Pak. J. Bot., 45(4): 1417-1421, 2013.

PROTOCOL OPTIMIZATION FOR EXTRACELLULAR LIPASE PRODUCTION BY *TRICHOPHYTON* SPP. (MBL 23) UNDER SOLID STATE FERMENTATION

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Abstract

The present study was conducted in the Laboratory of Mycology and Biotechnology, Department of Botany, Government College University, Faisalabad with the objective of process optimization for the production of extracellular lipases by *Trichophyton* sp. through solid-state fermentation (SSF) technique. Almond meal proved to be the best single substrate with enzyme production of 12.25±0.11U/mL, however, the production was further enhanced by manipulating the environmental variables like combined substrates, etc. Fifteen grams of mixed substrates (Almond+ Sunflower + Brassica) at 1:1:1 ratio was optimized for maximum extracellular lipase production (60.36 ± 0.10[a] U/mL). The extracellular lipase activity at 26°C after 48 h of incubation at diluent pH of 7 and by using 1 mL inoculum level was 68.65 ± 0.13[a] U/mL. Agricultural byproducts, nitrogen sources and carbon sources were also optimized for the maximum production of enzyme. Almond oil and Glucose were also found to be supportive carbohydrate & lipid additives to agro-wastes for hyperproduction of extracellular enzyme that improved the lipase production upto 73.34 ± 0.135[a] and 75.58 ± 0.083[a] U/mL, respectively. Maximum production of the extracellular lipase (76.42±0.036[a] U/mL) was obtained when peptone was used as an additional nitrogen source to the pre-optimized culture. From the present findings, it can be inferred that *Trichophyton* sp. (MBL 23) can be a prospective candidate for commercial lipase production. However, the stability analysis of the enzymes under stressful environmental parameters may be done before its final recommendation.

Introduction

Lipase (EC. 3.1.1.3) are esterases characterized by their unique ability to act upon emulsified substrate and hydrolyze glycerides to free fatty acids and glycerol (Gilbert, 1993). They are biocatalysts for the resolution of esterified secondary alcohols and for an enantioselective acyl transfer reaction, when the lipophilic substrate is deficient in water or even in organic solvent (Lacointe *et al*., 1996; Undurraga *et al.*, 2001). Lipases are ubiquitous cellular biomolecules. However, only microbial lipases have gained commercial credence (Hsu *et al*., 2002). Fungi characterized by being cosmopolitan in distribution are highly successful in survival because of their great plasticity and physiological versatility. Fungi thrive well in habitats with environmental extremes because of their efficient enzyme systems. Among the varied mechanisms for the adaptability of fungi to environmental extremes and for the utilization of their trophic niche, their ability to produce extracellular enzyme is of great survival value (Gopinah *et al*., 2005). Fungal species belonging to genera *Rhizopus*, *Mucor*, *Aspergillus*, *Fusarium* and *Penicillium* are preferable lipase sources (Gracheva *et al*., 1980; Guerra *et al*., 2003: Iftikhar *et al.*, 2008). 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). 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. The present study is aimed at optimizing the cultural conditions for the biosynthesis of extracellular lipases from a local isolate of *Trichophyton* sp.

Materials and Methods

Microorganism: The fungal culture under study was obtained from Laboratory of Mycology and Biotechnology, Department of Botany, GCU Faisalabad and then confirmed by Prof. Dr. Syed Qaiser Abbas. The isolated fungal strain were cultured and maintained on 4% potato dextrose agar (PDA) slants (Iftikhar *et al.*, 2008).

Substrates used: Different agricultural byproducts used in the present study such as almond meal, soybean meal, sunflower meal and coconut meal were obtained from the local market.

Fermentation technique: Production of fungal lipases was studied using solid state fermentation technique (Korn & Fujio, 1997). Fifteen grams of substrate (single or in combination) moistened with 7 mL of diluent (distilled water) was added in 250 mL conical flask. The flasks were autoclaved at 15-lb/inch² 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 sodium 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 h (Iftikhar *et al.*, 2008). The flasks were run parallel in triplicate.

Extraction of enzyme: After 48 h 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 titrimetrically as reported by Iftikhar *et al.*, (2008).

Statistical study: All the experimental data were analyzed by using co-stat software and figures were drawn by using MS. Excel 2007.

Results and Discussion

In the present study 5 different substrates including mustard meal, almond meal, soybean meal, sunflower meal and coconut meal were tested for the production of extracellular lipases by *Trichophyton* sp., through solid substrate fermentation technique (Fig. 1). Of all the substrates tested, almond meal showed maximum production of extracellular lipase ($12.25^{a} \pm 0.11$ U/mL) as compared to other substrates, while coconut meal gave the lowest extracellular lipase activity ($8.67^{e} \pm 0.26$ U/mL). This might be due to the reason that almond meal provides all required carbon, nitrogen, gum, asparagines and proteins (Haq *et al*., 2001). Specific activity of extracellular lipases was also found to be maximum for almond meal that further confirmed that almond meal carries all the basic nutritional requisites for the production of lipases. From this stand point, almond meal was taken as the basic substrate for the rest of the study.

Five different combinations of substrates, *i.e.,* almond + soybean+ sunflower meals (ASS), almond + soybean+ brassica meals (ASB), almond + sunflower + brassica meals (ASB*), coconut + soybean+ sunflower meals (CSS) and almond + coconut+ brassica meals (ACB), all in 1: 1: 1 ratio were subjected to solid state fermentation by inoculation with *Trichophyton* sp. for the production of extracellular lipase (Fig. 2). Amongst all the combinations, almond meal; sunflower meal; brassica meal (ASB*) showed highest extracellular lipase activity (60.36 ± 0.10^{a} U/mL) as compared to other combinations. The specific activity was also found to be maximum with this combination of tri-substrate which was 284.70 U/mg, while coconut+ almond +brassica meals (ACB) gave lowest enzyme production (47.8 ± 0.14^{d} U/mL). There is a considerable rise in the production of lipases with the use of three substrates in combination. It might be due to the reason that tri-substrate full fills the nutritional demands of the fungus. The data is in conformity with Edwinoliver *et al*., (2010) who reported a 3-fold increase observed in Tri substrate (TSF) as compared to the single substrate fermentation but in present work an five fold increase over the single substrate was observed. Therefore, the combination of almond meal; sunflower meal; brassica meal at the ratio of 1:1:1 was selected for further studies.

Fig. 3 shows the effect of different incubation temperature on the production of extracellular lipase by *Trichophyton* sp. through solid state fermentation technique. Different incubation temperatures including 22°C, 24°C, 26°C, 28°C and 30°C were tested for extracellular lipase production. The maximum extracellular activity of lipase (67.31 ± 0.14^{a} U/mL) was obtained at 26°C. Except 26°C, all incubation temperatures resulted in comparatively lower enzyme activities. A decrease in the lipase activity may be due to the fact that the enzyme denatured at higher temperatures. The optimum growth temperature for lipase production in this study is inline with the findings of Iftikhar *et al*., (2008). Hence, incubation temperature of 26°C was optimized for further studies. Amazingly, the specific activity of lipases went on an increasing trend that showed the lipase production of this fungus and its preference of lipid rich nutritional sources.

Table 1 shows the effect of different types of extractants such as tap water, distilled water, phosphate buffer and AC water on the production of lipase by *Trichophyton* sp. Maximum extracellular lipase activity (66.73 ± 0.15^{a} U/mL) was obtained by using phosphate buffer while other extractants showed lower enzyme activities. This might be due to the fact that PO_4^{-3} buffer increased the permeability of cell membrane causing extraction of enzyme from all fermented meal. This result is in accordance with Iftikhar *et al*., (2008). However, Mahadik *et al*., (2002) reported maximum enzyme extraction with sodium chloride (1%) and Triton X-100 (0.5%). Therefore, the phosphate buffer (pH 7) was optimized and selected for further studies.

Different substrate to diluent ratio have greater influence on the lipase activity by *Trichophyton* sp. through solid substrate fermentation (Fig. 4). Different volume of diluents ranging from 15-60 mL with an inter treatment difference of 15 mL was investigated during the study. Maximum extracellular lipase activity (68.28 ± 0.24^{a} U/mL) was observed when 15g of substrate was moistened with 60mL of moistening agent (1:4). Minimum lipase activity (48.33 ± 0.09^{d} U/mL) was observed when substrate to diluent ratio was 1:4. This could be due the fact that lipase production was decreased at very lower moisture content which may be ascribed to the decrease in moisture level and hence leading to suboptimal growth and less enzyme production as indicated by Silman *et al*., (1979). The present results are in accordance with the earlier reports (Mahanta *et al.*, 2008; Mateus *et al*., 2009). Therefore, 1:4 substrate to diluent (w/v) ratio was optimized for further studies.

Type of inoculum has great influence on extracellular lipases production. Table 2 showed the effect of different types of inoculum such as vegetative and spore inoculum on the production of extracellular lipase by *Trichophyton* sp. Maximum extracellular enzyme activity (67.67 ± 0.23^{a} U/mL) was obtained by using spore inoculum, while vegetative inoculum resulted in a lower activity of enzyme (45.37 ± 0.08^{b} U/mL). Size of inoculum has great influence on the production of lipase. Table 3 depicts that the effect of inoculum level on the production of enzyme. Different size of inoculum ranging from 1mL to 6mL with an interval of 1 mL was tested by *Trichophyton* sp. and maximum extracellular lipase production (68.61 ± 0.12^{a} U/mL) was obtained when 1.0 mL of spore inoculum was used. Further increase in inoculum size resulted in a gradual decrease in lipase activity. Ushio *et al*., (1996) also optimized 1.0ml of inoculum for maximum lipase production. Imandi *et al*., (2010) reported a 2mL of inoculum. Hence, 1.0 mL of spore inoculum was selected for further studies.

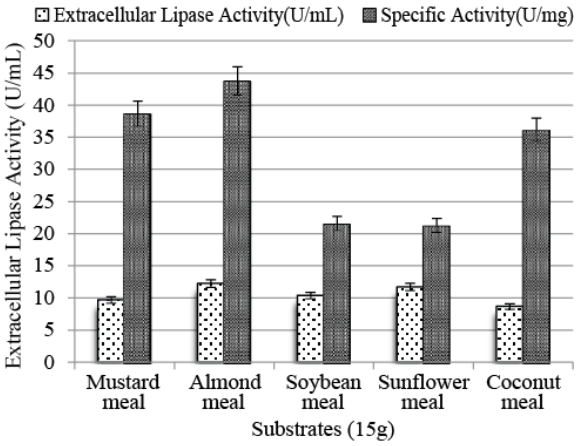
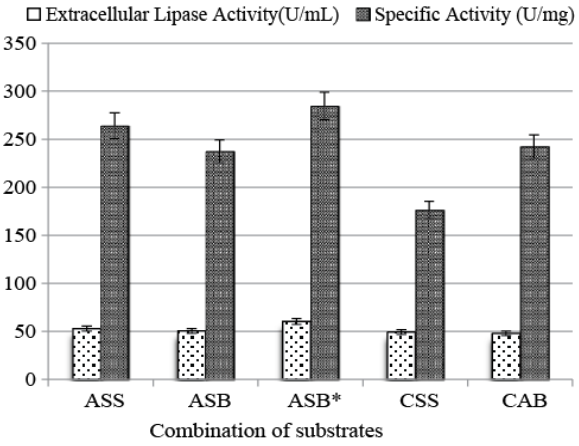


Fig. 1. Single substrate optimization for the production of extracellular lipases by *Trichophyton* sp.
*Temp. 28°C, Time 72h. Each value is an average of three replicates. Error bar indicates standard deviation among three replicates.



ASS = Almond + Soybean + Sunflower; ASB = Almond + Soybean + Brassica; ASB* = Almond + Sunflower + Brassica; CSS = Coconut + Soybean + Sunflower; CAB = Coconut +Almond + Brassica

Fig. 2. Comparison of Tri-substrate application for the production of extracellular lipases by *Trichophyton* sp.
*Temp. 28°C, Time 72h, Each value is an average of three replicates. Error bar indicates standard deviation among three replicates.

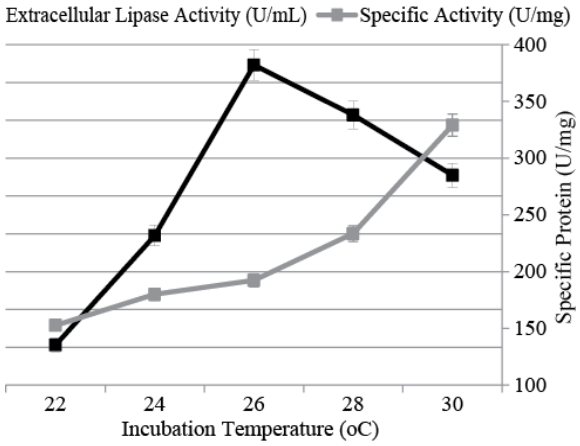


Fig. 3. Incubation temperature Vs production of lipases by *Trichophyton* sp.
* Time 72h, Substrate: ASB*, Each value is an average of three replicates. Error bar indicates Standard deviation among three replicates

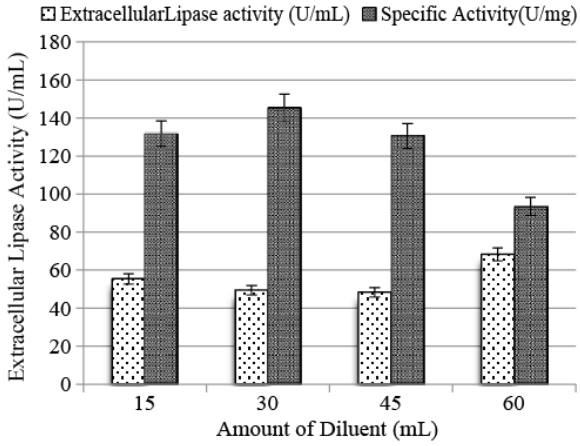


Fig. 4. Trisubstrate to diluent ratio and production of lipases by *Trichophyton* sp.
Time 72h, Substrate: ASB, Temp. 26°C, Each value is an average of three replicates error bar denotes standard deviation among replicates.

Table 1. Effect of different extractants on the production of lipase by *Trichophyton* sp.

Sr. No.	Moistening agents (15mL)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	Tap water	54.25 ± 0.06^{bc}	0.24 ± 0.02^{b}	226.04
2.	Distilled water	49.69 ± 0.17^{c}	0.17 ± 0.01^{c}	292.31
3.	Phosphate buffer	66.73 ± 0.15^{a}	0.35 ± 0.01^{a}	190.67
4.	AC water	59.67 ± 0.15^{b}	0.25 ± 0.01^{b}	238.69

* Time 72h, Substrate: ASB*, Temp. 26°C, Each value is an average of three replicates. ± denotes standard deviation among replicates

Table 2. Effect of type of inoculum on the production of lipase by *Trichophyton* sp. through solid substrate fermentation technique.

Sr. No.	Inoculum type (1mL)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	Spore inoculum	67.67 ± 0.23^{a}	0.27 ± 0.01^{b}	250.62
2.	Vegetative inoculum	45.37 ± 0.08^{b}	0.83 ± 0.03^{a}	54.66

Time 72h, Substrate: ASB, Temp. 26°C, Each value is an average of three replicates. ± denotes standard deviation among replicates

Table 3. Effect of inoculum size and different additives on the production of extracellular lipase by *Trichophyton* sp. through solid substrate fermentation.

Parameter	Inoculum size (mL)						Oil additives (1%)						Additional carbon additives (1%)					Additional nitrogen additives (1%)				
Variables	1.0	2.0	3.0	4.0	5.0	6.0	Olive Oil	Sunflower Oil	Soybean Oil	Coconut Oil	Almond Oil	Mustard Oil	Glucose	Sucrose	Starch	$KHCO_3$	Tween 80	$NaNO_3$	Yeast Extract	Urea	NH_4Cl	Peptone
Extracellular Lipase (U/mL/min)	68.61 ± 0.12^{a}	61.43 ± 0.05^{c}	60.34 ± 0.11^{b}	56.74 ± 0.13^{d}	52.22 ± 0.13^{e}	45.25 ± 0.14^{f}	67.66 ± 0.16^{e}	65.79 ± 0.18^{c}	61.39 ± 0.01^{d}	68.71 ± 0.17^{b}	73.34 ± 0.13^{a}	63.27 ± 0.19^{f}	75.58 ± 0.08^{a}	60.21 ± 0.07^{b}	51.4 ± 0.06^{d}	50.17 ± 0.14^{e}	58.67 ± 0.10^{c}	52.746 ± 0.13^{d}	62.223 ± 0.19^{b}	55.35 ± 0.07^{c}	51.816 ± 0.14^{e}	76.42 ± 0.03^{a}
Specific Activity (U/mg)	263.8	111.6	90.05	84.68	62.15	61.98	422.89	143.02	146.17	152.68	133.34	253.09	209.93	140.01	142.77	200.68	130.37	202.86	183	178.54	215.9	212.27

Each value is an average of three replicates. ± denotes standard deviation among replicates.

Incubation period has great influence on the production of extracellular lipase. The samples were incubated at different time intervals as 24, 48, 72, 96 and 120 h (Fig. 5). After 48 h of incubation, *Trichophyton* sp., showed maximum extracellular activity of lipase (68.65 ± 0.13^{a} U/mL). While above or below this time interval comparatively lower production of enzyme was obtained. The results are in line with the previously reported findings (Edwinoliver *et al.*, 2010; Kamini *et al.*, 1998). A decline in the biosynthesis of enzyme showed after 48h of cultivation as reported by Haq *et al.*, (2001). Hence, incubation period of 48h was optimized for further studies.

Table 3 reflects the consolidated data with respect to inoculum size, effect of different oils, carbon and nitrogen additives and their subsequent increasing or decreasing effects on the production of lipase by *Trichophyton* sp. According to the results, almond oil was found to be the best oil that gave maximum lipase activity (73.34±0.135^{a} U/mL) as compared to other oils. Mahadik *et al*., (2002) and D'Annibale *et al*., (2006) also investigated effect of different oils on lipase production and found that til oil, soybean oil, corn oil and olive oil to be the best inducers of lipase production. In the present study, almond oil was optimized and suggested for further studies. In connection with carbon sources, glucose was found to be the best carbon source that gave maximum lipase activity (75.58 ±0.08^{a} U/mL) as compared to other carbon sources that showed fewer activities. This might be due to the fact that supplementation of tri-substrate with additional carbon sources fulfilled the nutritional requirement of the fungus to some extent. This finding is in agreement with Imandi *et al.*, (2010) who also showed that glucose had inducing effect on enzyme production. Various organic and inorganic nitrogen additives tested are $NaNO_3$, yeast extract, urea, NH_4Cl and peptone. According to the results, peptone was found to be the best nitrogen source that gave maximum lipase activity (76.42 ± 0.04^{a} U/mL) as compared to other nitrogen sources that showed fewer activities. This might be due to the fact that peptone contains certain co-factors and amino acids, which fulfill physiological requirements of fungus for lipase biosynthesis (Freire *et al.*, 1997; Pokorny *et al*., 1994).

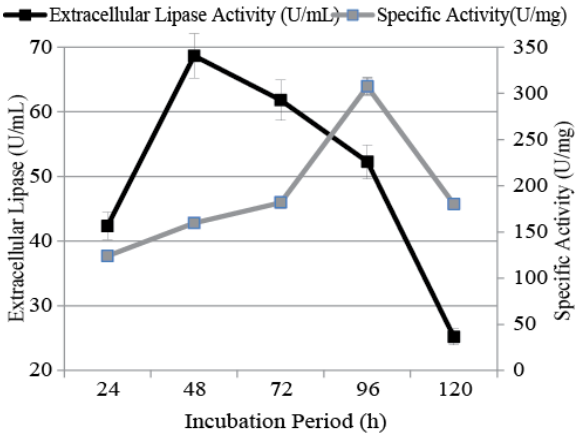


Fig. 5. Time course interval and production of lipase by *Trichophyton* sp. through solid substrate fermentation technique. *Temp. 26°C, Substrate: ASB*. Each value is an average of three replicates. ± denotes standard deviation among replicates

Acknowledgements

The present work is part of the thesis of Faiza Akram and is sponsored by Higher Education Commission, Pakistan as project entitled "Optimization of cultural conditions for the production of lipases by fungi isolated from different lipid rich environments and its characterization". The authors are also thankful to Prof. Dr. Syed Qaiser Abbas for helping in identification of the fungal strain.

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(Received for publication 20 December 2011)