



Gene Cloning, Heterologous Expression and Biochemical Characterization of A Novel Extracellular Lipase from *Rhizopus Oryzae* KU45

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Background: Lipases secreted from various *Rhizopus oryzae* strains were previously expressed in *Escherichia coli*, *Pichia pastoris*, and *Saccharomyces cerevisiae* and was shown to have distinct activities in response to different temperatures, metal ions, organic solvents, and specific substrates. However, until now, no other research biochemically characterized the functions of extracellular pro-lipase in a novel *Rhizopus oryzae* KU45.

Objectives: Characterization of a novel extracellular lipase from fungus *R. oryzae* KU45 after heterologous expression in *E. coli* BL21 (DE3) strain.

Materials and Methods: An extracellular lipase producing fungus was isolated from a soil sample and identified as a strain of *R. oryzae* by partial 18S rRNA gene sequencing. It was named as *R. oryzae* KU45. The lipase gene of KU45 was cloned into pET-28a expression vector and expressed in *E. coli* as inclusion bodies. The recombinant lipase was purified, refolded and characterized.

Results: The lipase exhibited maximum activity at 45°C, at slightly alkaline pH. It showed a broad substrate specificity acting on p-nitrophenyl esters with C₈-C₁₆ acyl groups as substrates and, many of the organic solvents and metal ions tested did not have any adverse effects on the enzyme activity.

Conclusions: High stability, broad substrate specificity and activity at mesophilic temperatures in the presence of organic solvents, and metal ions make the extracellular lipase of KU45 a candidate for various biotechnological applications.

Keywords: *Escherichia coli*, Extracellular lipase, Enzyme, Gene cloning and expression, Inclusion body, *Rhizopus oryzae*

1. Background

Lipases catalyze the hydrolysis of triglycerides to free fatty acids and glycerol under aqueous conditions. Lipases can also perform synthesis by transesterification reactions in the presence of organic solvents. Such properties of lipases make them popular for biotechnological applications (1).

Lipases are produced by organisms from all domains of life, but microbial lipases hold a special interest in biotechnology due to their ease of discovery and production. Some of the lipases that are exploited for commercial purposes are fungal origin and include lipases from *Mucor miehei*, *Humicola lanuginosa*, *Aspergillus niger*, and *Rhizopus* species (2).

The lipase secreted from *R. oryzae* is synthesized as a precursor form with a pre-sequence (26 amino acid residues) and a pro-sequence (97 amino acid residues) at the N-terminal side of the mature portion (269 amino acid residues) (3). In its natural host, the pre-sequence acts as a signal peptide to facilitate the secretion of the lipase, while pro-sequence aids in the correct folding

of the lipase which in its fully mature form lacks both the pre- and pro-sequences (4). However, heterologous expression studies in *Escherichia coli* have previously shown that pro-lipase but not the mature lipase had activity indicating the importance of pro-sequence in bacterial expression (5). In brief, enzyme activity of the *R. oryzae* lipase is modulated by the pro-sequence to synthesize the enzyme in non-destructive form. In addition, cysteine residue in pro-sequence with its neighboring amino acids plays catalytically important role in the enzyme folding (5) whereas the presence of pre-sequence delays the facilitated folding of proenzymes (3).

Previously, successful functional expression of *R. oryzae* lipase was demonstrated in *E. coli* (3, 5, 6), *Pichia pastoris* (7, 8) and *Saccharomyces cerevisiae* (4, 9). Although pro-lipase was formed as insoluble inclusion body in *E. coli* expression system, obtaining active form of the enzyme requires a refolding process (3). In addition, *E. coli* expression system has the great capacity to produce large amounts of recombinant

protein without background lipase or esterase due its high cell-density cultivation (6).

2. Objectives

In the present study, we report the cloning, expression in *E. coli*., purification, and characterization of a pro-lipase from a novel species of *Rhizopus* isolated in our laboratory (10) This is the first study to characterize a recombinant *Rhizopus* pro-lipase expressed and purified in *E. coli*.

3. Material and Methods

3.1. Bacterial, and Fungal Strains, and Media

Recombinant *E. coli* strains DH5 α and BL21 (DE3) were grown in Luria-Bertani (LB) medium, supplemented with 100 mg.l⁻¹ ampicillin or 30 mg.l⁻¹ kanamycin when required. IPTG (1 mM) was added for the induction of gene expression from plasmid pET28a. *R. oryzae* KU45 was grown in LB medium at 25°C in shaking flasks at 100 rpm.

3.2. Genotypic Identification of the Fungus by 18S rRNA Gene Sequence Analysis and BLAST Search

Genomic DNA was purified from the fungal mycelium grown in LB broth, using DNeasy Plant Mini Kit (Qiagen, Germany), and used as a template for the amplification of the 18S rRNA gene by polymerase chain reaction (PCR) using the primer pair, BMB-C (11) and SR-10R (12). The amplified DNA fragment was cloned into the pTZ57R/T cloning vector using the InsTAclone™ PCR Cloning Kit (Thermo Scientific, USA) according to the manufacturer's instructions and the transformation of *E. coli* DH5 α cells was carried out by heat shock method. Transformation mixture was plated on LB agar media containing 100 μ g.ml⁻¹ ampicillin and incubated overnight at 37°C. Several colonies were screened by colony PCR using primers M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGACC-3') and amplified fragments corresponding to the size of the 18s rRNA gene were sequenced from both directions using the same primers at the Biotechnology and Bioengineering Central Research and Application Laboratories, Izmir Institute of Technology. Phylogenetic association of the fungus was determined by Basic Local Alignment Search Tool (BLAST) (13) analysis of the sequenced 18S partial rRNA gene.

3.3. Cloning and Expression of the Encoding Gene for Extracellular Pro-Lipase

Total RNA was isolated from fungal mycelium using RNeasy Plant Mini Kit (Qiagen, Germany). Then

mRNAs were purified from total RNA by using Oligotex mRNA Mini Kit (Qiagen), and cDNA's were synthesized from this mRNA pool using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Fungal lipase cDNA was amplified using Pfu polymerase with previously defined primers RsLipF (5'-CCGATGGTTTCATTCATTTCCATTTCTC-3') and RsLipR (5'-GCTTACAAACAGCTTCCTTCGTT-3') (14). 1.2 kb Lipase cDNA was cloned into the pJET1.2/blunt cloning vector using CloneJet PCR Cloning kit (Thermo Scientific, USA), and sequenced from both directions at the Biotechnology and Bioengineering Central Research and Application Laboratories, Izmir Institute of Technology using pJET 1.2 forward and reverse primers (Thermo Scientific, USA). Lipases from *Rhizopus* species are produced as a pre-pro-enzyme containing a 26 amino acid long secretion signal at the N-terminal (15) which is not functional in *E. coli*. Therefore, lipase gene without this sequence was amplified using primers LipproF which contained a start codon prior to and in frame with the pro sequence (5'-CCATGGTTCCTGTTTCTGGTAAA-3') and LipR (5'-GGATCCTTACAAACAGCTTCCTTCG-3') (underlined sequences in LipproF and LipR primers are the recognition sequences for the restriction enzymes *NcoI* and *BamHI*, respectively). After purification and restriction digestion with *NcoI* and *BamHI*, the pro-enzyme fragment was cloned into pET-28a (Merck KGaA, Germany) expression vector, digested with the same restriction enzymes. *E. coli* BL21 (DE3) (Merck KGaA, Germany) was transformed with the recombinant plasmid pET-28a-Lip, and the recombinant colonies were selected with 30 μ g.ml⁻¹ kanamycin.

3.4. Purification of the Recombinant Pro-Lipase

Recombinant pro-lipase (without a secretion signal peptide) was not secreted by *E. coli* and thus was purified as inclusion bodies and then renatured. 2 ml overnight culture of the *E. coli* BL21 (DE3) pET-28a-Lip was used to seed 200 ml culture medium supplemented with kanamycin and incubated overnight at 37°C until an OD₆₀₀ of 0.6. Lipase expression was induced by 1mM IPTG and after an overnight incubation, cells were harvested by centrifugation (10,000 g, 10 min, 4°C) and the cell pellet was resuspended in 2 ml Tris-HCl buffer (100 mM, pH 8) containing 1 mM EDTA. Following seven cycles of 30 s sonication, cell lysate was centrifuged at 12,000 g for 20 min. Pellet, which contained the inclusion bodies was washed three times with Tris-HCl buffer and resuspended in 10 ml of denaturation buffer (50 mM Tris-HCl pH 8, 8

M urea, 10 mM DDT) and incubated at 37°C for 60 minutes with mild stirring. Following centrifugation (12,000 g, 20 min, 4°C), the supernatant containing the pro-lipase was renatured by dialysis against Tris–HCl buffer (16) and then concentrated by using a 20 kDa molecular weight cut off protein concentrator (Thermo Scientific, USA). Final protein sample concentration was determined as previously described (17), and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3.5. Assay of Lipase Activity and Determination of the Substrate Specificity

Both the lipase activity, which was assayed at spectrophotometrically using *p*-nitrophenyl laureate as a substrate, and the determination of substrate specificity were performed according to the protocol described previously (17) except that the incubations were performed at 45°C, pH 8.0.

To find out the impact of various fatty acid chain lengths on substrate specificity of the pro-lipase, activity towards *p*-nitrophenyl esters (*p*-nitrophenyl butyrate (C4), *p*-nitrophenyl caproate (C6) *p*-nitrophenyl caprylate (C8) *p*-nitrophenyl caprate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14) *p*-nitrophenyl palmitate (C16), *p*-nitrophenyl stearate (C18)) at 50mM, pH 8.0 and 45°C was assayed (16).

3.6. Effect of Temperature on Pro-Lipase Activity and Stability

The enzyme activity was measured in the range of 5°C–65°C with intervals of 10°C under standard assay conditions. To examine the thermal stability of the purified pro-lipase, the enzyme was allowed to stand at pH 8.0 for 1 hour at different temperatures (5 to 85°C). Then the residual activity was measured at pH 8.0 and 45 °C according to standard assay conditions (17).

3.7. Effect of pH on Pro-Lipase Activity and Stability

In order to find the optimum pH for pro-lipase activity, the enzyme activity was tested at 45 °C in pH range between 4.0–12.0 with different buffers (sodium acetate (pH 3.0–5.0), potassium phosphate (pH 5.0–7.0), Tris–HCl (pH 7.0–9.0) and glycine–NaOH (pH 9.0–12.0)). To determine the effect of pH on purified pro-lipase stability, pro-lipase was pre-incubated at 45 °C for 1 hour in buffers of pH 5.0–11.0 (17). The residual lipase activity was measured under standard assay conditions.

3.8. Effect of Different Metal Ions on Pro-Lipase Activity

To test the effect of different metal ions on purified pro-lipase activity, enzyme was incubated with different

metal ions (Ca²⁺, Mg²⁺, K⁺, Na⁺, Mn²⁺, Ni²⁺, Zn²⁺ and metal chelator EDTA at 1 mM) at 45°C, pH 8.0 for 1 hour. Then, the relative activity was evaluated by regarding to control (no metal ions in the mixture) as 100%.

3.9. Effect of Different Organic Solvents on Pro-Lipase Activity

The effect of different organic solvents on purified pro-lipase activity was tested by incubating the enzyme with ethanol, methanol, acetone, propanol, n-hexane, dimethylformamide and Dimethyl sulfoxide (DMSO) at a concentration of 30% (v/v) at 45°C, pH 8.0 for 1 hour (17). Then, the relative activity was evaluated by regarding the control (no organic solvents in the mixture) as 100%.

4. Results

4.1. Genotypic Identification of the Extracellular Lipase Producing Fungus

BLAST analysis of the partial 18S rRNA gene sequence of the fungus showed a sequence identity of 100% with *R. oryzae* and 99% with *R. stolonifer*. Thus, the fungus was referred to as *R. oryzae* KU45. The partial 18S rRNA sequence has been deposited in GenBank with accession number KY131949.

4.2. Cloning and Sequence Analysis of the Encoding Gene for Pro-Lipase

Based on the consensus sequence of lipases from other *Rhizopus sp.*, about 1.2-kb fragment was amplified by PCR using *R. oryzae* KU45 mRNA specific cDNA as template (**Fig. 1**). The lipase gene was homologous to lipases from *R. oryzae* (99% identity), *R. niveus* (97% identity), and *R. delemar* (97% identity), consistent with the 18s rRNA gene sequence results. Only a single amino acid differed *Rhizopus* KU45 lipase from lipases of two different strains of *R. oryzae* whose sequences were available on databases; DSM 853 (accession GQ502721.1) (3) and Hu3005 (accession AF229435.1) (18), which differed by two amino acids from each other (**Fig. 2**). The differences were all located at the mature lipase region of the lipases. Both KU45 and DSM8543 lipases contain valine at position 130, while Hu3005 contains alanine at the same position. On the other hand, KU45 and Hu3005 lipases contain isoleucine at position 250, while DSM853 lipase contains valine at the same position. KU45 lipase is phylogenetically more closely related to lipases from strains DSM853 and Hu3005 than lipases from these two strains are related to each other, suggesting the diversion of the latter two from KU45 lipase.

ATGGTTTCATTTCATTTCATTTCCTCAAGGTGTAGTCTTTGTCTTCTTGTCTCTTCCATGATGCTCGGTTTCATCT
GCTGTTCTCTGTTTCTGGTAAATCTGGATCTTCCACTACCGCGTCTCTGCATCTGACAATTCTGCCCTCCCTCCT
CTCATTTCCAGCCGTTGTGCTCCTCCTTCTAACAAGGGAAGTAAAAGCGATCTCAAGCTGAACCTTACTACATG
CAAAAGAATACAGAATGGTATGAGTCCCATGGTGGCAACTTGACATCCATCGGAAAGCGAGATGACAATTTGGTT
GGTGGCATGACTTTGGATTTACCTAGCGATGCTCCTCCTATCAGCCTCTCTGGATCTACCAACAGCGCCTCTGAT
GGTGGTAAGGTTGTGCTGCTACTACTGCTCAAATTCAGAGTTACCAAGTATGCTGGTATCGCTGCCACTGCC
TACTGTCTGTTCTGTTGCCCTGGTAACAAGTGGGACTGTGTCCAATGTCAAAGTGGGTTCTGATGGCAAGATC
ATCACTACCTTTACCTCCTTGTCTTTCCGACACAAATGGTTACGCTCTTGAGAAGTGATAAACAAAAGACCATTAT
CTTGTCTTCCGTTGGTACCAACTCCTTCAGAAGTGCCATCAGTATCGTCTTCAACTTTTCCGACTACAAGCCT
GTCAAGGGGCCAAGGTTATGCTGGTTTCTTCTTATGAGCAAGTTGTCAATGACTATTTCCCTGTCTATC
CAAGAACAAGTACCGCTAACCTACTTACAAGGTCATCGTTACCGGTCACCTACTCGGTGGTGCACAAGCTTTG
CTTGCCGGTATGGATCTCTACCAACGTGAACCAAGATTGTCTCCCAAGATTGAGCATCTTCACTGTTGGTGGT
CCTCGTGTGGTAACCCACCTTTGCTTACTATGTTGAATCTACCGGTTCTCTTCCACGTACCGTTCACAAG
AGAGATATCGTCTCACGTTCTCCTCAATCCTTCGGATTCTTTCATCCCGGTTGTAATCTTGGATCAAGTCC
GGTACCTCCAACGTTCAAATCTGTACTTCTGAAATTGAAACCAAGGATTGCAGTAACCTTATCGTTCCTTTACC
TCTCTCCTTGATCACTTGAGTTACTTTGATATCAACGAAGGAGCTGTTTGTA

Figure 1. Sequence of the cloned lipase cDNA Sequences encoding pre, pro, and mature lipase amino acids are shown in dark gray, light gray, and white background respectively. Underlined sequences are the primer binding sites for pro-lipase sequences which are shown in bold letters.

K	MVSFISISQGVSLCLLVSSMMLGSSAVPVSGKSGSSTTAVSASDNSALPPLISSRCAPPS
H	MVSFISISQGVSLCLLVSSMMLGSSAVPVSGKSGSSTTAVSASDNSALPPLISSRCAPPS
D	MVSFISISQGVSLCLLVSSMMLGSSAVPVSGKSGSSTTAVSASDNSALPPLISSRCAPPS

K	NKGSKSDLQAEPPYMQKNTEWYESHGNNLTSIGKRDDNLVGGMTLDLPSDAPPISLSGST
H	NKGSKSDLQAEPPYMQKNTEWYESHGNNLTSIGKRDDNLVGGMTLDLPSDAPPISLSGST
D	NKGSKSDLQAEPPYMQKNTEWYESHGNNLTSIGKRDDNLVGGMTLDLPSDAPPISLSGST

K	NSASDGGKVVAAATTAQIQEFTKYAGIAATAYCRSVVPGNKWDCVQCQKWVPDGKIITFT
H	NSASDGGKVVAAATTAQIQEFTKYAGIAATAYCRSVVPGNKWDCVQCQKWVPDGKIITFT
D	NSASDGGKVVAAATTAQIQEFTKYAGIAATAYCRSVVPGNKWDCVQCQKWVPDGKIITFT

K	SLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVNFSDYKPVKGAKVHAGFLSSYE
H	SLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVNFSDYKPVKGAKVHAGFLSSYE
D	SLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVNFSDYKPVKGAKVHAGFLSSYE

K	QVVNDYFPVQEQLTANPTYKVIIVTGHSLGGAQALLAGMDLYQREPRLSPKNLSIFTVGG
H	QVVNDYFPVQEQLTANPTYKVIIVTGHSLGGAQALLAGMDLYQREPRLSPKNLSIFTVGG
D	QVVNDYFPVQEQLTANPTYKVIIVTGHSLGGAQALLAGMDLYQREPRLSPKNLSIFTVGG

K	PRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQSFGLHPGVESWIKSGTSNVQICTS
H	PRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQSFGLHPGVESWIKSGTSNVQICTS
D	PRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQSFGLHPGVESWIKSGTSNVQICTS

K	EIETKDCSNSIVPFTSLLDHLSYFDINEGSCL
H	EIETKDCSNSIVPFTSLLDHLSYFDINEGSCL
D	EIETKDCSNSIVPFTSLLDHLSYFDINEGSCL

Figure 2. Comparison of the amino acid sequences of *R. oryzae* lipases from strains KU45, DSM 853 and Hu3005 Pre, pro, and mature lipase regions are shown with dark gray, light gray and white respectively. Sequences with bold letters represent amino acids that differ between at least two lipases.

4.3. Purification and Refolding of the Recombinant Extracellular Pro-Lipase from *E. coli*

Rhizopus lipases are initially synthesized as a pre-pro-enzyme consisting of an N-terminal 26-amino acid long export signal peptide, followed by a 97 amino acid long pro-peptide fused to the amino terminus of the 269 amino acids of the mature lipase enzyme (15). The pro-peptide serves as an intramolecular chaperone involved in the correct folding of the mature lipase in fungi (4). However, pre-sequence is not functional in *E. coli* (5). Therefore, *R. oryzae* KU45 pro-lipase excluding the signal peptide was over-expressed in *E. coli* (see materials and methods) with an N-terminal tag coded by the expression vector pET28-a. However, over-expressed *R. oryzae* KU45 pro-lipase formed inactive inclusion bodies in *E. coli* (data not shown) and hampered our attempts to purify the recombinant lipase by affinity chromatography utilizing the N-terminal tag. Thus, purification of the pro-lipase was achieved by inclusion body isolation followed by renaturation using urea. The molecular weight and purity of the renatured pro-lipase were confirmed by SDS-PAGE, and the molecular mass of purified lipase was estimated

to be approximately 39 kDa (**Fig. 3a**). The activity of the enzyme at 45°C, pH 8 was calculated as 256 U.mg⁻¹, using 4-nitrophenyl laureate as substrate.

4.4. Substrate Specificity of the Recombinant Extracellular Pro-Lipase

To find out the effect of fatty acid chain length on the specificity of the purified pro-lipase, various *p*-nitrophenyl esters were tested (**Fig. 3b**). The lipase maximally acted on *p*-nitrophenyl caprate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14) as substrate with relative activities of 91%, 100%, and 83% respectively. The activity was 50% with *p*-nitrophenyl caprylate (C8) and 32% with *p*-nitrophenyl caproate (C6). Although the activity of the enzyme decreased on long chain substrates (>14 carbon atoms), it still had 20% activity on *p*-nitrophenyl palmitate (C16), while there was no or little activity against *p*-nitrophenyl butyrate (C4), and *p*-nitrophenyl stearate (C18). These results showed that the pro-lipase *R. oryzae* KU45 preferentially acted on medium chain fatty acid ester substrates (18).

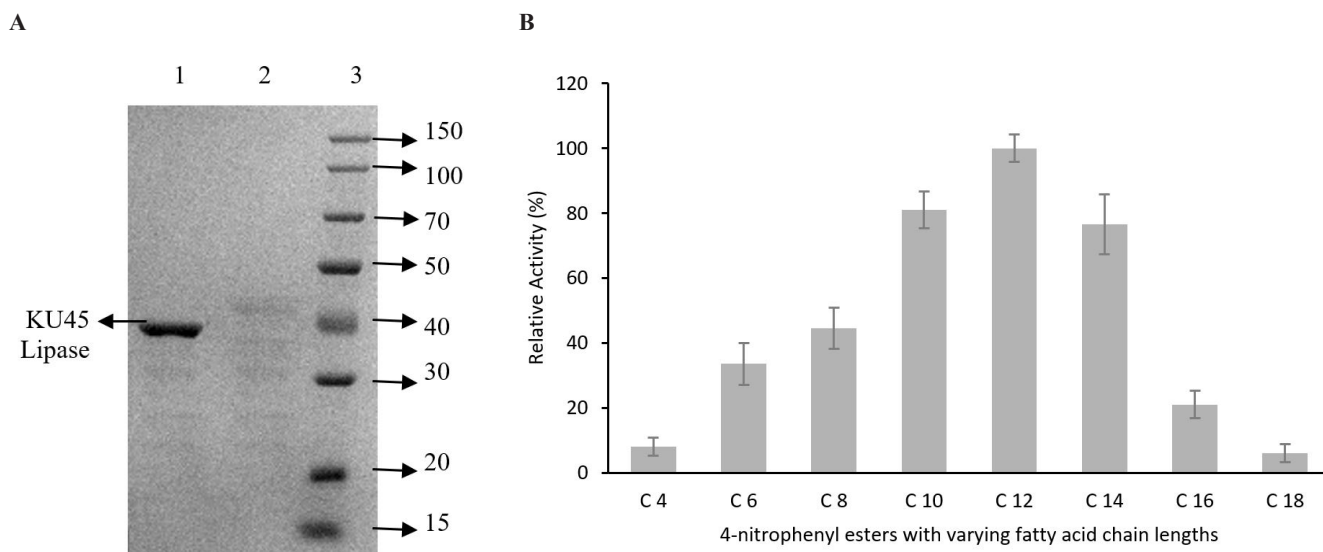


Figure 3. SDS-PAGE analysis and substrate specificity of purified lipase from *R. oryzae* KU45 (**A**) The protein was stained with Coomassie brilliant blue. Lane 1: Purified lipase as inclusion bodies. Lane 2: Inclusion body isolation from *E. coli* harboring the empty expression vector. Lane 3: Protein size marker (kDa). (**B**) Lipase activity assay was performed at 45°C, pH 8.0, using *p*-nitrophenyl esters of varying fatty acid chain as substrates. The activity towards *p*-nitrophenyl laurate (C12), which was calculated as 256 U.mg⁻¹ was taken as 100%.

4.5. Effect of Temperature on the Activity and Stability of Recombinant Extracellular Pro-Lipase

Lipase activity (17) was examined in the range of 5°C-65°C, in Tris-HCl buffer, pH 8.0, using *p*-nitrophenyl laurate as substrate and the maximum activity was observed at 45°C (**Fig. 4a**). The enzyme was also active at cold temperatures retaining 30% of its activity

at 25°C. It did not show any activity at 0°C. At 55°C *R. oryzae* KU45 lipase lost 95% of the maximum activity, and no activity was observed at 65°C. Thus, temperatures above 45°C adversely affected the lipase activity. The thermal stability of the purified lipase was investigated by pre-incubating the lipase at different temperatures for 1 hour in Tris-HCl buffer, pH 8.0. at

different temperatures (5 to 85°C) and then residual activity was measured using p-nitrophenyl laurate as substrate (**Fig. 4a**). The enzyme was highly stable at 5 to 45°C with almost no loss in activity, and it retained around half of its activity at 55 and 65°C, and a quarter of its activity at 75°C. However, no activity remained at 85°C. These results indicated that *R. oryzae* KU45 extracellular lipase was highly stable at temperatures up to its optimum temperature.

4.6. Effect of pH on the Activity and Stability of the Recombinant Extracellular Pro-Lipase

The lipase activity was assayed in buffers of different pH values at 45°C, using p-nitrophenyl laurate as substrate. The buffer systems were sodium acetate buffer (pH 3.0-5.0), potassium phosphate buffer (pH 5.0-7.0), Tris-HCl buffer (pH 7.0-9.0) and glycine-NaOH buffer (pH 9.0-12.0). The optimum activity of the lipase was observed at pH 8.0 (**Fig. 4b**) and it had

70% and 90% of its maximum activity at pH 7.0 and 9.0, respectively. In the acidic and alkaline range, a significant reduction in enzyme activity was observed. For instance, at pH 6.0 the lipase retained only 15% of its maximum activity. The enzyme lost all its activity below pH 6.0 and above pH 11.0. In order to investigate the pH stability, the enzyme was allowed to stand for 1 hour at different pH values (pH 5.0-11.0) at 45°C, and then the residual activity at each pH was measured using p-nitrophenyl laurate as substrate at 45°C (**Fig. 4b**). The purified lipase showed maximum stability at pH 8.0 retaining its 100% activity. After incubation at pH 6.0 and 7.0, the enzyme retained high activity (80% and 90% respectively). However, it started to lose activity after incubation below pH 6.0. On the alkaline range, the enzyme retained more than half of its maximum activity after incubation at pH 9.0 and 10.0 but lost its activity above pH 10.0.

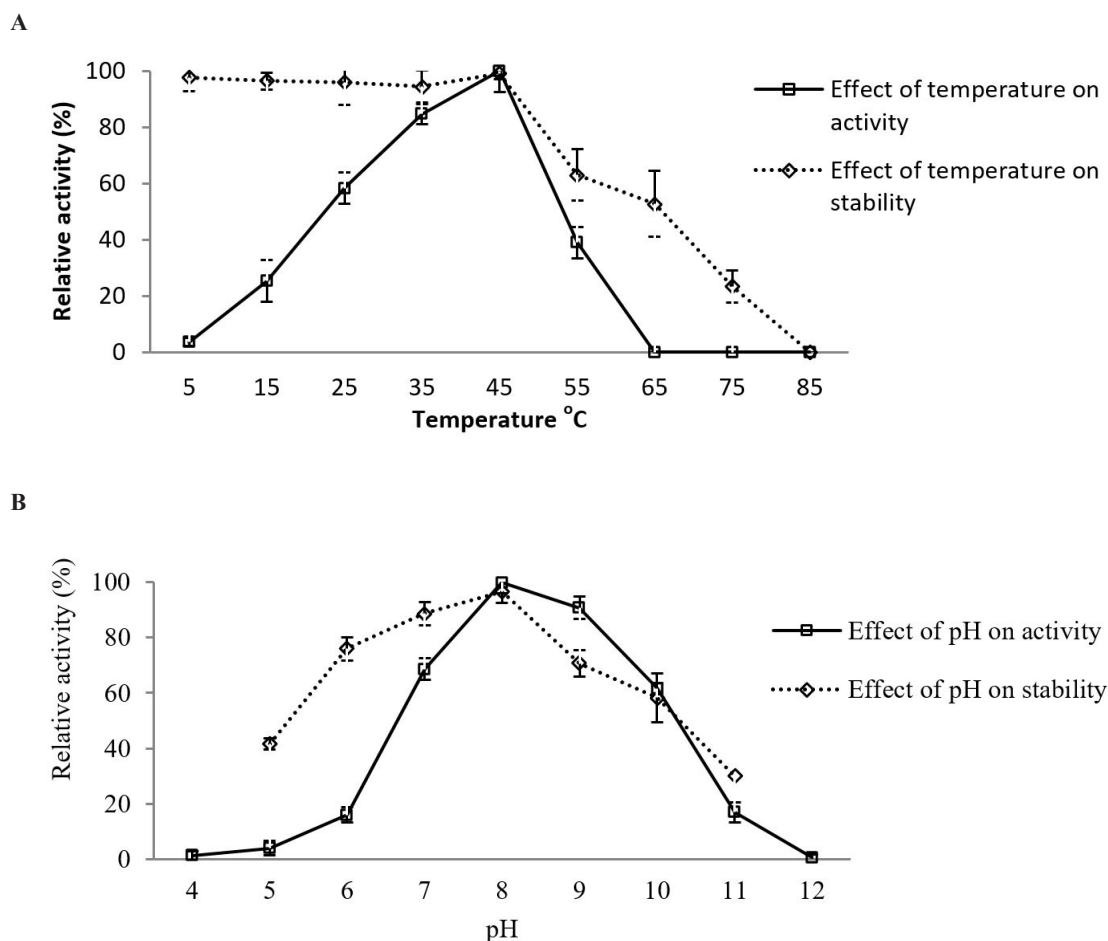


Figure 4. Effect of temperature (**A**) and pH (**B**) on activity and stability of *R. oryzae* KU45 recombinant lipase. Lipase activities were monitored at indicated reaction temperatures and pH values. The thermal stability of KE38 lipase was investigated by pre-incubating the lipase at different temperatures for 1 hour and then assaying the residual lipase activity (a). The pH stability of KE38 lipase was investigated by pre-incubating the lipase at different pH for 1 hour and then assaying the residual lipase activity (b).

4.7. Effect of Metal Ions on the Recombinant Extracellular Pro-Lipase Activity

To find out the effects of different metal ions on the pro-lipase, enzyme activities were investigated in the presence of Ca^{2+} , Mg^{2+} , K^{1+} , Na^{1+} , Mn^{2+} , Ni^{2+} , Zn^{2+} , and metal chelators of EDTA at 1 mM (**Table 2a**). Among divalent cations, Mg^{2+} , Mn^{2+} , and Ni^{2+} didn't have any considerable effect on the enzyme activity. However, in the presence of Ca^{2+} and Zn^{2+} almost half and a quarter of the activity of the enzyme was lost respectively.

Our results suggested that none of the metal ions tested were required for the enzyme activity. This was further emphasized by EDTA, a strong chelator of metals, which had no effect on the activity of the enzyme suggesting that *R. oryzae* KU45 pro-lipase did not require any metal cofactors.

4.8. Effect of Various Organic Solvents on the Recombinant Extracellular Pro-Lipase Activity

To investigate the effect of organic solvents on the lipase activity, ethanol, methanol, acetone, propanol, n-hexane, dimethylformamide and DMSO were used at a 30% (v/v) (**Table 2b**). DMSO and methanol activated the enzyme by 123% and 117%, respectively. The enzyme retained about 80% of its activity in the presence of ethanol. However, acetone, propanol, and dimethyl formamide strongly inhibited the lipase activity whereas in the presence of n-hexane activity was completely inhibited. High lipase activity of the *R. oryzae* KU45 recombinant pro-lipase in the presence of methanol and ethanol is a distinguishing feature among other *R. oryzae* lipases since the latter show decreased activity in those organic solvents (**table 1**). This property of *R. oryzae* KU45 recombinant pro-lipase makes it a strong candidate as a catalytic tool for the synthesis of biodiesels (19).

Table 1. Comparison of *R. oryzae* lipases from different strains.

Lipase	Expression Host	M.wt (kDa)	Opt. temp. (°C)	Opt. pH	Metal Ions tested	Organic Solvents tested	Substrates used	Optimal Substrate Specificity
<i>R. oryzae</i> KU45 proL ^a	<i>E. coli</i>	39	45	8.0	$\text{Ca}^{2+ (*)}$, $\text{Zn}^{2+ (*)}$, $\text{Na}^{+ (*)}$, $\text{K}^{+ (*)}$	Methanol ^(x) , DMSO ^(x)	pNEs	C 10-14
<i>R. oryzae</i> 3005 proL ^b	<i>P. pastoris</i>	35	40	9.0	$\text{Ca}^{2+ (*)}$, $\text{Mg}^{2+ (*)}$, $\text{Na}^{+ (*)}$, $\text{K}^{+ (*)}$, $\text{Zn}^{2+ (*)}$, $\text{Hg}^{2+ (*)}$, $\text{Ag}^{+ (*)}$	Methanol ^(*) , Ethanol ^(*)	TG	C 8-12
<i>R. oryzae</i> DSM 853 proL ^c	<i>E. coli</i>	n.m	55	8.0	n.m	n.m	n.m	n.m
<i>R. oryzae</i> HU3005 proL ^d	<i>P. pastoris</i>	35	30	8.0	n.m	n.m	n.m	C 4 and C 10
<i>R. oryzae</i> HU3005 matL ^d	<i>P. pastoris</i>	30	30	8.0	n.m	n.m	n.m	C 10
<i>R. oryzae</i> DSM853 proL ^e	<i>P. pastoris</i>	32	35	7.0	$\text{Ca}^{2+ (*)}$, $\text{Mg}^{2+ (*)}$	Methanol ^(*) , Ethanol ^(*) , DMSO ^(x)	n.m	n.m
<i>R. oryzae</i> WPG matL ^f	<i>E. coli</i>	29	37	8.0	n.m	n.m	TG	Short and long chain
<i>R. oryzae</i> proL ^g	<i>S. cerevisiae</i>	46	30	8.0	n.m	n.m	pNEs	C 12

(*) In its presence, pro-lipase activity decreased.

(x) In its presence, pro-lipase activity increased.

^a this work, ^b (8), ^c (3), ^d (18), ^e (19), ^f (28), ^g (9)

matL: maturelipase, proL: pro-lipase, n.m: not mentioned, pNE: *p*-nitrophenyl esters, TG: tryglicerids

Table 2. Effect of various metal ions (A) and various organic solvents (B) on extracellular lipase activity from *R. oryzae* KU45 Lipase activity assay was performed at 45°C, pH 8.0 in the presence of 1 mM various metal ions. The activity of control (no metal ions in the mixture) was taken as 100% (a). Lipase activity assay was performed at 45°C, pH 8.0, in the presence of 30% (v/v) various organic solvents. The activity of control (no organic solvents in the mixture) was taken as 100% (b).

A		B	
Metal ion	Relative activity (%)	Solvent	Relative activity (%)
None (Control)	100 ± 3	None	100,00 ± 3
CaCl ₂	55 ± 4	Ethanol	80,33 ± 7
MgCl ₂	98 ± 12	Methanol	117,66 ± 14
MnCl ₂	94 ± 7	Acetone	30,33 ± 4
NiCl ₂	103 ± 18	Propanol	37,00 ± 6
ZnCl ₂	78 ± 8	n-Hexane	0 ± 0
KCl	70 ± 10	Dimethyl formamide	39,00 ± 4
NaCl	59 ± 7	DMSO	123,33 ± 10
EDTA	98 ± 6		

5. Discussion

This study revealed that *R. oryzae* KU45 pro-lipase has high stability, broad substrate specificity and activity at mesophilic temperatures in the presence of various organic solvents, and metal ions.

Molecular weight of the purified recombinant pro-lipase from fungus *R. oryzae* KU45 was approximately found as 39kDa and slight changes was observed from other *R. oryzae* pro-lipases (see **Table 1**). Although lipases from *R. oryzae* strains including KU45, DSM853, and Hu3005 contain the same number of amino acids, post-translational modifications occurring in yeast host expression system might be the reason of such slight changes in molecular weights. Prediction of *R. oryzae* KU45 pro-lipase molecular weight using ExpASY bioinformatics tool (20)²*O.c* (http://web.expasy.org/compute_pi/) was in agreement with that of cloned *R. oryzae* KU45 pro-lipase (39.5 kDa and 39 kDa respectively).

The Pro-lipase from *R. oryzae* KU45 showed maximal activity on *p*-nitrophenyl caprate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14) indicating that medium chain fatty acid ester substrates were more preferred. Our findings are similar to earlier findings for other *R. oryzae* pro-lipases (**Table 1**) with slight differences which could be due to the differences in substrates used in enzyme assays. For instance, strain 3005 pro-lipase showed the highest activity on C 8-16 and C 12-18 when TGs and FAMES were used as substrates respectively (8).

The maximum enzyme activity of KU45 pro-lipase was present at 45°C. Similar to our isolate, most mesophilic or psychrophilic microbial lipase producers have been shown to have lipases with maximum activity

at much higher temperatures than the optimal growth temperatures of their source organisms (21). Several *R. oryzae* lipases were reported to have maximum activities in the range of 30- 55°C (**Table 1**). In addition, enzyme was highly stable up to 45°C and enzyme activity was gradually lost when 85°C was reached. The high stability of *R. oryzae* KU45 pro-lipase should be appeared as an important property especially for the enzymes intended to be used for long periods of time in industrial applications. KU45 lipase showed a higher and lower optimum temperature compared to Hu 3005 (30-40°C) and DSM853 (55°C) lipases which could be attributed to the amino acid differences among these three lipases (**Fig. 2**).

The *R. oryzae* KU45 pro-lipase showed its maximum activity and stability at pH 8.0 and its activity on the alkaline range was retained. These results showed that the enzyme was an alkaline lipase similar to other *R. oryzae* lipases (**Table 1**). However, it showed high resistance against significant pH changes.

The presence of metal ions has distinct and important roles in the function and structure of enzymes. For instance, metal ions may stabilize or destabilize enzyme conformation resulting in alteration of its activity, they may act as cofactors in catalysis reactions or metal ions form bonds with some of the amino acid side chains in the proteins resulting in denaturation in the active sites (22). Our results demonstrated that the presence of Ca²⁺ and Zn²⁺ leads to decrease in the enzyme activity. In contrast to our results, Wang *et al.*, (2013) reported that in the presence of Ca²⁺, Na⁺ and K⁺ ions, lipase activity of *R. oryzae* strain 3005 increased (**Table 1**). Although metal ions influence lipase activity in different ways, it was previously reported that Ca²⁺ inhibited lipase

activity by forming complexes with ionized fatty acids and changing their solubility and behaviors at oil-water interfaces (23). Monovalent cations such as Na⁺ and K⁺ caused a decrease in the catalytic activity of pro-lipase KU45. Binding of these monovalent cations to amino acid side chains of pro-lipase might have caused competitive inhibition which further led to decreased enzymatic activity (24).

Organic solvent stability of enzymes is a desirable feature especially in synthesis reactions such as esterification, and transesterification (25). In addition, enzyme activity is influenced by water miscibility of solvents, functional groups and molecular structures present in the organic solvents (22). KU45 pro-lipase showed high activity in the presence of DMSO, methanol, and ethanol whereas presence of acetone, propanol, dimethyl formamide and n-hexane resulted in inhibition of the enzyme activity. In contrast to our result, *R. oryzae* strains including 3005 and DSM853 were found to show low pro-lipase activity in the presence of methanol and ethanol (**Table 1**) (8, 19). Although water-miscible solvents such as methanol and ethanol were previously shown to increase *Rhizopus* sp. lipase activity, other factors such as enzyme structure and presence of molecular structure in the solvents might have been the reason of such varying results obtained for the effect of organic solvents on lipase activity (22). As observed in this study, high activity of the purified pro-lipase in the presence of methanol and ethanol makes it a strong candidate to be used for the synthesis of biodiesel which requires transesterification of fatty acids with short chain alcohols (26). It can also have a potential to be used in organic synthesis and chiral resolution reactions which involve the use of DMSO, ethanol, and methanol (27).

6. Conclusion

In the present study, a novel extracellular pro-lipase from *R. oryzae* KU45 was cloned and expressed in *E. coli*. The characteristics of novel *R. oryzae* KU45 pro-lipase indicates high temperature stability across a wide pH range, resistance to the presence of different metal ions, increased activity on methanol, ethanol, and DMSO, and a wide range of substrate specificity. These properties of the *R. oryzae* KU45 pro-lipase make it an excellent candidate for organic synthesis and transesterification reactions as well as utilizing biodiesel production in bioenergy industry.

Conflict of interest

The authors declare no conflict of interests.

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