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Original Research Article

Isolation of a lipase producing bacteria for enzyme synthesis in shake flask cultivation

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K e y w o r d s

Olive oil; Lipase; Submerged fermentation; Coconut cake filtrate; *Bacillus sp.*

A B S T R A C T

The present investigation describes the attempt of obtaining potential lipase producing isolate from oil mill soil. One of the strains, identified as *Bacillus* sp. (SRBIT-05) through microscopic observation was predominately studied for the lipase producing ability in submerged fermentation. The production was carried out using media containing heterogeneous specific substrates and sole specific substrate, in which sole specific substrate (olive oil) shown highest production (8.75 U/ml) at 36 hours. Coconut cake filtrate extract too yielded better lipase titer when used as sole specific substrate. Presence of surfactant was favoring better lipase activity. Biomass accumulation by the isolate (*Bacillus* sp.) was more pronounced when coconut cake filtrate was used as specific substrate, indicating that lipase production by *Bacillus* sp., is not growth associated. pH of the medium recorded an increase during the progress of fermentation and 36 hours of fermentation ensured maximum release of enzyme activity.

Introduction

Lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at an oil–water interface (Kamini *et al*. 2000). Lipases can also transesterify triacylglycerols or synthesize ester bonds in non-aqueous media (Macrae and Hammond 1985; Bornscheuer1995). The many applications of lipases include organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures and chemical analyses (Sharma *et al.* 2001). However, the major contributions of microbial lipases are in the detergent formulations. The main reason for the steadily growing interest in lipases is

because of their enantio-selective, regio-selective and chemo-selective nature (Vulfson *et al.* 1994). Microorganisms have been reported as potential lipase producers exhibiting the capability to utilizing most agricultural residues (Salihu *et al.* 2012). Among the agricultural residues, oil cakes stand as the most widely utilized substrate (Singh *et al.* 2010). Olive-mill wastewater also was used as a growth medium for lipase production (Brozzoli *et al.* 2009). The production of microbial lipases is generally induced by fat-related carbon sources (Breuil *et al.* 1978). Recently, several works have showed that Tween 80 is a suitable carbon source for production of microbial lipases (Dalmau *et al.* 2000). The inducers of lipases synthesis are many, wheat bran, rice bran, dextrins, sugarcane bagasse, coconut cake, olive oil cake, and gingley oil cake constitute a few of such successful inducers (Christen *et al.* 1993; Kamini *et al.* 1998). The purpose of the present study is bio-prospection of lipase producing microbes from oil mill soil aiming at the attainment (through submerged or solid state fermentation) of a lipase formulation for industrial applications. The better enzyme producing bacterial isolate for its ability to produce the enzyme was investigated under diverged nutrient conditions using submerged batch cultivation.

Materials and Methods

Materials

All the chemicals used were of analytical grade and procured from Renkem, New Delhi, Himedia, Mumbai and Merck, Mumbai, India. Soil samples were collected from around a local oil mill in Sathyamangalam, Tamilnadu, India.

Isolation of microbes producing lipase:

Soil samples transported to laboratory under sterile conditions were serially diluted up to 10^4 dilutions and plated in MYDP medium (yeast extract- 0.2 %, peptone-0.3%, malt extract-0.6%, glucose-0.05%, agar- 2% and pH- 6.3 0.2). The plates were incubated at 30°C for 24 hours in order to isolate individual colonies and test the same for their ability to produce lipase enzyme.

Culture maintenance

The selected isolates were grown and maintained in agar slants made of Groundnut Cake Extract Agar (GNCEA) medium (yeast extract-1%, peptone-1%, glucose- 0.3%, NaCl- 0.25%, KH_2PO_4-0.25%, agar-2% and pH- 6.5 0.2) and stored at 4°C for further use. This medium was chosen for selectively isolating colonies capable of growing on a lipid enriched medium.

Screening of Lipase producing microbial isolate through Submerged Fermentation (SmF)

For inoculum preparation, 2 loops of all the selected isolates were separately transferred from agar slants to 10 ml each of lipase production medium (yeast extract - 0.5%, KH_2PO_4-1%, $MgSO_4.7H_2O$-0.1%, Olive oil-1 % (v/v) and pH- 6.3 ± 0.2) held in test tubes. The culture tubes were incubated in rotary shaker at 120 rpm and 30°C for 24 hours. After 24 hours of fermentation, 5 ml each of the samples were collected for each isolate and they were centrifuged at 6000 rpm for 10 min and the corresponding supernatants were assayed for the enzyme activity.

Lipase assay

Lipase activity of cell less broths was determined by titrimetric method (using olive oil as substrate) (Yamada *et al.* 1962). One unit of lipase activity is defined as the amount of enzyme required to release 1 µmol of fatty acid at 37°C under standard assay conditions (pH 6.5, reaction time 20 min).

Microscopic observation

A thin smear of the better lipase producing microbial isolate was made on a clean glass slide which was later stained with safranin as simple stain. The smear after air drying was observed under the microscope at 100 X withimmersion oil.

Fermentation studies

Inoculum

For inoculum preparation, 2 loops of the selected isolate (SRBIT05) was transferred to 10 ml of MYDP broth medium (yeast extract- 0.2 %, peptone- 0.3%, malt extract- 0.6%, glucose- 0.05%, agar- 2% and pH- 6.3$\pm$ 0.2)held in test tubes and the same was incubated on a rotary shaker at 120 rpm and 30°C for 24 hours.

Production of lipase through SmF

The selected isolated culture was transferred (10% v/v) to 100 ml each of lipase production media (listed in table 1) and incubated for 96 hours at 30°C & 120 rpm. After every 24 hours of fermentation interval, 5 ml each of the samples were collected and centrifuged at 6000 rpm for 10 min at 4°C and the corresponding supernatants were assayed for the enzyme activity.

Analytical methods

Samples collected during fermentation were checked for pH of the cell free brothandthe same was examined by using digital pH meter. Biomass was measured as cell growth of different samples and was estimated for 5ml of samples. Samples werecentrifuged at 6000 rpm for 10 minutes (4°C). The supernatant were discarded and the pellet weight was reported as wet weight (g/ml) of cells.

Results and Discussion

Isolation and screening of microbial cultures capable of exhibiting lipase activity

The oil mill soil samples employed for isolating lipase producing cultures in lipid enriched agar (GNCEA medium) plates gavel even isolated colonies which were tested for their ability to produce lipase activity. Screening of these isolates carried out using typical lipase production medium gave one better isolate (6.75 U/ml of lipase activity) and this isolate was found to be *Bacillus* sp., by microscopic observation.

Production of lipase through SmF

During submerged cultivation of *Bacillus sp.*, in different fermentation media containing varying sources of nitrogen, carbon and inducers (table 1), maximum yield (8.75 U/ml) was observed at 36 hours in medium C1(Figure 1).The next highest production was in medium B1(8.5 U/ml).The presence of olive oil in both media appeared to have enhanced the production of lipase and this finding is corroborating the observations reported earlier (Sugihara *et al*., 1991). Additional presence of surfactant (tween-60) in the

medium C1 could have enhanced the inducibility of lipase production by *Bacillus sp.* Little enzyme activity was observed in the absence of olive oil even after prolonged cultivation (Sugihara *et al*., 1991). Lipase synthesis in the presence of palm oil at a concentration of 2% was found to yield 12-fold high titers than that obtained with fructose medium (Papaparaskevas *et al*., 1992). High production of lipase was recorded with specific activity of 7395 U/mg protein for alkaline lipase (pH 8.5) produced by *P. fluorescens* S1K WI in a medium which contained emulsified olive oil as the carbon source (Lee *et al*., 1993).There are also reports indicating higher lipase activities when vegetable oils (olive, soybean, sunflower, sesame, cotton seed, corn, and peanut oil) were used as the carbon source with maximum lipase production reported for olive oil incorporation (Sztajer *et al*., 1993, Essamri *et al*., 1998).

Several other studies confirm enhanced lipase production when oils were used as enzyme inducers. Lin *et al*. (1996) produced an alkaline lipase from *P. pseudoalcaligenes* F-111 in a medium that contained both olive oil (0.4%) and Triton X-100 (0.2%). The addition of Triton X-100 enhanced the alkaline lipase production by 50-fold compared to using olive oil alone. This is in line with our findings where we reported better lipase activity of 8.75 U/ml through Tween-60 incorporation in medium C1.

Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used with an exception reported by Papaparaskevas *et al*., (1992). Although good growth of *Rho.glutinis* obtained with organic nitrogen sources (e.g., yeast extract and tryptone), an inorganic nitrogen source such as ammonium phosphate yielded better lipase production (Papaparaskevas *et al*., 1992). A decrease in yeast extract concentration reduced the attainable lipase activity and replacement of the same with ammonium sulfate led to diminished lipase production (Pimentel *et al*., 1994).In our findings the production medium containing coconut cake filtrate supplemented with other supplements (Yeast extract, peptone, glucose, sodium chloride, potassium di-hydrogen phosphate) had not shown better production of lipase. The biomass production, on the contrary was prominently supported by coconut cake filtrate medium (A). Biomass yield of media without coconut cake filtrate (B1 & C1) was not on par with the biomass obtained in the presence of coconut cake filtrate (Figure 2). Whenever the heterogeneous substrates were used in the production medium (B & C), the lipase activity was not as pronounced as when olive oil alone used as substrate (B1 & C1) or coconut cake filter cake alone used as substrate (A). Olive oil as a sole specific substrate gave better lipase value (8.75 U/ml) than the medium containing heterogeneous substrates (5.5 U/ml). Our finding indicated that the single specific substrate incorporation is a preferred option for better lipase production. Among the two specific substrates tested, olive oil produced better enzyme titers. During the fermentation process there was slight increase in the pH from 6.8 to 8.2 detected during 96 hours (Figure 3). Similar repot was indicated were pH was increased from 6.5 to 8.8 in 96 hour of production of lipase using *P. camembertii*. When the pH was higher than 7.5, lipase production was reduced (Tan *et al*. 2004). Brozzoli *et al*. (2009) have reported an initially decline in pH from 6.5 to 4.3 and then increase in the pH up to 7.8 in 288 hours of fermentation

Table.1 Different lipase production media

S.No	Medium designation	Chemicals	Quantity used
1.	Production medium-**A** (pH -6.8± 0.2)	yeast extract	1
		peptone	1
		glucose	0.3
		NaCl	0.25
		KH_2PO_4	0.25
		coconut cake filtrate	10 (v/v)
2.	Production medium-**B** (pH - 6.8± 0.2)	yeast extract	0.5
		KH_2PO_4	1
		$MgSO_4.7H_2O$	0.1
		olive oil	1 (v/v)
		coconut cake filtrate	10 (v/v)
3.	Production medium-**C** (pH - 6.8± 0.2)	yeast extract	0.5
		KH_2PO_4	1
		$MgSO_4.7H_2O$	0.1
		olive oil	1 (v/v)
		tween-60	0.01(v/v)
		coconut cake filtrate	10 (v/v)
4.	Production medium-**B1** (pH - 6.8± 0.2)	yeast extract	0.5
		KH_2PO_4	1
		$MgSO_4.7H_2O$	0.1
		olive oil	1 (v/v)
5.	Production medium-**C1** (pH - 6.8± 0.2)	yeast extract	0.5
		KH_2PO_4	1
		$MgSO_4.7H_2O$	0.1
		olive oil	1 (v/v)
		tween-60	0.01 (v/v)

Figure.1 Estimated lipase titers produced by the selected microbial isolate (Bacillus sp.) in different growth media through SmF

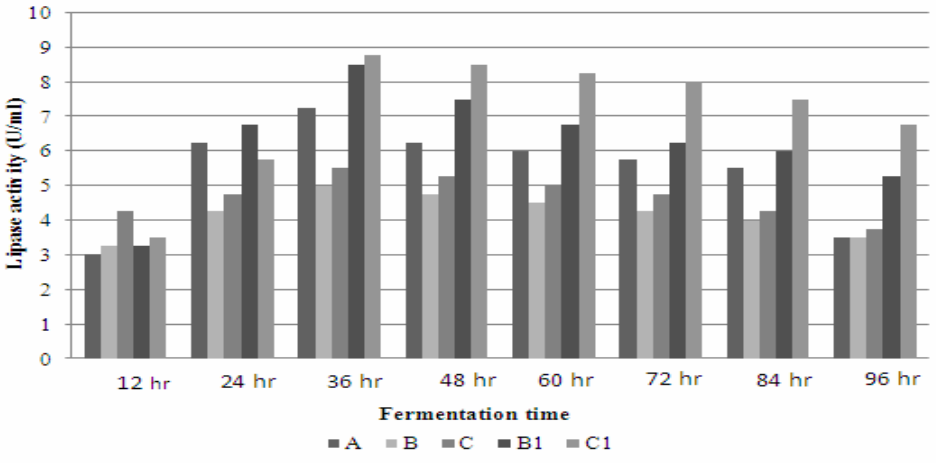


Figure.2 Change in Biomass concentration during the growth of microbial isolate on different medium by Smf

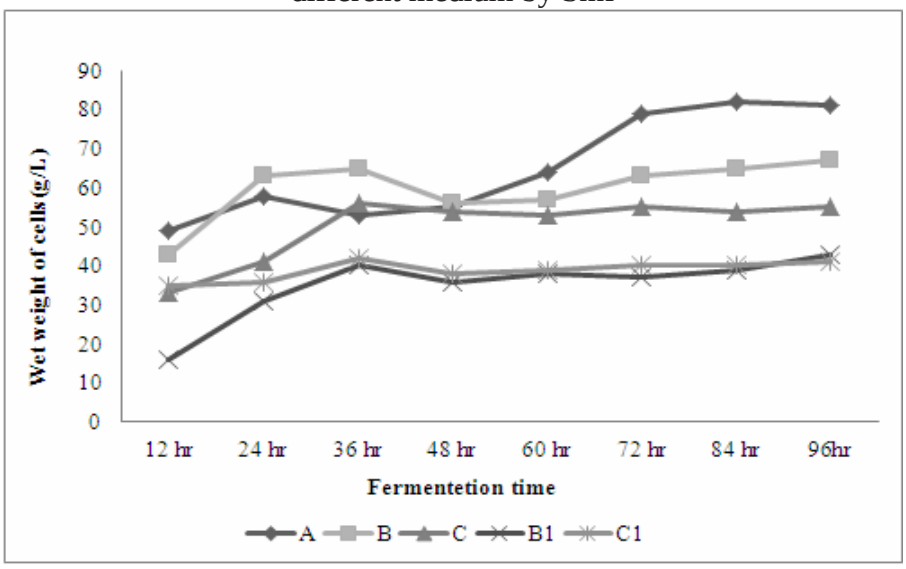
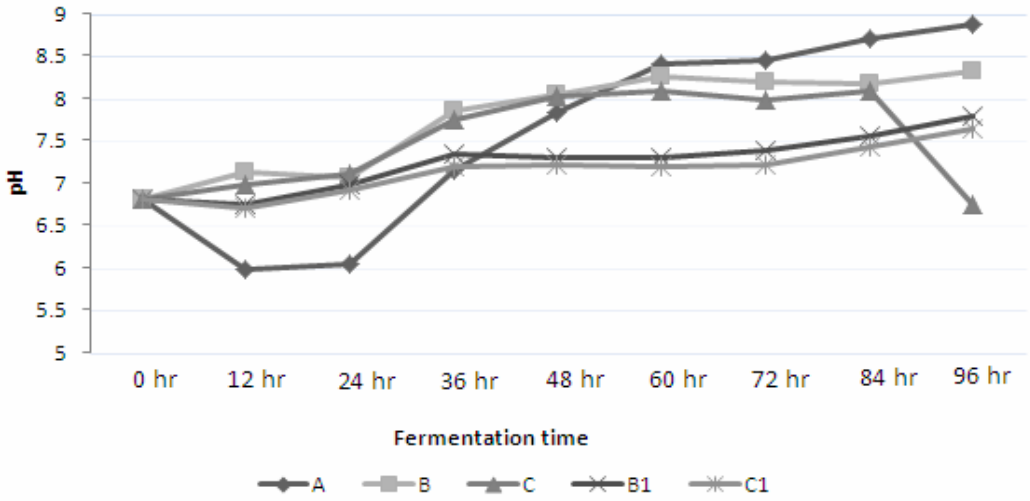


Figure.3 pH changes detected during lipase production by the selected microbial isolate in different growth media through SmF



for the lipase production by *C. cylindracea* in bench-top reactor using olive-mill wastewater as a growth medium. Contradicting report was indicated by Valero *et al.*, (1988) describing an initial steep decline from 6.1 to 3.2 in an olive oil containing medium.

Oil mill soil sample appeared to be good specific substrates incorporated separately, olive oil gave better results. Presence of source of lipase producing microbes. The short listed and selected isolate of *Bacillus* species gave better lipase activity during 36 hours of fermentation in submerged cultivation. Lipase production appeared to be induced better when single specific substrate is provided for enzyme production rather than heterogeneous substrate incorporation. Among the two surfactants favored better lipase production. From the findings we also

concluded that in about 36 hours, maximum secretion of lipase occurs in the medium.

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References

Bornscheuer, U.T. 1995. Lipase-catalyzed synthesis of monoglycerols.Enzyme Microbial Technology.17:578–86.

Breuil, C., D.B.Shindler, J.S.Sijher and D.J.Kushner.1978. Stimulation of lipase production during bacterial growth on alkanes. Journal of Bacteriology.133:601–606.

Brozzoli, V., S.Crognale, I.Sampedro, F.Federici, A.D'annibale and M.Petruccioli. 2009. Assessment of olive-mill wastewater as a growth medium for lipase production by *Candida cylindracea* in bench-top reactor. Journal of Bioresource Technology. 100:3395-3402.

Christen, P., R. Auria, C. Vega, E. Villegas and S. Revah. 1993. Growth of *Candida utilis* in solid-state fermentation. Biotechnology Advances.11:549–57.

Dalmau, E., J.L. Montesinos, M. Lotti andC. Casas. 2000. Effect of different carbon sources on lipase production by *Candida rugosa*. Enzyme Microbial Technology. 26:657–663.

Essamri, M., D. Valerie andC. Louis. 1998. Optimization of lipase production by *Rhizopusoryzae* and study on the stability of lipase activity in organic solvents. Journal of Biotechnology.60:97–103.

Kamini, N.R., T. Fujii, T. Kurosu andH. Iefuji. 2000. Production, purification and characterization of an extracellular lipase from the yeast, *Cryptococcus Sp*. S-2. Process Biochemistry.36:317-324.

Kamini, N.R., J.G.S. Mala andR. Puvanakrishnan. 1998. Lipase production from *Aspergillus niger* by solid-state fermentation using Gingelly oil cake.Process Biochemistry.33:505–11.

Lee, Y.P., G.H. Chung andJ.S. Rhee. 1993. Purification and characterization of *Pseudomonas fluorescens* SIK WI lipase expressed in *Escherichia coli*.BiochimicaetBiophysicaActa. 1169:156–64.

Lin, S.F., C.M. Chiou, C. Yeh and Y.C. Tsai. 1996. Purification and partial characterization of an alkaline lipase from *Pseudomonas pseudoalcaligenes* F-111. Applied Environmental Microbiology.62:1093–1095.

Macrae, A.R. andR.C. Hammond. 1985. Present and future applications of lipases. Journal of Biotechnology GeneticEngineering. 3:193–217.

Papaparaskevas, D., P. Christakopoulos, D. Kekos and B.J. Macris. 1992. Optimizing production of extracellular lipase from *Rhodotorulaglutinis*. Biotechnology Letters. 14:397–402.

Pimentel, M.C., N. Krieger,L.C. Coelho, J.O. Fontana, E.H. Melo, W. M. Ledingham andJ.L. Lima Filho. 1994. Lipase from a Brazilian strain of

Penicilliumcitrinum. Applied Biochemistry Biotechnology.49:59–74.

Salihu, A., Z.M. Alum,I.M. Abdulkarim andH.M. Salleh. 2012. Lipase production: An insight in the utilization of renewable agricultural residues. Resource Conservation and Recycling. 58:36-44.

Sharma, R., Y. Chisti andU.C. Banerjee. 2001. Production, purification, characterization, and applications of lipases. Biotechnology Advances.19:627-662.

Singh, M., K. Saurav, N. Srivastava and K. Kannabiran. 2010. Lipase Production by *Bacillus subtilis* OCR-4 in Solid State Fermentation Using Ground Nut Oil Cakes as Substrate. Current Research Journal of Biological Sciences. 2(4):241-245.

Sugihara, A., T. Tani andY. Tominaga. 1991. Purification and characterization of a novel thermostable lipase from *Bacillus Sp.*.Journal of Biochemistry. 109:211–216.

Sztajer, H., U. Menge and R. D. Schmid. 1993. Purification and properties of a lipase from *Penicilliumexpansum*. BiochimicaetBiophysicaActa. 1168:181–189.

Tan, T., M. Zhang, J. Xua, J. Zhang.1988. Optimization of culture conditions and properties of lipase from *Penicilliumcamembertii* Thom PG. Process Biochemistry.39:1495–1502.

Valero, F., F. Ayats, J. Lópezsantín,M. Poch. 1988. Lipase production by *Candidarugosa*: fermentation behaviour. Biotechnology Letters. 10:741–744.

Vulfson, E.N. 1994. Industrial applications of lipases. In: Woolley P, Petersen SB, editors. Lipases their structure, biochemistry and application. Cambridge, UK: Cambridge University Press,271–288.

Yamada, K., U. Ota, andH. Machinda. 1962. Studies on the production of lipase by microorganisms. Quantitative determination of lipase.Agricultural and Biological Chemistry, 26:63-69.

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