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ECOLOGICAL SCREENING OF LIPOLYTIC CULTURES AND PROCESS OPTIMIZATION FOR EXTRACELLULAR LIPASE PRODUCTION FROM FUNGAL HYPERPRODUCER

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Abstract

Present investigation describes the biosynthesis of extracellular lipases by various local fungal strains isolated from various lipid rich habitats of Faisalabad. The isolated cultures of *Aspergillus niger, Penicillium chrysogenum, Rhizopus microsporus, Mucor mucedo, Alternaria alternata, Trichophyton* sp., *Fusarium semitectum*, E (un-identified), *Curvularia* sp., *Aspergillus flavus*, G (un-identified), F (*Mucor* sp.) and H (Synnematous) were identified and screened for the extracelluler lipases production. Different environmental parameters such as pH, temperature, inoculum size, amount of substrate and incubation time were optimized for the selected hyper producer. It was found that maximum production of lipases by *Trichophyton* sp., was obtained after 48 h of batch fermentation. Similarly, the diluent pH of 7.0 and incubation temperature of 30°C were found optimum for enzyme production by the microorganism. The maximum production of lipases during the course of present studies was 65.20 ± 1.13^{a} U/g.

Introduction

Lipases are special kind of esterases characterized by its unique ability to act upon emulsified substrate and hydrolyze glycerides to free fatty acids and glycerol (Gilbert, 1993). Ester synthesis is carried out in aqueous media in the presence of various lipases (Lacointe *et al*., 1996). Lipases occur widely in nature, but only microbial lipases are commercially significant (Mark *et al*., 2001; Hsu *et al*., 2002). It is well known that lipases are the most widely used enzymes in organic synthesis and more than 20% biotransformations are performed with lipases (Gitlesen *et al*., 1997). In addition to their role in synthetic organic chemistry, these also find extensive applications in chemical, pharmaceutical, food and leather industries (Gulati *et al*., 2005; Gunstone, 1999). Fungi characterized by being ubiquitous in distribution are highly successful in survival because of their great plasticity and physiological versatility (Immanuel *et al.*, 2008; Iftikhar *et al.*, 2010a; Iftikhar *et al.*, 2010c). Fungi thrive well in habitats with environmental extremes because of their efficient enzyme systems. Among the varied mechanisms for fungi adaptability to environmental extremes and for the utilization of their trophic niche, their ability to produce extracellular enzyme is of great survival value (Gopinath *et al*., 2005).

Fats and oils are recognized as essential nutrients in both human and animal diet. Good health and life require dietary fats to provide a major source of energy, essential fatty acid, a vehicle for fat soluble vitamins and important components of cell membrane. Among all the microorganisms, fungi especially *Rhizopus* sp., *Mucor* sp., *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp., are preferable lipase sources. (Gracheva *et al*., 1980; Iftikhar & Hussain, 2002; Iftikhar *et al.*, 2003; Iftikhar *et al.*, 2007; Iftikhar *et al.*, 2008; Iftikhar,

2009). *Aspergillus niger* is among the well known lipase producer, mainly used in the in the industry (Pokorny *et al*., 1994; Undurraga *et al*., 2001. The present study is aimed to exploit local extreme conditions of Faisalabad for the isolation and screening of lipolytic fungal strains. It is further aimed to optimize the cultural conditions for the biosynthesis of extracellular lipases.

Materials and Methods

Isolation of microorganisms: Lipase producing fungal strains was isolated from various lipid rich habitats by serial dilution technique (Akano & Atanda, 1990). Tween 80-agar plates g/L (Peptone, 10.0; NaCl, 5.0; $CaCl_2.2H_2O$, 0.1; Tween 80, 10.0; Agar-agar'20.0; pH: 6.0) were used for the isolation of lipolytic fungi. Tween 80 was separately sterilized and added into the rest of the autoclaved medium and the pH was adjusted to 6.0 as reported by Gopinath *et al.,* (2005). About 10 mL medium was poured into each petri dish and inoculated. Lipolytic activity was indicated by the appearance of a visible precipitate, resulting from the deposition of crystals of the calcium salt formed by the fatty acid liberated by the enzyme, or as a clearing of such a precipitate around a colony due to complete degradation of the salt of the fatty acid. At regular intervals of 24 h incubation, each plate was examined and measurement of zones were taken to monitor lipolytic activity (Data not given).

Screening of lipolytic fungi: Isolated strains were screened for the biosynthesis of extracellular lipases by shake flask fermentation. The screening was carried out in 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium g/L (Soybean meal, 10.0; Olive oil, 20.0; Glucose, 10.0; K_2HPO_4, 2.0; $NaNO_3$, 0.5; $MgSO_4.7H_2O$, 0.5; pH 7.0). The strain showed the highest lipase activity was selected for further studies (Iftikhar *et al.,* 2010a).

Identification of selected strains: Microscope (MEIJI Model: ML2100) was calibrated and various measurements were taken for the identification after morphological examination of strains after Kirk (2009). Identifications were then confirmed by Prof. Dr. Syed Qaiser Abbas at the Mycology and Biotechnology Research Laboratory, Department of Botany, GC University, Faisalabad and assigned the codes accordingly (Ahmad, 1956; Ahmad, 1960 & Ahmad, 1962). The isolated fungal cultures were maintained on 4% potato dextrose agar (PDA) slants (Iftikhar *et al.,* 2003).

Substrates used: Different agricultural byproducts used in the present study such as brassica meal, almond meal, coconut meal, rice husk and wheat bran was obtained from the local market.

Fermentation technique: Production of fungal lipases was studied through solid state fermentation (Korn & Fujio, 1997). Ten grams of substrate with 7 mL of diluent (distilled water) was added in 250 mL cotton wool plugged conical flask. The flasks were autoclaved at 15-lb/inch^2 pressure (121°C) for 15 minutes and cooled at room temperature. One mL of the spore suspension prepared in monoxal O.T (Di-octylester of sulfosuccinic acid) was aseptically transferred to each cotton wool plugged conical flask and flasks were then placed in an incubator at 30 ± 2°C for 48 hours (Iftikhar *et al.,* 2010b). The flasks were run parallel in triplicate.

Buffer preparation: Buffers of various pH were prepared in the required composition, after consulting the web site (http://delloyd.50megs.com/moreinfo/buffers2.html#acetate).

Extraction of enzyme: After 48 hours 100 mL of phosphate buffer (pH 7.0) was added to each flask. The flasks were rotated on the rotary shaker at 150 rpm for one hour at 30°C. After one hour the ingredients of the flask were filtered and filtrate was used for estimation of lipase activity. Lipase activity in the fermented meal was determined spectrophotometrically as reported by Iftikhar *et al.,* 2010a.

Statistical study: All the experimental data were analysed by co-stat software.

Results and Discussion

In the present study out of 50 fungal cultures observed, only 13 fungi showed good extracellular lipase activity, which were then identified as *Aspergillus niger*, *Penicillium chrysogenum*, *Rhizopus microsporus*, *Mucor mucedo*., *Alternaria alternata*, *Trichophyton* sp, *Fusarium semitectum*, E (un-identified), *Curvularia* sp, *Aspergillus flavus*, G (un-identified), F (*Mucor* sp.) and H (Synnematous) and was confirmed by Prof. Dr. Syed Qaiser Abbas, Mycology and Biotechnology Research Laboratory, Department of Botany, GC University, Faisalabad. Few of the cultures remain unidentified due to the lack of literature. Selected and identified culture was then used for further studies (Table 1). Out of all the fungi examined, one of the culture showed good extracellular lipase production i.e., *Trichophyton* sp., (38.1 ± 0.01^{a} U/g). Other cultures did not exhibit considerable lipase activity, presumably because the enzyme activity was associated with cell growth (Gutarra *et al.*, 2007). Therefore *Trichophyton* sp. was selected for further studies.

Different agricultural by-products such as brassica meal, almond meal, and coconut meal and rice husk and wheat bran were used as substrate and examined with regard to their effect on the extracellular lipases production by *Trichophyton* sp. (Table 2). It was found that production of extracellular lipases ranged from 12.43 ± 1.08^{d} U/g to 46.5 ± 1.13^{a} U/g. Brassica meal gave significantly highest enzyme activity (46.5 ± 1.13^{a} U/g), as compared to other substrates. Other substrates may not fulfill the nutritional needs of the organism (Gunstone, 1999). Therefore brassica meal was used for further studies.

Size of inoculums has great influence on extracelluler lipase production (Table 3) indicated the effect of size of inoculum on production of extracelluler lipase. The size of inoculums ranged from 0.5 to 2.5 ml with an interval of 0.5 mL. Maximum lipase production (48.7 ± 0.13^{a} U/g) was obtained in case of 1.0 mL of inoculum. As the amount of mycelium increased, it consumed majority of the substrate for growth purpose, hence enzyme synthesis decreased (Iftikhar *et al.*, 2003). At low level of inoculum the insignificance of the results might be due to the fact that the growth of the fungus was not proper and the time required to reach in the stationary phase of growth was increased (Yasser *et al.*, 2002). Hence 1.0 mL of inoculum was optimized for further studies.

The effect of rate of fermentation on the extracellular lipase production by T*richophyton* sp. was studied (Fig. 1). The samples were incubated at different time interval for 12, 24, 36, 48, 60, 72, 84 and 96 hours. After 48 hrs of incubation extracellular lipase production reached at its maximum (50.12 ± 1.99^{a} U/g). With the increase of incubation period there was gradual decrease in lipase production. It might be due to the exhaustion of nutrients in substrate, which results in the inactivation of enzyme (Sztajar & Maliszewska, 1988; Korn & Fujio, 1997). Hence incubation period of 48 hours was optimized for further studies.

Table 1. Screening of various fungal strains for extracellular lipase production through solid state fermentation*.

Strain code	Name of fungi	Extracellular lipase activity (U/g)
MBL-36	*Aspergillus niger*	32.2 ± 0.28^{c}
MBL-37	*Penicillium chrysogenum*	22.8 ± 0.64^{d}
MBL-38	*Rhizopus microsporus*	14.85 ± 1.61^{e}
MBL-32	*Mucor mucedo*	30.58 ± 0.25^{c}
MBL-34	*Alternaria alternata*	26.42 ± 0.08^{cd}
MBL-35	*Trichophyton* sp.	38.01 ± 0.01^{a}
MBL-33	*Fusarium semitectum*	33.9 ± 0.57^{b}
MBL-39	E (Un-identified)	23.16 ± 0.22^{d}
MBL-40	*Curvularia* sp.	16.9 ± 0.78^{e}
MBL-41	*Aspergillus flavus*	11.9 ± 1.54^{f}
MBL-42	G (Un-identified)	14.8 ± 0.76^{e}
MBL-43	F (Un-identified)	28.1 ± 0.84^{c}
MBL-44	H (Synnematous)	12.9 ± 0.10^{ef}

Each value is an average of three replicates ± denotes standard deviation among replicates; Numbers followed by different letters differ significantly at $p \leq 0.05$
*Coconut meal, 10 g

Table 2. Effect of agricultural byproducts on the production of extracellular lipase by *Trichophyton* sp. through solid state fermentation.

Agriculture by products*	Extracellular lipase activity (U/g)
Wheat bran	12.43 ± 1.08^{e}
Rice husk	25.7 ± 1.20^{d}
Coconut meal	39.9 ± 1.20^{c}
Almond meal	44.82 ± 2.40^{b}
Brassica meal	46.5 ± 1.13^{a}

*Amount of substrate: 20 g

Table 3. Effect of inoculum size on the production of extracellular lipase by *Trichophyton* sp. through solid state fermentation.

Inoculum size (mL)	Extracellular lipase activity (U/g)
0.5	39.09 ± 2.23^{b}
1	48.7 ± 0.13^{a}
1.5	31.89 ± 1.25^{c}
2.0	27.56 ± 3.62^{d}
2.5	19.05 ± 1.48^{e}

Each value is an average of three replicates ± denotes standard deviation among replicates; Numbers followed by different letters differ significantly at $p \leq 0.05$

Table 4. Effect of amount of substrate* on the production of extracellular lipase by *Trichophyton* sp., through solid state fermentation.

Amount of substrate (g)	Extracellular lipase activity (U/g)
10	29.23 ± 1.73^{e}
15	36.02 ± 2.68^{d}
20	49.34 ± 1.72^{b}
25	58.46 ± 1.06^{a}
30	41.34 ± 1.89^{c}

*Substrate used: Brassica meal

Amount of substrate has great influence on extracellular lipase production (Table 4). Amount of substrate was ranged from 10 g to 30 g. Maximum extracellular lipase production was obtained in case of 25 g of brassica meal *i.e.*, 58.46 ± 1.06[a] U/g. As the amount of substrate increased, the lipase production was decreased (Iftikhar & Hussain, 2002). Hence 25g of substrate was optimized for further studies.

Temperature also plays an important role in the metabolic processes of an organism. A range of 20°C to 50°C was employed in the present study (Table 5). Maximum production of lipase (59.12 ± 1.41[a] U/g) was reached when flask was incubated at 30ºC. By further increase in temperature, the enzyme activity was decreased 35.61± 0.17[c] U/g at 50ºC. It might be because lipase is sensitive to temperature (Iftikhar *et al.*, 2003). Decrease in extracellular lipase production can be associated to either decrease in fungal growth or inactive nature of enzyme itself (Lui *et al.*, 1995). Thus the incubation temperature of 30 ± 2°C was optimized for further studies by solid-state fermentation.

pH plays an important role in metabolic process of an organism. The production of lipase by *Trichophyton* sp. was studied at different pH of diluents (3-9). Maximum lipase production was obtained in case of pH 7 (Table 6). As the pH increased, lipase production gradually decreased because production of enzyme is very sensitive to the pH. Ohnishi *et al.*, 1994 reported optimal pH (7.0 -10.0) for maximum production of lipase. In present study pH 7.0 was selected for the maximum production of lipase (65.2 ± 1.13[a] U/g). It might be due to the organism required neutral pH for the growth as well as for the production of lipase. Hence pH 7 of diluent was optimized for extracelluler lipase production.

Table 5. Effect of temperature on the production of lipase by *Trichophyton* sp. through solid state fermentation.

Temperature (ºC)	Extracellular lipase activity (U/g)
20	47.76 ± 0.90[c]
25	52.36 ± 1.19[b]
30	59.12 ± 1.41[a]
35	43.58 ± 0.74[d]
40	41.61 ± 2.794[e]
45	44.06 ± 0.74[cd]
50	35.61 ± 0.17[f]

Table 6. Effect of pH of diluent on the production of lipase by *Trichophyton* sp. through solid state fermentation.

pH	Extracellular lipase activity (U/g)
3	44.82 ± 2.40[e]
4	48.12 ± 1.48[d]
6	59.46 ± 1.06[b]
7	65.2 ± 1.13[a]
9	53.01 ± 1.42[c]

Each value is an average of three replicates ± denotes standard deviation among replicates; Numbers followed by different letters differ significantly at $p \leq 0.05$

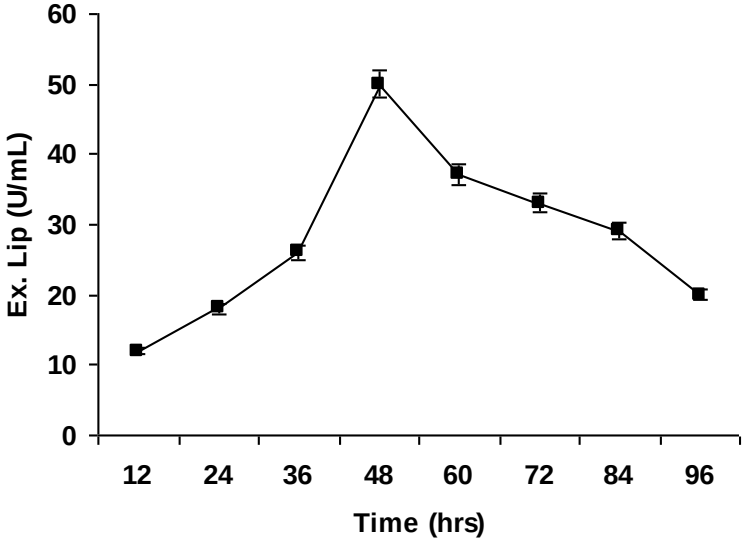

Fig. 1. Effect of the rate of fermentation on extracellular lipase production by *Trichophyton* sp. through solid state fermentation.

References

Ahmad, S. 1956. Fungi of Pakistan *Biol. Soc. Pak. Lahore, Monogr*., 1: 1-126.

Ahmad, S. 1960. Further Contributions to the fungi of Pakistan. I. *Biologia*, 6: 117-136.

Ahmad, S. 1962. Further Contributions to the fungi of Pakistan. Ð. *Biologia*, 8: 123-150.

Akano, D.A and O.O. Atanda. 1990. The present level of aflatoxin in Nigerian groundnut cake ("Kulikuli"). *Lett. Appl. Microbiol*., 10: 187-189.

Gilbert, E.J. 1993. *Pseudomonas* lipase biochemical properties and molecular cloning. *Enzyme and Microbial Technology*, 15: 634-645.

Gitlesen, T., M. Bauer and P. Adlercreutz. 1997. Adsorption of Lipase on polypropylene powder, *Biochimica et Biophysica Acta*., 1345: 188-196.

Gopinath, S.C.B., P. Anbu and A Hilda. 2005. Extracellular enzymatic activity in fungi isolated from oil rich environments. The Mycological Society of japan and springer verlag Tokyo. *Mycoscience*, 46: 119-126.

Gracheva, I.M., T.G. Mukhamedzhanova and L.T. Voino. 1980. Regulation of lipase biosynthesis in the cultivation of *Rhizopus oryzae. Biology Khim*, 16: 303-305.

Gulati, R.I.J., V. Kumar, A.K. Parsad, V.S. Parmar and R.K. Saxena. 2005. Production of a novel alkaline lipase by *Fusarium globulosum* using Neem oil, and its applications. *Pure and Applied Chemistry*, 77: 251-262.

Gunstone, F.D. 1999. Enzymes as biocatalysts in the modification of natural lipids. *Journal of the Science of Food and Agriculture*, 79: 1535-1549.

Gunstone, F.D. 1999. Enzymes as biocatalysts in the modification of natural lipids. *Journal of the Science of Food and Agriculture*, 79: 1535-1549.

Gutarra, M.L.E., M.G. Godoy, L.R. Castilho and D.M.G. Freire. 2007. Inoculum strategies for *Penicillium simplicissimum* lipase production by solid-state fermentation using a residue from the babassu oil industry. *J. Chem. Technol. Biotechnol*., 82: 313-318.

Hsu, A., K. Jones, T.A. Foglia and W.N. Marmer. 2002. Immobilized lipase catalyzed production of alkyl esters of restaurants grease as biodiesel. *Biotechnology and Applied Biochemistry*, 36: 181-186.

Iftikhar, T. 2009. Studies on the Biosynthesis of Lipases by Rhizopus oligosporus. Ph,D. Thesis GC University, Lahore.

Iftikhar, T. and A. Hussain. 2002. Effect of nutrients on the extracellular lipase production by the mutant strain of *R. oligosporous* T^{uv}-31. *Biotech*., 1(1): 15-20.

Iftikhar, T., I.U. Haq and M.M. Javed. 2003. Optimization of cultural conditions for the production of lipase by submerged fermentation of *Rhizopus oligosporous* T^{uv} 31. *Pak. J. Bot.,* 35(4): 519-525.

Iftikhar, T., M. Niaz, M. Afzal, I.U. Haq and M.I. Rajoka. 2008. Maximization of intracellular lipase production in a lipase-overproducing mutant derivative of *Rhizopus oligosporus* DGM 31: A kinetic study. *Food Technol. Biotechnol.,* 46: 402-412.

Iftikhar, T., M. Niaz, S.Q. Abbas, M.A. Zia, I. Ashraf, K.J. Lee and I.U. Haq. 2010a. Mutation induced enhanced biosynthesis of lipases by *Rhizopus oligosporus* var. *microsporus*. *Pak. J. Bot*., 42(2): 1235-1249.

Iftikhar, T., M. Niaz, M.A. Zia, M.A. Qadeer, S.Q. Abbas, M. I. Rajoka and I.U. Haq. 2007. Screening of media and Kinetic studies for the production of lipase from a mutant strain of *Rhizopus oligosporus*, *Biotechnologies International Symposium "Which biotechnologies for south countries*" *Proceedings of Biotech World 2007*. Oran Algerie, 24-25 November 2007.

Iftikhar, T., M. Niaz, Y. Hussain, S.Q. Abbas, I. Ashraf and M.A. Zia. 2010b. Improvement of selected strains through gamma Irradiation for enhanced lipolytic potential. *Pak. J. Bot*., 42(4): 2257-2267.

Iftikhar, T., M. Niaz, M.A. Zia and I.Q. Haq. 2010c. Production of extracellular lipases by *Rhizopus oligosporus* in a stirred fermentor. *Braz. J. Microbiol*., 41(4): 1124-1132.

Immanuel, G., P. Esakkiraj, A. Jebadhas, P. Iyapparaj and A. Palavesam. 2008. Lipase Production by *Serratia rubidaea, Food Technol Biotechnol.,* 46(1): 60-65.

Korn, M.S. and F. Fujio. 1997. Effect on the degree of maceration of soybean fermented by *Rhizopus* strains. *Journal of Faculty of Agriculture*, 41: 231-237.

Kirk, P. 2009. CABI. Index Fungorum. *Bioscience Database*.

Lacointe, C.E. Dubreucq and P. Galzy. 1996. Ester synthesis in aqueous media in the presence of various lipases. *Biotechnology Letters*, 18: 869-74.

Lui, G., S.Y. Lu, Jiang and Y. Wu. 1995. Production and characteristics of thermostable lipolytic enzyme from *Monascus fulginosus. Acta Microbiology*, 35: 109-114.

Mark, S.G.J., R.K. Numbi and R. Puvanakrishnan. 2001. Strain improvement of *Aspergillus niger* for enhanced lipase production, 19: 108-21.

Ohnishi K, Y. Yoshida and J. Sekiguchi. 1994. Lipase production of *Aspergillus oryzae*. *Journal of Fermentation and Bioengineering*, 77: 490-5.

Pokorny, D., J. Friedrich and A. Cimerman. 1994. Effect of nutritional factors on lipase biosynthesis by *Aspergillus niger*. *Biotechnology Letters*, 16: 363-366.

Sztajer, H., I. Maliszawsika and J. Weizore. 1988. Producton of Exogenous lipase by bacteria, fungi and actinomycetes. *Enzyme Microbiology and Technology*, 10: 492-7.

Undurraga, D.A., Markovits and S. Erazo. 2001. Cocoa butter equivalent through enzymic interesterification of palm oil mid fraction. *Process Biochemistry*, 36: 933-9.

Yasser, R., A.N. Abdel-Fattahl, A. Soliman, A. Ahmed, S.A. Gaballa, Sabry and I.E. Ahmed. 2002. Lipase production from a Novel Thermophilic *Bacillus* sp.: Application of Plackett-Burman design for evaluating culture conditions affecting enzyme formation. *Acta Microbiologica Polonica*, 51: 353-366.

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