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MOLECULAR CLONING AND CHARACTERIZATION OF NOVEL THERMOSTABLE LIPASE FROM *SHEWANELLA PUTREFACIENS* AND USING ENZYMATIC BIODIESEL PRODUCTION

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ABSTRACT

A novel thermostable lipase from *Shewanella putrefaciens* was identified, expressed in *Escherichia coli*, characterized and used in biodiesel production. Enzyme characterization was carried out by enzyme assay, SDS-PAGE and other biochemical reactions. The recombinant lipase was found to have a molecular mass of 29 kDa and exhibited lipase activity when Tween 80 was used as the substrate. The purified enzyme showed maximum activity at pH 5.0 and at 80°C. The recombinant lipase was used for the transesterification of canola oil and waste oil. The enzyme retains 50% of its activity at 90°C for 30 minutes. It is also able to retain 20% of its activity even at 100 °C for 20 minutes. These properties of the obtained new recombinant thermostable lipase make it promising as a biocatalyst for industrial processes.

Keywords: Cloning, lipase, thermostable, *Shewanella putrefaciens*, biodiesel production

INTRODUCTION

Lipase (EC 3.1.1.3) is a kind of lipolytic enzyme, which is known to catalyze the hydrolysis of long-chain triglycerides. Microbial lipases are known to have diversity in their properties and substrate specificities, which further improve their biotechnological importance **(Mhetras *et al.*, 2009)**. Lipases are used to hydrolyze ester bonds of a variety of nonpolar substrates at high activity, stereoselectivity, and regioselectivity. Furthermore, they catalyze different amide and ester bonds formation in nonpolar solvents. The reaction can be designed and optimized to get a wide range of novel products. This can be done by making changes in substrate structure, additives, water activity, solvent, temperature, pressure and the biocatalyst itself. Selection of lipase being used in each application is based on its activity, stability and selectivity **(Dizge *et al.*, 2009; Ebrahimpour *et al.*, 2011; Hasan *et al.*, 2006)**. They are widely used in the dairy and oleo chemical industries, in the synthesis of structured triglycerides, surfactants, pharmaceuticals and agrochemicals, and in polymers and household detergents **(Hasan *et al.*, 2006).** Extracellular lipases are produced by a variety of microorganisms, including fungi **(Cardenas *et al.*, 2001**) and bacteria ***(Eggert et al.*, 2002; Rashid *et al.*, 2001; Yumoto *et al.*, 2003)**. Lipases of microbial origin represent the most extensively used class and are receiving increasing attention due to their relative ease of production and potential applications in biotechnology **(Shi *et al.*, 2010; Bornscheuer *et al.*, 2002; Schmid and Verger, 1998; Shibatani *et al.*, 2000)**.
The important requirements for industrial application of enzymes is thermal stability. Although many lipases have already been studied, the requirement for new lipases with more improved thermal stability and substrate specificity is still an important field of research **(Gutarra *et al.*, 2009; Haki and Rakshit, 2003).** As compared to mesophilic enzymes thermostable enzymes show higher resistance to chemical denaturants and are able to withstand higher substrate concentrations. These thermostable enzymes catalyze the reactions at higher process rates due to a decrease in viscosity and an increase in diffusion coefficient **(Ebrahimpour *et al.*, 2011)**. Therefore, thermostable lipases have got wide spread interest for potential application in the detergent, pharmaceutical, dairy, oil and fat industries due to their extreme stability at high temperatures and in organic solvents **(Emtenani *et al.*, 2013).** So thermostable lipase also catalyzed biodiesel production has received attention due to several advantages such as mild reaction conditions, low energy consuming, and environment friendly ***(Arai et al., 2010; Dwiarti et al.*, 2010; Yan *et al.*, 2011)**.

The aim of this work is to obtain novel thermostable lipase gene from *Shewanella putrefaciens*. The lipase gene from *Shewanella putrefaciens* was not defined in the GenBank database and the biochemical characterization of the purified enzyme from *Shewanella putrefaciens* has yet not been reported the subject of any study which may be a good contribution to GenBank database especially including lipase gene from *Shewanella putrefaciens*. In this contribution, firstly, the lipase gene was cloned, then, soluble expression was obtained and used biodiesel production. The obtained genes were characterized with using different parameters such as the effects of pH and temperature on the enzyme activity. Also, other biochemical characterizations were carried out. Therefore, this is the first report related to *Shewanella putrefaciens* lipase so far. A new bacterial lipase gene with industrial usage is likely to be obtained after further studies.

MATERIAL AND METHODS

Chemicals and microorganisms

All the chemicals and reagents were analytical grade and were purchased from commercial sources. Restriction endonucleases, other modifying enzymes and DNA polymerase were obtained from Thermo (USA). *Shewanella putrefaciens* was purchased from ATCC (USA) number of 8071. *E. coli* XL-1 Blue was used as the host for recombinant plasmids. The pET-28a (Novagen, Madison, WI, USA) was used as an expression vector to produce the target protein. *E.coli* BL21 (DE3-pLysS, Novagen) was used as the host for expression of lipase gene under the control of the T7 promoter. *E. coli* transformants were grown at 37 °C in Luria–Bertani (LB) broth and the LB medium was supplemented with 100 µg/mL kanamycin.

Isolation and cloning of the lipase gene

Genomic DNA isolation from Shewanella putrefaciens was performed using a High Pure PCR Template preparation kit (Roche, Germany), according to the manufacturer's instructions. In order to amplify the lipase gene of Shewanella putrefaciens, a nested PCR strategy was used. Suitable forward and reverse primers were designed which including the appropriate restriction enzyme site at the 5' end. The PCR product was purified and then digested with EcoRI and HindIII enzyme and subsequently cloned into the expression vector pET28a (Novagen, USA) that had been previously digested with the same enzymes.

Expression of the lipase gene and purification of the recombinant protein

The lipase gene was cloned in pET28a plasmid vector and transformed into E.coli BL21 (DE3, pLysS) cells for expression. The E.coli cells transformed with this plasmid and plated on a LB agar containing 100 µg/ml kanamycin. The transformat was grown in a 10 ml flask containing 5 ml LB medium supplemented with 100 µg/ml kanamycin at 37°C shaking at speed of 250 rpm overnight. The next day, a 1:100 dilution overnight culture was put into 50 ml flask containing 20 ml LB without antibiotic. After reaching to 0.6 optical density at 600 nm, cells were induced with 1mM IPTG (isopropyl-*b*-D-thiogalactoside) to induce the target protein expression. After incubation at 37°C for 4 h with shaking at 250 rpm, cells were harvested by centrifugation (8,000×g for 20 min at 4°C) and washed twice with 50 mM cold potassium phosphate buffer (pH7.6), and the cell pellet was stored at -20°C for later purification. All purification steps were performed according to the instruction of B-PER 6xHis Fusion Protein Purification Kits (Thermo, USA) and previous study **(Sinirlioglu *et al.*, 2013)**. Shortly, the cells were resuspended in 10 mL B-PER Reagent by either vortexing or pipetting up and down for homogenous suspension. The mixture was gently shaken at room temperature for 10 minutes. Lysate was centrifuged at 14,000×g for 15 min to remove debris then the supernatant was collected in a new tube. The column was prepared by adding 10 ml of B-PER Reagent. The column was loaded with prepared extract and then washed with a wash buffer. The bound protein was eluted with elution buffer. The purity of the enzyme in the eluted fractions was checked by %10 SDS-PAGE. Protein concentration was determined by the Bradford method **(Zhu *et al.*,2012)** and bovine serum albumin was used as standard for calibration. Enzyme samples were stored at -20°C until further use.

Sequence analysis of lipase enzyme

The DNA sequence analysis, enzyme characteristics and multiple sequence analysis was performed using the CLC-BIO main workbench like previous study **(Sinirlioglu *et al.*, 2013).**

Enzyme assay

Free lipase activity was determined using Tween80 as the substrate. The activity with Tween 80 as substrate was measured spectrophotometrically at 450 nm using 1% (w/v) Tween 80 in 20 mM Tris-HCl (pH 7), containing 80 mM $CaCl_2$. After the addition of the enzyme, the increase in absorbance was monitored as a function of time. The increase in absorbance was the result of increasing turbidity of the in situ formed calcium oleate. Lipase activity were monitored UV-Vis Spectrophotometer. The reaction mixture which contained 500 µl Tris-HCl buffer, 200 µl Tween 80 solution (1% w/v), 200 µl $CaCl_2$ solution (80 mM) and 100 µl enzyme, loaded into spectrophotometer and measured at 450 nm for 20 minutes of reaction time at room temperature. One unit (U) of lipase activity was defined as 1.0 µmol of product formed per minute under the assay conditions.

Characterization of lipase enzyme

The influence of temperature on lipase activity was studied spectrophotometrically. The reaction buffer contained 500 µl Tris-HCl buffer, 200 µl Tween80 solution (1% w/v), 200 µl $CaCl_2$ solution (80 mM) and 100 µl enzyme. The effect of temperature on lipase activity was determined by incubating reaction buffer at various temperatures ranging 30ºC to 100°C. The influence of pH on lipase activity was also studied spectrophotometrically. The pH dependence was determined with Tween80 solution (1% w/v) as the substrate. The reaction buffer contained 500 µl Tris-HCl buffer, 200 µl Tween80 solution (1% w/v), 200 µl CaCl2 solution (80 mM) and 100 µl enzyme. pH of the reaction buffer was adjusted from 2 to 10 using 50 mM glycine HCl buffers (pH 2.0 to 3.0), 50 mM sodium acetate buffers (pH 4.0 to 5.0), 50 mM sodium phosphate buffers (pH 6.0 to 7.0) and 2M NaOH (pH 8.0 to 10.0). The enzyme stability against pH was determined by measuring the activities of enzyme after incubation at 4°C for 1 h in the buffers. Thermostability was determined by incubating at various temperatures (30-100°C) at pH 5.0 for 30 min. The enzymatic assays were carried out under defined enzyme assay conditions. All characterizations were performed triplicate for both pH and temperature effects.

Effect of metal ions and chemical agents

The effect of metal ions was determined by detecting the residual activity after incubating enzyme solution containing 5 mM and 10 Mm various metal ions at 80 °C for 1 h. The sample without metal ions was taken as control. Effect of chemical agents was determined by comparing the residual activity after incubating lipase solution containing 5 mM and 10 mM chemical agents at 80 °C for 1 h. The enzyme solution containing distilled water instead of chemical agents was taken as control.

Transesterification reaction (biodiesel production)

The transesterification reactions were carried out in a 50 ml screw-capped vessel and heated to 65 °C in a shaking incubator in 150 r/min. The compositions of the reaction mixtures were 15 g of canola oil or waste oil, 100 IU recombinant lipase and methanol (a 1:4 molar ratio of oil/methanol). After a 24 h reaction, 100 µl sample was taken from the reaction mixture and centrifuged to obtain the upper layer. Methyl heptadecanoate which served as the internal standard and an aliquot of the upper layer were measured and mixed thoroughly for GC analysis to determine fatty acid methyl esters (FAMEs) and glycerides content. For the time course studies, an aliquot of 100 µl of reaction medium was taken at various time intervals and was diluted in n-heptane for GC analysis. The methyl ester contents of reaction mixture were measured on a gas chromatograph (Agilent Technologies Model 6890N). Helium was used as carrier gas with a flow rate of 1.2ml/min.

RESULTS AND DISCUSSION

Sequence analysis and molecular characterization of the lipase from *Shewanella putrefaciens*

Using the primer pair of F- (5'-AGAATTCATGAATTATGTTTCCTCAG-3') and R- (5'-TTAAGCTTTTGGCACATTACCTTGC-3'), 896-bp open reading frame (ORF) was amplified from the genomic DNA of *Shewanella putrefaciens.* The nucleotide sequence of lipase gene from *Shewanella putrefaciens* was not defined in the GenBank database and the biochemical characterization of the purified enzyme from *Shewanella putrefaciens* has yet not been reported the subject of any study. Gene for cloning were selected which encodes a putative lipase protein with 294 amino acids. The predicted molecular weight was 29 kDa. A sequence analysis with a BLAST search in the database NCBI showed that the putative lipase gene from *Shewanella putrefaciens* was a member of the PGAP1 super family protein and also the metX super family which belongs to the α, β-fold hydrolase domain. The conserved pentapeptide Gly-X-Ser-X-Gly in most microbial lipases was also found in the recombinant lipaseas given in Figure1. This peptide is thought to play an important role in substrate recognition and binding **(Tan and Miller, 1992).**

MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSEFMNYVSSGQGHAVLIIHGLFGNLDNLKGLGQVL
ESQHQVIRVDVPNHGLSEHWDHMDYPRLAHAMIDLLDNLDIAHAHVIGHSMGGKIAMATALAFPERII
SMIAADIAPVAYEPRHDIVFAALESLPLEGHTDRRFALNHLINHGIDEATAQFLLKNLQRTDTGFRWK
MNLSGLKTCYPNIIGWHNQPPNPVLSYSGPSLFIRGGDSNYVNSEHRDAIMAQFPTAQAKTLEGCGHW
LHAQKPAIFNRIVSEFIDKQAM

Figure 1 The recombinant *Shewanella putrefaciens* lipase amino acid sequence. The conserved sequence G-X-S-X-G in common lipases is indicated by the bold shadowed box.

The expression of recombinant lipase was observed by SDS-PAGE analysis. The SDS PAGE of bacterial lysate of *E. coli* strain BL21 (DE3) pLysS containing recombinant pET28a/lipase vector. The recombinant lipase is a monomeric protein with a predicted molecular weight of 29 kDa (Fig.2).

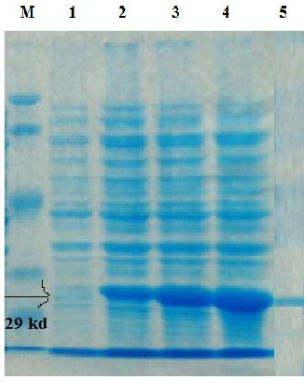

Figure 2 SDS-PAGE analysis: Marker is prestained protein molecular weight marker (Fermentas), First lane shows before IPTG induction. Second line shows after 1 hour IPTG induction, third line shows after 2 hours IPTG induction and fourth lane shows after 3 hours IPTG induction.Last one is purified enzyme.

Biochemical characterization of recombinant lipase

Effects of temperatures on enzymatic activity of recombinant lipase were measured in a temperature range between 30 °C and 100 °C when Tween 80 was used as the substrate. The obtained protein exhibited the maximum activity at 80 °C and the relative activity still reached to 50% even at 90 °C and 20% activity at

100 °C. The enzyme was thermostable at 90 °C and 100 °C for 30 and for 20 minutes, respectively (Fig.3). According to the presently prevalent definition **(Nakamura *et al.*, 1992; Schmidt-Dannert *et al.*, 1994)**, the obtained protein can be defined a thermophilic and thermostable enzyme. Among the studied thermostable lipases known so far, lipase from *Aspergillus terreus* showed excellent temperature tolerance (15-90°C) and was able to retain 100% activity at 60°C for 24 hours, but it does not worked at higher temperatures **(Yadav *et al.*, 1998).** Previously, a study showed that *Pseudomonas sp*. lipase was stable at 100 °C **(Adams and Brawley, 1981)**. As far as our knowledge, there is no other lipase which shows any activity at 100 °C. But, the obtained lipase from *Shewanella putrefaciens* was able to retain 20% of its activity even at 100 °C for 20 minutes.

To investigate the influence of pH on recombinant lipase, the activity was measured at various pHs. The recombinant lipase was active in a pH range of 4.0–6.0, with the maximal activity at pH 5.0 (Fig.3). The pH stability of lipase was explored by pre-incubating lipase in buffer with different pH at 80 °C for 1h. The recombinant lipase completely lost its activity at pH 8.0. It retained approximately 70% of its activity at pH 4.0 and 6.0. But it retained only 30% of its activity at pH 7.0.

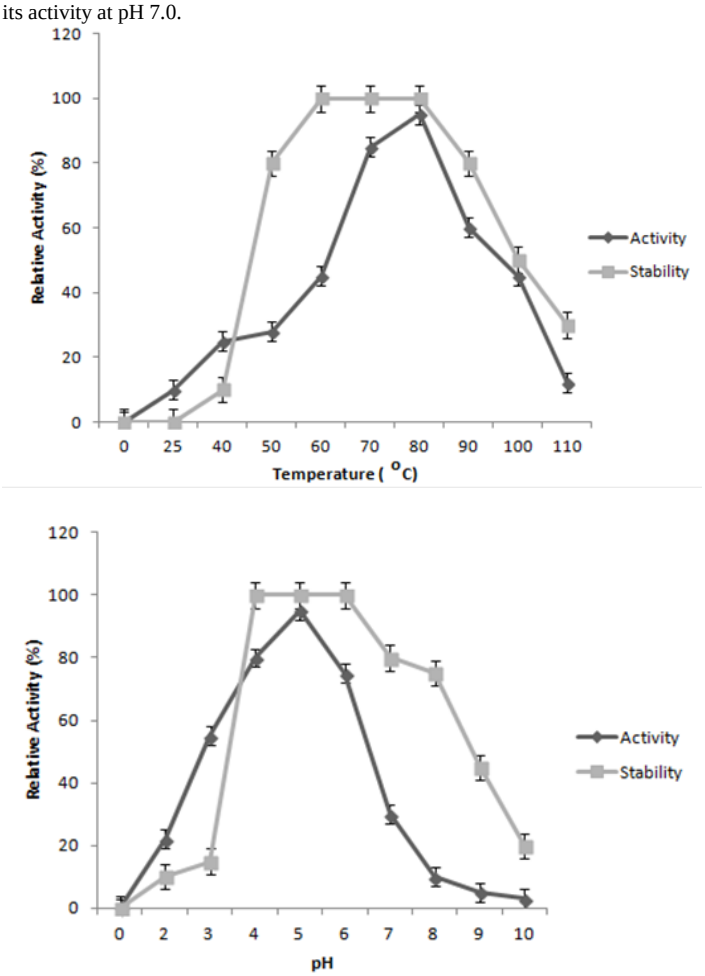

Figure 3 Effects of temperature and pH on the enzyme activity.Also it shows enzyme stability under different temperature and pH condition.

Metal ions play important roles in the structure and function of enzymes. Generally, metal ions might bind to some of the amino acid chains of protein and the enzyme activity can change by stabilizing or destabilizing its conformation (**Ebrahimpour *et al.*, 2011**). The effect of some metal ions and chemical agents on the activity of recombinant lipase was investigated (Fig. 4). The effect of metal ions might be attributed to change in the solubility and behavior of ionized fatty acids upon complex formation with metal ions and the direct inhibition of enzyme catalytic function**(Emtenani *et al.*, 2013; Kambourova *et al.*, 2003)**. Both of the tested metal ions Ca^{2+} and Mn^{2+} could meaningfully enhance the lipase activity but Zn^{2+} dramatically reduced the activity and also Fe^{2+} 10 mM ions droped the enzyme activity. Other metal ions did not influence the lipase activity to much. Chemical agents such as EDTA did not affect the activity so this enzyme not a metalloenzyme **(Sathish Yadav *et al.*, 2011).** Also presence of other chemical agents showed the same activity like EDTA.

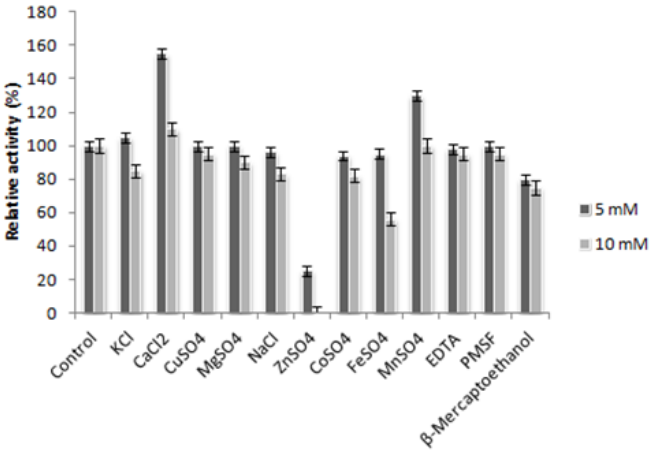

Figure 4 Effects of metal ions and chemical agents on the enzyme activity.

Recombinant lipase catalyzed biodiesel production in the presence of methanol and canola oil or waste oil. For complete conversion of oil to biodiesel, nearly 3 M of methanol need to be added into the reaction mixture. But in the transesterification system catalyzed by lipase, methanol usually is added stepwise, otherwise methanol could result in denaturation of lipase (**Ranganathan *et al.*, 2008**). The production of high level at around 9 h and 15 h in order to waste oil or canola oil (Fig. 5) and our lipase showed a good biodiesel yield % 55 and % 65. Reaction stability remained up to 10 h. We used 1:4 methanol in the reaction stepwise because in transesterification system using lipase, methanol is added stepwise due to inhibition of the lipase enzyme **(Li *et al.*, 2011; Ranganathan *et al.*, 2008)**.

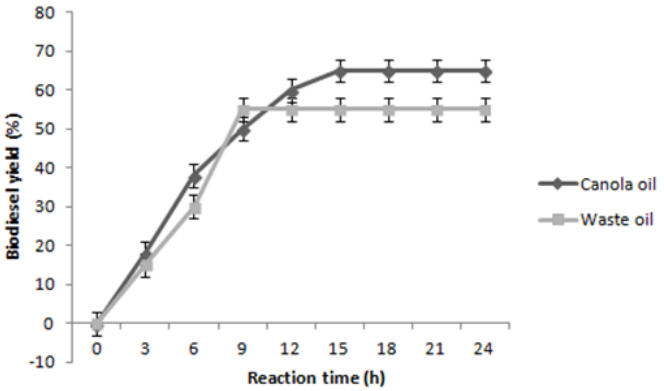

Figure 5 Biodiesel production using methanol from canola oil and waste oil by recombinant lipase.

CONCLUSION

In this study, we report the cloning of a novel lipase gene from *Shewanella putrefaciens* ATCC 8071. Biochemical characterization showed that the recombinant lipase was a novel thermostable lipase and possessed several attractive characteristics. The obtained lipase showed high activity in broad ranges of pH and temperature. Recombinant lipase is most active at 80 °C and even able to retain 50% and 20% activity at 90 °C and 100 °C, respectively, which makes it different from other typical lipases. This lipase also catalys the biodiesel. These properties showed that it might be used for many biotechnological applications.

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