^{b}	0.6 ± 0.05^{cd}	23.42
4	20	52.27 ± 0.74^{c}	0.7 ± 0.04^{bc}	18.01
5	25	32.86 ± 0.41^{e}	0.8 ± 0.12^{b}	13.35
6	30	15.1 ± 0.6^{f}	1.0 ± 0.01^{a}	9.77
	LSD	1.2	0.13	

Every value use in triplicates.

Selection of substrate

Six different substrates including taramera meal, almond meal, soybean meal, coconut meal, khaskhas meal and til meal were tested for the production of extracellular lipases (Table 1). Taramera meal gave highest extracellular enzyme activity (77.26±2 U/ml) while almond meal was found to show least production of extracellular lipase activity (11.14U/ml). Taramera gave maximum production of lipase as it fulfill the nutritional needs of the organism while other substrates may not fulfill these needs.

Table 3. Effect of inoculum size on production of lipase by *Penicillium sp*, through solid substrate fermentation technique.

SL	Inoculum size (mL)	Lipase activity (U\mL)	Proteins (mg\mL)	Specific activity (U/mg)
1	0.5	42.43±0.48^{e}	0.4±0.1^{e}	26.11
2	1	77.26±0.46^{a}	0.3±0.01^{f}	56.92
3	1.5	68.11±0.10^{b}	0.5±0.05^{d}	26.69
4	2	55.6±0.52^{c}	0.6±0.5c^{d}	18.0126.24
5	2.5	50.8±0.72^{d}	0.7±0.08^{c}	13.2517.23
6	3	33.4±0.46^{f}	0.8±0.04^{b}	9.7716.43
7	3.5	13.28±4.98^{g}	0.9±0.005^{a}	9.87
	LSD	3.5	0.11	

Every value use in triplicates.

Effect of different quantities of substrates

Effect of different quantity of substrates such as 5g, 10g, 15g, 20g, 25g 30g, and 35g were tested for maximum extracellular lipase production by *Penicillium* sp. Maximum extracellular (76.98±0.8U/ml) lipase production was obtain with 10g of substrate (table 2). The level above or below did not support the production of enzyme. Perhaps it is due to the reason of less porosity and decrease in moistening level.

Table 4. Effect of incubation period on the production of lipase by *Penicillium* sp, through solid state fermentation technique.

SL	Time of incubation	Lipase activity (U\mL)	Proteins (mg\mL)	Specific activity (U/mg)
1	24	56.6±0.52^{c}	0.972±0.04^{a}	13.61
2	48	68.53±0.50^{b}	0.8±0.01^{b}	17.34
3	72	77.2±0.52^{a}	0.57±0.04^{d}	29.94
4	96	53.33+1.13^{d}	0.59±5.77^{d}	24.88
5	120	48.63±0.60^{e}	0.47±0.01^{e}	24.19
6	144	45.3±0.43^{f}	0.66±0.01^{c}	15.71
7	168	42.56±0.51^{g}	0.36±0.03^{f}	25.71
	LSD	1.21	0.04	

Every value use in triplicates.

Effect of inoculum

Table 3 shows the effect of different inoculum levels on the lipase production. Different inoculum sizes ranging 0.5ml– 3.5 ml were tested for maximum lipase production by *Penicillium sp* and maximum lipase production (77.26±0.26U/ml) was obtained with 1.0 ml inoculum. It might provide optimum amount of mycelium. Inoculum of 1.0 ml concentration for maximum lipase production has been reported by Ushio *et al.*, 1996.

Effect of incubation time

The samples were incubated at different time intervals as 24, 48, 72, 96, 120, 144 and 168 h (Table 4).

After 72 h of incubation. *Penicillium* sp showed maximum extracellular activity (77.2±0.52U/ml) of lipase. With increase of incubation period there was decrease in lipase production. It might be due to the collapse of nutrients in substrate in long period of time, moisture is low and due to low moisture the action of enzyme is also low (Kamini *et al.,* 1998; Edwinoliver *et al.,* 2009).

Table 5. Effect of incubation temperatures on extracellular lipase production by *Penicillium* sp. Through solid substrate fermentation.

SL	Temperature (c)	Lipase activity (U\mL)	Proteins (mg\mL)	Specific activity (U/mg)
1	20	54.4±0.56^{c}	0.659±0.00^{c}	22.0
2	30	77.0±0.41^{a}	0.50±0.02^{d}	40.05
3	40	67.95±0.070^{b}	0.80±0.10^{b}	19.47
4	50	49.9±0.14^{d}	0.99±0.00^{a}	17.60
5	60	44±0^{e}	0.76±0.02^{bc}	16.086
6	70	40.85±0.21^{f}	0.88±0.01^{ab}	11.14
	LSD	0.73	0.12	

Every value use in triplicates.

Effect of incubation temperature

Different incubation temperatures including 20°C, 30°C, 40°C, 50°C and 60°C were tested for extracellular lipase production. Maximum extracellular lipase activity (77±0.41 U/ml) was achieved at 30°C (Table 5). Temperature plays an important role in the metabolic processes of an organism. Increasing temperature increased the rate of all physiological processes but when it increases the limit it denatures the enzyme and hence productivity decrease. In the present study, lipase showed maximum activity at 30°C. This temperature is considered as optimum temperature for activity of lipase (Silva *et al*., 2005). The optimum growth temperature for lipase production in this study is in line with the findings of other workers (Iftikhar *et al.,* 2010).

Table 6. Effect of different moistening agents on the production of lipase by *Penicillium* sp. through solid substrate fermentation technique.

SL	Moistening agents	Lipase Activity (U\mL)	Proteins (mg\mL)	Specific activity (U/mg)
1	Tap water	90.13±0.321^{a}	0.49±0.03^{c}	36.30
2	P buffer	77.16±0.28^{b}	0.59±0.04^{b}	28.74
3	Distilled water	60.06±0.40^{c}	0.34±0.04^{d}	45.49
4	A buffer	54.6±0.5^{d}	0.67±0.01^{a}	21.14
	LSD	0.48	0.050	

Every value use in triplicates.

Effect of different moistening agents

Table 6 shows the effect of various types of moistening agents such as tap water, distilled water, phosphate buffer and AC water on the production of lipase by *Penicillium* sp. Maximum extracellular lipase activity (90.13U/ml) was obtained in case of tap water. The enzyme exhibited hyper production by using tap water as it is a proper source for its action.

Effect of pH of diluents on lipase production

Table 7 show the effect of different of pH such as 3, 5, 7, 9, and 11 on the production of lipase by *Penicillium* sp. Maximum extracellular enzyme production (89.86±0.21U/ml) was obtained in case of 9 pH. Enzyme action decrease when pH level increases or decreases from optimum pH level. In acidic pH the majority of enzymes showed maximum activity. The upper and lower pH from this limit did not support the maximum production of enzyme (Yaqoob *et al*., 1999). It seems that enzyme is stable at this pH.

Effect of different carbon sources

Table 8 shows the effect of various carbon sources such as lactose, sucrose, glucose, dextrose and galactose on the production of lipase by *Penicillium* sp. and maximum extracellular lipase activity (98.9±0.14U/ml) was obtained in case of lactose.

Table 7. Effect of varying pH of diluents on the production of extracellular lipase by *Penicillium*sp, through solid state fermentation technique.

SL	pH of diluents	Lipase Activity (U\mL)	Proteins (mg\mL)	Specific activity (U/mg)
1	3	54.35±0.49^{e}	0.54±0.03^{d}	23.16
2	5	66.5±0.0.70^{d}	0.43±0.01^{d}	36.73
3	7	68.8±0.28^{c}	0.65±0.00^{c}	21.66
4	9	89.85±0.21^{a}	0.77±0.01^{b}	14.72
5	11	77.06±0.8^{b}	0.94±0.07^{a}	10.91
	LSD	1.12	0.11	

Every value use in triplicates.

Effect of nitrogen sources

Table 9 shows the effect of different nitrogen sources such as $(NH_4)_2SO_4$, Urea, trypton, soybean and yeast extract on the production of lipase by *Penicillium* sp. The maximum extracellular lipase activity (98.7±0.42U/ml) was obtained with $(NH_4)_2SO_4$. This result is in line with the previous findings (Iftikhar and Hussain, 2002).

Table 8. Effect of different carbon sources on the production of lipase by*Penicillium*sp, through solid state fermentation technique.

SL	Carbon sources 1%	Lipase Activity (U\mL)	Proteins (mg\mL)	Specific activity (U/mg)
1	Lactose	98.9+0.14^{a}	0.45±0.00^{e}	31.96
2	Sucrose	80.5±0.70^{b}	0.64±0.03^{d}	17.66
3	Dextrose	75.5±0.70^{c}	0.99±0.00^{a}	9.98
4	Galactose	73.02±0.0.28^{d}	0.85±0.02^{b}	15.47
5	Glucose	67.9±0.14^{e}	0.74±7.07^{c}	16.21
	LSD	0.92	0.03	

Every value use in triplicates.

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