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Full Length Research Paper

Production of lipase from genetically improved *Streptomyces exfoliates* LP10 isolated from oil-contaminated soil

Magda M. Aly[1,2]*, Sanaa Tork[1,3], Salel M. Al-Garni[1] and Lubna Nawar[1]

[1]Biology Department, Faculty of Science, King Abdulaziz University, Saudi Arabia.
[2]Botany Department, Faculty of Science, Kafr El-Sheikh University, Egypt.
[3]Microbial Genetics Department, National Research Center, Dokki, Cairo, Egypt.

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Lipases (triacyl glycerol acyl hydrolase) catalyze hydrolysis and syntheses of ester formed from glycerol and long-chain fatty acids. They have many industrial applications, especially in food and detergent industries. Out of 33 bacterial isolates, a group of 20 bacterial isolates produced lipase enzyme on tributyrin agar and Tween 80 agar media. In liquid medium, the lipase activity was ranged from 1.5 to 6.9 IU/ml. Among the evaluated bacteria, the isolate LP10 that was isolated from soil collected from fuel station. It was the most active isolate in lipase production (6.9 IU/ml). Using morphological, physiological and biochemical studies, it was identified as an isolate belonging to the genus *Streptomyces* and identified as *Streptomyces exfoliates* LP10. Identification was confirmed using 16S rDNA analysis. Growth of the selected bacterium in medium containing tributyrin and Tween 60 at initial pH 6 in addition to incubation at 37°C for three days yielded the maximum lipase production. The molecular weight of the purified enzyme was 60 kDa, determined using gel electrophoresis. Improvement of lipase production was carried out between *Streptomyces exfoliates* LP10 and *Streptomyces niveus* using protoplast fusion. Five fusants were obtained. Fusant LP3 was the best lipase producer (3 times higher) compared to its parents.

Key words: Lipase, *Streptomyces exfoliates*, tributyrin, protoplast fusion, 16S rDNA.

INTRODUCTION

Lipolytic enzymes are one of the most important groups of biocatalysts for biotechnological application (Jaeger and Eggert, 2002). The enzyme include esterase and lipases which catalyze the hydrolysis and synthesis of short chain (≤10) and long chain acylglycerol, respectively (Arpigny and Jaeger, 1999; Sharma et al., 2001). Lipases (EC 3.1.1.3) hydrolyze triacylglycerols to fatty acids, di-acylglycerols, monoacyl glycerols and glycerol whatever under certain conditions, catalyze reverse reactions such as esterification and trans-esterification (Nini et al., 2001; Park and Mori, 2005).

Lipase enzyme has many potential applications in different industrial sectors and biotechnology such as detergent manufacturing, food ingredient production, wastewater treatment, paper processing, pharmaceutical production, fine chemical synthesis and manufacture of pesticides, cosmetics and single cell protein (Jaeger et al., 1999). They can be used as catalyst for synthesis of esters and for trans-esterification of the oil for the production of biodiesel (Gunstone, 1999; Poonam et al., 2005; Gulati et al., 2005).

The interest in microbial lipase production has increased due to many useful features such as broad substrate specificity, the versatility of the molecular structure, and stability in organic solvents (Jaeger et al., 1994; Elibol and Ozer, 2000). Lipases secreted into the culture medium by many fungi and bacteria recently have attracted considerable attention owing to their biotechnological potential (Aires-Barros et al., 1994, Mori et al., 2009). Bacterial lipases were observed in the strains of *Serratia marescens, Pseudomonas aeruginosa*

*Corresponding author. E-mail: magdammali@hotmail.com. Tel: 00966546407565.

(Hasan et al., 2006; Madan and Mishra, 2010) and *Bacillus* species (Ramchuran et al., 2006; Dutta and Ray, 2009; Sangeetha et al., 2010). Other genera like *Acinetobacter, Staphylococcus, Streptococcus, Burkholderia, Achromobacter, Arthrobacter, Alcaligenes, Chromobacterium* and *Streptomyces* (Abramic et al., 1999; Riaz et al., 2010; Sangeetha et al., 2011) have been studied as lipase producer. The number of bacterial lipases that have been purified and characterized and whose genes have been sequenced is increasing (Sommer et al., 1997) especially for lipases of *Staphylococcus, Bacillus,* and *Streptomyces* species (Dartois et al., 1992; Cruz et al., 1994). Although *Streptomyces* strains were recognized through their high exogeneous lipolytic activity, lipases of this genus were not studied as intensively as were those from some other bacteria (Jaeger et al., 1994). *Streptomyces exfoliatus* extracellular lipase is the only lipase from the *Streptomyces* genus whose crystal structure has been determined. Its chain length specificity has not been characterized (Wei et al., 1998). However, only a few streptomycete lipases have been characterized until now. From *S. exfoliatus* and *Streptomyces albus* two very homologous lipases have been characterized genetically (Perez et al., 1993; Cruzet et al., 1994) and the three-dimensional structure of the *S. exfoliatus* enzyme has been elucidated (Wei et al., 1998). In addition, an extracellular lipase-encoding gene from *Streptomyces cinnamomeus* was cloned and sequenced in addition no similarity to the two *Streptomyces* lipase sequences was found (Sommer et al., 1997). This suggests much higher variability of lipases than expected in this bacterial group.

Because of huge variation in applications, the availability of lipases with specific characteristics is still a limiting factor. Thus to search for new lipases with different characteristics and improve lipase production continue to be important research topics. Generally, the enzymes of industrial interest were produced in the presence of inducers like triacylglycerols, surfactants, vegetable oils, oil industry wastes or their hydrolysis products in the culture medium. Inducers as Tween 80 and oils of soybean and olive have been evaluated for lipase production (Li et al., 2004).

This paper dealing with the screening of bacterial isolates obtained from oil-contaminated habitats for lipase production, identification of the most active isolate, optimization of growth conditions for maximum enzyme production, molecular weight determination of the pure enzyme and enhancement of lipase production by protoplast fusion.

MATERIALS AND METHODS

Isolation and purification of the bacterial isolates

Soil samples were collected from oil-contaminated places; sand, marine water, wastewater, plant roots and marine shrimps from Jeddah city, Saudi Arabia. Serial dilutions were prepared from soil, sand and water, 0.1 ml of the last dilution was spread on starch nitrate agar medium. Plant roots and marine animals were cut into small parts and were put on the surface of the previous medium as described by Aly et al. (2011). The inoculated plates were incubated at 30°C for 4 days. The developed colonies were purified, and then were maintained on nutrient agar slants at 4°C.

Screening for lipase production by the isolated bacteria

All the previous isolated bacteria were streaked on solid tributyrin agar medium (Smibert and Krieg, 1981) containing (g/L), peptone from meat 2.5; peptone from casein 2.5; yeast extract 3.0; agar-agar 15.0 and 10.0 ml tributyrin (glycerol tributyrate), pH was adjusted to 7.5. The presence of clear zone around the colonies indicated lipase production. The diameter of clear zone was measured for each colony. All lipase-producing bacteria were screened on Tween 80 agar medium (Gopinath et al., 2005) and were individually grown in Basal Broth medium and lipase activity was assayed.

Production of lipase in liquid media

The cultures were prepared by dispensing 48 ml of Basal Broth medium or any tested broth medium in 250 ml Erlenmeyer flasks. The basal medium for lipase production contained olive oil (standard) 7% (v/v), dextrose 1% (w/v) peptone 3% (w/v) pH 7. After sterilization, about 2ml (4×10^6 CFU/ml) of bacterial suspension, previously grown in nutrient broth medium for 2 days at 30°C, were inoculated into each flask. The inoculated flasks were shook on a rotatory shaker (150 rpm) at 30°C for 5 days. The cells were harvested by centrifugation at 10,000 rpm for 15 min and the supernatant was used for lipase assay. All steps were carried out under aseptic conditions.

Enzyme assay by titrimetic method

The enzyme assay was performed with the cell free supernatant as the crude enzyme source. One ml sample solution was added to the assay substrate containing 10 ml of 10% homogenized olive oil in 10% gum acacia, 2 ml of 0.6% $CaCl_2$ solution and 5 ml of 0.2 mol/L phosphate buffer pH 7.0. The enzyme substrate mixture was incubated on an orbital shaker with a shaking speed of 100 rpm at 37°C for 1 h. To stop the reaction, 20 ml ethanol/acetone mixture (1:1v/v) was added to the reaction mixture. Liberated fatty acids were titrated with 0.1 M NaOH and the appearance of pink color was taken as the endpoint. One lipase unit (IU) is defined as the amount of enzyme that liberated 1 µmol ρ nitrophenol/min under the assay conditions.

Taxonomical studies

Morphological, physiological and biochemical characterization

The best lipase producing actinomycete was characterized and identified. It was grown on different agar media. Morphological studies were conducted using light and electron microscopy. Carbon and nitrogen utilization in addition to sensitivity of the selected bacterium to different antibiotics were carried out. Analysis of the isomer of diaminopimelic acid (DAP) of the cell wall and the whole-cell sugar composition were determined as described by Hasegawa et al. (1983). Phospholipid types and fatty acid after transforming to methyl esters (Butte, 1983) were determined using two-dimensional thin-layer- and gas chromatography (Hoischen et al., 1997), respectively.

Figure 1. Lipase activity of Lp10 grown on (A) Tributyrin agar medium and lipolytic activity is indicated by a halo around the colony, (b) Tween 80 medium and lipolytic activity is indicated by a precipitate around the growth.

Phylogenetic analysis of 16S rDNA sequence

Genomic DNA from the selected isolate LP10 was obtained using QIAamp DNA Mini Kit (Aly et al., 2011). The primers were designed based on the highly conserved region of 16S rDNA from various bacteria (Weisberg et al., 1991; Tork et al., 2010). The 16S rDNA gene was amplified by PCR. The PCR products were purified using QIAquick PCR purification kit (QIAGEN) and sequenced using ABI PRISM 310 genetic analyzer (Perkin Elmer, USA). The DNA sequence was compared to the GenBank database in the National Center for Biotechnology Information (NCBI) using BLAST program.

Optimization of lipase production

The growth (A_{550}) and lipase production of the selected bacterium were determined using different media with varying composition after 5 days of growth at 30°C and 150 rpm. The media used were medium A comprised of 0.2% glucose, 0.5% yeast extract, 0.5% tributyrin and 0.5% sodium chloride, 0.04% calcium chloride, 0.2% magnesium chloride, 0.1% magnesium sulphate and 0.1% potassium dihydrogen phosphate. Medium B had 0.5% peptone, which replaced yeast extract in medium A, medium C had 0.5% Tween 80 added to medium A, medium D had 0.5% Tween 80 added to medium B. Medium E was composed of g/L: Nutrient Broth 13, $CaCl_2.2H_2O$ 0.1, Tween 80 (10 ml), medium F as described by Lesuisse et al. (1993), medium G as described by Sugihara et al. (1991) and medium G Basal Broth medium that was used as control. The pH of all the media was adjusted to 7.0. All the experiments were carried out in triplicate and the averages were reproduced. The best medium for optimization studies was selected based on maximum lipase production

Growth and lipase production at different temperatures (20, 25, 30, 35, 37 and 40°C) by the selected bacterium LP10 in medium C (pH 7) were determined after 5 days of growth at 150 rpm. Similarly, the effect of different incubation periods (1 to 7 days) at 150 rpm on growth and lipase production by the selected bacterium LP10 in medium C was measured after incubation at 40°C. Effect of different initial pH values on growth and lipase production by the isolate LP10 was studied using medium C that was prepared with different initial pH values (5 to 9). Acetate buffer was used in the pH range of 4 and 5, phosphate buffer was used for pH 6 and 7 and finally Borate buffer was used for 7 to 9. After incubation of the isolate LP10 at 40°C and 150 rpm for 3 days, growth and lipase production was measured as described earlier.

Purification and molecular weight determination of lipase enzyme

LP10 was grown in 2000 ml of Medium C (pH6), divided in 250 ml Erlenmeyer flasks. Each flask contained 48 ml of the medium and inoculated with 2ml of the pre-culture ($4x10^6$ CFU/ml). After 3 days of growth at 40°C and 150 rpm, the cells were collected after centrifugation at 5000 rpm for 15 min. Solid ammonium sulphate was added to the cold bacterial filtrate with stirring to 80% saturation. The dialyzed solution was concentrated under vacuum and applied to diethylaminoethyl-cellulose (DEAE cellulose) followed by Sephadex G75 column chromatography. The purified enzyme was collected, lyophilized and analyzed. The molecular weight was also determined by SDS-PAGE according to the method of Laemmli (1970) and Sugihara et al. (1990).

Protoplast fusion and antibiotic pattern for the two selected bacteria

Protoplast fusion was carried out between *S. exfoliatus* (LP10) and *Streptomyces niveus* described by Aly et al. (2003). Protoplast fusion was performed to obtain fusant highly producer for lipases. The antibiotic patterns for the two tested bacteria detected on Mueller Hinton medium using paper disc diffusion assay were as follows: *S. exfoliatus* (LP10) was *strep.* $^-$ *tet.* $^+$ and *S. niveus* was *strep.* $^+$ *tet.* $^-$

Statistical analysis

Each reading had three replicates. Means of variable and standard deviation were recorded. Data were subjected to statistical analysis and difference between mean values determined by the Student's t-test. Differences were considered significant when probability was less than 0.05.

RESULTS

Bacterial isolates used in this study were obtained from different sources including oil-contaminated soils, sand, wastewater, marine animals and roots. Thirty-three bacterial isolates were obtained on starch nitrate agar medium. All the isolates were screened on tributyrin agar medium. Out of the 33 isolates, 20 isolates hydrolyzed the lipid material producing a clear zone around the growth (Figure 1a). The activity in lipase production for each isolate was measured by the diameter of the clear zone. Lipase producing isolates were grown on agar medium containing Tween 80. The presence of white precipitate around the colonies indicated lipase production (Figure 1b). The activity of lipase produced by each isolate, detected in liquid medium was ranged from 1.5 to 6.9 IU/ml (Table 1). Based on the screening data,

Table 1. Source of isolation, colony color and lipase production by the isolated bacteria.

No. of bacterial isolate	Source of isolation	Type of the isolate	Color of the colony	Lipase detection			
				Solid medium			Liquid medium (IU /ml)
				Presence of precipitate around the colony	Diameter of the clear zone (mm)		Lipase activity
LP1	Contaminated soil	FB	White	+++	20		4
LP2	Contaminated soil	TB	White	+++	22		4.1
LP3	Contaminated soil	TB	White	++	14		3.7
LP4	Contaminated soil	TB	Yellow	+	12		1.7
LP5	Contaminated soil	TB	Pink	+	12		1.9
LP6	Contaminated soil	TB	creamy	+	10		1.9
LP7	Contaminated soil	FB	Pale gray	++	20		2.4
LP8	Contaminated soil	FB	White	++	20		2.3
LP9	Sand	FB	Green	+	15		1.4
LP10	Waste water	FB	Pale yellow	+++	28		6.9
LP11	Waste water	TB	orange	+++	11		4.4
LP12	Waste water	TB	Creamy	++	10		2.5
LP13	Waste water	TB	Pale yellow	+++	16		4.6
LP14	Waste water	FB	Pale gray	++	12		2.5
LP15	Waste water	FB	Brown	+	8		1.6
LP16	Waste water	FB	Pink	+	8		1.5
LP17	Plant roots	TB	Creamy	+	10		1.6
LP18	Marine water	TB	Gray	+	8		1.7
LP19	Marine shrimp	TB	Gray	+	8		1.7
LP20	Marine shrimp	FB	Brown	+	8		1.5

+: little precipitate, ++: moderate precipitate, +++: strong precipitate, TB : true bacteria, FB: filamentous bacteria.

the isolate LP10, obtained from oil contaminated soil sample was the best lipase producer and it was selected for more detail experiments.

The isolate LP10 was characterized by morphological, physiological, biochemical properties and composition of cell wall constituents in addition to the characteristics lipids, sugars and fatty acids. The growth of the isolate LP10 on different growth media was ranged from heavy, moderate to poor (Table 2). Table 3 summarized the morphological characters of the selected isolate. Microscopic observation of the isolate LP10 (Figure 2) showed substrate and aerial mycelia bearing a straight chain of conidia which had smooth surface. The diameter of the spore was ranged from 4 to 5 and 6 to 9 µm. No zoospore, sporangium, sclerichia or fragment hyphae were present. Some physiological characters were recorded in Table 4. No melanin pigment was detected and temperature growth range was approximately 15 to 45°C. The isolate LP10 was resistant to a wide variety of antibiotics, including, penicillin, cephalosporin, rifampin and kanamycin (Table 4). It was sensitive to tetracyclines. The bacterium grew aerobically, strongly catalase and oxidase positive.

Glucose, glycerol, mannitol and sucrose were good carbon sources whereas the suitable nitrogen sources were ammonium chloride, sodium nitrate and amino acids (Table 5). Analyses of whole cell wall hydrolysates, revealed the

Table 2. Cultural characteristics of the actinomycete isolate LP10, grown on different agar media at 30°C.

Media	Growth	Color of aerial mycelium	Color of substrate mycelium	Presence of soluble pigment
Starch-nitrate agar	Heavy	Yellow	Yellowish-white	+
Glucose Asparagine agar	Heavy	Dark yellow	Yellow	+
In-organic salts-starch iron agar (ISP-4)	Moderate	Pale yellow	Yellowish- white	+
Tyrosine agar (ISP-7)	Scanty	Yellow	Pale yellow	+
Yeast extract-malt extract agar (ISP-2)	Moderate	Yellowish brown	Yellow	+
Oatmeal agar (ISP-3)	Moderate	White	Pale yellow	+
Glycerol asparagine agar (ISP-5)	Moderate	Dark yellow	Yellow	+
Bennet agar	Scanty	Yellow	Yellow	+
Omura agar	Heavy	Yellow	Dark yellow	+

+ : Soluble pigment present

Table 3. Morphological characters of the selected isolate LP10.

Tested character	Results
Gram stain	Gram positive
Source of isolation	Oil contaminated soil
Motility of spore	Absence
Shape of spore	Cylindrical (5-7 and 6-7 µm)
Spore chain	Straight chain
Spore Surface	Smooth
Number of spore/ chain	5-25
Aerial and substrate mycelia	Well developed
Zoospore, sporangium, sclerichia and fragmented mycelium	Absence

presence of only L-isomer of diaminopimelic acid were (L-DAP), in addition to glucose. Whole cell sugar pattern was glucose and analysis of phospholipids of the isolate indicated the presence of phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Some saturated branched and non branched fatty acids were detected in the strain LP10 and no mycolic acids were present (Table 6).

The 16S rDNA sequence was compared to the GenBank database in the National Center for Biotechnology Information (NCBI) using the BLAST program. The 16S rDNA sequence of this isolate LP10 showed high levels of sequence similarity with members of the genus *Streptomyces*, such as *S. exfoliates* (95%) as shown in Figure 3.

The growth and lipase production varied with medium used, incubation temperature, incubation period and initial pH of the medium. Eight different media for lipase production by the selected bacterium were used and lipase was measured (Figure 4). The best medium for lipase production was medium C (7.55 IU/ml) followed by medium H (7.0 IU/ml). The effect of temperature on lipase production was carried out by culturing *S.*

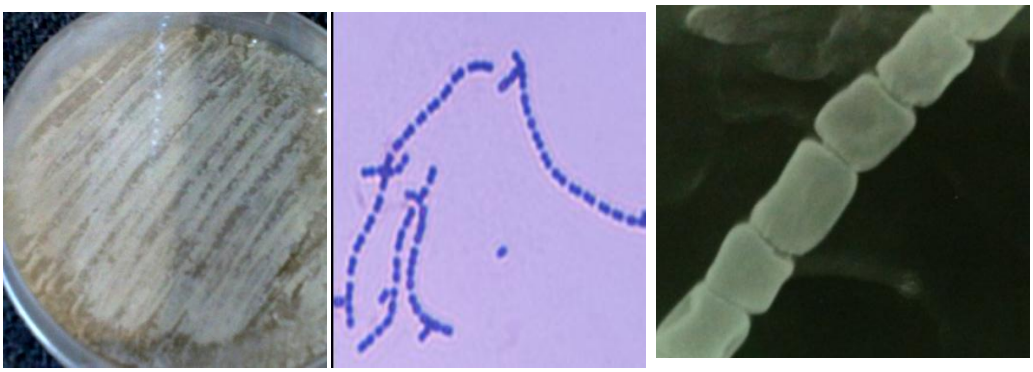

Figure 2. The selected actinomycete isolate LP10, A: grown on starch nitrate agar medium, B: under light microscope x1000, C: under scanning electron microscope (X 20 000).

Table 4. Physiological characteristics of the isolate LP10.

Character	Reaction	Resistance to antibiotics	Result
Melanin pigment production on tyrosine agar and iron agar	+ve	Penicillin	+
H_2S production	-ve	Cephalosporine	+
Enzyme activities		Kanamycin	+
Lecithinase,	-ve	Rifampin	+
Chitinase, gelatinase, pectinase and proteinase	+ve	Tetracyclines	-

-ve: Negative results, +ve: Positive results, -: Sensitive, +: Resistance.

Table 5. Utilization of different carbon and nitrogen sources by the isolate LP10.

Carbon source	Utilization	Nitrogen source	Utilization
Negative control (No carbon source)	-	Na NO_3	++
Positive control (Glucose)	++	NH_4NO_3	++
D-xylose	++	NH_4Cl	++
D-mannitol	++	Lysine	+
Glycerol	++	Valine	+
Raffinose	-	Phenyl alanine	++
D- galactose	-	Tryptophan	+
Sucrose	++	Glutamate	+

++: Good utilization, + : Utilization, -: No utilization.

exfoliates LP10 at temperatures from 10 to 45°C on medium C (Figure 5). The most suitable temperature for growth and lipase production was 37°C (9.7 IU/ml). Effect of different incubation periods on growth and lipase production by the *S. exfoliates* LP10 was illustrated in Figure 6. The maximum lipase activity of the enzyme was recorded after 3 days of growth (9.96 IU/ml).

The effect of pH of medium on lipase production was shown in Figure 7. The maximum lipase activity was obtained at initial pH 6.0 (11.7 IU/ml) and lipase production dropped significantly at pH 9.0 (Figure 7). No production of lipase was observed at pH 9.5.

S. exfoliates LP10 which was isolated from oil contaminated soil collected from Jeddah was selected for production of lipase. It was grown using the best conditions detected for lipase production. The enzyme was precipitated with 80% ammonium sulfate, purified using different column chromatography and the profile of elution was determined. The active fractions that showed maximum lipase activity were collected, lyophilized and used for enzyme characterization and molecular weight determination. The molecular weight was determined to be 60 kDa using gel electrophoresis (Figure 8).

Genetic improvement was carried out using protoplast

Table 6. The biochemical tests (sugar, amino acid, phospholipids, and fatty acid composition of the cell wall or cell hydrolysate) of the isolate LP10.

Type of the reaction	Results
Sugar in the cell hydrolysate	
Glucose	+
Amino acids in the cell wall	
Diaminopimelic acid (DAP)	+ (L-Form)
Glutamic acid	+
Glycine	+
Alanine	+
Lysine	+
Phospholipids	
Phosphatidylethanolamine (PE)	+
Phosphatidylinositolmannoside (PIM)	+
Phosphatidic acid (PA)	+
Phosphatidylinositol (PI)	+
Fatty acids	Saturated Iso- and antiso fatty acids.

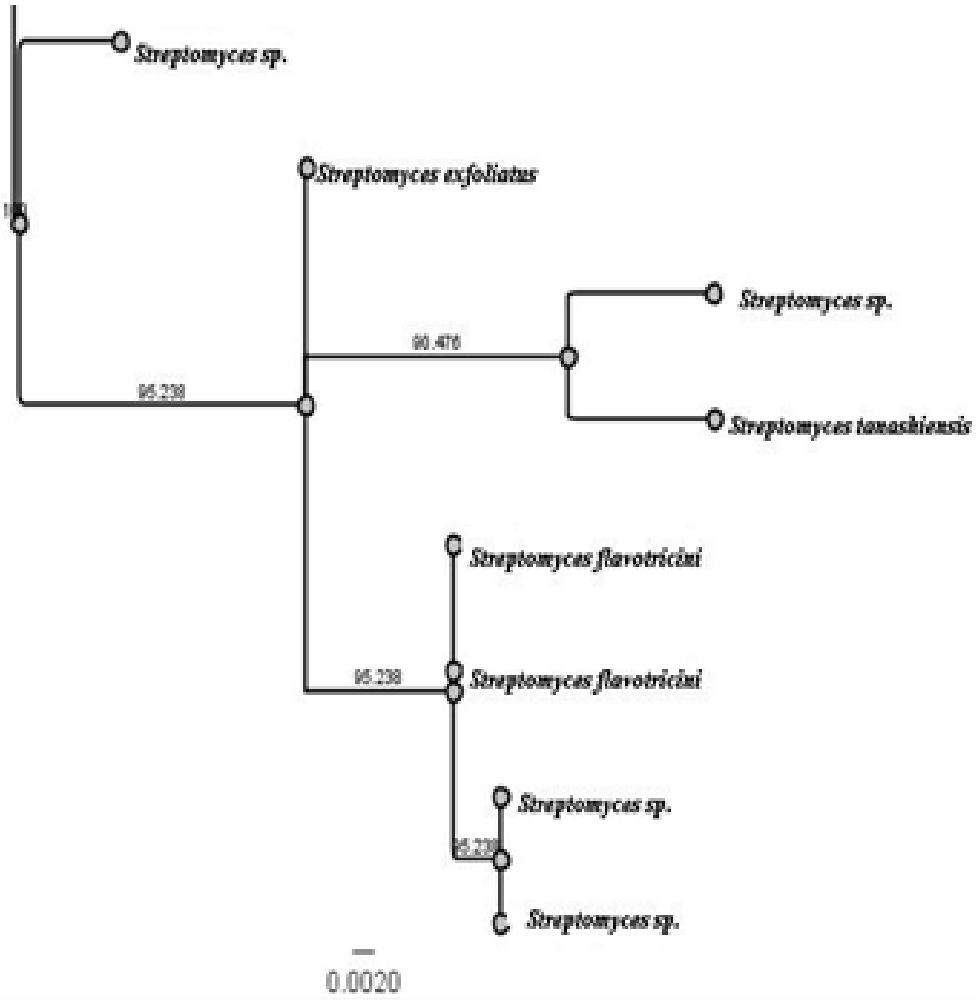

Figure 3. Phylogenetic tree based on 16S rDNA sequence comparisons of *Streptomyces* LP10, using neighbor joining tree method, maximum sequence difference =0.002.

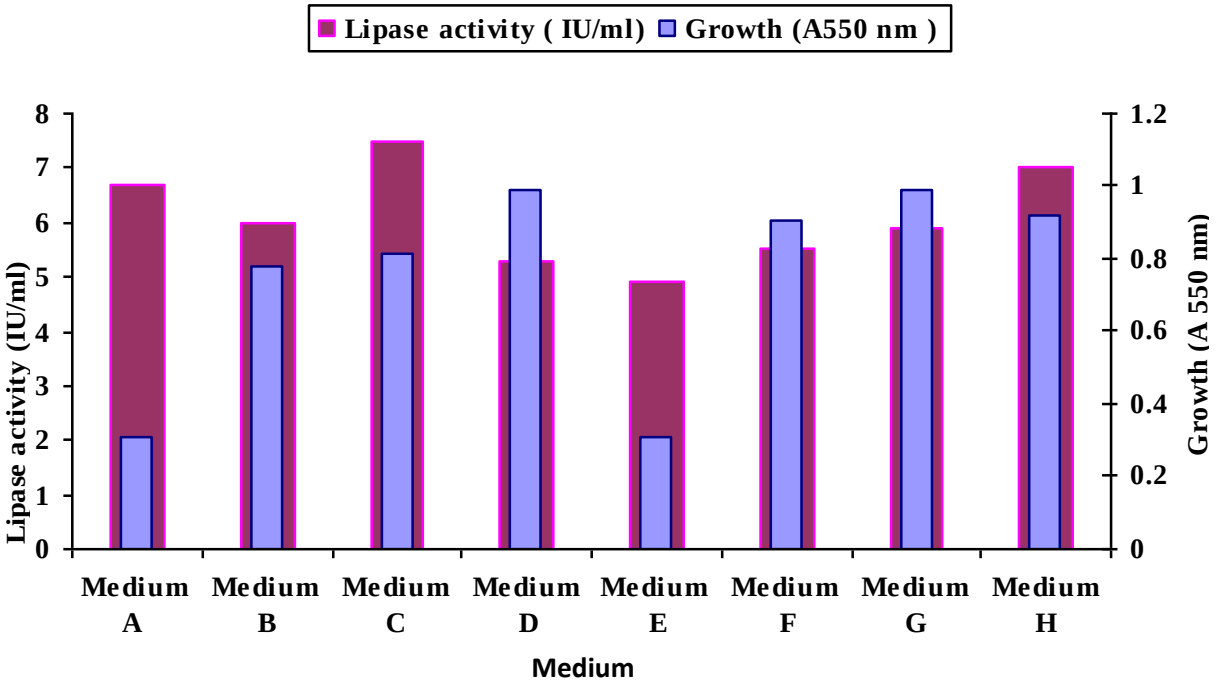

Figure 4. Effect of different media on growth (A_{550}) and lipase production (IU/ml) by *Streptomyces exfoliates* LP10.

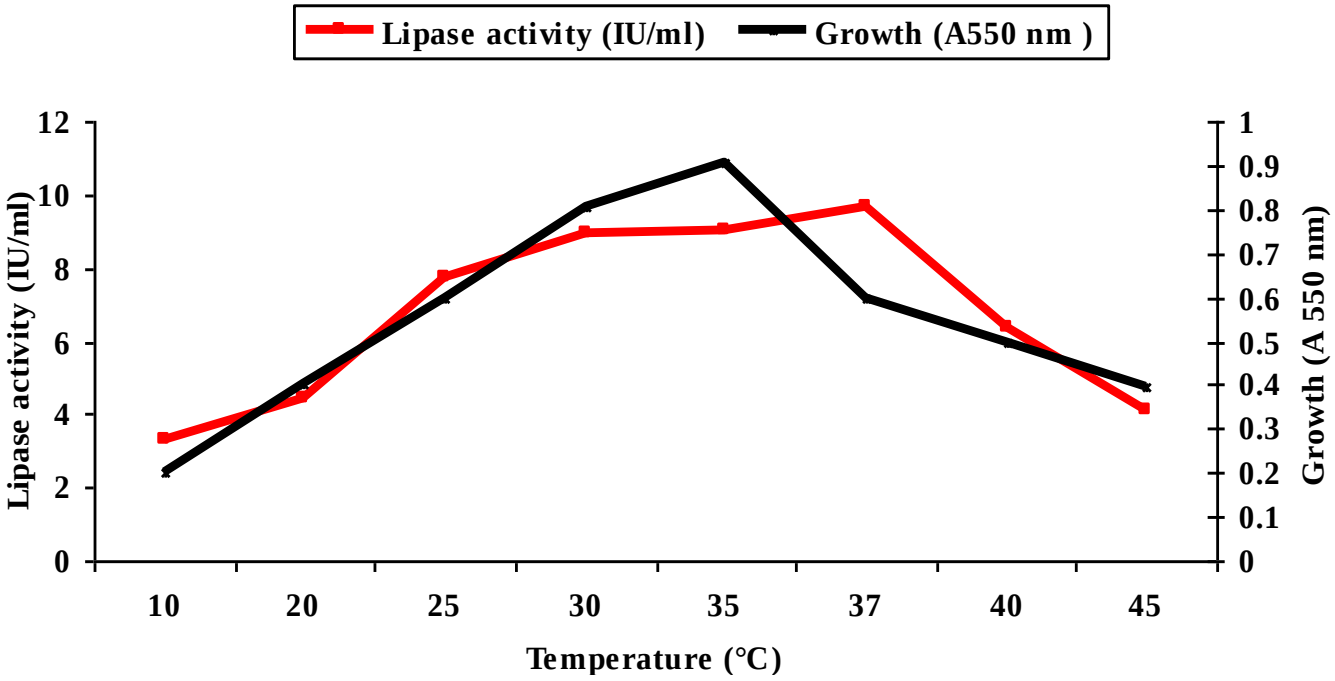

Figure 5. Efffect of different temperature on growth (A_{550}) and lipase production (IU/ml) by the selected bacterium LP10.

fusion between *S. exfoliates* LP10 and *S. niveus*. For *Streptomyces* LP10, 86% of cells converted into protoplast and could regenerate successfully but 78% of cells of *S. niveus* yielded real protoplast and could regenerate successfully. Protoplasts of the two parents of *Streptomyces* were fused using 25% PEG 6,000 and then plated on minimal regeneration medium containing both streptomycin and tetracycline. The developed

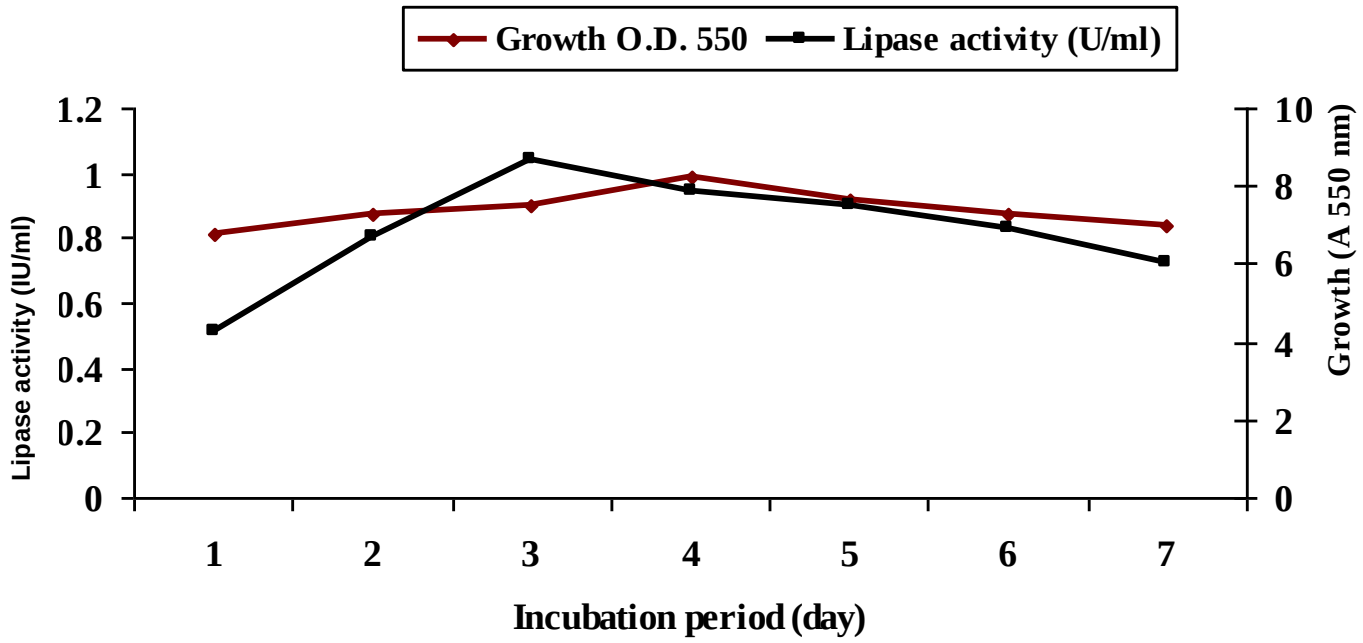

Figure 6. Effect of different incubation peroid on growth (A_{550}) and lipase production (IU/ml) by the selected bacterium LP10.

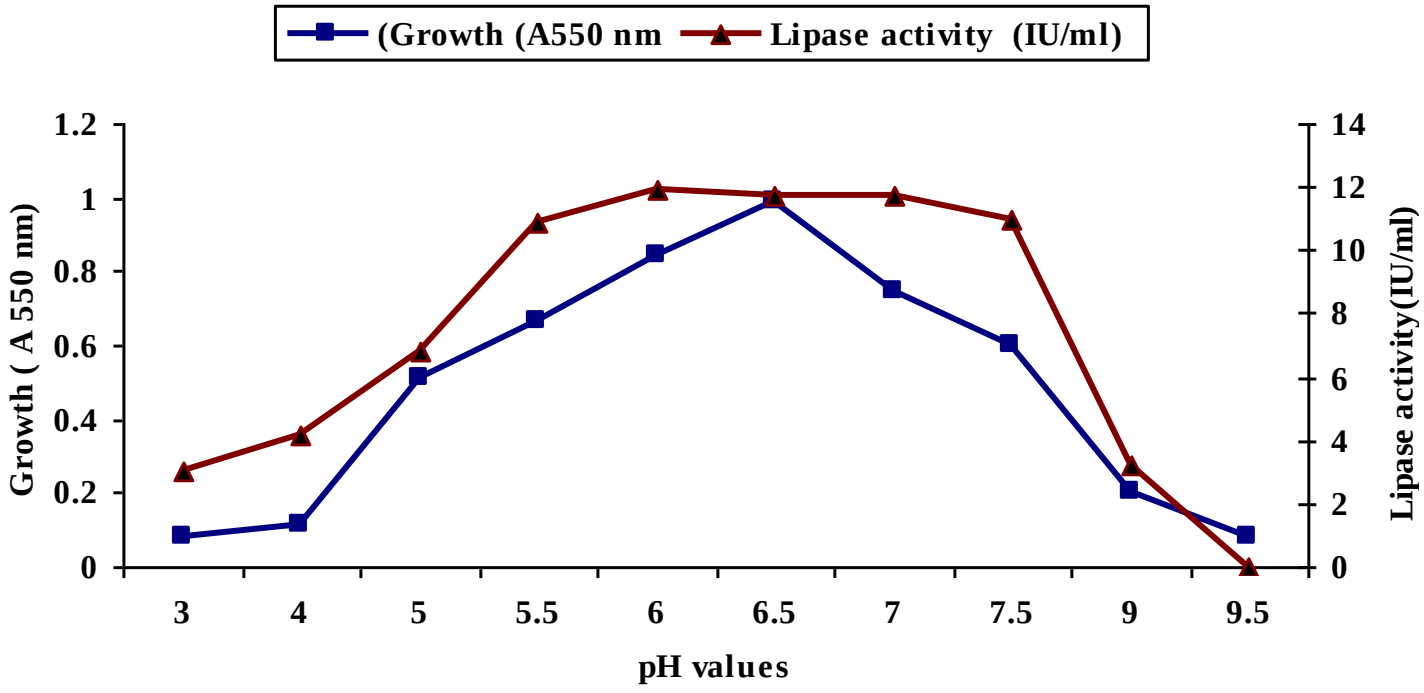

Figure 7. Effect of different pH on growth (A_{550}) and lipase production (IU/ml) by the selected *Streptomyces exfoliates* LP10.

colonies were only the heterocaryons or prototrophic recombinants (*strep.* $^{+}$ *tet.* $^{+}$). Five fusants were successfully isolated and screened for lipase production. One of them (Fusant LP 3) had higher lipase production (3 times) than its parent (Figure 9).

DISCUSSION

Microbial lipases are currently receiving much attention with the rapid development of enzyme technology (Karam and Nicell, 1997). In this study, 33 bacterial isolates were

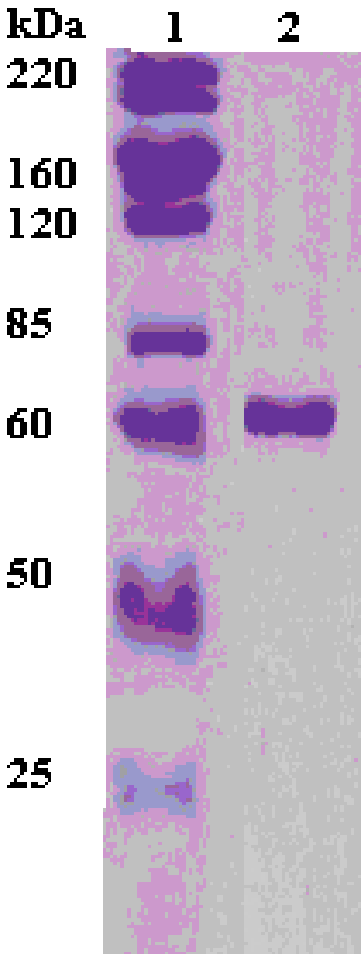

Figure 8. Molecular weight of the purified lipase isolated from *Streptomyces exfoliates* by SDS-PAGE. Lane 1: Protein standard (220-15 K Da), Lane 2: Purified lipase.

screened for lipase production. 20 bacterial isolates (60%) produce lipase on solid growth medium containing either 0.5% tributyrin or Tween 80 (polyoxyethylene sorbitan monooleate), using plate detection methods. Tributyrin is a triglyceride naturally present in butter and can be described as a liquid fat. The plates contained a lipid substrate and lipolysis was observed as clear halos or opaque zones around the lipase producing culture. Hun et al. (2003) and Nair and Kumar (2007), recommended these methods of lipase detection on agar plates. Similarly, *Streptomyces rimosus* R6-554W was identified as a lipase producer after culturing on agar plates containing tributyrin (Abramic et al., 1999). Chaturvedi et al. (2010) used tributyrin agar as selective medium for isolation of lipase producing *Bacillus subtilis*. Wu and Tsai (2004) studied the hydrolysis rate of tributyrin with crude lipase from *Pseudomonas fluorescenes* where they reported the use of tributyrin in lipase detection.

In liquid medium, the quantities of lipase production by the 20 bacterial isolates were ranged from 1.5-6.9 IU/ml using the titrimetric method. The titrimetric method employed the neutralization of the free fatty acids that are released after lipolysis and the volume of the base consumed indicated the extent of lipolysis (Shukla and Gupta, 2007). The quantities of lipase detected by Gowland et al. (1987) for *Bacillus* sp. was 4 IU/ml in lipid containing medium. Abdul Hamid et al. (2003) reported similar lipolytic activity of 4.58 IU/ml for *Bacillus* sp., 3.51 IU/ml for *Ralstonia paucula* and 1.80 to 2.62 IU/ml for other unidentified bacteria.

The most active lipase- producing bacterium was the isolate LP10 (6.9 IU/ml), which was obtained from oil contaminated soil, collected from fuel station. Rajan (2010) observed that oil contaminated soils from oil grinding mill, automobile service stations, and restaurant waste dumping sites are the richest sources for the isolation of oil degrading bacteria and the biggest clear zones forming bacteria on trybutyrin plates were obtained. He added that these oil-destroyer bacteria produce extracellular lipase which utilizes oil, and hence it can be used for the self-remediation of lipid contaminated soils and water bodies.

The selected bacterium LP10 was Gram positive and had filamentous structure. Analyses of cell wall hydrolysates indicating a wall chemotype IV, whole cell sugar as type A. Analysis of lipids of the selected isolate indicated phospholipids PII pattern and presence of saturated fatty acids. The isolate LP10 was identified as *Streptomyces* sp. according to Bergey's manual of systematic bacteriology (Williams et al., 1989) and the results was confirmed using 16S rDNA as recommended by Weisburg et al. (1991), Tork et al. (2010) and Aly et al. (2011).

Many lipase producers identified as *R. paucula, B. subtilis, Bacillus thermoglucosidasius, Bacillus stearothermophilus* and *Bacillus coagulans* were obtained by Abdul et al. (2003) from the hot spring in Malaysia. Haba et al. (2000) screened 47 strains of the genera *Pseudomonas, Bacillus, Candida, Rhodococcus*, and *Staphylococcus* for lipolytic activity on the waste oils and the highest producers were *Pseudomonas* sp. (2.748 IU/ml) and *P. aeruginosa* (1.703 IU/l). Vishnupriya et al. (2010) detected a potent lipase producer named *Streptomyces griseus* using different type of oils including olive, palm and sunflower oil. The quantities of lipase measured were generally lower than that detected for fungi like *Geotricum* that showed maximum lipase production (146 IU/ml) when urea was used as nitrogen source (Ginalska et al. 2007).

Factors affecting production and accumulation of lipase were studied. It was known that temperature, pH, incubation period, carbon and nitrogen sources in addition to presence of inducer and its concentration are factors affecting lipase production (Immanuel et al., 2008). In the present study, it was found that maximum

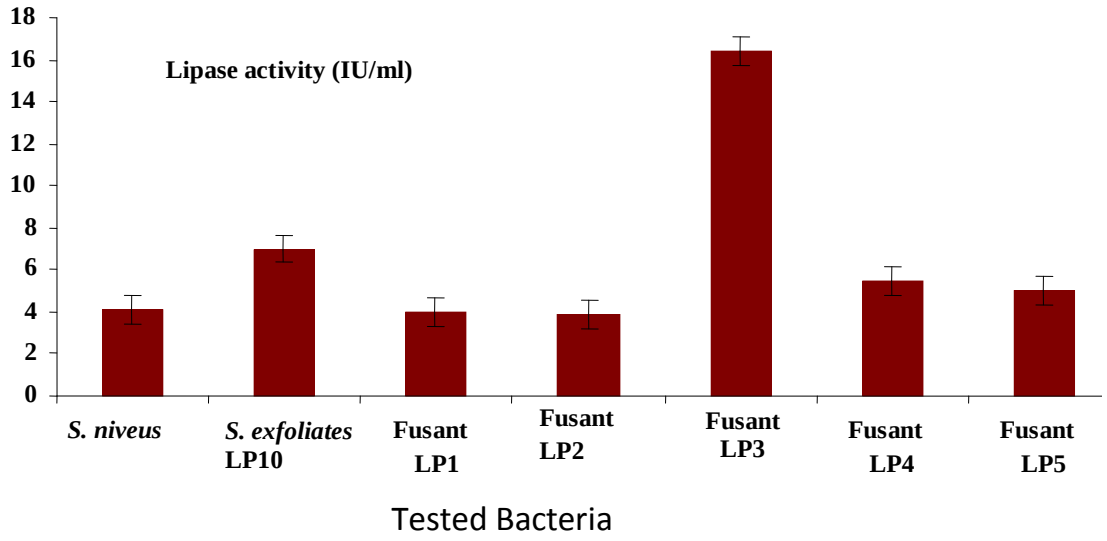

Figure 9. Lipase activity (U/ml) by five obtained fusants in relation to their parent (*Streptomyces niveus* and *S. exfoliates* LP10).

lipase production by the selected bacterium LP10 was obtained in medium C, which contained tributyrin and Tween 80 as a source of lipid and served as both a carbon source and an inducer for lipase production. Lipase synthesis thus appears to be inducible and a very low level of lipase was observed by Gowland et al. (1987) when bacteria was grown in a medium containing a carbon source like glucose.

Bacterial lipases are mostly extracellular; where the biggest factor in the expression of lipolytic activity has always been the requirement of an adequate carbon source. Tween 80 did not stimulate the growth but assisted in enzyme production (Hasan et al., 2006). Rowe and Gilmour (1982) found that the medium contained Tween 80 gave the maximum lipase productivity, thus confirming the fact that Tween 80 is the best inducer for the production of lipase. Similarly, Espinosa et al. (1990) found that Tween 80 appeared to be the best inducer and suggested a double effect of Tween 80 can serve as both inducer because of its chemical nature that was similar to some natural lipid substrates and a surfactant, stimulating the enzyme release. Induction of lipases by Tween 80 has also been reported for *Bacillus* sp. CM7 (Emanuilova et al., 1993). Similar results were obtained in case of lipase obtained from *Bacillus* strain H1 (Handelsman and Shoham, 1994). Tween 80 has been found to increase the lipolytic activity of *Bacillus* sp. (Sidhu et al., 1998), *Bacillus stearothermophilus* (Gowland et al., 1987) as well as that of other micro-organisms such as *Rhizopus delemar* (Espinosa et al., 1990). Song et al. (2001) found that the surfactants or Tween 80 could be helpful to lipase production, as in case of *Candida rugosa*. The best medium observed by Handelsman and Shoham (1994) for lipase production from *Bacillus* sp. was basal salt medium supplemented with 1% Tween 80. On contrast, Rathi et al. (2002) observed that olive oil is the most used lipid substrate to induce lipase production by bacteria however other carbon sources like molasses have been found to completely inhibit the lipase production by *Bacillus* sp. (Eltaweel et al., 2005).

Lipase production by the selected *Streptomyces* isolate was enhanced 2 times after growth for 3 days in medium C (initial pH 6) containing tributyrin and Tween 80 as inducer at 37°C. Lipases production influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, the dissolved oxygen concentration and incubation period which can vary from a few hours to several days depending on the bacteria and environmental conditions (Elibol and Ozer, 2000). An excellent extracellular lipase of *Hendersonula toruloidea* was produced in shake-flask cultures contained 0.2%; olive oil, at an initial culture pH of 6·0 during the late logarithmic phase after 120 h. (Odibo et al., 1995).

The lipase from *Giotricum candidum* showed the highest activity when the initial pH of the medium was adjusted to pH 7.0 (Baillargeon et al., 1989). On the contrary, lipase production from the newly isolated strain of *Geotrichum* sp. was at maximum at pH 5.0 (Macedo et al., 1997). Ginalska et al. (2007) found that maximum lipase production was at pH 6 by a newly isolated strain of *Geotrichum* like R59. However, Vishnupriya et al. (2010) reported that there are no significant changes obtained in the pH range. Therefore, the maximum lipase activity at the particular pH could not be affected with initial pH value. They added that another important parameter for lipase activity was incubation period where the lipase yields were optimum at 72 h by using olive oil as a substrate. Lipase production was at maximum at

initial pH 7.0 and incubation at 37°C by an isolate identified as *Streptomyces* sp. (Sirisha et al., 2010).

The best conditions for culture of the selected *Streptomyces* LP10 and maximum quantity of lipase enzyme was applied and enzyme was extracted. After enzyme precipitation, it was purified using different column chromatography and its molecular weight was determined to be 60 kDa. Abdul et al. (2003) purified extracellular lipases with approximately 67 kDa using ultra filtration of cell-free culture supernatant of *Bacillus* sp. Lower molecular weight of 50 kDa lipase was purified from the culture filtrate of *Streptomyces cinnamomeus* (Sommer et al., (1997). From the literatures, the molecular weight of lipase for *Pseudomonas* (Chartrain el al., 1993) and *Pseudomonas putida* (Lee and Rhee, 1993), were between 29 to 45 kDa but Abdul Rahim et al. (2004) detected larger molecular weight of 60 and 61 kDa.

Enhancement of lipase production was carried out using protoplast fusion between two S*treptomyces* isolates. During protoplast fusion, chromosomes of the two cells cut into pieces and randomly reassembled to form a new haploid chromosome. The haploid colony containing the hybrid haploid chromosome can grow only on selective medium supplemented with tetracycline and streptomycin as double selective marker. Every gene that is responsible for bacterial proliferation, morphological characteristics, sporolation rate and/or metabolite synthesis and secretion could be recombined after protoplast fusion. However, since the fusants or recombinant progenies were chosen based on their survival on double selective media, only cells that carried recombination between marker genes became screenable. Fusant 3 was the best one for lipase production. Similarly, Aly et al. (2011) enhanced chitinase production by two isolates of the genus *Streptomyces* using this technique. The five obtained fusants (LP1-LP5) showed a diversity of lipase production. Only one fusant LP3 had higher rates than those of the two parental isolates. It did not have any noticeable difference with its parents regarding properties such as morphology, sporolation and growth speed. In fact, the growth curve of LP3 was similar to that of the *S. exfoliates* LP10. Lipase production by LP3 showed a significant increase in comparison to its two parents, *S. exfoliates* LP10 and *S. niveus*. More detail studies concerning the molecular characterization of the fusant LP3 will be carried out**.**

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