Pak. J. Bot., 43(4): 2201-2206, 2011.

OPTIMIZATION OF CULTURAL CONDITIONS FOR THE BIOSYNTHESIS OF LIPASES BY *PENICILLIUM CHRYSOGENUM* (MBL 22) THROUGH SOLID STATE FERMENTATION

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Abstract

The present study was conducted in the Laboratory of Biotechnology and Mycology, Department of Botany, GC University, Faisalabad with the objective to study the production of extracellular lipases by *Penicillium chrysogenum* through solid- state fermentation (SSF). *Penicillium chrysogenum* was screened for the extracellular lipase production. The maximum extracellular lipase activity was obtained at 28ºC after 72 h by using 1 mL inoculum level. 15g of mixed substrates (Almond+ Soybean+ Sunflower) was optimized for maximum extracellular lipase production. Agricultural by products, nitrogen sources and carbon sources were also optimized for the maximum production of enzyme. Maximum production of the extracellular lipase (64.77±0.155^a U/mL) was obtained when olive oil was used as additional oil. Tween 80 and yeast extract were optimized as the best additional carbon and nitrogen sources respectively.

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 (Iftikhar *et al.,* 2010a; Iftikhar *et al.,* 2010c). Fungi thrive well in habitats with environmental extremes because of their efficient enzyme systems (Akano & Atanda, 1990). 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* spp., *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., are preferable lipase sources. (Gracheva *et al*., 1980; Ohnishi *et al.,* 1994; Lui *et al.*, 1995; 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 industry (Pokorny *et al*., 1994; Undurraga *et al*., 2001. The present study is aimed to optimize the cultural conditions for the biosynthesis of extracellular lipases by a locally isolated strain of *Penicillium chrysogenum* (MBL-22).

Materials and Methods

Microorganism: The fungal culture under study was obtained from Mycology and Biotechnology research laboratory, GCU Faisalabad. 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. The isolated fungal cultures were maintained on 4% potato dextrose agar (PDA) slants (Iftikhar *et al.,* 2003).

Substrates used: Different agricultural by products used in the present study such as Almond meal, Soybean meal, Sunflower meal, Brassica meal and Coconut meal were 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/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 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).

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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 30ºC. 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.,* 2003.

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

Results and Discussion

In the present study five different substrates including brassica meal, Almond meal, Soybean meal, Sunflower meal and Coconut meal were tested for the production of extracellular lipases by *Penicillium chrysogenum* through solid substrate fermentation technique (Table 1). Of all the substrates tested, Mustard meal gave significantly highest extracellular enzyme activity (7.42±0.087^{a} U/mL) as compared to other substrate while Almond meal was found to be least significant for the production of extracellular lipase activity (5.14±0.156^{c} U/g). The production of enzyme following growth of organism was found to be ($P\leq0.05$) in Almond meal. Thus, it was found to be the best source of carbon and nitrogen. Other substrates may not fulfill the nutritional needs of the organism. This work is not in conformity with work reported by (Rao *et al.*, 1993; Kamini *et al.,* 1998; and Haq *et al.,* 2001) who utilized other substrates and obtained higher activities of lipase.

Five different combinations of substrates named as C1, C2, C3, C4 and C5 were checked for the production of extracellular lipase by *Penicillium chrysogenum* (Table 2). Of all the substrate tested, C1 gave significantly highest extracellular enzyme activity (56.81±0.132^{a} U/mL) as compared to other substrates. However, C3 was found to be least significant for the production of extracellular lipase activity (31.14±0.167^{e} U/mL). The production of enzyme following growth of organism was found to be highly significant ($P\leq0.05$) in C1;. Therefore, the combination C1 (Almond meal; Sunflower meal; Soybean meal) at the ratio of 1:1:1 was selected for further studies. It might be due to the reason that tri substrate full fills the nutritional demands of the fungus. Edwinoliver *et al.*, (2010) reported a 3-fold increase in TSF as compared to the single substrate fermentation, while in present work an increase of 8–folds over the single substrate was observed.

Different amounts of substrate to diluent ratio have greater influence on the lipase activity by *Penicillium chrysogenum* through solid substrate fermentation (Table 3). Different volumes of diluents ranging from 15 -60mL at an interval of 15 mL was investigated during the study. The maximum extracellular (56.49± 0.156^{a} U/mL) production of lipase was attained by using 15mL of the diluent. Minimum lipase activity (37.80±0.172^{d} U/g) was observed when substrate to diluent ratio was 1:4. Therefore 1:1 substrate to diluent ratio was optimized for further studies. This could be due the fact that lipase production was decreased at very higher moisture content which may be ascribed to the decrease in porosity and hence decrease in gaseous exchange leading to suboptimal growth and less enzyme production as indicated by Silman *et al.*, (1979). Less lipase activity was observed at lower moisture content due to the reduction in the solubility of nutrients of the substrate, lowers the degree of swelling and creates higher water tension as suggested by Guerra *et al.*, (2003). The present results are in accordance with the results of (Mahanta *et al.*, 2008; Mateus *et al*., 2009).

Size of inoculum has great influence on the production of lipase. Table 4 shows the effect of different inoculum levels on the production of lipase. Different inoculum sizes ranging 1mL- 6mL with an interval of 1 mL were tested for maximum extracellular lipase production by *Penicillium chrysogenum* and maximum extracellular (58.72±0.170^{a} U/mL) lipase production was obtained in case of 1.0 ml of inoculum. As the size of inoculum was increased, the lipase production gradually decreased (Gutarra *et al.*, 2007). Hence 1.0 mL of inoculum was selected for further studies. Ushio *et al*., 1996 also optimized 1.0ml of inoculum for maximum lipase production. Imandi *et al.,* (2010) reported a 2mL of inoculum. Table 5 shows the effect of different types of inoculum such as vegetative and spore inoculum on the production of extracellular lipase by *Penicillium chrysogenum*. Maximum extracellular (56.74±0.204^{a} U/mL) lipase activity was obtained in case of spore inoculum, while vegetative inoculum resulted in a lower activity of enzyme (40.81±0.134^{b} U/mL). Hence spore inoculum was optimized and selected for further studies.

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 hours (Table 6). Maximum (56.70±0.177^{a} U/mL) extracellular activity of lipase obtained after 72 hours of inoculation. While above or below this time interval comparatively lower production of enzyme was obtained. Hence incubation period of 72 h was optimized for further studies. It might be due to the exhaustion of nutrients in substrate, which resulted in the inactivation of enzyme. Decline in exponential curve might be due to the exhaustion of nutrients or loss of moisture. The results are in line with the findings of (Edwinoliver *et al.,* 2010 and Kamini *et al.*, 1998). A decline in the biosynthesis of enzyme showed after 48h of cultivation as reported by Haq *et al*. (2001).

Table 7 shows the effect of different incubation temperatures on the production of extracellular lipase by *Penicillium chrysogenum*. Different incubation temperatures including 22ºC, 24ºC, 26ºC, 28ºC and 30ºC were tested for extracellular lipase production. Maximum extracellular (59.81±0.134^{a} U/mL) lipase activity was achieved at 28°C. Further increase or decrease in temperature, the enzyme activity was greatly inhibited. Hence incubation temperature of 28ºC was optimized for further studies. The lower and higher temperature didn't support the maximum enzymatic activity. 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.,* (2010c). Table 8 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 *Penicillium chrysogenum.* Maximum extracellular (58.22±0.066^{a} U/mL) lipase activity was obtained in case of PO^{-}_{4} buffer while other extractants showed lower enzyme activities. Therefore the phosphate buffer (pH 7) was optimized and selected for further studies.

Table 1. Selection of substrate for the production of extracellular lipase by *Penicillium chrysogenum* through solid substrate fermentation technique.

S #	Substrate (g)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	Brassica meal	7.42 ± 0.087[a]	0.250 ± 0.03[a]	29.84
2.	Almond meal	5.14 ± 0.156[c]	0.246 ± 0.040[b]	21.42
3.	Soybean meal	5.32 ± 0.116[c]	0.156 ± 0.040[c]	34.10
4.	Sunflower meal	6.11 ± 0.070[b]	0.153 ± 0.020[d]	39.20
5.	Coconut meal	5.35 ± 0.119[c]	0.146 ± 0.030[e]	36.64
	LSD	0.21	0.04	

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 2. Selection of Tri-substrate for the production lipases by *Penicillium chrysogenum* through solid substrate fermentation technique.

S.#	Combinations of substrates (C) (1:1:1)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	C1 (Almond + Soybean+ Sunflower)	56.81 ± 0.132[a]	0.15 ± 0.01[e]	378.75
2.	C2 (Almond + Soybean+ Brassica)	37.24 ± 0.201[d]	0.18 ± 0.01[c]	206
3.	C3 (Almond+ Sunflower + Brassica)	31.14 ± 0.167[e]	0.25 ± 0.01[b]	124.58
4.	C4 (Coconut +Soybean + Sunflower)	48.31 ± 0.117[b]	0.16 ± 0.01[d]	301
5.	C5 (Coconut + Almond+ Brassica)	47.32 ± 0.120[c]	0.28 ± 0.01[a]	169.02
	LSD	0.25	0.02	

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 3. Effect of substrate to diluent ratio on the production of lipase by *Penicillium chrysogenum* through solid substrate fermentation technique.

S.#	Substrate to diluent ratio		Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
	Substrate (g)	Diluent (ml)			
1.	15.0	15.0	56.49± 0.156 [a]	0.36±0.01[c]	154.35
2.	15.0	30.0	45.77±0.130[c]	0.35±0.03[d]	129.66
3.	15.0	45.0	54.35±0.11[b]	0.55±0.02[b]	98.81
4.	15.0	60.0	37.80±0.172[d]	0.76±0.02[a]	49.74
	LSD		0.20	0.04	

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 inoculum size on the production of lipase by *Penicillium chrysogenum* through solid substrate fermentation technique.

S.#	Inoculum size (mL)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	1.0	58.72 ± 0.170[a]	0.173 ± 0.01[f]	345.41
2.	2.0	57.81 ± 0.137[b]	0.73 ± 0.03[d]	79.19
3.	3.0	48.78 ± 0.136[c]	0.65 ± 0.02[e]	75.04
4.	4.0	42.69 ± 0.280[d]	0.83 ± 0.02[a]	51.43
5.	5.0	34.40 ± 0.102[e]	0.75 ± 0.02[c]	45.86
6.	6.0	30.19 ± 0.08[f]	0.76 ± 0.03[b]	39.72
	LSD	0.32	0.04	

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 9 shows the effect of different nitrogen sources such as $NaNO_3$, yeast extract, urea, NH_4Cl and peptone on the production of lipase by *Penicillium chrysogenum*. According to the results, the maximum extracellular lipase activity (59.81± 0.20[a] U/mL) was obtained when yeast extract was added to the fermentation medium. Thus yeast extract as nitrogen source found to be best for optimum production of lipases. Our work is in accordance with D'Annibale *et al.,* (2006) whereas Edwinoliver *et al.,* (2010) found that additives have no effect on lipases production. The results are also inline with Ramini *et al.,* (2010) who found that urea has inhibitory effect on lipase production whereas urea showed higher lipolytic activity (Imandi *et al.,* 2010; Imandi *et al.,* 2010[d]).

Table 5. Effect of type of inoculum on the production of lipase by *Penicillium chrysogenum* through solid substrate fermentation technique.

S.#	Inoculum type (1mL)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	Spore Inoculum	56.74 ± 0.204^{a}	0.22 ± 0.02^{b}	257.90
2.	Vegetative Inoculum	40.81 ± 0.134^{b}	0.68 ± 0.01^{a}	60.01
	LSD	0.32	0.07	

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 6. Effect of incubation period on the production of lipase by *Penicillium chrysogenum* through solid substrate fermentation technique.

S.#	Incubation period (h)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	24	30.70 ± 0.164^{d}	0.15 ± 0.02^{d}	204.72
2.	48	54.34 ± 0.13^{b}	0.25 ± 0.02^{c}	217.36
3.	72	56.7 ± 0.177^{a}	0.44 ± 0.02^{a}	128.86
4.	96	51.21 ± 0.116^{c}	0.33 ± 0.02^{b}	155.19
5.	120	25.67 ± 0.295^{e}	0.13 ± 0.03^{e}	197.46
	LSD	0.37	0.03	

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 7. Effect of incubation temperature on the production of lipase by *Penicillium chrysogenum* through solid substrate fermentation technique.

S.#	Incubation temperature (°C)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	22	29.29 ± 0.169^{e}	0.17 ± 0.01^{e}	172.32
2.	24	34.59 ± 0.0916^{d}	0.35 ± 0.03^{b}	98.82
3.	26	55.34 ± 0.115^{b}	0.24 ± 0.02^{d}	230.60
4.	28	59.81 ± 0.134^{a}	0.43 ± 0.03^{a}	139.1
5.	30	52.19 ± 0.085^{c}	0.26 ± 0.03^{c}	200.74
	LSD	0.24	0.03	

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 8. Effect of different extractants on the production of lipase by *Penicillium chrysogenum* through solid substrate fermentation technique.

S.#	Moistening agents (15mL)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	Tap water	34.32 ± 0.137^{d}	0.26 ± 0.01^{d}	132
2.	Distilled water	54.25 ± 0.111^{b}	0.35 ± 0.01^{c}	155
3.	Phosphate buffer	58.22 ± 0.066^{a}	0.44 ± 0.02^{a}	132.31
4.	AC water	44.32 ± 0.117^{c}	0.36 ± 0.01^{b}	123.11
	LSD	0.20	0.01	

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 10 shows the effect of different carbon sources such as glucose, sucrose, starch, $KHCO_3$ and Tween 80, on the production of lipase by *Penicillium chrysogenum*. The production of enzyme following growth of the organism was found to be maximum (60.23 ± 0.12^{a} U/mL) as Tween 80 was supplemented to the fermentation and varied significantly ($P<0.05$) than the other carbon sources. While other carbon sources didn't show an increase in lipases activity, that could possibly be due to the contamination problems and also increase the cost of production as reported by Imandi *et al.,* (2010). The present work is supported by the work of (Iftikhar *et al.,* 2010d).

Table 11 shows the effect of different additional oils such as olive oil, sunflower oil, soybean oil, coconut oil, almond oil and mustard oil on the production of lipase by *Penicillium chrysogenum.* For this purpose 1% of different oils such as olive oil, coconut oil, almond oil, mustard oil, soybean oil and sunflower oil were added to the fermentation medium as an additional source. The maximum extracellular lipase activity (64.77 ± 0.155^{a} U/mL) was obtained when olive oil was added to the fermentation medium while soybean oil and sunflower oil showed an inhibitory effect. The present results are in accordance with D'Annibale *et al.,* (2006) but in his work every oil has an inducing effect (5-7-fold increase) whereas in present study soybean oil and sunflower oil has an inhibitory effect over lipase production.

Table 9. Effect of different nitrogen sources on the production of lipase by *Penicillium chrysogenum*.

S.#	Nitrogen sources (1%)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	$NaNO_3$	34.60 ± 0.101^{d}	0.35 ± 0.02^{d}	98.82
2.	Yeast Extract	59.81 ± 0.200^{a}	0.54 ± 0.02^{a}	110.76
3.	Urea	29.30 ± 0.172^{e}	0.26 ± 0.02^{e}	112.67
4.	NH_4Cl	52.20 ± 0.103^{c}	0.45 ± 0.02^{c}	115.98
5.	Peptone	55.34 ± 0.114^{b}	0.46 ± 0.01^{b}	120.3
	LSD	0.25	0.03	

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 10. Effect of different carbon sources on the production of lipase by *Penicillium chrysogenum* through solid substrate fermentation.

S.#	Carbon sources (1%)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	Glucose	58.23 ± 0.094^{b}	0.35 ± 0.02^{e}	166.38
2.	Sucrose	55.32 ± 0.115^{d}	0.36 ± 0.03^{d}	153.66
3.	Starch	29.36 ± 0.083^{c}	0.56 ± 0.03^{b}	52.42
4.	$KHCO_3$	55.70 ± 0.238^{e}	0.46 ± 0.03^{c}	121.09
5.	Tween 80	60.23 ± 0.12^{a}	0.63 ± 0.03^{a}	95.60
	LSD	0.29	0.05	

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 11. Effect of different additional oils on the production of lipase by *Penicillium chrysogenum* through solid substrate fermentation.

S.#	Oils (1%)	Extracellular lipase activity (U/mL)	Total protein estimation (mg/mL)	Specific activity (U/mg)
1.	Olive Oil	64.77 ± 0.155^{a}	0.17 ± 0.02^{e}	381
2.	Sunflower Oil	38.33 ± 0.178^{e}	0.53 ± 0.02^{a}	72.31
3.	Soybean Oil	30.27 ± 0.190^{f}	0.14 ± 0.02^{f}	216.21
4.	Coconut Oil	56.35 ± 0.153^{c}	0.36 ± 0.02^{c}	156.52
5.	Almond Oil	61.28 ± 0.133^{b}	0.45 ± 0.02^{b}	136.18
6.	Mustard Oil	52.74 ± 0.180^{d}	0.34 ± 0.02^{d}	155.11
	LSD	0.33	0.03	

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

Acknowledgment

The authors are thankful to Higher Education Commission (HEC) Pakistan for providing funds to work on "Optimization of cultural conditions for the production of lipases by fungi isolated from different lipid rich environments and its characterization"

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(Received for publication 30 December 2009)