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Partial Purification, Characterization and Biodiesel Application of *Streptomyces lienomycini* Lipase

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BURAK SEN[1], NURDAN SARAC[1]*, AYSEL UGUR[2]
[1]*Department of Biology, Faculty of Sciences, Mugla Sitki Kocman University, Mugla, Turkey*
[2]*Section of Medical Microbiology, Department of Basic Sciences, Faculty of Dentistry, Gazi University, Ankara, Turkey*
***Address correspondenceto:** Department of Biology, Faculty of Sciences, Mugla Sitki Kocman University, Mugla, Turkey, Tel. +90 252 211 3278, Fax: +9 0252 211 1472, e-mail: sarac_63@hotmail.com*

Abstract

The lipase from Streptomyces lienomycini 350-2 (LipS350-2) was partially purified, characterized, and its transesterification capacity was determined. Lipase activity was measured using p-nitrophenylpalmitate (p-NPP) as a substrate. LipS350-2 was partially purified using ammonium sulfate precipitation, dialysis and gel filtration chromatography. The lipase was found to have a specific activity of 1466.8 U/mg and a molecular mass of approximately 52 kDa. Optimal pH and temperature for LipS350-2 was 9.0 and 40°C, respectively. The lipase exhibited good stability at pHs from 7-11 and at temperatures ranging from 4°C-40°C. The enzyme conserved approximately 69% of its activity at the end of 1 h in the presence of isopropanol. Hydrolytic activity of the enzyme was highest towards p-NPP (C_{16}) among the various p-nitrophenol esters tested and towards waste edible oil among the natural substrates tested. With regard to storage stability, LipS350-2 enzyme can be stored at +4°C for 30 days.Biodiesel was then produced using the lipase as a catalyst, and the fatty acid alkyl ester content of the biodiesel product was analyzed. The measurements of transesterification indicated LipS350-2 to be a potential catalyst for biodiesel production due to its high activity under thermophilic conditions and stability in the presence of various alcohols.

Keywords: biodiesel, characterization,lipase, *Streptomyces*

1. Introduction

Today's move towards renewable energy has been prompted by rising prices of crude oil, rapidly diminishing fossil fuel sources, and the environmental consequences of their continuing use. Recently, much attention has begun being paid to biodiesel, a green, nontoxic, environmentally safe and renewable fuel (1).Biodiesel, i.e. fatty acid methyl esters, is a biodegradable and nonpolluting fuel that represents an environmentally acceptable alternative fuel for diesel engines. From an environmental point of view, biodiesel demonstrates a number of clear advantages over conventional fuel: it comes from renewable sources, and hence does not contribute to new carbon dioxide emissions; it is biodegradable; and because its combustion products contain relatively low levels of particulates and sulphur, carbon and nitrogen oxides, it significantly reduces pollution (2, 3).Transesterification, also referred to as 'alcoholysis', is a catalyzed reaction in which one alcohol is used to displace another alcohol (preferably methanol or ethanol) from an ester to yield fatty acid alkyl esters and glycerol as a by-product (4). Particularly over the past decade, considerable attention has been paid to the use of transesterification of virgin plant oils as well as low-quality waste oils with short-chain alcohols in biodiesel production (5). However, a number of problems are associated with both

the acid and alkali methods of catalysis that are most commonly used in producing biodiesel, namely downstream processing costs, by-products recovery and environmental problems (6). Enzymatic conversion of triglycerides has been suggested as a realistic, environmentally more attractive alternative to conventional transesterification methods (7) due to its relatively lower processing temperature and greater selectivity (8,9).Widely used in industry, lipases (E.C.3.1.1.3) are capable of catalyzing a variety of reactions such as hydrolysis, alcoholysis, esterification, and transesterification(10). However, despite the importance of the *Streptomyces* genus in terms of secondary metabolites, including antibiotics, *Streptomyces* enzymes and lipases have rarely been examined in the literature (11-14), and to our knowledge, the literature includes no reports on the *S. lienomycini* lipase. Therefore, this paper presents the partial purification, characterization, and biodiesel application of the enzyme produced from *S. lienomycini* 350-2.

2. Materials and Methods

2.1. Enzyme production

The *S. lienomycini* 350-2 strain, 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 (15). 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. Fifteen250 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.Lipase assay was performed according to Winkler et al. (16), with some modifications (17). The substrate and enzyme was incubated at 130 rpm, 30°C for 30 min, and the absorbance was measured (λ=410nm).

2.2. Enzyme purification

The supernatant was precipitated with ammonium sulfate (90% saturation and pH 8.0); the mixture was then centrifuged, the pellet was dissolved in 35 ml 50mM Tris-HCl buffer (pH 8) and dialyzed overnight against 2 liters of the same buffer. In addition, that enzyme (55.5 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.

2.3. Enzyme characterization

2.3.1. Effects of pHandtemperature on lipaseactivityandstability

Todeterminetheeffect of pH on lipase activity and stability, various pH buffer system swereused: citrate phosphate buffer (pH 5.0-6.0), Tris-HCl buffer (pH 7.0-9.0), glycine-NaOH buffer (pH 10.0-11.0). The enzyme activity was determined using a standard spectro-photometric 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. 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 evaluate the pH stability, aliquots of enzyme samples were pre-incubated at 30°C for 1h and 2h with the respective pH buffers. Remaining enzyme activity was measured by using the standard *p*-NPP method and calculated while considering the initial activity as 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 wase valuate dbymea suring the residual activities at different time intervals (1-2 h) after incubating the enzyme solution at various temperatures (4-70ºC). There sidual activity was measured according to the *p*-NPP method as described earlier. The initial lipase activity was considered to be 100%.

2.3.2. Alcohol stability of LipS 350-2

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 rpmfor 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.

2.3.3. Substratespecifity of lipase

Substrate specificity of the lipase was determined by measuring activity towards p-nitrophenylesters of varying chain length (p-nitrophenylbutyrate (C_4), p-NPP (C_{16}), and p-nitrophenyloleate (C_{18})) with spectrofotometric method, and towards triglycerides (oliveoil, sunflower oil and waste edible oil) with titrimetric method.

2.3.4. 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.

2.4. Biodiesel production potential of lipase

Biodiesel production was studied according to Yang et al. (18) with minor modifications (19). 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 oleate (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. This reaction was also made with ethanol. In addition, the biodiesel reaction was made with sunflower oil and waste edible oil. The biodiesel reaction was also made with Novozym 435 (*Candidaantarctica* lipase B) and the product was also analysed with TLC.

3. Results and Discussion

Given that lipase-catalyzed biodiesel synthesis requires relatively small amounts of alcohol as well as relatively small amounts of water and energy for product isolation, this study investigated the purification of an alkaline, thermoactive lipase produced by the *S. lienomycini* strain 350-2, its characterization and its potential application in biodiesel production. *S. lienomycini* 350-2 was incubated until the optimum time, and the culture supernatant was used in the purification stage. LipS350-2 was partially purified using ammonium sulfate precipitation and gel-filtration chromatography was performed using Sephacryl S-100HR. Results are summarized in Table1. Approximately 1.56-fold purification with 1.170% recovery was achieved. The enzyme was found to have a specific activity of 1466.8 U/mg, and SDS-PAGE analysis indicated a molecular mass of 52 kDa.

Table 1. Summary of LipS350-2 purification

Purification Step	Total Protein (mg)	Total Activity (Unit)	Spesific Activity (U/mg)	Yield (%)	Purification (fold)
Crudeextract (supernatant)	12.53	11782	940.303	100	1
Ammoniumsulphatepre cipitation+ dialysis	6.314	7785.96	1233.06	66.08	1.308
Gel filtrationchromatograph y	0.094	137.88	1466.8	1.170	1.56

The pH and temperature profiles of LipS350-2 were determined using buffers of different pH values and various temperatures. The enzyme was found to exhibit maximum activity at pH 9.0 and 40°C and remained highly stable at pH ranges of 7.0-11.0 (Fig. 1) and at temperatures ranging between 4°C and 40°C (Fig. 2).

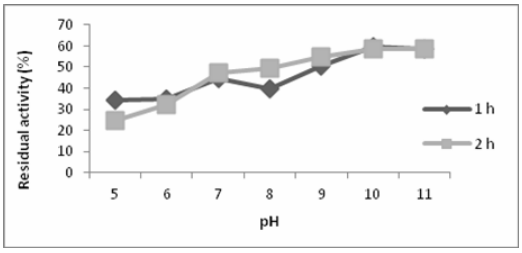

Figure 1. pHstability of LipS350-2.

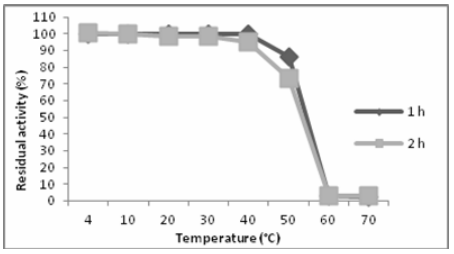

Figure 2. Temperature stability of LipS350-2.

Previous studies have reported streptomycetes lipases to have pH stability ranging between pH 4.0-10.0 (20), pH 6.0-11.0 (21), and pH 4.0-9.5 (22). Reports on the thermostability of streptomycetes lipases have reported them to be fully thermostable (20), stable between 15°C and 60°C (21), and stable between 55°C and 65°C (22). Stability in the presence of various alcohols is a prerequisite of any enzyme to be used in biodiesel production. Therefore, this study examined LipS350-2 stability in various concentrations of methanol, ethanol and propanol. The effects of methanol/ethanol concentration and incubation period on lipase stability are shown in Figures 3 and 4, respectively. LipS350-2 had maximum stability in 15% concentrations of methanol (91.95%) and ethanol (84.48%).

Moreover, LipS350-2 retained approximately 45% of its initial activity after 30 days' storage at 4°C (data not shown). Along with its other characteristics, LipS350-2's alcohol stability demonstrates its potential usefulness in transesterification and as a biocatalyst for biodiesel production.

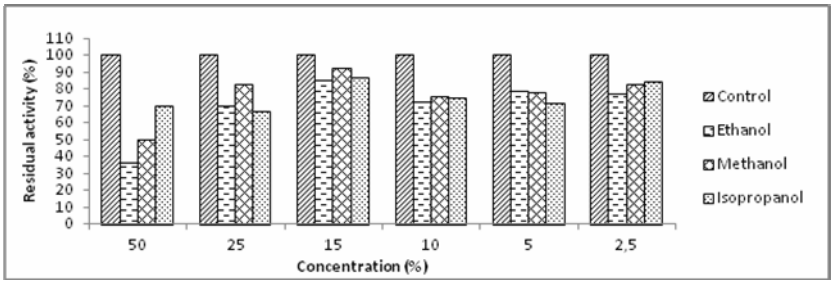

Figure 3. LipS350-2's stability at various alcohol concentrations.

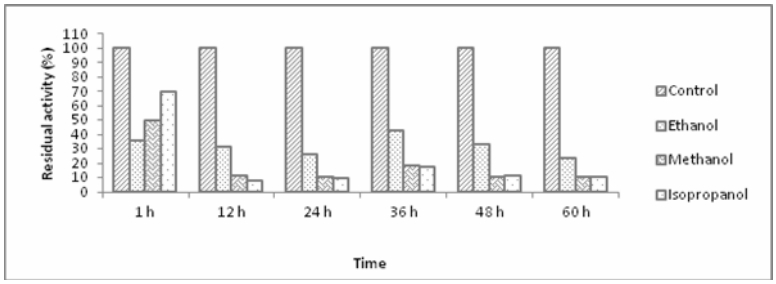

Figure 4. LipS350-2's stability at various time intervals in the presence of ethanol, methanol, and isopropanol.

In terms of substrate specificity, LipS350-2 was highly active against *p*-NPP and waste edible oil (Figure 5). Moreover, LipS350-2 catalyzed transesterification with olive oil in both methanol and ethanol (Figure 6) and with sunflower oil and waste edible oil in ethanol (Figure 7). Reaction products were identified using TLC. LipS350-2's ability to catalyze biodiesel production with various substrates, including waste oils, would be highly applicable in the commercial production of biodiesel.

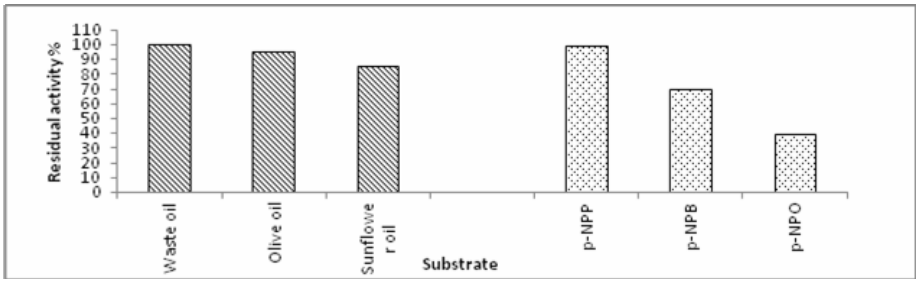

Figure 5. Substratespecifity of LipS350-2.

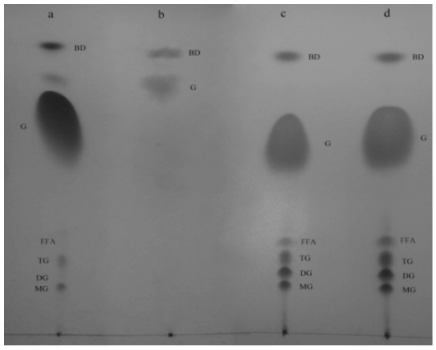

Figure 6. LipS350-2 catalyzed biodiesel production with olive oil, TLC plate. a: Novozyme 435 catalysed biodiesel production, b: standard (methyl oleate, Sigma Chemicals, 99% Pure), c: Reaction samples (with olive oil and methanol), d: Reactionsamples (with olive oil and ethanol), BD: biodiesel, G: glycerol, FFA: free fatty acid, TG: triglyceride, DG: diglyceride, MG: monoglyceride.

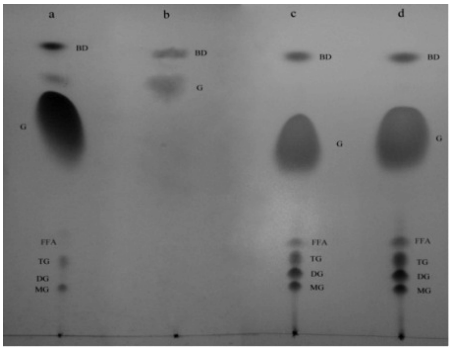

Figure 7. LipS350-2 catalyzed biodiesel production with sunflower oil and waste edible oil, TLC plate. a: Novozyme 435 catalysed biodiesel production, b: standard (methyloleate, Sigma Chemicals, 99% Pure), c: Reactionsamples (with sunflower oil and ethanol), d: Reactionsamples (withwasteedibleoilandethanol), BD: biodiesel, G: glycerol, FFA: free fatty acid, TG: triglyceride, DG: diglyceride, MG: monoglyceride.

4. Conclusion

Biochemical characterization of LipS350-2 showed this lipase to possess a number of characteristics of importance for industry, namely high activity and stability at alkaline pH and various temperatures, including relatively thermophilic conditions. Moreover, given its alcohol tolerance and its ability to catalyze biodiesel from a variety of substrates, including different waste edible oils, LipS350-2 has a potentially valuable role to play in biodiesel production.

Acknowledgment

ThisworkisBurak Sen'smaster of sciencethesis.

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