Pak. J. Bot., 50(4): 1571-1578, 2018.

OPTIMIZATION PROCESS FOR ENHANCED EXTRACELLULAR LIPASES PRODUCTION FROM A NEW ISOLATE OF *ASPERGILLUS TERREUS*AH-F2

AROOSH SHABBIR AND HAMID MUKHTAR*

Institute of Industrial Biotechnology, Government College University, Lahore-54000, Pakistan
**Corresponding author's email: hamidwaseer@yahoo.com*

Abstract

Lipases are the enzymes of choice for applications in a large number of industries. Present study describes the isolation of potent lipolytic fungal strains and their subsequent production by using shake flasks fermentation technique. The selected isolate was identified as *Aspergillus terreus*, based on morphological features and 18s rRNA sequencing. Seven different culture media were analyzed for the extracellular lipase production employing *A. terreus* as the production organism and it was found that M6 gave maximum lipase production *i.e.*, 5.0 U/mL/min in the medium containing (% w/v) $MgSO_4$, 0.05; $NaNO_3$, 0.05; KCl, 0.05; KH_2PO_4, 0.2; olive oil, 1.0; pH 6.0. Maximum production of lipase (7.66 U/mL/min) was found at a medium pH of 6.0 and an incubation temperature of 30ºC after 72 hrs of incubation period. An increase in the lipase production (7.99 U/mL/min) was observed when the production medium was provided with 1.0% peptone, 2.0% glucose and 1.0% olive oil. Ammonium sulphate precipitation was carried out for the partial purification of the lipase and maximum purification of the enzyme was found at 80% saturation level of ammonium sulphate with 1.812 fold purification.

Key words: Lipase, Shake flasks fermentation, Optimization, Purification, Fermentation parameters.

Introduction

Microbial lipases currently occupy prominent place amongst biocatalysts because they can catalyze different types of reactions in aqueous and non-aqueous media (Dominguez *et al.,* 2005). When the lipase catalyzes a reaction it acts in the interface between the oil and the aqueous phases due to opposite polarity between the enzymes and their substrates (Sethi *et al.,* 2016). These can be obtained from animals, plants and microbes such as fungi and bacteria but due to high productivity, genetic variability and low expenditure, the majority of the enzymes which are commercially important, are produced from microbial sources (Treichel *et al.,* 2010). Owing to the reason that lipases obtained from different microorganisms may have different specificities for substrates, pH and temperature optima, screening for extracellular enzyme activities has extraordinary significance in discovering new lipases for large scale process development reasons.

Filamentous fungi are familiar as good producers of lipases and many fungal enzymes are exploited in different processes in food and other industries (Aravindan, 2007). Fungi are considered as inexpensive sources of lipase production and that is why they have gained considerable attention as well as importance in the recent past (Iftikhar *et al*., 2012). Species which belong to *Rhizopus, Mucor, Penicillia* and *Aspergilli,* are found to be important in production of lipases which have major commercial significance (Rekha *et al.,* 2012).

Lipase production is affected by three main factors which include media composition, type of fermentation and bioreactor design. Moreover, suitable physiological parameters like temperature, pH and oxygen quantity are the most significant factors that decide the production of lipase in a culture medium from filamentous fungi (Treichel *et al.,* 2010; Griebeler *et al.,* 2011). Cultural parameters optimization can increase the production of lipases to many folds which are highly valued products of commercial importance. The utilization of different substrates and cultural strategies for the production of lipase still remains a promising area of research (Gupta *et al.,* 2004).

Lipases have incredible applications in industries like organic synthesis, detergent formulation, dairy, textiles, tea, food & nutrition and paper (Ghosh *et al.,* 1996). One of the most conventional lipases' applications has been flavor enhancement in cheese which has now been extended to a range of other dairy foodstuffs and confectionaries such as milk-chocolate, butter and sweets which is achieved by careful triglycerides hydrolysis to liberate free fatty acids that act as flavor originators (Sharma & Kanwar, 2014). Lipases also have applications in alcoholysis, acidolysis and hydrolysis reactions. Biodiesel production is one of the striking applications of lipases because they acts as a biocatalyst in biodiesel production and it is estimated that lipase mediated biodiesel will soon replace conventional diesel fuel because it has important features such as non-toxicity, less emissions of particulate matter, unburned hydrocarbons and carbon monoxide (Livia *et al.,* 2016). The main aim of the present study is to optimize fermentation conditions for the enhanced lipase production in submerged fermentation from *A. terreus* using different oils as substrates.

Materials and Methods

Isolation and screening of lipolytic fungi: Different strains of fungi were isolated from soil and oily samples collected from various areas of Lahore which included Shalamar town, Nasir Bagh and Thokar Niaz Baig. Isolation was performed by serial dilution method and then the preliminary screening of lipolytic fungi was carried out by using Tween 80 agar plates and phenol red staining technique (Singh *et al.,* 2006). The isolates which formed bigger zones of hydrolysis were selected and shifted to the slants of PDA for growth and maintenance. Screening of best lipase producing isolates was done by using shake flasks fermentation technique.

Fermentation technique: Spore suspension was used for the inoculation of flasks carrying fermentation medium. The inoculum preparation was performed by adding 10 mL of autoclaved distilled water in the slant and scratching off the spores to make a uniform spore suspension.

The fungal isolates were cultured in 250 mL conical flasks which contained 50 mL of production medium composed of (g/L) $CaCl_2$, 0.1; Yeast extract, 10.0; glucose, 0.5; KH_2PO_4, 1.0; $(NH_4)_2SO_4$, 1.5 and olive oil, 10.0 (pH 6.0). This medium was sterilized by autoclaving at 121ºC for 15 min at 15 lbs pressure and then inoculated with fungal spore suspension after cooling down to room temperature. Afterwards these flasks were incubated at 30ºC and 200 rpm in an incubator shaker for 72 hrs. All the experiments were performed in triplicate (Zouaoui & Bouziane, 2011).

After fermentation, centrifugation of culture broth was performed for 15 min at 6000 rpm. Centrifugation separated the cell mass from supernatant. Supernatant was used for lipase assay and the dry cell mass was determined by using pallets.

Lipase assay: The activity of the enzyme was determined according to the procedures suggested by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Biotechnology (Stoytcheva *et al.,* 2012). It was determined by incubating 1mL of clear fermentation broth added to 10 mL of olive oil (10%) homogenized in gum acacia (10%). 5 mL of pH 7.0 phosphate buffer and 2 mL of 6.0% $CaCl_2$ in a 250 mL conical flask. Then this flask was incubated at 30ºC for 60 min which contained the reaction mixture. 20 mL of ethanol:acetone mixture (1:1) was added in the flask to stop the reaction after 60 min of incubation. Two phenolphthalein indicator (0.075%) drops were added in the flask and mixed. The content of the flasks were titrated with 0.2N NaOH which was taken in the burette and noted the differences in the values of control and experimental flasks. One unit of lipase activity is defined as "the amount of enzyme which releases one µmol fatty acids per minute under specified assay conditions."

Lipase units can be determined as follows:

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

whereas,
ΔV= V2 – V1
V1= Volume of NaOH used against control flask
V2= Volume of NaOH used against experimental flask
N= Normality of NaOH
V (Sample) = Volume of enzyme extract

Determination of dry mycelial mass: The pallets of fungal culture obtained after centrifugation were used to determine the dry mycelial mass and for this purpose, these were washed by adding 10 mL of distilled water in each centrifuge tube which were pre-weighed and centrifuged again for 10 min at 6000 rpm. The pellet was dried at 70ºC for 2 to 3 hrs while the supernatant was discarded.

Screening of culture media: Seven different fermentation media were screened for the lipase production by the selected fungal isolate. The recipe of these culture media can be summed up as follows:

Medium M1: (% w/v) Glucose, 0.2; $(NH_4)_2SO_4$, 0.5; Peptone, 0.1; Yeast extract, 2.0; K_2HPO_4, 0.1; Olive oil 2.0; pH 7.2 (Bindiya & Ramana., 2012).

Medium M2: (% w/v) Peptone, 1.0; KH_2PO_4, 0.1; yeast extract, 0.1; $MgS0_4$, 0.025; $NaNO_3$, 0.025; olive oil, 0.5; pH 6.0 (Rakesh *et al.,* 2011).

Medium M3: (% w/v) Na_2HPO_4, 0.12; Yeast extract, 0.5; KH_2PO_4, 0.7; olive oil, 0.25; $MgSO_4$. $7H_2O$, 0.02; Peptone, 2.5; pH 5.0 (Yongxian *et al.,* 2013).

Medium M4: (% w/v) glucose, 0.05; KH_2PO_4, 0.1; $CaCl_2$, 0.01; $(NH_4)_2SO_4$, 0.15; yeast extract, 1.0; olive oil, 1.0; pH 6.0 (Mohamed *et al.,* 2010).

Medium M5: (% w/v) peptone, 0.5; Glucose, 2.0; yeast extract, 0.3; Malt extract, 0.3; olive oil, 1.0; pH 6.0 (Afshin *et al.,* 2013). Medium (M6): (% w/v) $MgSO_4$, 0.05; olive oil, 1.0; $NaNO_3$, 0.05; KCl, 0.05; KH_2PO_4, 0.2; pH 6.0 (Deyaa *et al.,* 2016).

Medium M7: (%w/v) NaH_2PO_4, 1.2; KH_2PO_4, 0.2; $MgSO_4$, 0.03; $CaCl_2$, 0.025; olive oil, 1.0; pH 6.0 (Alexandra *et al.,* 2014).

Partial purification of lipase: In order to attain partially purified lipase, ammonium sulphate fractionation was carried out at different saturation levels *i.e.,* 40 to 85%. The pellets were dissolved individually in a little quantity of 0.2 M phosphate buffer (pH 7.0), then dialysis was performed against the same buffer. Lipase activity assay was performed for all the fractions and the fractions showing the maximum activity of lipase were pooled, freeze dried and stored at -80ºC for further use.

Protein was estimated by performing Bradford assay (Bradford, 1976) using the standard curve of BSA.

Results and Discussion

Different lipolytic fungal strains were isolated from the different samples, collected from various places. These fungal strains were isolated on the basis of zones of clearance on the tween 80 agar plates and their lipid degrading ability was confirmed by using phenol red staining technique. By the production of lipase, the color of phenol red dye changed into yellow. Four out of 45 isolates were screened for lipase production in shake flasks using submerged fermentation. The isolate (F2) showed maximum activity of lipase *i.e.,* 5.0 U/mL/min while F1, F3 and F4 showed 3.6, 4.6 and 3.3 U/mL/min, respectively. All the other isolates showed lesser amount of lipase production. So, F2 isolate was selected and used for the production of lipase in further experiments (Fig. 1).

For the identification of best fungal strain, PCR was performed for the amplification of ITS region of F2 and then agarose gel electrophoresis was used for the analysis of the PCR product as shown in Fig. 2. The length of ITS region of F2 was 608 bp. The sequence sent by Axil Scientific First BASE Laboratories, Singapore, is shown in Fig. 3. BLAST of the gene sequence was performed for the identification of the strain which indicated that F2 was very much related to *Aspergillus* species. Afterward, multiple alignment of this sequence was performed in which 8 other known sequences of *Aspergillus* species were compared. These 8 sequences were obtained from NCBI data bank. The multiple alignment results were expressed in dendrogram as shown in Fig. 4. The results confirmed that the selected isolate was *Aspergillus terreus*.

Seven different culture media viz., M1, M2, M3, M4, M5 M6 and M7 were screened for the lipase production from *Aspergillus terreus*. The results showed that M6 medium containing (% w/v) $MgSO_4$, 0.05; $NaNO_3$, 0.05; KCl, 0.05; KH_2PO_4, 0.2 and olive oil, 1.0 (pH 6.0) was found to be the finest medium for the extracellular lipase production in the submerged fermentation (5.0 U/mL/min) while all the other media M1, M2, M3, M4, M5 and M7 showed 2.6, 2.3, 2.9, 1.0, 2.9 and 2.3 U/mL/min, respectively. The growth of *Aspergillus terreus* as shown by dry cell mass was also highest *i.e.*, 57.1 mg/ mL in M6 medium (Fig. 5).

The maximum production of lipase in the M6 medium may occurred because it is a modified Czapek Dox medium and contain $NaNO_3$ which is an effective source of nitrogen easily metabolized by fungi (Chahinian *et al.*, 2000). The other fermentation media did not support the fungal growth because they either lack these nutrients or ingredients may not be present in the appropriate amounts.

Effect of six different organic nitrogen sources including peptone, yeast extract, trypton, meat extract, urea and casein and five different inorganic nitrogen sources including $NaNO_3$, $(NH_4)_2PO_4$, $(NH_4)_2SO_4$, $(NH_4)_2NO_3$ and NH_4Cl_2 was studied on the production of lipase by the selected fungal isolate. The results showed that peptone gave the maximum production of extracellular lipase *i.e.*, 5.99 U/mL/min so it was the best source of organic nitrogen while ammonium chloride was found to be the best inorganic source of nitrogen for the purpose so both of these were selected for further studies. The growth of the *Aspergillus terreus* seemed directly connected to the nitrogen source used as the maximum dry cell mass *i.e.*, 59.4 mg/mL was observed in a medium containing peptone and ammonium chloride (Fig. 5.1). Various concentrations of peptone varying from 0.5 to 3.5% were also checked for their effect on enzyme production and 1.0% peptone was observed to be the best amount for the extracellular lipase production *e.g.*, 6.33 U/mL/min and there was a considerable decrease in the production of lipase by further increasing the peptone concentration (Fig. 5.2).

The presence of peptone as a source of nitrogen in the production medium favored the lipase production because peptone is easily metabolized by fungi as compared to other nitrogen sources. Peptone also contains some amino acids and co-factors which enhance the lipase production while these are absent in other sources of nitrogen so peptone was proved to be best source of nitrogen in the present study. Many reports have shown that production of lipase could be enhanced by inorganic sources of nitrogen while the growth of cell was improved by organic sources (Boonchaidung & Papone, 2013) that is why ammonium chloride enhanced the production of lipase without significantly affecting the fungal growth (Fig. 5.1). This report supported the present study in which ammonium chloride and peptone were employed as inorganic and organic sources of nitrogen, respectively.

Effect of six different carbon sources including glucose, maltose, sucrose, fructose, starch and lactose was evaluated on the production of extra cellular lipase from *A.terreus*. It was found that *A. terreus* gave maximum production of extracellular lipase 6.49 U/mL/min while using glucose as the source of carbon. Similarly, sucrose and fructose showed 61.0 and 60.3 U/mL/min respectively while all the other carbon sources including maltose, starch and lactose had shown lesser activity. The growth of *A. terreus* was also maximum *i.e.*, 63.2 mg/mL by using glucose as the carbon source (Fig. 5.3). Amount of glucose ranging from 0.5 to 2.5% was also optimized and it was found that 2.0% glucose concentration resulted in the maximum lipase production *i.e.*, 6.99 U/mL/min while by further increasing or decreasing the concentration of glucose, the production of lipase was decreased. The growth of *A.terreus* was also maximum in the concentration of 2.0% glucose which reached up to 63.8 mg/mL.

Carbohydrate carbon sources enhance the cell growth at the beginning of the fermentation while afterwards they improve the lipase production (Bindiya & Ramana, 2012) that is why various carbon sources were evaluated for the production of lipase. Glucose was proved to be the best carbon source among all the carbon sources studied, as it is easy for the microorganism to utilize glucose rather than fructose, sucrose, maltose, starch and lactose and improves the lipase production. Similar results were reported by Boonchaidung & Papone (2013) in which the production of lipase was improved when the production medium was supplemented with glucose.

Different substrates like acacia oil, olive oil, Indian goose berry oil, soap berry oil, coconut oil, used cooking oil, nigella oil, sesame oil, canola oil, sunflower oil, cottonseed oil and coriander oil were evaluated and their effect on lipase production. The results proved that the best substrate for the lipase production (7.49 U/mL/min) was olive oil by *A. terreus* while sesame oil, cottonseed oil, Indian gooseberry oil, nigella oil and used cooking oil showed 6.33, 5.66, 5.0, 6.0 and 5.0 U/mL/min, respectively (Fig. 5.5). All the other substrates showed lesser lipase production so olive oil was selected for lipase production in further studies. Different olive oil concentrations ranging from 0.5 to 2.5% were also optimized and it was found that 1.0% olive oil concentration was best for the extracellular lipase production (7.66 U/mL/min) while growth of *A. terreus* was also maximum at 1.0% olive oil concentration in the production medium as depicted by the dry cell mass of 71.8 mg/mL (Fig. 5.6).

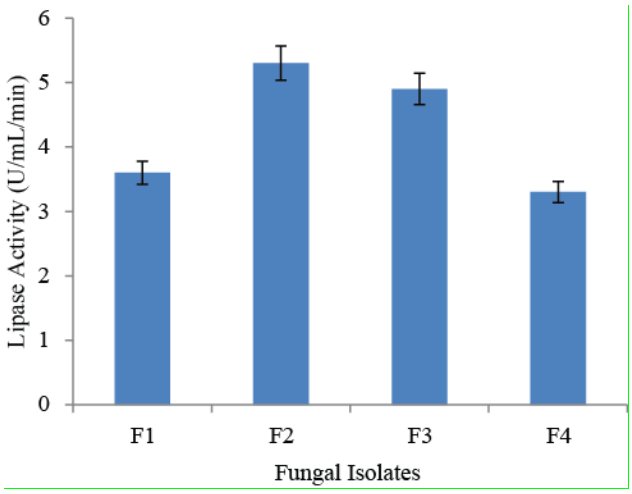

Fig. 1. Screening of lipolytic fungi.

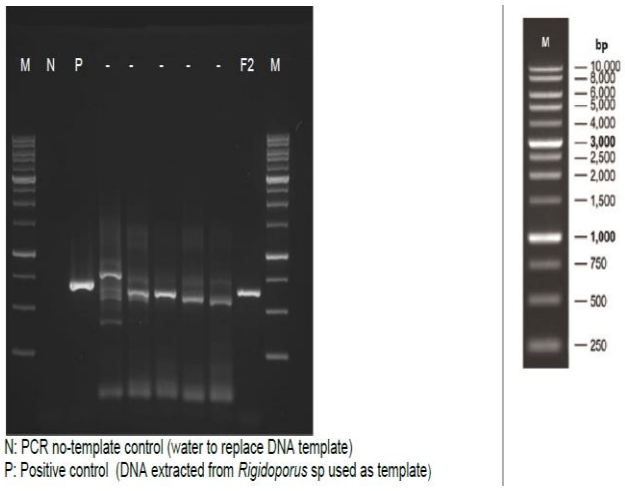

Fig. 2. Gel Electrophoresis profile of PCR product (F2).

```
>F2_608bp
TCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGGTCTTTATGGCCCAACCTCC     60
CACCCGTGACTATTGTACCTTGTTGCTTCGGCGGGCCCGCCAGCGTTGCTGGCCGCCGGG    120
GGGCGACTCGCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACATGAACCCTGTTCTGAAA    180
GCTTGCAGTCTGAGTGTGATTCTTTGCAATCAGTTAAAACTTTCAACAATGGATCTCTTG    240
GTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCA    300
GTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTG    360
TCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCTCGTCCCCCGGCTC    420
CCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGC    480
TTCGTCTTCCGCTCCGTAGGCCCGGCCGGCGCCCGCCGACGCATTTATTTGCAACTTGTT    540
TTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA    600
GCGGAGGA                                                        608
```

Fig. 3. Gene sequence of F2.

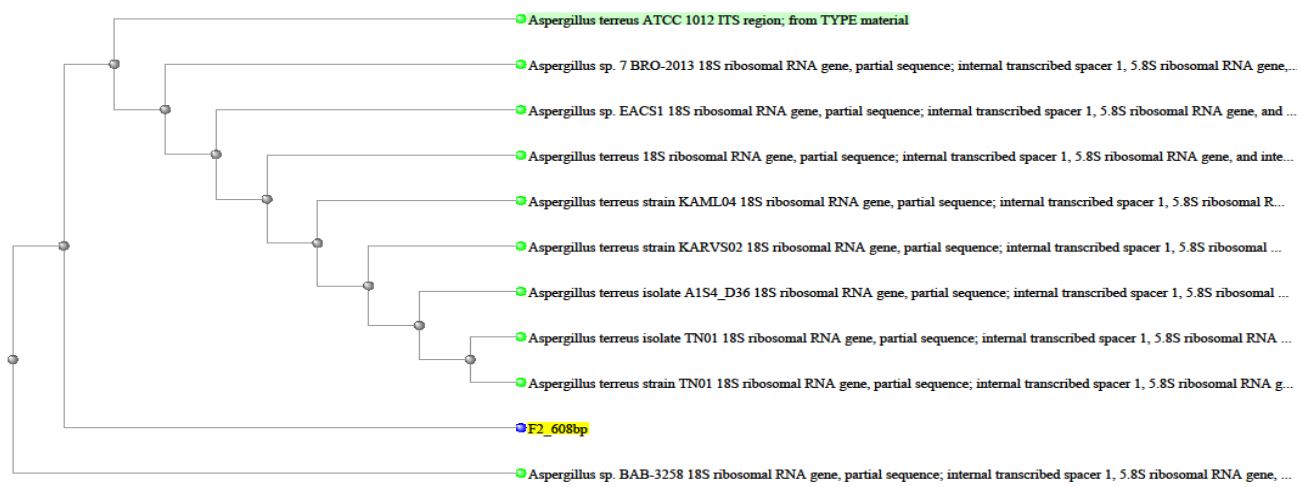

Fig. 4. Phylogenetic tree of selected fungal strain (F2).

Evaluation of various lipidic substrates showed that almost all the oils increased the lipase production but maximum production was achieved by utilizing olive oil as the substrate. Olive oil has the ability to increase the secretion of lipase from the fungal cells because lipase production increases by increasing the total quantity of C18: n fatty acid esters in the oil and olive oil is highly unsaturated with the presence of C18: n fatty acids so olive oil is an important lipase inducer (Lakshmi *et al.*, 1999). As also reported by Pera *et al.*, (2006), maximum lipase activity was observed by using olive oil as a substrate by *Aspergillus niger*. Adinarayana *et al.*, (2004) reported that maximum activity of lipase was observed when 1% olive oil was utilized as a substrate for lipase production by *Aspergillus* sp. An increase in the olive oil concentration has an inhibitory effect on the production of lipase because the microbial growth and metabolism is affected by higher concentration of olive oil.

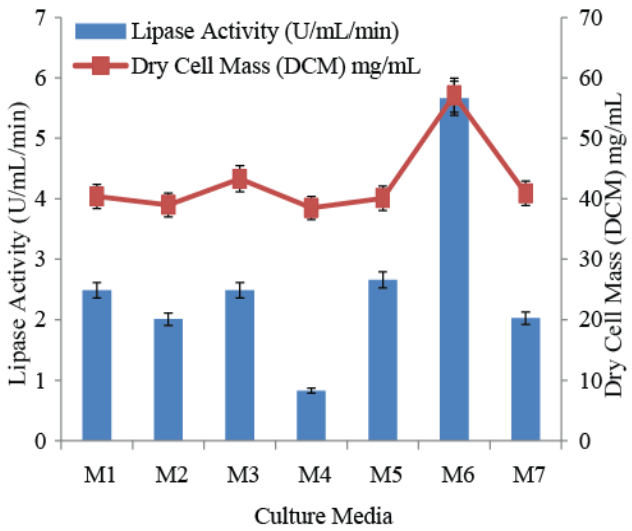

Fig. 5. Screening of different culture media for lipase production by *Aspergillus terreus* in submerged fermentation*.

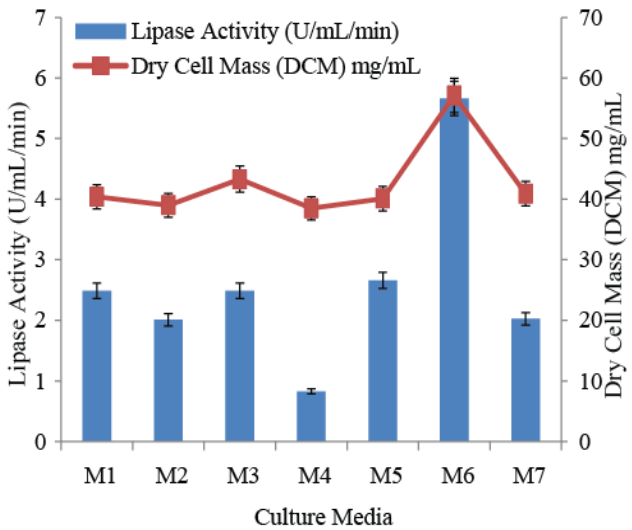

Fig. 5.1. Screening of different organic nitrogen sources for lipase production by *Aspergillus terreus* in submerged fermentation*.

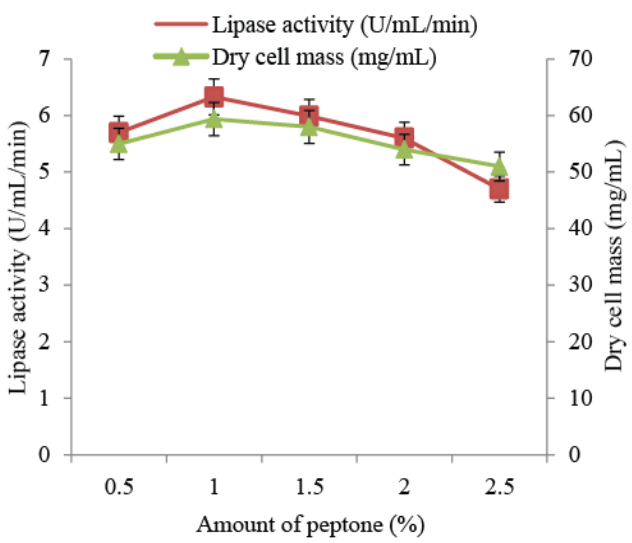

Fig. 5.2. Effect of different concentrations of peptone on lipase production by *Aspergillus terreus* in submerged fermentation*.

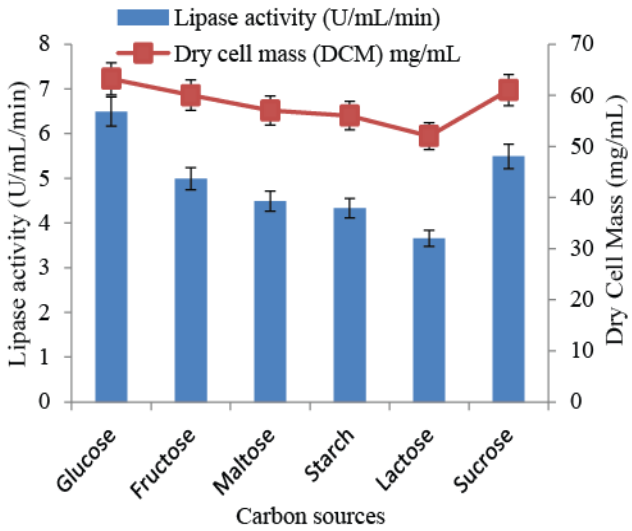

Fig. 5.3. Screening of different carbon sources for lipase production by *Aspergillus terreus* in submerged fermentation*.

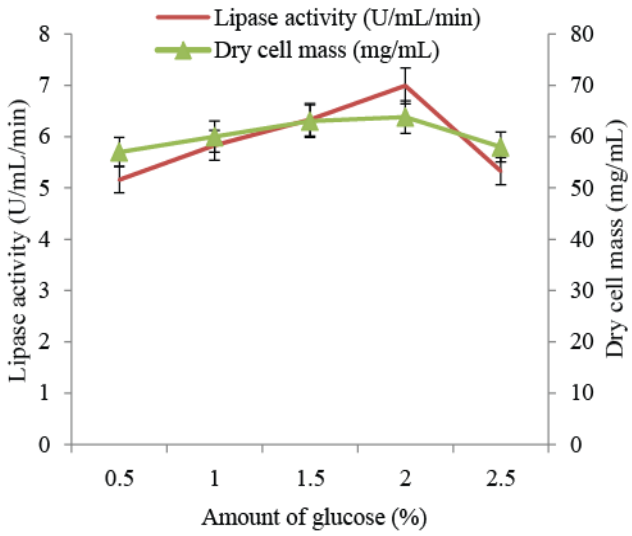

Fig. 5.4. Effect of different concentrations of glucose on lipase production by *Aspergillus terreus* in submerged fermentation*.

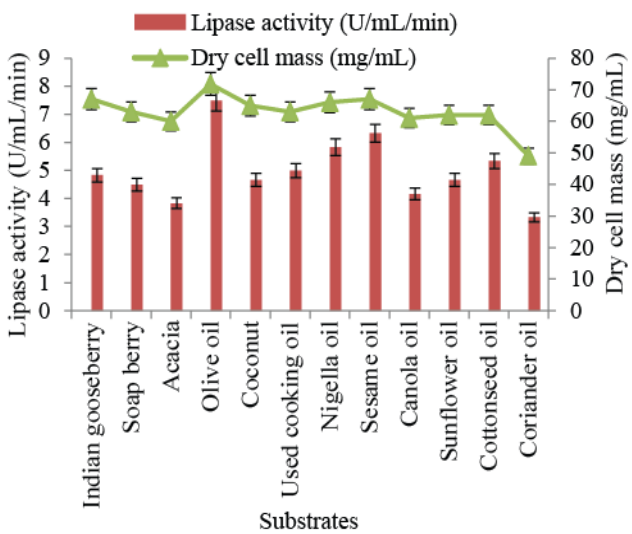

Fig. 5.5. Screening of different substrates for lipase production by *Aspergillus terreus* in submerged fermentation*.

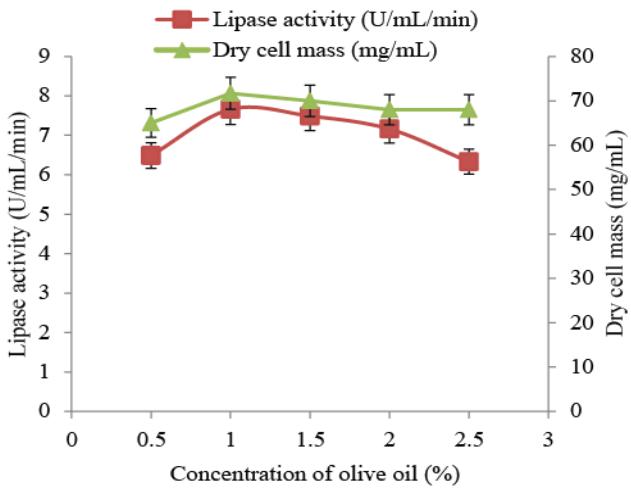

Fig. 5.6. Effect of different concentrations of olive oil on lipase production by *Aspergillus terreus* in submerged fermentation*.

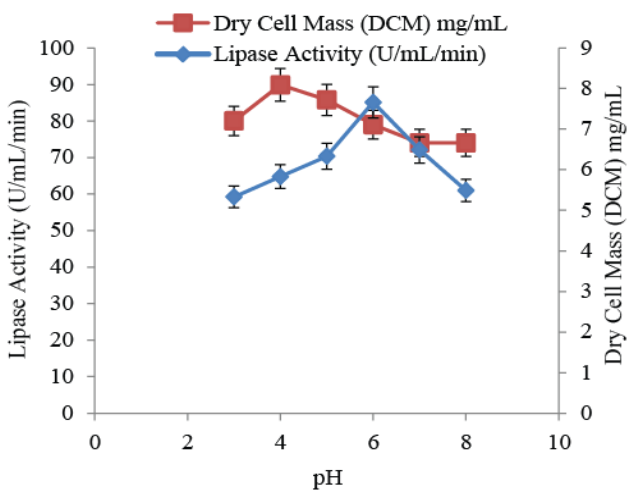

Fig. 5.7. Effect of initial pH on lipase production by *Aspergillus terreus* in submerged fermentation*.

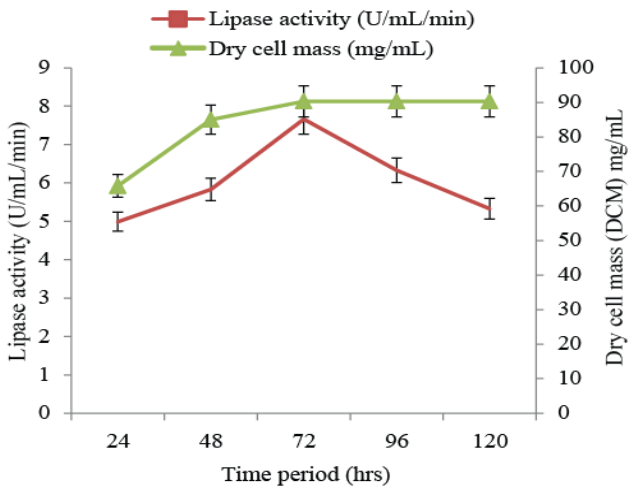

Fig. 5.8. Effect of different incubation periods on lipase production by *Aspergillus terreus* in submerged fermentation*.

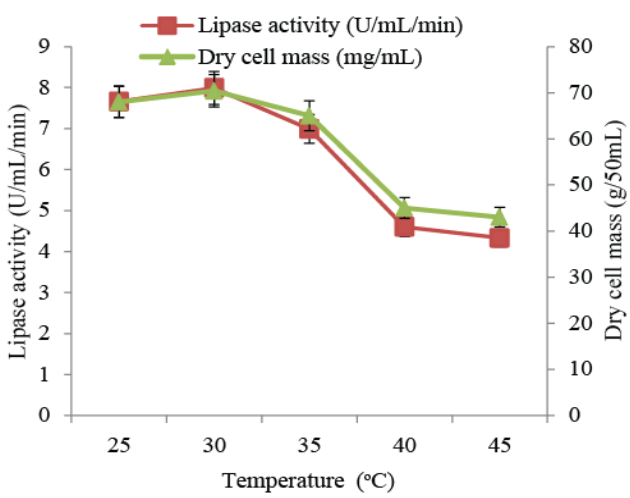

Fig. 5.9. Effect of different incubation temperatures on lipase production by *Aspergillus terreus* in submerged fermentation*.

Table 1. Partial purification of lipase produced by *A. terreus*.

Percentage saturation	Total units	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme extract	7990	2030	3.935	100	1
30	330	2000	0.165	4.130	0.041
40	490	1870	0.262	6.132	0.066
50	830	1620	0.512	10.387	0.130
60	1330	1430	0.930	16.645	0.236
70	1990	1090	1.825	24.906	0.463
80	6990	980	7.132	87.484	1.812
85	300	410	0.731	3.754	0.185

Impacts of starting pH of culture medium on the extracellular lipase production by *A.terreus* during submerged fermentation was evaluated and the outcomes showed that pH 6.0 was ideal for the production of lipase *i.e.*, 7.66 U/mL/min. Above and below this pH, the productivity of lipase was decreased. Dry cell mass was found to be directly related to pH of the medium as the maximum growth of the *A. terreus* was also observed at pH 6.0 *i.e.*, 89.9 mg/ mL (Fig. 5.7). Mostly, fungi are acidophiles and grow in low pH ranges but they also have the ability to change the pH of the production medium because they uptake various anions and cations present in the culture medium as reported by Sethi *et al.*, (2013). Maximum production of lipase by *Candida lipolytica* and *Trichoderma* sp has also been reported at a medium pH of 6.0 (Sidra *et al.*, 2016; Toscano *et al.*, 2014).

The effect of different incubation periods ranging from 24 to 120 hrs on the lipase production by *A. terreus* was observed (Fig. 5.8). The results demonstrated that after 72 hrs of incubation, the production of lipase was

highest *i.e.,* 7.66 U/mL/min but on further increasing this time period, the lipase production was decreased. So, 72 hrs was chosen as the best incubation period for the following studies. At an incubation period of 72 hrs, highest dry cell mass of *A. terreus* was also obtained *i.e.,* 90.3 mg/mL while further increase in the time period was not increased the cell mass. Lipase production at an optimum incubation period of 72 hrs from *Aspergillus* sp. has been observed by Mukesh *et al.,* (2012). Similarly, highest lipase activity was achieved after 72 hrs of incubation from *Mucor racemosus* as reported by Nadia *et al.,* (2010). It was observed that after 72 hrs, there was a reduction in the activity of lipase and this decline in the activity of lipase may be due to the maximum consumption or utilization of nutrients in the production medium or by the production and accumulation of many toxic by-products.

Different incubation temperatures *i.e.,* 25 to 45°C were evaluated to find out the best temperature for the maximum lipase production. The maximum lipase activity was achieved at an incubation temperature of 30°C which was 7.99 U/mL/min as shown by the results (Fig. 5.9). Hence, 30°C was used as an incubation temperature for the extracellular lipase production in the further studies. Dry cell mass was also found to be maximum at 30°C e.g., 70.4 mg/mL. A higher temperature had inhibitory effect on fungal growth so lipase production was also affected that's why maximum production of lipase was observed at 30°C as also reported by Hosseinpour *et al.,* (2011). Similarly, low temperature also affected the production of lipase by delaying the uptake of nutrients present in the culture medium (Sirisha *et al.,* 2010). These reported results support our findings in which maximum production of lipase was observed at the incubation temperature of 30°C. So, an incubation temperature of 30°C was selected for further experiments.

Ammonium sulphate at different saturation levels was used for the fractional precipitation of lipase and the results depicted that at 80% saturation, maximum activity of lipase was observed *i.e.,* 6.99 U/mL/min while all the other fractions showed very little no enzyme activity. The specific activity of the lipase was 7.132 U/mg at 80% saturation level of ammonium sulphate with 1.812 fold purification (Table 1). Due to the high solubility of ammonium sulphate and several other advantages, it is used in the precipitation of most of the proteins. Ammonium sulphate also maintains the structure and activity of enzymes and proteins that is why it is widely used for the said purpose (David *et al.,* 2009).

Conclusion

Present study concludes that *Aspergillus terreus,* a local isolate was found to be a good producer of extracellular lipase during submerged fermentation. By optimization of different fermentation parameters, the production of enzyme was enhanced.

References

Adinarayana, K., K.V.V.S.N.B. Raju, M.I. Zargar, R.B. Devi, P.J. Lakshmi and P. Ellaiah. 2004. Optimization of process parameters for production of lipase in solid-state fermentation by newly isolated *Aspergillus* species. *Indian J. Biotechnol.,* 3: 65-69.

Afshin, F., M. Ghasemi and E.A. Salehi. 2013. Using fermentation processes for production of lipase by *Aspergillus niger.* 2nd Intl. *Conference on Environ. Agriculture and Food Sci., (ICEAFS'2013)* August 25-26, 2013 Kuala Lumpur (Malaysia).

Alexandra, K., B. Nemeth, C. Vagvolgyi, T. Papp and M. Tako. 2014. Screening for extracellular lipase enzymes with transesterification capacity in mucoromycotina strains. *Food Technol. Biotechnol.,* 52(1): 73-82.

Sidra, T. Iftikhar, M. Ali, F. Latif, R. Abdullah and M.I. Ka. 2016. Mutagenic strain improvement of *Aspergillus niger* (MBL-1511) and optimization of cultural conditions for boosted lipolytic potential through submerged fermentation. *Pak. J. Bot.,* 48(6): 2539-2548.

Aravindan, R., P. Anbumathi and T. Viruthagiri. 2007. Lipase applications in food industry, *Indian J. Biotechnol.,* 6(2007): 141-158.

Bindiya, P. and T. Ramana. 2012. Optimization of lipase production from an indigenously isolated marine *Aspergillus sydowii* of Bay of Bengal. *J. Biochem Tech.,* 3(5): 203-211.

Boonchaidung, T. and T. Papone. 2013. Effect of carbon and nitrogen sources on lipase production by isolated lipase-producing soil yeast. *J. Life Sci. Technol.,* 1(3): 176-179.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*., 72: 248-54.

Chahinian, H., G. Vanot, A. Ibrik, N. Rugani, L. Sarda and L.C. Comeau. 2000. Production of extracellular lipases by *Penicillium cyclopium* purification and characterization of a partial acylglycerol lipase. *Biosci. Biotechnol. & Biochem.,* 64(2): 215-22.

David, L., M. Vierros, G. Hamon, S. Arico and C. Monagle. 2009. Marine genetic resources: a review of scientific and commercial interest. *Mar Policy*., 33: 183-194.

Deyaa, M.A.F., R.A. Bayoumi, and M.A. Hassan. 2016. Production of thermoalkaliphilic lipase from *Geobacillus thermoleovorans* DA2 and application in leather industry. Hindawi Publishing Corporation. *Enzyme Research*, 2016: 1-9.

Dominguez De María, P., C. Carboni-Oerlemans, B. Tuin, G. Bargeman, A.V.D. Meer and R.V. Gemert. 2005. Biotechnological applications of *Candida antarctica* lipase A: State-of-the-art. *J. Mol. Catalysis B*: *Enzymatic.,* 37: 36-46.

Ghosh, P.K., R. Saxena, K. Gupta, R.P. Yadav and W.S. Davidson. 1996. Microbial lipases: production and applications. *Sci. Progress*, 79: 119-157.

Griebeler, N., A.E. Polloni., D. Remonatto, F. Arbter, R. Vardanega, J.L. Cechet, M.D. Luccio., D.D. Oliveira, H.T. Rogerio, L. Cansian, E.R. Jorge and L. Ninow. 2011. Isolation and screening of lipase-producing fungi with hydrolytic activity. *Food Bioprocess Technol.,* 4(4): 578-586.

Gupta, R., N. Gupta and P. Rathi. 2004. Bacterial lipases: An overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.,* 64: 763-781.

Hosseinpour, M.N., G.D. Najafpour, H. Younesi and M. Khorrami. 2011. Submerged culture studies for lipase production by *Aspergillus niger* NCIM 584 on soya flour. *Middle East J. Sc. Res.,* 7(3): 362-366.

Iftikhar, T., M. Niaz, M.A. Zia, M. Sadaf and R. Jabeen. 2012. Production potential of locally isolated strain of *Fusarium solani* (MBL 24) for extracellular lipases. *Pak. J. Bot.,* 44: 393-397.

Lakshmi, B.S., P. Kangueane, B. Abraham and G. Pennathur. 1999. Effect of vegetable oils in the secretion of lipase from *Candida rugosa* (DSM 2031). *Letters in Appl. Microbiol.,* 29: 66-70.

Livia, T.A., I. Souza, A. Adriano, F. Heizir and D. Castro1. 2016. Selection of lipases for the synthesis of biodiesel from *Jatropha* oil and the potential of microwave irradiation to enhance the reaction rate. *Bio-Med. Research Int.*, 2016: 1-13.

Mohamed, S.T., L. Zaghloul, H.M. Saeed, M.M. Berekaa and Y.R. Abdel-Fattah. 2010. Enhanced production of lipase by the thermophilic *Geobacillus stearothermophilus* strain-5 using statistical experimental designs. *New Biotechnol.*, 27(4):

Mukesh, K., D.J.R. Rejitha, S. Devika, M.D.B. Immaculate, N. Rebecca and P.T. Kalaichelvan. 2012. Production, optimization and purification of lipase from *Bacillus* sp. MPTK 912 isolated from oil mill effluent. *Adv. Appl. Sci. Res.*, 3: 930-938.

Nadia, N., Z.A. Nehad., A.E. Elsayed., M.A. Essam and M.A. Hanan. 2010. Optimization of lipase synthesis by *Mucor racemosus* production in a triple impeller bioreactor. *Malaysian J. Microbiol.*, 6: 7-15.

Pera, L.M., M.C. Romero, M.D. Baigori and G.R. Castro. 2006. Catalytic properties of lipase extracts from *Aspergillus niger*. *Food Technol. Biotechnol*., 44 (2): 247-252.

Rakesh, K., S. Mahajan, A. Kumar and D. Singh. 2011. Identification of variables and value optimization for optimum lipase production by *Bacillus pumilus* RK31 using statistical methodology. *New Biotechnol.*, 28(1):

Rekha, K.S.S., M.V.V.C. Lakshmi, V. Sridevi and M. Manasa. 2012. An overview of microbial lipases. *J. Chem. Biol. Phys. Sc. Section*, 2: 1379-1389.

Sethi, B.K., J.R. Rout., R. Das., P.K. Nanda and S.L. Sahoo. 2013. Lipase production by *Aspergillus terreus* using mustard seed oil cake as a carbon source. *Annals Microbiol.*, 63(1): 241-252.

Sethi, B.K., P.K. Nanda and S. Sahoo. 2016. Characterization of biotechnologically relevant extracellular lipase produced by *Aspergillus terreus* NCFT 4269.10. *Brazilian J. Microbiol.*, 143-149.

Sharma, S. and S.S. Kanwar. 2014. Organic solvent tolerant lipases and applications. *The Sci. World J.*, 1-15.

Singh, R., N. Gupta,V.K. Goswami and R. Gupta. 2006. A simple activity staining protocol for lipases and esterases. *Appl. Microbiol. Biotechnol.*, 70(6): 679-682.

Sirisha, E., N. Rajasekar and M.L. Narasu. 2010. Isolation and optimization of lipase producing bacteria from oil contaminated soils. *Adv. Bio. Res.*, 4(5): 249-252.

Stoytcheva, M., G. Montero, R. Zlatev, J.A. Leon and V. Gochev. 2012. Analytical methods for lipases activity determination: A review. *Current Analytical Chem.*, 8: 400-407.

Toscano, L., G. Montero, M. Stoytcheva, V. Gochev, L. Cervantes, H. Campbell, R. Zlatev, B. Valdez, C. Perez and M. Gil-Samaniego. 2014. Lipase production through solid-state fermentation using agro-industrial residues as substrates and newly isolated fungal strains. *Biotechnol. Biotechnol. Equipment.*, 7(5): 4074-4077.

Treichel, H., D. de Oliveira, M.A. Mazutti, M. Di Luccio and J.V. Oliveira. 2010. A review on microbial lipases production, *Food Bioprocess Technol.*, 3: 182-196.

Yongxian, F., L. Shang and J. Qian. 2013. Screening, identifying and medium optimization of a lipase-producing filamentous fungus from soil for high chiral resolution of 1-phenylethanol. *African J. Microbiol. Res*., 7(33): 4235-4243.

Zouaoui, B and A. Bouziane. 2011. Isolation, optimisation and purification of lipase production by *Pseudomonas Aeruginosa. J. Biotechnol. Biomaterial.*, 1(7): 1-4.

(Received for publication 18 October 2017)