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A Green Alternative for Biodiesel Production: Transesterification with *Streptomyces* sp. AU-1 Lipase

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Abstract

This study entailed the partial purification and characterization of a lipase produced from Streptomyces sp AU-1 (LipSAU-1). Lipase activity was measured with p-nitrophenyl palmitate (p-NPP) as a substrate. Ammonium sulphate precipitation, dialysis and gel filtration chromatography were used in the partial purification of LipSAU-1. SDS-PAGE showed the purified LipSAU-1 to be homogeneous, with a molecular mass of approximately 66 kDa. LipSAU-1 was found to have an optimal pH of 9.0 and an optimal temperature of 50°C, with high stability at all pH and temperature values tested in this study. The enzyme conserved approximately 55% of its activity at the end of 1 h in the presence of methanol. Its hydrolytic activity was found to be highest towards p-NPP among the various p-nitrophenyl esters and towards olive oil among the various natural substrates investigated. Thin layer chromatography (TLC) and gas chromatography (GC) were used to analyze the fatty acid alkyl ester content of the biodiesel produced by the reaction using LipSAU-1 as a catalyst. LipSAU-1's transesterification activity indicated it to be a potential biocatalyst for biodiesel production, given its high activity under thermophilic conditions and stability in the presence of various alcohols.

Keywords: *Streptomyces*, characterization, transesterification, biodiesel

1. Introduction

A steady increase in fossil-fuel consumption has led to a decline in traditional energy reserves along with concerns about environment pollutants, including green-house emissions, that has in turn sparked an interest in the development of biofuel [1]. Biodiesel is a biodegradable, non-toxic, sulfur-free form of renewable energy synthesized from renewable resources such as vegetable oils, animal fats, and recycled restaurant greases that has become the subject of intensive study as a possible alternative to fossil fuels [2-5]. While chemical synthesis of biodiesel is both cost-effective and efficient, its development has been restricted by a number of downstream difficulties, such as glycerol recovery, removal of inorganic salts, risk of soap formation, and difficulties in separating the catalyst and unreacted methanol from the biodiesel. These difficulties have prompted researchers to begin searching for alternative pathways for biodiesel production [2,6,7]. One process suggested as a realistic alternative to conventional physiochemical methods of biodiesel production is the enzymatic transesterification of triglycerides [8]. Not only is enzyme catalyzation a more environmentally attractive option, it is also preferable for its reusability, specificity, ability, thermostability and ability to operate under mild reaction conditions and with substrates not

possible with conventional methods [8,9-12]. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that act at the ester-water interface to hydrolyze long-chain triglycerides into diacylglycerol and carboxylate, or, conversely, to synthesize esters from fatty acids and glycerol [13]. In terms of industrial applications, lipases are used mainly in the hydrolysis of fats and oils [14]. Although the use of lipases in transesterification of oils for biodiesel synthesis has increased in recent years [15,16], the widespread use of lipase catalyzation in the industrial production of biodiesel has been prohibited by relatively high costs and short operational lifespan [17]. Still, when compared to acid or alkaline catalyzation, lipase catalyzation of biodiesel synthesis offers greater potential because of the relatively simpler process for purifying fatty acid methyl esters, which can be accomplished under milder conditions and without producing soap as a by-product [18-20]. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases [21]. The *Streptomyces* species' significant role in medical science, ecology and biotechnology has made it one of the best-studied, best-characterized species of bacteria [22]. Despite the fact that *Streptomyces* strains are known for their high exogenous lipolytic activity, lipases of this genus have not received the great attention that has been given to those of certain other bacteria [23]. There are some reports about the biodiesel production with *Streptomyces* lipases [24-28]. This paper addresses the partial purification, characterization, and application in biodiesel production of the lipase obtained from *Streptomyces* sp. AU-1.

2. Materials and Methods

2.1. Enzyme production The *Streptomyces* sp. AU-1 isolate, which have lipolytic activity, was inoculated on ISP2 agar medium and incubated at 30°C for 7 days. After the incubation, 0.01% (v/v) Tween 80 solution was used to harvest the spores [29]. For this purpose, Tween 80 solution was added onto the surface of sporulated isolate under aseptic conditions. After 10 minutes, the spores crossed the solution, and the solution was taken in a sterile tube. Fifteen 250 ml Erlenmeyer flasks with 100 ml ISP2 broth were inoculated with 2% spore solution ($5x10^6$ spore/ml) and incubated at 130 rpm, 30°C for 7 days. After the incubation, the cells were filtrated with Whatman filter paper No: 42, and the supernatant was used as lipase solution.

2.2. Lipase Assay Lipase activity was determined according to Winkler et al. [30] with some modifications as detailed elsewhere [31]. The substrate and enzyme was incubated at 130 rpm, 30°C for 30 min, and the absorbance was measured (λ=410nm) spectrofotometrically (Shimadzu spectrofotometer). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol/min of *p*-nitrophenol under the standard assay conditions which given above.

2.3. Enzyme Purification The supernatant was precipitated with ammonium sulfate (90% saturation and pH 9.0); the mixture was then centrifuged, the pellet was dissolved with 50mM Tris-HCl buffer (pH 8) and dialyzed overnight against 2 liters of the same buffer. In addition, that enzyme solution (48 ml) was concentrated with an ultrafiltration membrane. After dialysis, the enzyme solution was put on a Sephacryl S-100 HR column (pre-equilibrated with 50mM Tris-HCl buffer (pH 8)). The sample was eluted with 50mM Tris-HCl buffer (pH 8). Four-milliliter fractions were collected and assayed for lipase activity. The protein concentrations were determined according to the method of Bradford by using bovine serum albumin reagent (BSA, Sigma Chemicals) as the standard. The molecular mass of the enzyme was determined with SDS-PAGE electrophoresis.

2.4. Enzyme Characterization

2.4.1. Effects of pH and temperature on lipase activity and stability To determine the effect of pH on lipase activity and stability, various pH buffer systems were used: citrate phosphate buffer (pH 5.0-6.0), Tris-HCl buffer (pH 7.0-9.0), glycine-NaOH buffer (pH 10.0-

10.6). The enzyme activity was determined using a standard spectrophotometric method (*p*-NPP as the substrate).

p-NPP was unstable at pH above 9.0. For this reason, for determined the optimum pH of the enzyme, the activity was also measured titrimetrically by using olive oil as the substrate at pH between 8.0 and 11.0. The reaction mixture was prepared by emulsifying 10% (w/v) olive oil in 10% (w/v) Triton X-100 in buffers with different pH values ranging between 8.0 and 12.0. A total of 1 mL of the crude lipase was added to each of the 10-mL reaction mixture at different pH and incubated for 30 min at 30ºC and 130 rpm in a shaker incubator (Zhicheng). The reaction was stopped by adding 1 mL of 1:1 acetone/ethanol solution. Subsequently, 2–3 drops of phenolphthalein indicator were added to each of the reaction mixture. The contents of each reaction mixture were titrated with 0.05 M NaOH solution. The lipase activity was calculated as micromoles of free fatty acids formed from olive oil per mL of the crude lipase.

To determining the pH stability, aliquots of enzyme samples were pre-incubated at 30°C for 1h and 2h with different pH buffers. The lipase activity was determined by using the standard *p*-NPP method and the remaining activity was calculated (the initial activity was take 100%).

To determining the effect of temperature on lipase activity, the enzyme reaction at different temperatures in the range of 4-70ºC at pH 9.0 was observed (the optimum pH of LipSAU-1). Similarly the thermal stability of enzyme was evaluated by measuring the residual activities at different time intervals (1-2 h) after incubating the enzyme solution at various temperatures (4-70ºC). The residual activity was measured according to the *p*-NPP method as described earlier. The initial lipase activity was considered to be 100%.

2.4.2. Alcohol stability of Lipase The effects of various alcohols (methanol, ethanol, and isopropanol) on the enzyme activity were investigated. The lipase was incubated in the presence of alcohol (50% v/v) at 30°C, 130 rpm for 1 h. The control was the sample incubated without an alcohol in the presence under the same experimental conditions. Residual activity was measured using a standard method with *p*-NPP as the substrate. Residual activity was expressed by taking the activity without any alcohol as 100%.

Besides this, the effects of various concentrations of methanol, ethanol, and isopropanol and the incubation period of presence of these alcohols were similarly measured.

2.4.3. Substrate specifity of lipase Substrate specificity of the lipase was determined by measuring activity towards p-nitrophenyl esters of varying chain length (*p*-nitrophenyl butyrate (C_4), *p*-NPP (C_{16}), and *p*-nitrophenyl oleate (C_{18})) with spectrofotometric method, and towards triglycerides (olive oil, sunflower oil and waste edible oil) with titrimetric method.

2.4.4. Storage stability of lipase The storage stability of the lipase was evaluated by measuring its activity (towards *p*-NPP) for 30 days at various time intervals of storage at 4ºC.

2.5. Biodiesel production potential of lipase Biodiesel production was studied according to Yang et al. [32] with minor modifications [33]. Olive oil (7.89 ml) and methanol (0.99 ml) were kept in screw-capped glass tubes, mixed with a lipase sample (2.6 ml), and incubated at 40°C with shaking at 220 rpm for 48 h. After incubation, 200 µl of samples were taken from the reaction mixture and diluted with 1 ml of *n*-hexane for 2 min.

Afterward, the samples were centrifuged at 10 000 rpm for 15 min, and 10 µl of the upper layer was applied to a TLC plate. Methyl oleat (Sigma, purity 99%) was spotted as reference biodiesel. After developing the plate in *n*-hexane/ethyl acetate/acetic acid (90:10:1), the spots were visualized with iodine vapor after air drying for a short time. The biodiesel reaction was also made with sunflower oil and waste edible oil.

The fatty acid alkyl ester contents of the products were determined by gas chromatography (GC) analyzer (Agilent 7890 GC) according to EN 14105 standard. For this purpose, at the end of the enzymatic reaction, 20 ml of samples were taken from the reaction mixture and

diluted with 100 ml of *n*-hexane for 2 min. Afterward, the samples were centrifuged at 10 000 rpm for 15 min, and the upper layer was used for GC analysis.
The biodiesel reaction was also made with Novozym 435 (*Candida antarctica* lipase B) and the product was also analysed with TLC and GC analysis.

3. Results and Discussion

Lipase catalyzation of biodiesel synthesis has a number of advantages over chemical synthesis, including relatively short reaction times and relatively small amounts of alcohol, water and energy required for product isolation. This study investigated the purification, characterization and potential use in biodiesel production of an alkaline, thermoactive lipase produced by the *Streptomyces* sp. AU-1 isolate. *Streptomyces* sp. AU-1 was incubated until the optimum time, and the culture supernatant was used in purification. LipSAU-1 was partially purified with ammonium sulfate precipitation and gel-filtration chromatography performed using Sephacryl S-100HR (Table 1). Purification of approximately 1.62-fold was achieved, with 0.26% recovery. The enzyme was found to have a specific activity of 140 U/mg, and SDS-PAGE determined the enzyme molecular mass to be 66 kDa.

Table 1. Summary of LipSAU-1 purification

Purification Step	Total Protein (mg)	Total Activity (Unit)	Spesific Activity (U/mg)	Yield (%)	Purification (fold)
Crude extract (supernatant)	11.23	970	86.38	100	1
Ammonium sulphate precipitation+ dialysis	0.39	51.70	132.56	5.33	1.54
Gel filtration chromatography	0.018	2.52	140	0.26	1.62

The pH and temperature profiles of the LipSAU-1 were determined using buffers of varying pH and temperature values. The enzyme exhibited maximum activity at pH 9.0, and was stable at pH 10.0-11.0, remaining highly stable at these pH values at the end of 2 h (Figure 1). In terms of temperature, the lipase was fairly stable between 4°C and 50°C, retaining over 90% of its activity (Figure 2), and the optimum temperature was 50°C.

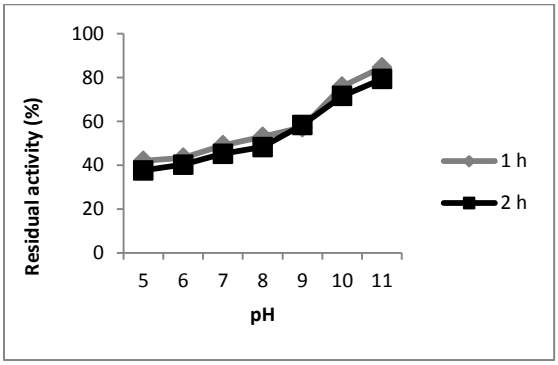

Figure 1. pH stability of LipSAU-1.

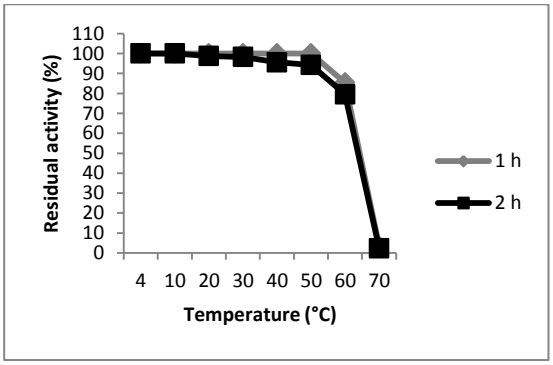

Figure 2. Temperature stability of LİPSAU-1.

The high stability of the LipSAU-1 found in this study is in line with findings for *Streptomycetes* lipases reported by previous studies. Abramic et al. [34] reported stability at pH 4.0-10.00 and full thermosability; Côté and Shareck [35] reported stability at pH 6.0-11.0 and temperatures ranging between 15°C and 60°C; and Bielen et al. [36] reported stablity at pH 4.0-9.5 and temperatures ranging from 55°C and 65°C.

Stability in the presence of various alcohols is a prerequisite of any enzyme to be used in biodiesel production; therefore, this study examined LipSAU-1's stability in methanol,

ethanol and propanol of various concentrations (Fig. 3) and at various time periods (Fig.4) LipSAU-1 was found to be stable in the presence of both methanol (55.72%) and ethanol (46.46%). Along with its other characteristic properties, the alcohol stability of LipSAU-1 demonstrates its usefulness in transesterification and as a biocatalyst for biodiesel production.

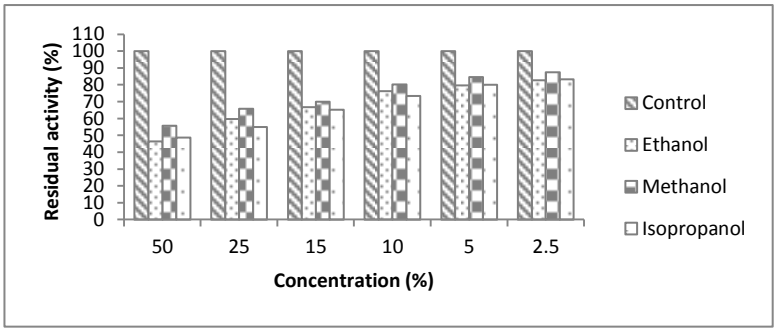

Figure 3. LipSAU-1's stability at various alcohol concentrations.

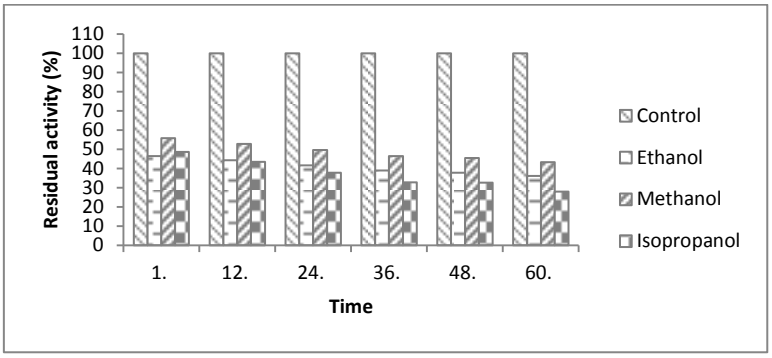

Figure 4. LipSAU-1's stability at various time intervals in the presence of ethanol, methanol, and isopropanol.

In terms of substrate specifity, LipSAU-1 was found to be highly active towards *p*-NPP and olive oil (Figure 5). In addition, LipSAU-1 retained over 60% of its initial activity after 30 days' storage at 4°C (data not shown).

The biodiesel reaction was catalyzed with LipSAU-1 with olive oil, sunflower oil and waste oil in the presence of methanol. Biodiesel production was also catalyzed with olive oil, sunflower oil and waste oil using the commercial lipase Novozyme 435 in the presence of methanol. GC (Table 2) was used to analyze the fatty acid alkyl ester contents of the various reactions. In addition, TLC (Figure 6) was used to analyze the fatty acid alkyl ester contents of the reaction with olive oil. The ability of LipSAU-1 to catalyse biodiesel production with various substrates, including waste oils, suggests it has high applicability in commercial biodiesel production.

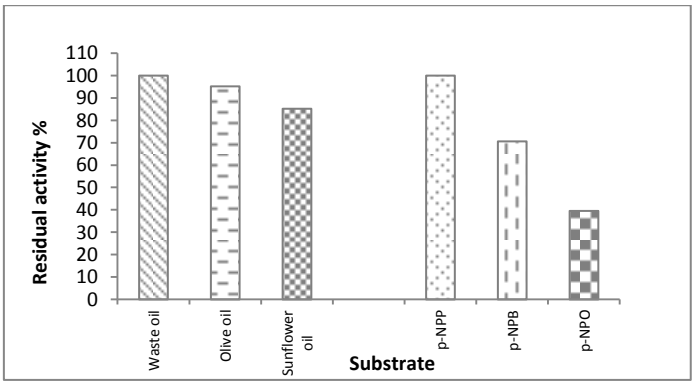

Figure 5. Substrate specifity of LipSAU-1.

Table 2. Fatty acid alkyl ester yield of biodiesel product

Product	Total fatty acid alkyl ester yield (% m/m)
LipSAU-1 (Olive oil)	5.22
Novozyme (Olive oil)	3.71
LipSAU-1 (Sunflower oil)	3.89
Novozyme (Sunflower oil)	3.74
LipSAU-1 (Waste edible oil)	4.69
Novozyme (Waste edible oil)	5.51

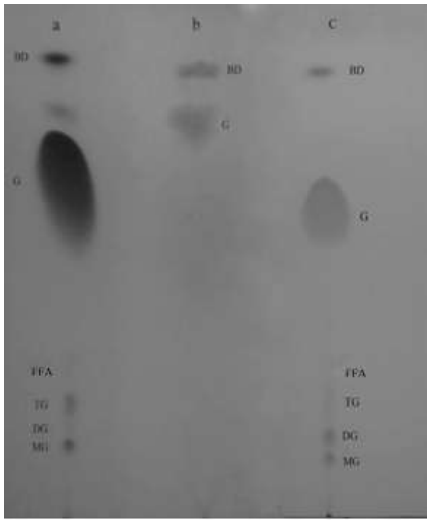

Figure 6. LipSAU-1 catalyzed biodiesel production, TLC plate. a: Novozyme 435 catalysed biodiesel production, b: standard (methyl oleate, Sigma Chemicals, 99% Pure), c: Reaction samples (with olive oil and methanol), BD: biodiesel, G: glycerol, FFA: free fatty acid, TG: triglyceride, DG: diglyceride, MG: monoglyceride.

4. Conclusion

Biochemical characterization showed the enzyme LipSAU-1 to possess a number of industrially important characteristics. Not only is LipSAU-1 highly active and stable at alkaline pHs and a range of temperatures, including thermophilic conditions, it is alcohol-tolerant, a characteristic that suggested LipSAU-1 could be used as a catalyst in biodiesel production. Further investigation demonstrated LipSAU-1's capacity to catalyze biodiesel synthesis from various substrates, including waste oils, suggesting that LipSAU-1 has a potentially important role to play in the commercial production of biodiesel from waste edible oils.

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References

1. I.KAZANCEVA, V.MAKAREVIČIENĖ, K.KAZANCEVA, Application of Biotechnological Method to Biodiesel Fuel Production using n-butanol. Environ. Res. Eng. Manag.,2,35,42(2011).
2. H.FUKUDA, A.KONDO, H.J.NODA, Biodiesel fuel production by transesterification of oils. Biosci. Bioeng.,92,405,416(2001).
3. Ö.KÖSE, M.TÜTER, H.A.AYSE, Immobilized *Candida antartica* lipase-catalyzed alcoholysis of cotton- seed oil in a solvent-free medium. Biores. Technol., 83,125,129(2002).
4. Y.SHIMADA, Y.WATANABE, T.SAMUKAWA, A.SUGIHARA, H.NODE, H.FUKUDA, Y.TOMINGA, Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. J. Am. Oil Chem. Soc.,76,789,793(1999).
5. Y.SHIMADA, Y.WATANABE, A.SUGIHARA, Y.TOMINGA, Enzymatic alcoholysis for

biodiesel fuel production and application of the reaction to processing. J. Mol. Catal. B: Enzym.,17,133,142(2002).
6. F.MA, M.A.HANNA, Biodiesel production: a review. Biores. Technol.,70,1,15(1999).
7. B.K.BARNWAL, M.P.SHARMA, Prospects of biodiesel production from vegetable oils in India. Renew. Sust. Energy,9,363,378(2005).
8. H.NOUREDDINI, X.GAO, R.S.PHILKANA, Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. Biores. Technol.,96,769,777(2005).
9. C.C.AKOH, S.W.CHANG, G.C. LEE, J.F.SHAW, Enzymatic approach to biodiesel production. J. Agric. Food Chem.,55,8995,9005(2007).
10. S.AL-ZUHAIR, F.W.LING, L.S.JUN, Proposed kinetic mechanism of the production of biodiesel from palm oil using lipase. Process Biochem.,42,951,960(2007).
11. M.K.MODI, J.R.C.REDDY, B.V.S.K. RAO, R.B.N.PRASAD, Lipase-mediated conversion of vegetable oils into biodiesel using ethyl acetate as acyl acceptor. Biores. Technol.,98,1260,1264(2007).
12. N.OGNJANOVIC, D.BEZBRADICA, Z.KNEZEVIC-JUGOVIC, Enzymatic conversion of sunflower oil to biodiesel in a solvent-free system: process optimization and the immobilized system stability. Biores. Technol.,100,5146,5154(2009).
13. J.L.ARPIGNY, K.JAEGER, Bacterial lipolytic enzymes: classification and properties. Biochem. J.,343,177,183(1999).
14. V.R.MURTY, J.BHAT, P.K.A.MUNISWARAN, Hydrolysis of oils by using immobilized lipase enzyme: a review. Biotechnol. Bioprocess Eng.,7,57,66(2002).
15. L.FJERBAEK, K.V. CHRISTENSEN, B.NORDDAHL, A review of the current state of biodiesel production using enzymatic transesterification. Biotech. Bioeng.,102,1298, 1315(2009).
16. W.PARAWIRA, Biotechnological production of biodiesel fuel using biocatalysed transesterification: a review. Critic. Rev. Biotechnol.,29,82,93(2009).
17. Y.X.LI, J.W.YANG, F.L.HUI, W.W.FAN, Y.YANG, Optimization of biodiesel production from rice bran oil via immobilized lipase catalysis. Afric. J. Biotechnol., 10,16314,16324 (2011).
18. D.H.LEE, J.M.KIM, H.Y.SHIN, S.W.KANG, S.W.KIM, Biodiesel production using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases. Biotechnol. Bioprocess.,11,522,525(2006).
19. D.J.JEON, S.H.YEOM, Two-step bioprocess employing whole cell and enzyme for economical biodiesel production. Korean J. Chem. Eng.,27,1555,1559(2010).
20. L.A.NELSON, T.A.FOGLIA, W.N.MARMER, Lipase catalyzed production of biodiesel. J. Am. Oil Chem. Soc.,73,1191,1195(1996).
21. R.SHARMA, Y.CHISTI, U.C.BANERJEE, Production, purification, characterization, and applications of lipases. Biotechnol. Adv.,19,627,662(2001).
22. S.D.BENTLEY, K.F.CHATER, A.M.CERDEÑO-TÁRRAGA, G.L.CHALLIS, N.R. THOMSON, K.D.JAMES,et al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature,417,141,147(2002).
23. K.JAEGER, S.RANSAC, B.W.DIJKSTRA, C.COLSON, M.VAN HEUVEL, O.MISSET, Bacterial lipases. FEMS Microb. Rev.,15,29,63(1994).
24. S.S.CHO, d.J.PARK, J.R.SIMKHADA, J.H.HONG, J.K.SOHNG, O.H.LEE, J.C.YOO, A neutral lipase applicable in biodiesel production from a newly isolated *Streptomyces* sp. CS326. Bioprocess Biosyst. Eng., 35(1-2),227,234(2012).
25. P.MANDER, S.CHO, J.SIMKHADA, Y.CHOI, D.PARK, J.HA, J.YOO, An organic solvent-tolerant alkaline lipase from *Streptomyces* sp. CS268 and its application in biodiesel production. Biotechnol. Bioprocess Eng.,17(1),67(2012).
26. P.MANDER, H.-Y.YOO, S.W.KIM, Y.H.CHOI, S.S.CHO, J.C.YOO, Transesterification of waste cooking oil by an organic solvent-tolerant alkaline lipase from *Streptomyces* sp. CS273. Appl. Biochem. Biotechnol.,172(3),1377,1389(2014).
27. A.UGUR, N.SARAC, R.BORAN, B.AYAZ, O.CEYLAN, G.OKMEN, New lipase for Biodiesel Production: Partial Purification and Characterization of LipSB 25-4. ISRN Biochem.,1,7(2014).
28. B.AYAZ, A.UGUR, R.BORAN, Purification and characterization of organic solvent-tolerant lipase from *Streptomyces sp.* OC119-7 for biodiesel production. Biocat. Agric. Biotechnol.,4(1),103,108(2015).
29. B.HERNÁNDEZ-RODRÍGUEZ, J.CÓRDOVA, E.BÁRZANA, E.FAVELA- TORRES,

Effects of organic solvents on activity and stability of lipases produced by thermotolerant fungi in solid-state fermentation.J. Mol. Cat. B Enzym.,61,136,142(2009).

30. K.W.WINKLER, K.ULRICH, M.STUCKMANN, Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J. Bacteriol.,138,663,670(1979).
31. R.BORAN, A.UGUR,Partial purification and characterization of the organic solvent-tolerant lipase produced by *Pseudomonas fluorescens* RB02-3 isolated from milk.Prep.Biochem. Biotechnol.,40,229,241(2010).
32. K.S.YANG, J.SOHN, H.K.KIM,Catalytic properties of a lipase from *Photobacterium lipolyticum* for biodiesel production containing a high methanol concentration.J. Biosci. Bioeng.,107,599,604(2009).
33. H.YOO, J.R.SIMKHADA, S.S.CHO, D.H.PARK, S.W.KIM, C.N.SEONG, J.C. YOO,A novel alkaline lipase from *Ralstonia* with potential application in biodiesel production.Biores. Technol.,102,6104,6111(2011).
34. M.ABRAMÍC, I.LESCÍC, T.KORICA, L.VITALE, W.SAENGER, J.PIGAC, Purification and properties of extracellular lipase from *Streptomyces rimosus.*Enzyme Microb. Technol.,25(6),522,529(1999).
35. A.CÔTÉ, F.SHARECK,Cloning, purification and characterization of two lipases from *Streptomyces coelicolor* A3(2). Enzyme Microb. Technol.,42,381,388(2008).
36. A.BIELEN, H.CETKOVIĆ, P.F.LONG, H.SCHWAB, M.ABRAMIĆ, D.VUJAKLIJA, The SGNH-hydrolase of *Streptomyces coelicolor* has (aryl)esterase and a true lipase activity. Biochimie,91(3),390,400(2009).