

## SCREENING AND IDENTIFICATION OF TWO NEW POTENTIAL LIPASE PRODUCING FUNGI ISOLATED FROM SOIL

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### ABSTRACT

The utilization of microbial enzymes has found broad technological application in different industrial processes. A total of 336 fungal strains were isolated from soil and screened for lipase activity. Among them, 12 exhibited a greater clear zone than the others, indicating higher lipase activity, were cultivated under submerged fermentation. Two of the selected isolates, FSS48 and FSS241 were the best lipase producer. Therefore, these two strains were identified; the phylogenetic analyses based on the results of 5,8S rDNA gene sequencing revealed that FSS48 and FSS241 isolates were close in identity to *Penicillium italicum* and *Penicillium expansum* respectively. Results exhibit that better carbon source for lipase production by FSS48 is soya beans powder 3% and from FSS241 is beet pulp 2%. The optimum pH and temperature for lipase production by FSS48 were found to be 8 at 30°C, after 120 h of incubation, while FSS241 were found to be 7 at 25°C, after 120 h of incubation. Overall, the results of the present study demonstrate that the two new fungal isolate are promised to be good source for extracellular lipase production under submerged conditions.

**Key words:** Filamentous fungi, lipase, submerged fermentation, 5,8S rDNA.

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## INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze hydrolyse triacylglycerols to fatty acids, di-acylglycerols, mono-acylglycerols and glycerol and under certain conditions, catalyze reverse reactions such as esterification and trans esterification (Fernandes et al., 2007; Joseph et al., 2008). They are one of the most important classes of industrial enzymes and they have many applications in the food, dairy, detergent, and pharmaceutical industries (Babu & Rao, 2007). Since lipases are physiologically necessary for living organisms, they are ubiquitous and can be found from diverse sources such as plants, animals, and microorganisms (Pahoja & Sethar, 2002). More abundantly, however, they are found in bacteria, fungi, and yeasts (Haki & Rakshit, 2003). Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activity. Microbial enzymes are also more stable than corresponding plant and animal enzymes and their production is more convenient and safer (Thomasi & Kavitha, 2015). Considering the widespread use of these enzymes in different industries, nowadays many efforts are made to find enzymes with specific properties for optimal efficiency of industrial processes (Balan et al., 2010). Due to the wide range of applications, researchers have made attempts to isolate lipase producing fungi. The study of isolation and screening enlarges the number of species that potentially produce lipases, contributing to the development of innovative commercial strategies, once these new species can present different temperatures and pH ranges, stabilities, specificity to certain fatty acids, and enantio selectivity (Gupta et al., 2003). The present study was aimed to isolate and identify of lipase producing fungi collected from different regions of Syria.

### Materials and methods

#### Fungal isolates and spore suspension preparation

The fungal isolates used in this study isolated in our laboratory from different Syrian soil samples which collected from various region (Bakri et al., 2010). The isolates were grown on petri dishes containing potato dextrose agar and incubated at 25 oC. the isolates maintained on slant PDA at 4 oC and in glycerol 20% at -80 oC until needed. Fungal spores suspension prepared by harvesting spores with peptone water from PDA flask, and the count of spores in suspension calculated using hemacytomer and adjusted to be  $5 \times 10^7$  spores/ml.

#### Screening of lipase producing fungi

For selection lipase producing isolates, 10  $\mu$ l of fresh spore suspension for each fungi isolate was aliquoted onto tributyrin agar plates which consist of: agar 1.2%, yeast extract 1% and tributyrin 1%, incubated for 72 hr at 30 oC and screened for lipase synthesis. Positive lipolytic isolates were selected according to the clear zones of hydrolysis on the tributyrin. Twelve isolates having the highest clearing zone were selected and grown in liquid culture for selected highest lipase producing fungi isolates. Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 50 ml of basal culture medium (g/l): peptone 2%, yeast

extract 0.5%, NaCl 0.5%, Na<sub>2</sub>CO<sub>3</sub> 0.025% and 2% olive oil. The pH was adjusted to pH6 before sterilization. The amount of lipase produced by each isolate was determined from the extract culture filtrate.

### **Submerged fermentation**

The production of lipase by best fungi isolates studied in submerged fermentation. The experiments were conducted in 250 ml Erlenmeyer flasks containing 50 ml of culture medium. The culture medium used for lipase production was composed of agriculture wastes 2% (w/v), mineral salts (NaCl 0.5%, Na<sub>2</sub>CO<sub>3</sub> 0.025%) and nitrogen source 1%. The flasks were sterilized at 121 °C for 20 min. 1 mL fresh spore suspension (containing around 5×10<sup>7</sup> spores/ml) was added to the sterilized medium and the flasks were incubated in an orbital shaker at 30 °C and 150 rpm for 5 days.

### **Effect of carbon and nitrogen sources**

To investigate the effect of carbon sources on lipase production, olive oil was replaced by other waste agriculture as sole carbon sources such as wheat bran, sugar beet pulp, tomato pulp, soya cake, cotton seed cake, olive pulp and wheat straw at 2% (w/v) and the effect of concentration for the best waste agriculture was studied.

The effect of organic and inorganic nitrogen sources on lipase production was studied by replacing the nitrogen source in basal salt solution with 1% of yeast extract, peptone, tryptone, corn steep, hydrolysis casein, ammonium chloride, ammonium nitrate and ammonium ortho phosphate. The optimum concentration of the best nitrogen source for lipase production was investigated.

### **Effect of initial pH and temperature**

The effect of initial pH was studied by adjusting the pH of the lipase production medium at different pH as 3, 4, 5, 6, 7, 8, 9, and 10. Similarly, the effect of incubation temperature was studied at 15°C, 20°C, 25°C, 30°C and 35°C.

### **Enzyme assay**

Lipolytic activity was determined titrimetrically on the basis of olive oil hydrolysis (Macedo et al., 1997) with some modifications. Olive oil emulsion substrate was prepared by mixing 10 mL olive oil and 90 ml from 5% Arabic gum solution. The reaction mixture contained 5 mL olive oil emulsion substrate, 4 ml buffer and 1 mL of crude enzyme. The reaction mixture was incubated in water bath with a shaking speed of 150 rpm at 50°C for 20 min. To stop the reaction, 10 ml ethanol acetone mixture (1:1) was added to the reaction mixture. Liberated fatty acids were titrated with 0.05 mol/L NaOH. One lipase unit (U) was defined as the amount of the enzyme that released one µmol fatty acid per min.

Molecular identification of fungi

### **Genomic DNA isolation**

Total genomic DNA of selected isolates was extracted from 48 h growing cultures medium (2% glucose, 1% yeast extract and 1% peptone). 1.5 ml of cultured cells was collected by centrifugation at 13 000 rpm for 5 minutes. Cells were washed with distilled water and digested in 750 µl enzymatic lysis solution (10 µl of proteinase k 20 mg/ml, 2% SDS, 1% 2-mercaptoethanol, 1% CTAB and 10 mM EDTA in 50 mM Tris pH 8 buffer) and incubated for

30 minutes at 60°C. The lysate was extracted with phenol: chloroform: isoamyl alcohol (25:24:1) three times. The extract was purified by adding 1/10 of the volume of sodium acetate 3 M and 1 ml ethanol. The mix was vortexed and placed for 15 minutes on ice. Sediment high molecular weight DNA was obtained after 5 minutes of centrifugation at 13000 rpm washed with 70% ethanol and air-dried. The final DNA pellet was dissolved in 50 µl hydration solution and stored at -20°C. DNA concentration was estimated by measuring the absorbance at 260 nm. The quality of the isolated genomic DNA was calculated by the ratio OD260nm /OD280nm.

#### **PCR amplification of the 5.8 S rDNA**

PCR was used to amplify 5.8 S rDNA gene of α-amylase producing isolate. Primers used for PCR and DNA sequencing are ITS1 (5`-TCC GTA GGT GAA CCTGCG G-3`) and ITS4 (5`-TCC TCC GCT TATTGA TATGC-3`). The standardized PCR conditions were as follows: one cycle of denaturation at 95°C for 5 minutes followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 2 minutes, and one cycle of extension at 72°C for 10 minutes. PCR products were visualized by electrophoresis in 1% (w/v) agarose gel stained by ethidium bromide.

#### **5.8 S DNA sequencing**

PCR amplicons were purified using Microcon Y-100 filters (Millipore) and sequenced using ABI Prism® Big Dye® terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions. The sequencing products were purified by ethanol precipitation. Sequencing reactions were carried out on DNA Sequencer. The sequences obtained (Length, approximately 500 bp) were then assembled in silico (Vector NTI) using overlapping to form contiguous sequence.

#### **Phylogenetic analysis**

Phylogenetic analysis was realized by an alignment of consensus sequences of 5.8S genes collected in an international database (Genbank). The resultants were then expressed in percentage of homology between the submitted sequence and the most relevant sequences from the database.

#### **Results and Discussion**

##### **Screening of lipase producing fungi**

Screening of lipase producers on agar plates is frequently done by using tributyrin as a substrate (Cardenas et al., 2001) and clear zones around the colonies indicate to lipase activity (Figure 1). In this study three hundred and twenty fungal isolates were evaluated for lipase activity. Eleven of these isolates with a high clear zones diameter > 35 mm were selected and grown in liquid culture. Three isolates FSS1, FSS48 and FSS241 showed higher lipase production (4.36, 10.88, and 14.82 U/ml respectively) as shown in table 1. FSS48 and FSS241 were selected for further studies.

Table 1. Production of lipase by Fungi isolates under submerged fermentation.

| Fungi isolates | Clearing Zone (mm) | Lipase (U/ml) |
|----------------|--------------------|---------------|
| FSS1           | 37                 | 4.36          |
| FSS2           | 38                 | 2.25          |
| FSS46          | 38                 | 2.69          |
| FSS48          | 43                 | 6.98          |
| FSS78          | 40                 | 0.32          |
| FSS79          | 42                 | 0.87          |
| FSS80          | 37                 | 1.31          |
| FSS107         | 43                 | 0.63          |
| FSS187         | 38                 | 0.19          |
| FSS241         | 36                 | 8.75          |
| FSS295         | 50                 | 0.81          |

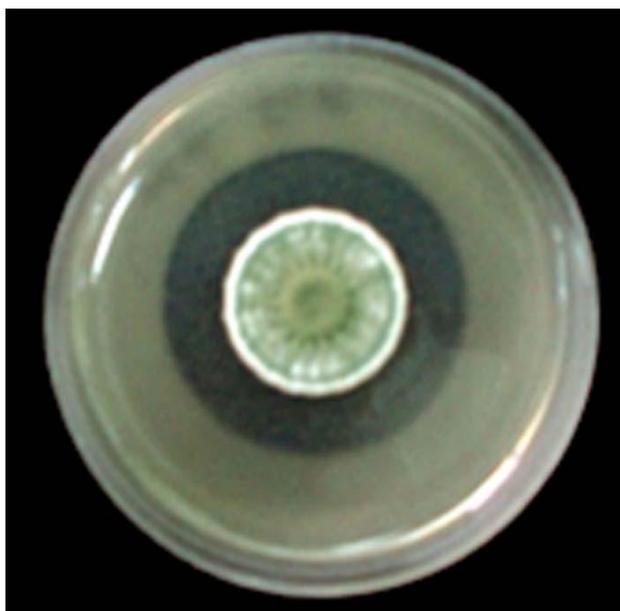


Fig.1. Clear zone around the colony of lipase producer fungus

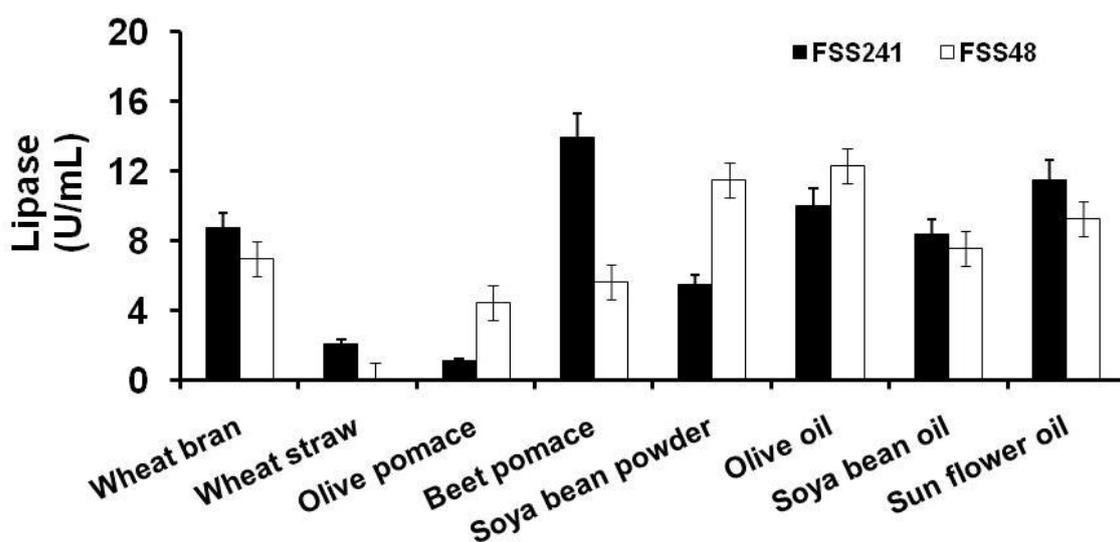
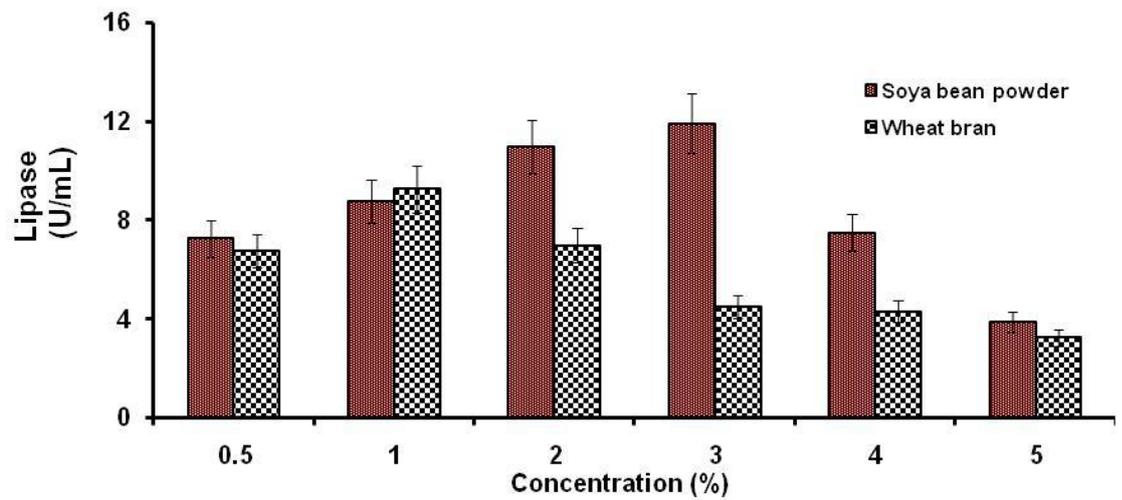
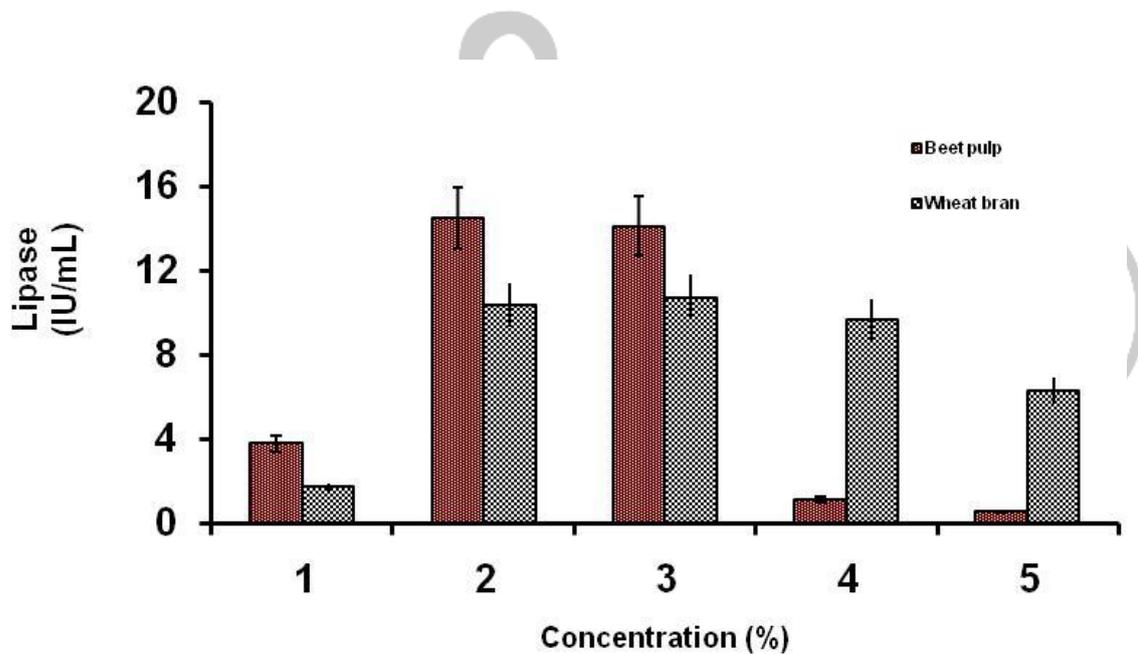


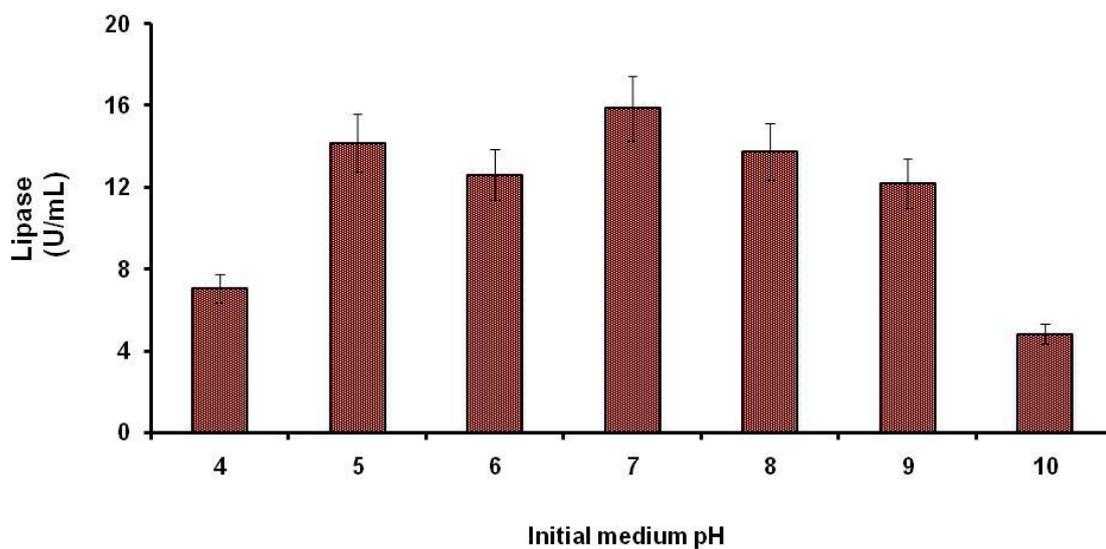
Fig.2. Effect of carbon sources on lipase produced by *Penicillium italicum* FSS48 and *Penicillium expansum* FSS241



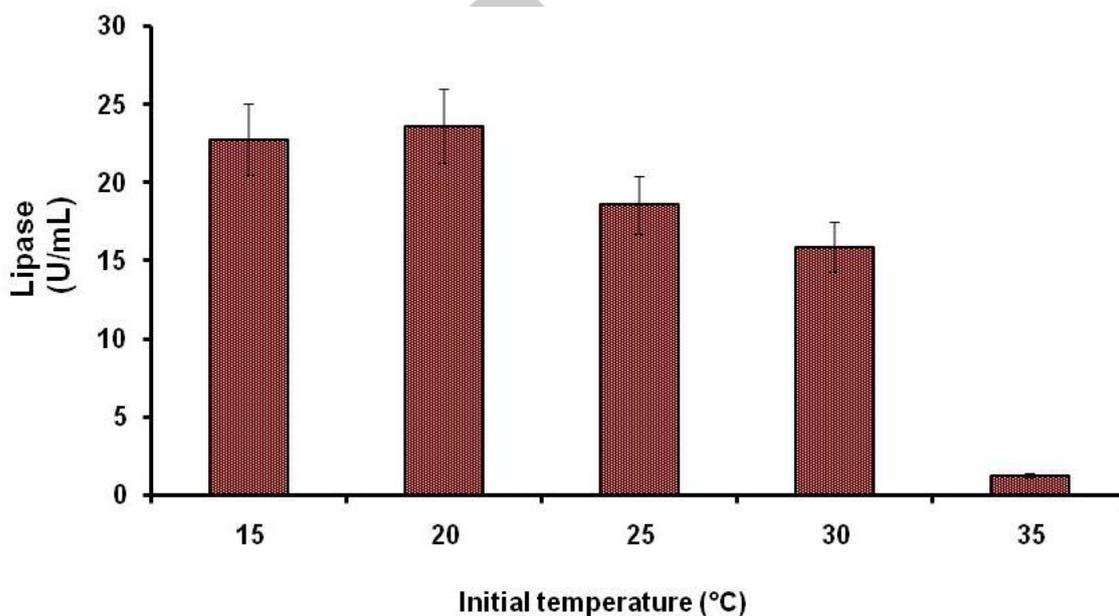
**Fig.3.** Effect of soya bean powder and wheat bran concentration on lipase produced by *Penicillium italicum* FSS48



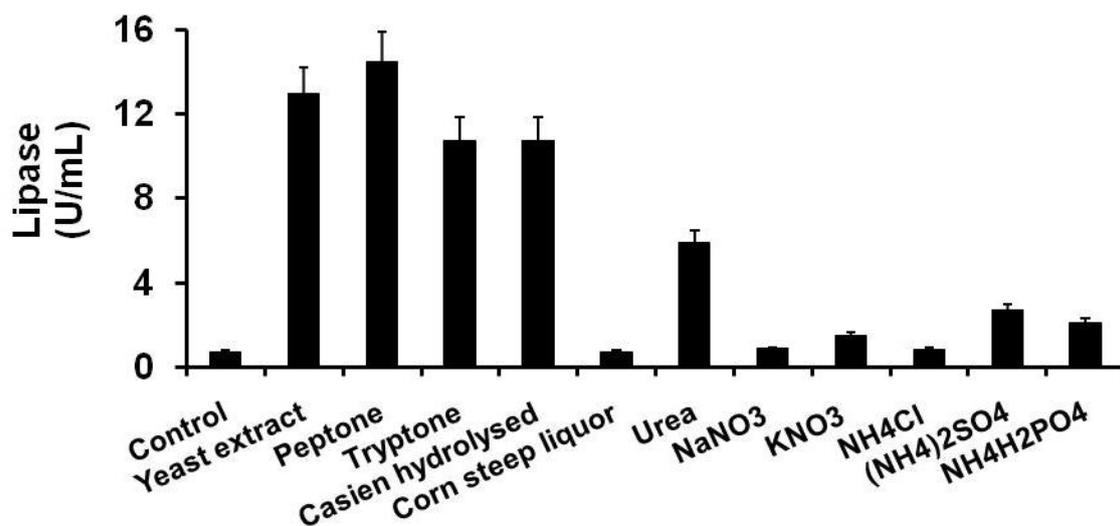
**Fig.4.** Effect of beet pulp and wheat bran concentrations on lipase produced by *Penicillium expansum* FSS241



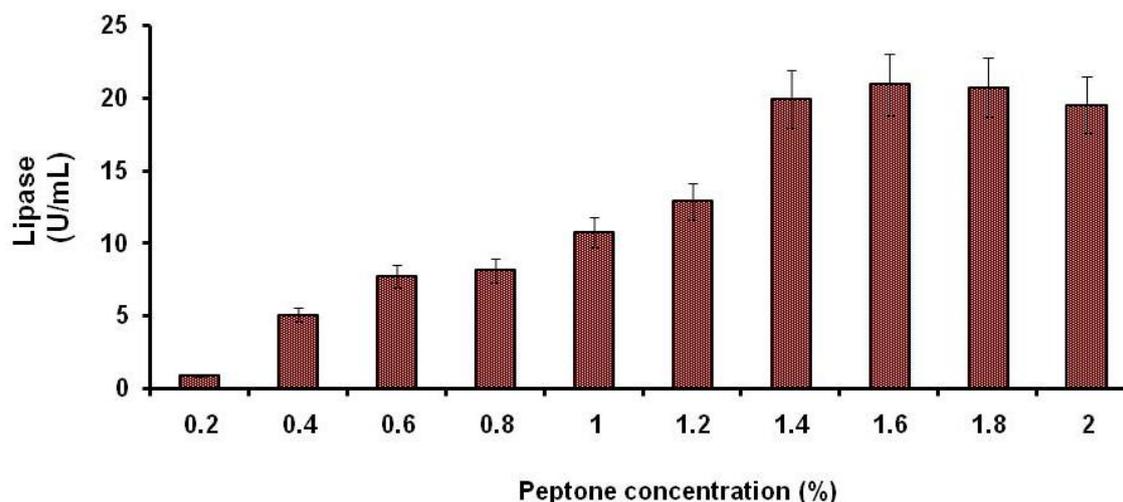
**Fig.5.** Effect of initial medium pH on lipase produced by *Penicillium expansum* FSS241



**Fig.6.** Effect of incubation temperature on lipase produced by *Penicillium expansum* FSS241



**Fig.7.** Effect of nitrogen sources on lipase produced by *Penicillium expansum* FSS241



**Fig.8.** Effect of peptone concentration on lipase produced by *Penicillium expansum* FSS241

making it possible to calculate the percentage of homology of sequence carried out this alignment per pairs of nucleotide. The nucleotide BLAST similarity search analysis, based on the 5.8S DNA gene sequence revealed that FSS48 and FSS241 isolates were closely related to *Penicillium italicum* and *Penicillium expansum*, respectively, with a 100 % homology.

**Identification of the selected isolates**

DNA sequencing of the internal transcribed spacer (ITS) regions, shows promise to identify a broad range of fungi to the species level (Li et al., 2007). The phylogenetic analysis is based on the sequence gene 5,8S DNA and was carried out by alignment of sequences consensus of genes 5,8S DNA collected in the Gene Bank database. An algorithm

production from FSS241 (figure 4) are 2 and 3%, respectively.

#### **Effect of initial medium pH on lipase production from *Penicillium expansum* FSS241**

Lipase production effected by initial pH of fermentation medium, so determining initial pH of lipase production medium is important for obtaining good lipase yield by *P. expansum* FSS241. Figure 5 showed that highest lipase production obtained at pH7 (15.125 U/ml). Gopinath *et al.*, (2003) reported optimum lipase production at pH7 by *Geotrichum candidum*, while optimum lipase production by *Penicillium expansum* PED-03 was at pH 5.5-6 (Dai & Xia, 2005).

#### **Effect of incubation temperature on lipase production from *Penicillium expansum* FSS241**

Temperature is one of the important parameters that determines the success of submerged fermentation system. Therefore, the effect of temperature on lipase production by *P. expansum* FSS241 was examined and the results obtained are shown in Figure 6. The production of lipase was maximum at the ambient temperature 20°C with an activity of 23.63 U/ml. This temperature was in agreement with those reported by Pinheiro *et al.*, (2008) who showed that the highest lipase production by *Penicillium verrucosum* was obtained at temperature 20°C in submerged fermentation.

#### **Effect of nitrogen sources on lipase production from *Penicillium expansum* FSS241**

The effect of nitrogen source supplementation on the production of

#### **Effect of carbon sources on lipase production from *Penicillium italicum* FSS48 and *Penicillium expansum* FSS241**

The production of primary metabolites by microorganisms are highly influenced by their growth, which is determined by the availability of the nutrients in the substrates. Therefore, lipase production is influenced by the type and concentration of carbon and nitrogen sources (Elibol & Ozer, 2000). Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield; however, a few authors have produced good yields in the absence of fats and oils as a carbon sources. The financial cost of medium preparation is a major role in determining the economic feasibility of the enzyme production process. A number of available agriculture wastes and relatively cheap price were tested. As it is shown in Figure 2, the carbon source exhibit better lipase production from FSS48 is soya beans powder followed by wheat bran and from FSS241 is sugar beet pulp followed by wheat bran.

Investigations of the carbon source effects have been done in order to find good lipase inducers and enhance lipase production (Prazeres, 2006; Andrade, 2013).

The concentration effect of the best carbon sources on lipase production from *Penicillium italicum* FSS48 and *Penicillium expansum* FSS241 were studied. Figure 3 showed that 3% soya beans powder and 1% wheat bran are the best concentration for lipase production from FSS48, and the best concentration in sugar beet pulp and wheat for lipase

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lipase by *Penicillium expansum* FSS241 was also examined with 2% sugar beet pulp. The results obtained using various nitrogen sources are shown in Figure 5. As shown in Figure 7, when compared to the cultivation in the absence of any nitrogen sources the presence of nitrogen sources have improved lipase production. Among the nitrogen sources tested, peptone was found to enhance the production of lipase at 1% (w/v) by about 94.8% compared to the cultivation without the addition of any nitrogen sources. This agreement with results of Ülker (2011) in which peptone was the best nitrogen source for lipase production by *Trichoderma harzianum*.

The effect of the concentration of peptone between 0.2 and 2% on the enzyme production was examined. The results obtained indicated that the optimum concentration was 1.6% (w/v) with the lipase production of about 20.9U/ml (Figure 8).

## Conclusion

Results obtained in the current study demonstrate that the local *Penicillium expansum* FSS241 strain is a promising source of lipase production using a cheap medium, and production have been increased with increment about 39.5% compare to values before optimization culture conditions and enzyme-substrate interaction conditions. Therefore, this strain could be alternative of the commercial strain. However, the process of lipase production from the new local *Penicillium expansum* FSS241 strain may be commercialized after further optimization for enhanced enzyme production in fermenter.

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