

Cloning of Synthetic Lipase Gene from *Rhizomucor miehei* with Original Signal Peptide in *Pichia pastoris*

MARTHA EKA CAHYANI¹, IS HELIANTI^{2*}, NIKNIK NURHAYATI², AND ABINAWANTO¹

¹Department of Biology, Faculty of Mathematics and Natural Science, Universitas Indonesia, Depok 16424, Indonesia;

²Center of Bioindustrial Technology, Agency for Assessment and Application of Technology (BPPT), Building 611, LAPTIAB-BPPT, Puspiptek Area, Tangerang Selatan 15314, Indonesia

Lipases (EC 3.1.1.3) are classified as hydrolases that hydrolyze lipids. These enzymes have potential application in biotechnology and industrial process. In previous study we have cloned the synthetic *Rhizomucor miehei* lipase gene using the vector pUC57 in *Escherichia coli* DH5 α , but only found the very low enzymes activity. This study aimed to clone synthetic *Rhizomucor miehei* lipase gene into *Pichia pastoris* expression plasmid for lipase expression with the original signal peptide. A DNA fragment with the original signal peptide had been obtained by PCR, cut by *Xho* I and *Xba* I and then ligated into pPICZ α A linearized with the same enzymes. The mixture of ligation reaction, then was transformed into *Escherichia coli* DH5 α . Zeocin-resistant transformants were selected and contained plasmid was analyzed by restriction enzymes analyses and DNA sequencing. As the result, a synthetic *Rhizomucor miehei* lipase gene (RMLip) with the size of 1132 bp was successfully cloned to pPICZ α A vector plasmid. The recombinant plasmid with the correct DNA sequence was transformed into *Pichia pastoris* X33. Cultivation of recombinant *P. pastoris* was carried out with the addition of 1.5% methanol every day with appropriate aeration. The recombinant lipase produced by *Pichia pastoris* X33 containing RMLip in its chromosomal DNA had optimal temperature and pH, 30 °C and 9.0, respectively.

Key words: *Pichia pastoris*, pPICZ α A, *Rhizomucor miehei*, synthetic lipase gene

Lipase (EC 3.1.1.3) diklasifikasikan sebagai enzim hidrolase yang menghidrolisis lipid. Enzim ini memiliki potensi dalam berbagai aplikasi di bidang bioteknologi dan industri. Dalam penelitian sebelumnya, kami telah mengklon gen lipase *Rhizomucor miehei* sintetik menggunakan vektor pUC57 dalam *Escherichia coli* DH5 α , dan mendapatkan aktivitas enzim yang sangat rendah. Penelitian ini bertujuan untuk mengklon gen lipase *Rhizomucor miehei* sintetik ke dalam plasmid ekspresi *Pichia pastoris* untuk mengekspresikan lipase dengan menggunakan peptida sinyal asli. Gen lipase sisipan diperoleh dengan PCR, dipotong dengan *Xho* I dan *Xba* I, kemudian diligasi ke dalam pPICZ α A yang telah dilinearisasi dengan enzim yang sama. Hasil ligasi kemudian ditransformasi ke dalam *Escherichia coli* DH5 α . Transforman yang tahan terhadap zeocin dipilih dan kandungan plasmidnya dianalisis dengan enzim restriksi dan pengurutan DNA. Hasilnya diperoleh gen lipase *Rhizomucor miehei* (RMLip) berukuran 1132 pb. Plasmid rekombinan yang tepat kemudian ditransformasikan ke *Pichia pastoris* X33. Kultivasi dilakukan dengan penambahan 1.5% metanol setiap hari dengan aerasi yang sesuai. Lipase rekombinan yang diproduksi oleh *Pichia pastoris* X33 yang mengandung RMLip di dalam DNA kromosomnya mempunyai temperatur dan pH optimal masing-masing 30 °C dan 9.

Kata kunci: gen lipase sintetik, *Pichia pastoris*, pPICZ α A, *Rhizomucor miehei*

Lipase (EC 3.1.1.3) is classified as hydrolase that hydrolyzes lipids. These enzymes are biocatalysts that can act on solid and liquid media (Brunel *et al.* 2004). This character makes lipase potential in a variety of applications in biotechnology and industry, including pharmaceutical, pesticide, biosensors, biodiesel, detergents, and various food products (Vakhlu and Kour 2006). Several techniques have been developed to obtain higher conversions, as highly specific enzymes for each application, improving the possibility of industrial applications for these biocatalysts (Franken *et al.* 2008; Soccol and

Vandenbergh 2003).

Lipase used commercially mainly come from microorganisms. Microbial lipases chosen because it has a more stable properties such as selectivity and broad substrate specificity (Dutra *et al.* 2008; Griebeler *et al.* 2011). One of the microorganisms produces lipase is *Rhizomucor miehei* (Sarma *et al.* 2001). *R. miehei* is a mold that has lipase with high activity and good stability under diverse conditions (Rodrigues and Lafuente 2010). Lipase of *R. miehei* has been used in ester hydrolysis, ester synthesis, and transesterification reaction (Huang *et al.* 2012). These lipase was able to successfully catalyze the synthesis of ethyl caproate in organic solvent displayed on the surface *Pichia pastoris* (Han *et al.* 2009), and also effective for the

*Corresponding author: Phone: +62-21-7560536 ext 7119, Fax: +62-21-7566922; Email: is.helianti@bppt.go.id

transesterification of vegetable oils and other raw materials containing higher fatty acid alkyl ester into derivative (Huang *et al.* 2014). Since then, industry has continually searched for new components to improve the benefit of this development along with its cost-efficiency.

Center of Bioindustrial Technology, LAPTIAB, BPPT has conducted cloning of synthetic *Rhizomucor miehei* lipase gene using the vector pUC57 in *Escherichia coli* DH5 α . Although the cloning process succeeded, but the enzyme activity obtained is very low. In this study we chose *Pichia pastoris* as a host to replace *E. coli*. *P. pastoris* is a methylotrophic yeast that has been considered as an excellent host for the production of heterologous proteins (Sreekrishna *et al.* 1997). *P. pastoris* has distinguished characteristics, yielding stable transformants of integration of foreign DNA into chromosomal DNA, have a high-density growth, have the ability to secrete high levels of foreign proteins (Adrio and Demain 2003), expressing heterologous protein can either be intracellular and extracellular (Balamurugan *et al.* 2007). In addition, they have the simplicity of techniques needed for the molecular genetic manipulation and the ability to modify the eukaryotic protein after translation, such as glycosylation, disulfide bond formation and proteolytic processes (Cereghino and Cregg 2000).

Expression of any foreign gene in *P. pastoris* requires three basic steps, which is the insertion of the gene into an expression vector, introduction of the expression vectors into the genome of *P. pastoris*, and examination of potential expression strains for the foreign gene product (Cereghino *et al.* 2000). Therefore, this study aimed to clone *R. miehei* synthetic lipase gene in *P. pastoris* expression plasmid for lipase expression with the original signal peptide.

MATERIALS AND METHODS

Strains, Plasmids, and Media. *E. coli* DH5 α (Invitrogen) was used as the host for the recombinant plasmid cloning and propagation. *P. pastoris* X33 was used as the host for lipase expression. Plasmid pUC57 containing the DNA of synthetic *R. miehei* lipase (Rmlip) has been described elsewhere (Hanniya 2015). The vector pPICZ α A was supplied from Invitrogen. *E. coli* DH5 α was grown at 37 °C in Luria Bertani (LB) low salt agar medium containing 25 μ g mL⁻¹ zeocin for selection of clones transformed with the recombinant vector plasmid. *P. pastoris* X33 was routinely grown in shaking flasks at 30 °C, in a rich

standard medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% (w/v) yeast nitrogen base, 4 x 10⁻⁵% (w/v) biotin, 1% (v/v) glycerol (Buffered Glycerol-complex (BMGY)) before induction, or 1.5% (v/v) methanol (Buffered Methanol-complex (BMMY)) for induction.

For maintaining cultures and plates, Yeast Extract Peptone Dextrose (YPD) medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) containing 50 μ g mL⁻¹ zeocin was used, and for selection of transformants, Yeast Extract Peptone Dextrose Sorbitol (YPDS) medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, 2% (w/v) agar) containing 50 μ g mL⁻¹ zeocin and Tributyrin Agar (TBA) medium (1% (w/v) yeast extract, 2% (w/v) special peptone, 2% (w/v) tributyrin, 2% (w/v) agar, 2% (w/v) dextrose) containing 50 μ g mL⁻¹ zeocin was used.

Construction of the Vector pPICZ α A-Rmlip. Construction of the vector pPICZ α A-Rmlip including cloning, transformation into *E. coli* and selection, were conducted as described by Sambrook and Russel (2001). The *E. coli* expression vector pUC57 containing the synthetic Rmlip gene served as a template for polymerase chain reaction (PCR). PCR was performed using forward primer (5'-GCATCCTCGAGAAAAGAGAGGCTGAAGCTATGGTGCTGAAACAGCGCGC-3') and reverse primer (5'-GCATCTCTAGAGCGGTGCACAGGCCGGTGTAAATG-3'). The forward primer containing an *Xho* I-site (underlined) and the reverse primer containing an *Xba* I-site (underlined) and the sequence that will detect 6xHis. The primers were ordered from Integrated DNA Technologies (Singapore). The PCR process is carried out by 30 cycles with predenaturation, denaturation, annealing, extantion, endextantion at 98 °C, 30 s; 98 °C, 10 s; 69 °C, 30 s; 72 °C, 30 s; 72 °C, 5 min, respectively. The obtained insert gene was digested with *Xho* I and *Xba* I. The restriction enzymes were purchased from Fermentas (Burlington, Canada) and Thermo Scientific (Waltham, USA), respectively. It was then ligated into pPICZ α A linearized with the same enzymes. The mixture of ligation reaction, then was transformed into *E. coli* DH5 α . Zeocin-resistant transformants which grown on LB low salt media were selected and analyzed by plasmid digestion with *Bgl* II, *Xho* I, and *Xba* I. Transformants showed the correct digestion pattern were sequenced to confirm the sequence.

Transformation into *P. pastoris*. Plasmid with the correct DNA sequence, were used for transformation into

P. pastoris X33. The yeast was transformed with 1 µg of *Sac* I-linearized pPICZα A-RMlip by the spheroplast method of Cregg *et al.* (1985) with the following modifications. A fresh colony of *P. pastoris* X33 was inoculated into 10 mL of YPD and grown with shaking at 250 rpm 30 °C overnight. On the following day, 5 mL sample of the overnite culture was added to 45 mL of fresh YPD as starter OD₆₀₀ of 0.2 and grown cells under the previous conditions until OD₆₀₀ reaches 1.4 to 1.6. The cells were harvested by centrifugation at 6000 x g for 5 min and washed twice with 10 mL of ice-cold miliQ H₂O and then suspended in 8 mL of freshly prepared T buffer (10 mM Tris-HCl pH 7.5, 0.1 M Lithium acetate, 10 mM DTT, 0.6 M sorbitol) and incubated at 30 °C for 30 min. The spheroplasts were washed twice in 10 mL of ice-cold 1 M sorbitol and suspended in 400 µL of ice-cold 1 M sorbitol. One hundred µL of the spheroplasts were mixed with 1 µg of *Sac* I-linearized pPICZα A-RMlip. Then, the transformation performed by electroporation, which is applied electric pulse to the mixture (1.5 kV, 25 µF, 200 Ω for Gene pulser, Bio-Rad, USA) The mixture was added by 1 mL of ice-cold 1 M sorbitol and incubated at 30 °C without shaking for 1 h. The mixture then was added by 1 mL of YPD and incubated at 30 °C with shaking at 250 rpm for 1 h. One hundred µL of the mixture were spreaded on TBA medium plates containing 50 µg mL⁻¹ zeocin and incubated at 30 °C. Transformants were selected after 2-3 days at 30 °C by plating cells on TBA medium. Lipase activity was detected by the appearance of clear zones around colonies. Zeocin-resistant transformants which showed a clear zone were selected and analyzed by colony PCR and continued to PCR product digestion.

Cultivation. Standard cultivation method was carried out according to method described in Hu *et al.* (2013) and Wang *et al.* (2016). A single positive colony was cultivated in 100 mL of BMGY medium at 30 °C overnite under constant agitation at 250 rpm, until an OD₆₀₀ = 2-5 was reached. Then, these culture was transfered into 900 mL of the BMGY medium for large-scale cultivation until an OD₆₀₀ reached 5. The cells were collected by centrifugation at 3800 rpm for 20 min, and resuspended in 200 mL of the BMMY medium. Cultures were induced with absolute methanol to a final concentration of 1.5% every 24 h. The expression culture supernatants was harvested every 24 h and stored at -4 °C before analysis.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The expression of lipase gene in the supernatant was analyzed by SDS-PAGE, which was conducted using a 12%

polyacrylamide gel on a vertical mini gel apparatus (40 A, 200 V, 45 min, Bio-Rad, USA), as described by Laemmli (1970). Protein molecular weight marker was purchased from Amersham (GE Healthcare, UK). Samples were mixed with 2× loading buffer, with a ratio 1:1. Then, the mixture was boiled at 100 °C for 5 min before electrophoresis. Proteins were stained with page blue staining solution (Thermo Scientific, Waltham, USA). Protein staining is done by polyacrylamide gel washing with miliQ H₂O three times, then incubated with page blue for 30 s in the microwave and continued incubation at 24 °C, 300 rpm, for 1 h. Furthermore, it was rinsed and soaked by miliQ H₂O overnight.

Characterization of Recombinant Lipase. The lipase properties of supernatants were determined by the alkali titration method, using olive oil as substrate, as described by Fang *et al.* (2014) with the following modifications. The reaction was conducted in a mixture of 4 mL of 50 mM Tris-HCl (pH 8.0), 5 mL of emulsion of olive oil (25% (v/v) olive oil emulsified with 1.5% (w/v) polyvinyl alcohol solution) and 1 mL of proper dilute enzyme solution at incubation 50 °C for 20 min in a shaker. Finally, 5 mL of methanol was added to terminate the reaction. The amount of liberated fatty acids was measured by titration with 50 mM NaOH using phenolphthalein as an indicator. One unit (U) of lipase activity was defined as the amount of lipase necessary to liberate 1 µmol per min of fatty acids from the olive oil. The optimal tempetarure was determined by examining the activity of the enzymes at Tris-HCl buffer pH 8.0 in the various temperatures incubation (30-80 °C). The optimal pH was determined by measuring the enzymatic activity at the optimal temperature at pH range 5.0-10.0 (pH 5.0; pH 6.3-8.0; pH 8.0-9.0; pH 9.4-10.0 using Na-citrate buffer 0.05 mM; Na-phosphate 0.05 mM; Tris-HCl 0.05 mM; Glycine-NaOH 0.05 mM, respectively). Each sample was assayed in duplicate and the average value was determined.

RESULTS

Construction of the Recombinant Plasmid pPICZα A-RMlip. A fragment DNA encoding synthetic *R. miehei* lipase gene (RMlip) with the total size 1132 bp has been obtained by PCR. The vector pPICZα A is a *P. pastoris* expression vector. These vector size of 3593 bp. In order to the vector and RMlip can be ligated, the vector was also restricted at *Xho* I and *Xba* I sites. The total size pPICZα A-RMlip after

ligated is 4621 bp. This ligated vector and inserted fragment was then transformed into *E. coli*. After transformation, there were four transformants grown on LB low salt agar medium. Of the four transformants, only three transformants can be grown in LB low salt broth medium. Three transformants cultures were verified by *Bgl* II, *Xho* I, and *Xba* I. *Xho* I and *Xba* I are the single cutting site located between the vector pPICZ α A and *Rmlip*, so that the DNA size obtained by cutting was 4621 bp (Fig 1A and 1B). While, *Bgl* II is the double cutting sites located at the vector pPICZ α A and *Rmlip*, so that the result was obtained by cutting the size of 2992 bp and 1629 bp (Fig 1A). Based on the verification by *Bgl* II, *Xho* I, and *Xba* I, showed that the three transformants are three positive colonies that carrying the vector pPICZ α A-*Rmlip* with a total size of 4621 bp.

Sequencing. Sequence analysis was conducted to confirm the accuracy of the target gene sequence, the correct fusion of lipase gene to the vector, and the rate of mutation that occurred during the selection process. From DNA sequencing result we found that the DNA was in frame with alpha factor. The sequences were processed using online software www.genome.jp/tools/clustalw/, later translated using software <http://web.expasy.org/translate/> into the protein sequence of samples. Based on the results of the sequencing, there are primers used (grey highlight), which is used as a marker of the original signal peptide, a marker of restriction enzymes *Xho* I and *Xba* I, as well as the marker His codon before the end translation process (Fig 2A). Sequence of synthetic *R. miehei* lipase is between the both primers. Based on the protein sequence, there are amino acid of the original signal

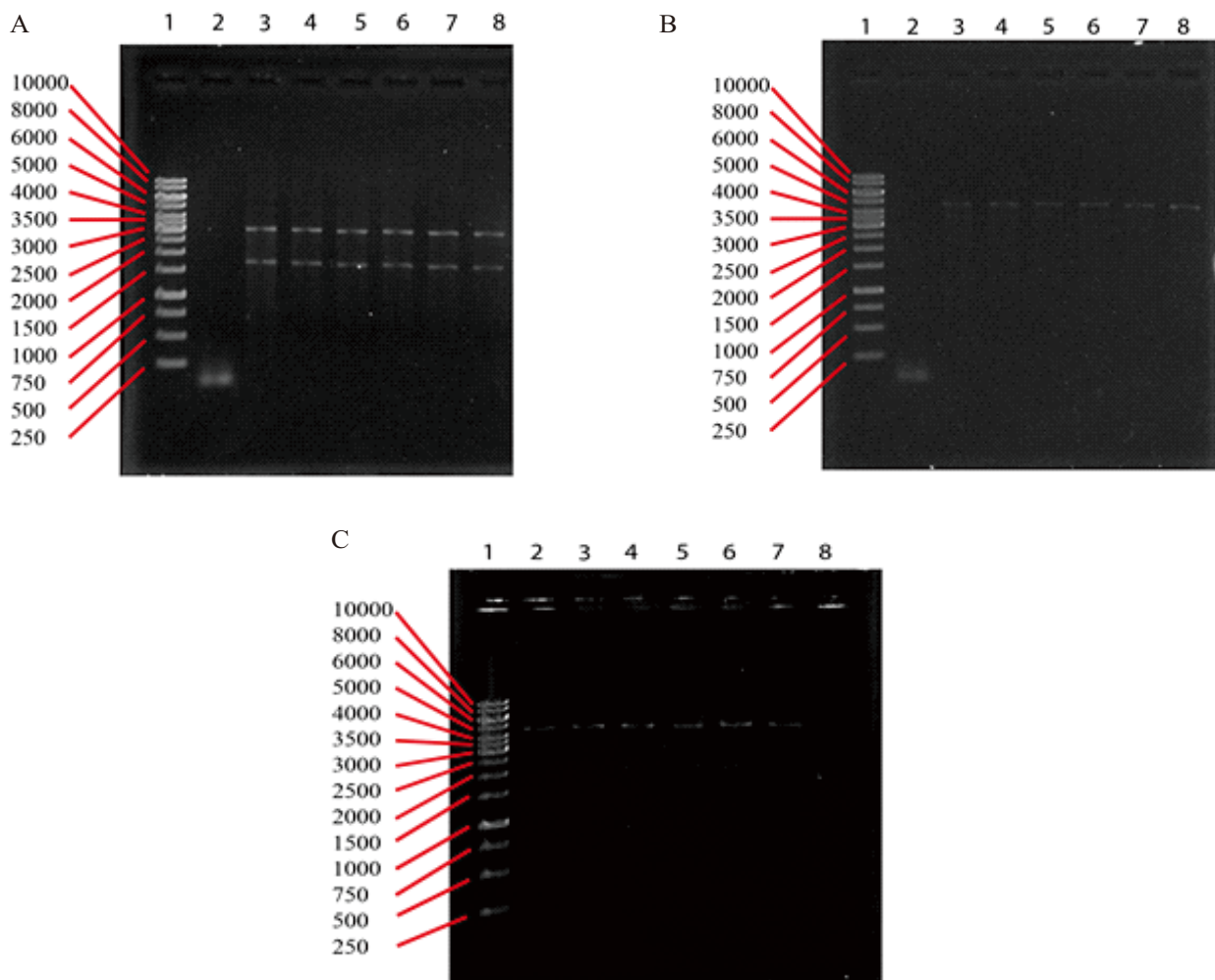


Fig 1 *E. coli* transformants verification. (A) Analysis of transformants by plasmid digestion with *Bgl* II. (B) Analysis of transformants by plasmid digestion with *Xho* I. Line 2: Negative control. Lines 3-8: Transformants analyzed with enzyme restriction, the sizes of 2992 bp and 1629 bp or 4629 bp, respectively. (C) Analysis of transformants by plasmid digestion with *Xba* I. Lines 2-7: Transformant analyzed with *Xba* I, the sizes of 4629 bp. Line 2: Negative control. Each transformant was screened in duplicate.

peptide, amino acid of the propeptides, and amino acid of the mature enzyme, and obtained also size lipase protein produced is 32.9 kDa (Fig 2B). The results of the translation are then alignment with international protein sequence databases available on the website <http://ncbi.nlm.nih.gov> by selecting Protein BLAST menu. Alignment is performed to find the sample sequence similarity to sequences contained in the databases. Protein BLAST results showed that the protein sequence pPICZ α -R*Mlip* transformed into *E. coli* DH5 α have similarities that are 100% identical to the protein lipase from *Rhizomucor miehei* in international database (data not shown).

Transformation into *P. pastoris* X33 and Selection of Positive Transformants. After transformation process of the correct sequence recombinant plasmid into *P. pastoris*, the recombinant colonies were grown. Transformants expressing lipase activity, as indicated by the formation of a clear zone around colonies on TBA medium. Several transformants were picked for confirmation by colony PCR and continued plasmid digestion with *Bgl* II. The total size of R*Mlip* after the colony PCR was 1132 bp and the total size of R*Mlip* after the restriction by *Bgl* II was 680 bp and 452 bp. Based on the confirmation by the colony PCR and *Bgl* II, showed that the transformants are positive colonies that carry the vector pPICZ α -R*Mlip* (Fig 3).

SDS-PAGE Electrophoresis. SDS-PAGE analysis showed that after induction by methanol, there was a band that appeared after induction with the measured molecular weight 32.9 kDa (Fig 4).

Characterization of Lipase Properties. Temperature and pH are two important reaction parameters for enzymes. Each enzyme has a distinct optimal temperature and pH levels. As well as recombinant lipase produced from recombinant *P. pastoris* X33 containing R*Mlip*. The optimal reaction temperature and pH of this recombinant lipase were 30 °C and 9.0, respectively.

DISCUSSION

Lipases has been successfully applied in various applications, such as in the food industry, organic synthesis, pharmaceutical (Houde *et al.* 2004; Diego *et al.* 2009) and in the field of biodiesel (Huang *et al.* 2012; Huang *et al.* 2014). The success is challenging us to keep looking for a better lipase expression with the aid of genetic engineering. This study aimed to clone *R. miehei* synthetic lipase gene into *P. pastoris* expression

plasmid for lipase expression with the original signal peptide.

In this study *R. miehei* synthetic lipase gene with signal peptide has been cloned in *P. pastoris* expression vector and transformed into *P. pastoris* X33. Based on the analysis sequencing, R*Mlip* has 363 amino acid residues, which consists of a 24 amino acid residues signal peptide, a 80 amino acids residues propeptida, and 269 amino acid residues of the mature enzyme. This is supported by Boel *et al.* (1988), which said that, R*Mlip* is synthesized as a precursor, which contained 70 amino acid residues propeptida before the 269 amino acid residues mature enzyme. Differences in the number of amino acids propeptides affected by signal peptides are used. That is because propeptides are C-terminal domain of signal peptides containing site of the cutting, which will be cleaved off in the protein maturation steps (Emanuelsson *et al.* 2007; Wang *et al.* 2016). Signal peptides and propeptides is a very important sequence in protein synthesis, because the functional region of the protein cannot form the native structure without the propeptide region (Anderson *et al.* 1999). In addition, the protein molecular weight of lipase in pPICZ α A-R*Mlip* is 32.9 kDa. This is reinforced by the statement Wu *et al.* (1996) which says that the molecular weight of R*Mlip* is 31.6 kDa and the statement Huge-Jensen *et al.* (1989) states that, the molecular weight of R*Mlip* is 32 kDa. So it is suspected that the molecular weight of lipase R*Mlip* is in the same range.

Temperature and pH is a very important parameter to determine the properties of the lipase expressed (Wang *et al.* 2015). In this study, R*Mlip* express lipase with high levels at 30 °C, ie at 8.33 U mL⁻¹ \pm 0.50. This is supported by the statement Huang *et al.* (2014), having said that, the temperature tolerance for R*Mlip* well expressed in *P. pastoris* X33 is at a temperature of 0-40 °C. For optimum pH, R*Mlip* express lipase with high levels at pH 9.0 in Tris-HCl buffer, ie at 8.44 U mL⁻¹ \pm 0.07. This is supported also by the statement Huang *et al.* (2014) which says that, pH tolerant for R*Mlip* in *P. pastoris* X.33 ie at pH 4.0—10.0. He *et al.* (2015) has been conducting research to increase the activity of lipase R*Mlip* by combining the AOX1 and GAP promoter whose expression using the α factor signal peptide of the *Saccharomyces cerevisiae* in *P. pastoris* GS115, lipase activity is generated is 140 U mL⁻¹. The result is higher of the control that only used the AOX1 and GAP promoter, which is 25 U mL⁻¹ and 20 U mL⁻¹, respectively. When compared with the control and the results of the such research, the lipase

A

5'GATCAAAAAACAACATAATTATTCGAAACGATGAGATTTCTTCAATTTTTACTGCTGTTTTATTTCGAGCATCCTC
 CGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCGGGCTGAAGCTGTCATCGGTTAC
 TCAGATTTAGAAGGGGATTTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTTATAAA
 TACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGGCTGAAGCTATGGTGCTG
 AAACAGCGCGCGAAGCTATCTGGGCTTTCTGATTGTGTTTTTACCAGCTTTCTGGTGGAAAGCGGTGCCGATTAACG
 CCAGAGCAACAGCACCGTGGATAGCCTGCCGCCGCTGATTCCGAGCCGACCAGCGCGCCGAGCAGCAGCCCGAG
 CACCACCGATCCGGAAGCGCCGGCGATGAGCCGCAACGGCCCGTCCGAGCGATGTGGAAACCAAATATGGCAT
 GCGCTGAACGCGACCAGCTATCCGGATAGCGTGGTGCAGGCGATGAGCATTGATGGCGGCATTCCGCGCGGCGAC
 CAGCCAGGAAATTAACGAACTGACCTATTATACCACCTGAGCGCGAACAGCTATTGCCGCACCGTGATTCCGGGC
 GCGACCTGGGATTGCATTATTGCGATGCGACCGAAGATCTGAAAATTATTAACCTGGAGCACCTGATTTATG
 ATACCAACGCGATGGTGGCGCGGGCGATAGCGAAAAACCATTATATTGTGTTTCGCGGCAGCAGCAGCATTTCG
 CAACTGGATTGCGGATCTGACCTTTGTGCCGGTGAGCTATCCGCCGGTGAGCGGCACCAAAGTGCATAAAGGCTTT
 CTGGATAGCTATGGCGAAGTGCAGAACGAACTGGTGGCGACCGTGCTGGATCAGTTTAAACAGTATCCGAGCTATA
 AAGTGGCGGTGACCGCCATAGCCTGGGCGCGCGACCGCGTCTGTGCGCGCTGGGCCTGTATCAGCGCGAAG
 AAGCCTGAGCAGCAGCAACCTGTTTCTGTATACCCAGGGCCAGCCGCGCTGGGCGATCCGGCGTTTGCAGAACTA
 TGTGGTGAACCGCATTCCGTATCGCCGACCGTGAACGAACGCGATATTGTGCCCATCTGCCGCCGGCGGCG
 TTTGGCTTTCTGCATGCGGGCGAAGAATATTGGATTACCGATAACAGCCCGAAACCGTGCAGGTGTGCACCAGCG
 ATCTGGAAACCAGCGATTGCAGCAACAGCATTGTGCCGTTTACCAGCGTGTGGATCATCTGAGCTATTTTGGCATT
 AACACCGGCTGTGCACCGCTCTAGAACAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATC
 ATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCTCAGTTCAAGTTGGGCACCTACGAGAAGACCGTCTG
 CAGTTAACGGC 3'

B

Met R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A
 Q I P A E A V I G Y S D L E G D F D V A V L P F S N S T N N G L
 L F I N T T I A S I A A K E E G V S L E K R E A E A **Met** V L K Q
 R A N Y L G F L I V F F T A F L V E A V P I K R Q S N S T V D S L
 P P L I P S R T S A P S S S P S T T D P E A P A **Met** S R N G P L P
 S D V E T K Y G **Met** A L N A T S Y P D S V V Q A **Met** S I D G G
 I R A A T S Q E I N E L T Y Y T T L S A N S Y C R T V I P G A
 T W D C I H C D A T E D L K I I K T W S T L I Y D T N
 A **Met** V A R G D S E K T I Y I V F R G S S S I R N W I A D L T
 F V P V S Y P P V S G T K V H K G F L D S Y G E V Q N E L V
 A T V L D Q F K Q Y P S Y K V A V T G H S L G G A T A L L C
 A L G L Y Q R E E G L S S S N L F L Y T Q G Q P R V G D P A
 F A N Y V V S T G I P Y R R T V N E R D I V P H L P P A A F G
 F L H A G E E Y W I T D N S P E T V Q V C T S D L E T S D C S
 N S I V P F T S V L D H L S Y F G I N T G L C T A L E Q K L I
 S E E D L N S A V D H H H H H H Stop

Part of the α factor of pPICZ α A

Region of signal peptide and propeptides

Region of *Rhizomucor miehei* lipase

Theoretical pI/Mw: 5.03 / 32917.74

Fig 2 Analysis sequencing. (A) In the DNA sequence, there are primers used (grey highlight), which is used as a marker of the original signal peptide, a marker of restriction enzymes *Xho* I and *Xba* I, as well as the marker His codon before the end translation process, sequence of synthetic *Rhizomucor miehei* lipase is between the both primers. (B) In the protein sequence, there are amino acid of the original signal peptide, amino acid of the propeptides, and amino acid of the mature enzyme. These components are very important in protein expression. The protein sequence analysis by software [http://web.expasy.org/ translate/](http://web.expasy.org/translate/) showed that the size of pPICZ α -RMLip lipase protein molecular weight is 32.9 kDa.

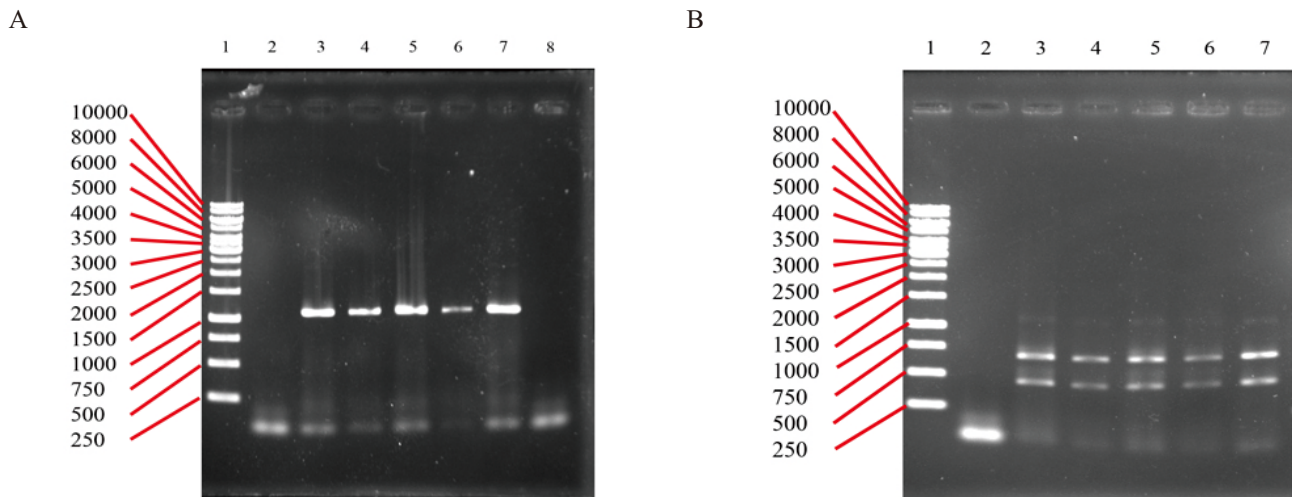


Fig 3 *Pichia pastoris* transformants confirmation. (A) *Rmlip* confirmation result of transformation by colony PCR. Line 1: GeneRuler DNA Ladder 1 Kb. Line 2: Negative control of PCR that used ddH₂O. Line 3: Positive control of PCR that used extraction *Rmlip*. Line 4-8: *Rmlip* result of transformation, the total size was 1132 bp. Almost all the positive colonies, from five colonies, only 1 negative colony. (B) *Rmlip* confirmation result of colony PCR by *Bgl* II. Line 1: GeneRuler DNA Ladder 1 Kb. Line 2: Negative control of PCR that used ddH₂O. Line 3: Positive control of PCR that used extraction *Rmlip*. Line 4-7: *Rmlip* result of transformation, the total size was 680 bp and 452 bp. Restriction results from four colonies, are all positive.

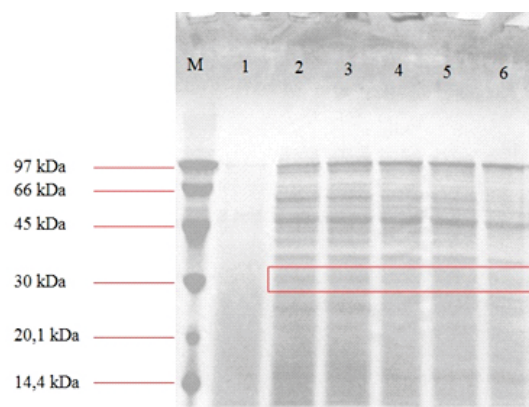


Fig 4 SDS-PAGE analysis of culture supernatants during the cultivation. The molecular weight of protein *Rmlip* is 32.9 kDa. Line M: Protein molecular weight marker. Line 1-6: Culture supernatant after 0, 24, 48, 72, 96, 120, and 144 h of cultivation.

activity generated in this study has not increased significantly. It may be caused by several factors, the most striking is the incorporation of the promoter and signal peptide used. The combined use of the *AOX1* and *GAP* promoters can be attributed to a positive effect on the expression level by increasing the copy number of the expression cassette. The transcription levels of the intracellular protein and secreted protein have been shown to increase greatly upon integration of multiple copies of the expression vector to an appropriate extent in *P. pastoris* (Scorer *et al.* 1994; Shen *et al.* 2012). Signal peptides also affect the expression of

recombinant lipase. There are three kinds of signal peptides that can be used directly in *Pichia pastoris* to secrete and express a foreign gene that is the native signal peptides, the *S. cerevisiae* α factor prepropeptides (α factor signal peptide) and the *P. pastoris* acid phosphatase (*PHO1*) signal. The original/native signal peptide is rarely used because there are still some weaknesses, one of which is described in the research Brocca *et al.* (1998) regarding the effect of the original signal peptides on the expression and secretion of industrial lipase Lip1 from *Candida rugosa*. Brocca *et al.* (1998) stated that the

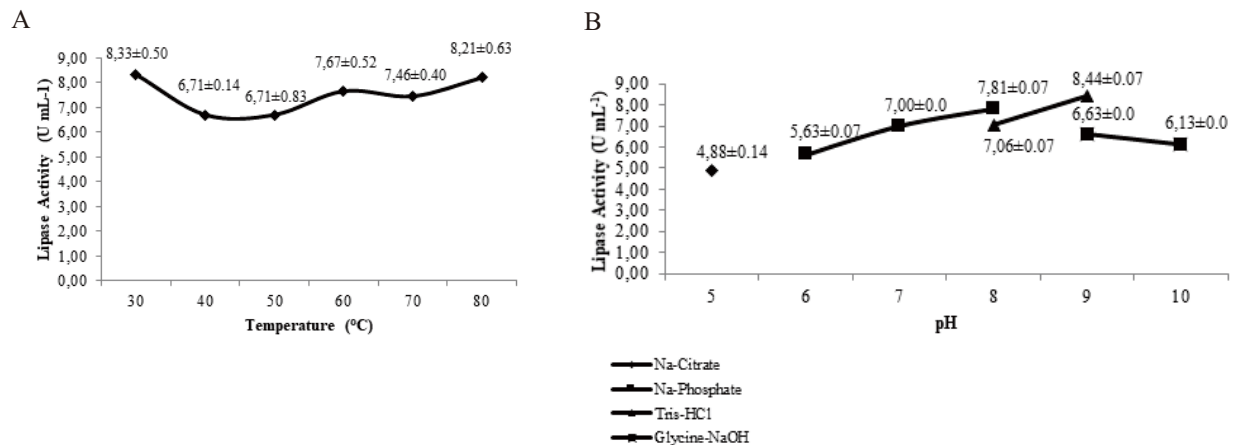


Fig 5 Characterization of properties lipase. (A) The optimal temperatures was determined by examining the activity of the enzymes at Tris-HCl buffer pH 8.0 in the various temperatures incubation (30–80 °C). The optimal reaction temperature of pPICZαA-RMlip in *P. pastoris* X33 is 30 °C, ie at 8.33 U mL⁻¹ ± 0.50. (B) The optimal pH was determined by measuring the enzymatic activity at the optimal temperature at pH range 5.0–10.0 (pH 5.0; pH 6.3–8.0; pH 8.0–9.0; pH 9.4–10.0 using Na-citrate buffer 0.05 mM; Na-phosphate 0.05 mM; Tris-HCl 0.05 mM; Glycine-NaOH 0.05 mM, respectively). The optimal reaction pH levels of pPICZαA-RMlip in *P. pastoris* X33 is pH 9.0 in buffer Tris-HCl, at 8.44 U mL⁻¹ ± 0.07.

original signal peptide can secrete lipase Lip 1, but its expression was hampered. There is an assumption that, in this study also experienced the same thing, signal peptide RMlip successfully constructed, but the expression of lipase inhibited. Cereghino and Cregg (2000) said that, although the original signal peptide and α factor prepropeptida (α factor signal peptide) from *S. cerevisiae* was adequate for foreign protein secretion and expression, but the highest levels of protein secretion was from clone with the full preprotein.

This study obtained that synthetic *Rhizomucor miehei* lipase gene with the size of 1132 bp was successfully cloned to pPICZα A vector with a total size of 4621 bp. DNA sequencing analyses showed that the synthetic lipase gene pPICZα A-RMlip are 100% identical to the *R. miehei* lipase. The gene pPICZα A-RMlip was successfully integrated into chromosome of *P. pastoris* X33. The enzymatic properties of pPICZαA-RMlip in *P. pastoris* X33 is to have the optimal reaction temperature is 30 °C and the optimal reaction pH is 9.0.

ACKNOWLEDGMENT

This study was funded by National Incentive for Consortium Research (Insinas) 2015—2016 from Ministry of Research, Technology, and Higher Education, Republic Indonesia.

REFERENCES

- Adrio JL, Demain AL. 2003. Fungal Biotechnology. Int. Microbiol. 6: 191-199. doi: 10.1007/s10123-003-0133-0.
- Anderson DE, Peters RJ, Wilk B, Agard DA. 1999. Aliphatic protease precursor: characterization of a structured folding intermediate. Biochemistry. 38: 4728-4735. doi: 10.1021/bi982165e.
- Balamurugan V, Reddy GR, Suryanarayana VVS. 2007. *Pichia pastoris*: a notable heterologous expression system for the production of foreign proteins-vaccines. IJBT. 6: 175-186.
- Brunel L, Neugnot V, Landucci L, Boze H, Moulin G, Bigey F, Dubreucq E. 2004. High-level expression of *Candida parapsilosis* lipase/acyltransferase in *Pichia pastoris*. J Biotechnol. 111: 41-50.
- Boel E, Hujjensen B, Christensen M, Thim L, Fiil NP. 1988. *Rhizomucor miehei* triglyceride lipase is synthesized as a precursor. Lipids. 23: 701-706. doi: 10.1007/BF02535672.
- Brocca S, Schmidt-Dannert C, Lotti M, Alberghinaand L, Schmid RD. 1998. Design, total synthesis, and functional overexpression of the *Candida rugosa* lip1 gene coding for a major industrial lipase. Protein Sci. 7: 1415-1422.
- Cereghino JL, Cregg JM. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24: 45-66.
- Cregg JM, Barringer KJ, Hessler AN, Madden KR. 1985. *Pichia pastoris* as a host system for transformations.

- Mol Cell Biol. 5: 3376-3385.
- De Diego T, Lozano P, Abad MA, Steffensky K, Vaultier M, Iborra JL. 2009. On the nature of ionic liquids and their effect on lipase that catalyze ester synthesis. *J Biotechnol.* 140: 234-242.
- Dutra JCV, Terzi SC, Bevilacqua JV, Damaso MCT, Couri S, Langone MAP, Senna LF. 2008. Lipase production in solid-state fermentation monitoring biomass growth of *Aspergillus niger* using digital image processing. *Appl Biochem Biotechnol.* 147: 63-75. doi: 10.1007/s12010-007-8068-0.
- Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Proto.* 2: 953-971. doi: 10.1038/nprot.2007.131.
- Fang Z, Xu L, Pan D, Jiao L, Liu Z, Yan Y. 2014. Enhanced production of *Thermomyces lanuginosus* lipase in *Pichia pastoris* via genetic and fermentation strategies. *J Ind Microbiol Biotechnol.* 41: 1541-1551.
- Franken LPG, Marcon NS, Treichel H, Oliveira D, Freire DMG, Dariva C, Destain J, Oliveira JV. 2008. Effect of treatment with compressed propane on lipases hydrolytic activity. *Food Bioproc Technol.* 3: 511-520. doi: 10.1007/s11947-008-0087-5.
- Griebeler N, Polloni AE, Remonato D, Arbter F, Vardanega R, Cechet JL, Luccio MD, de Oliveira D, Treichel H, Cansian RL, Rigo E, Ninow JL. 2011. Isolation and screening of lipase-producing fungi with hydrolytic activity. *Food Bioproc Technol.* 4: 578-586. doi: 10.1007/s11947-008-0176-5.
- Han Z, Han S, Zheng S, Lin Y. 2009. Enhancing thermostability of a *Rhizomucor miehei* lipase by engineering a disulfide bond and displaying on the yeast cell surface. *Appl Microbiol Biotechnol.* 85: 117-126. doi: 10.1007/s00253-009-2067-8.
- Haniyya. 2015. Isolasi plasmid pUC57-lip E. coli DH5 α klon F1.12 yang mengandung gen lipase sintesis *Rhizomucor miehei* dan analisis verifikasi kloningnya [Isolation of pUC57-lip plasmid in E. coli DH5 α clone F1.12 containing synthetic lipase *Rhizomucor miehei* gene and cloning verification analysis]. Laporan Kerja Praktik *unpublish*, Depok: xi+37 hlm.
- He D, Luo W, Wang Z, Pengmei Lv, Yuan Z. 2015. Combined use of GAP and AOX1 promoters and optimization of culture conditions to enhance expression of *Rhizomucor miehei* lipase. *J Ind Microbiol Biotechnol.* 42: 1175-1182. doi: 10.1007/s10295-015-1633-6.
- Houde A, Kademi A, Leblanc D. 2004. Lipases and their industrial applications. *Appl Biochem Biotechnol.* 118: 155-170. doi: 10.1385/ABAB:118:1-3:155.
- Hu H, Gao J, He J, Yu B, Zheng P, Huang Z, Mao X, Yu J, Han G, Chen D. 2013. Codon optimization significantly improves the expression level of a keratinase gene in *Pichia pastoris*. *PLOS ONE.* 8: 1-8. doi: 10.1371/journal.pone.0058393.
- Huang D, Han S, Han Z, Lin Y. 2012. Biodiesel production catalyzed by *Rhizomucor miehei* lipase-displaying *Pichia pastoris* whole cells in an isooctane system. *Biochem Eng J.* 63: 10-14. doi: 10.1016/j.bej.2010.08.009.
- Huang J, Xia J, Yang Z, Guan F, Cui D, Guan G, Jiang W, Li Y. 2014. Improved production of a recombinant *Rhizomucor miehei* lipase expressed in *Pichia pastoris* and its application for conversion of microalgae oil to biodiesel. *Biotechnol Biofuels.* 7: 111-121. doi: 10.1186/1754-6834-7-111.
- Huge-Jensen B, Andreasen F, Christensen T, Christensen M, Thim L, Boel E. 1989. *Rhizomucor miehei* triglyceride lipase is processed and secreted from transformed *Aspergillus oryzae*. *Lipid.* 4:781-785.
- Killian JA, de Jong AM, Bijvelt J, Verklei AJ, de Kruijff B. 1990. Induction of non-bilayer lipid structures by functional signal peptides. *EMBO J.* 9: 815-819.
- Knoche K, Kephart D. 1999. Cloning blunt end pfu DNA polymerase generated PCR fragment into pGEM-T vector system. *Promega Notes.* 70: 10-14.
- Laemmli UK. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.
- Rapoport TA. 1992. Transport of proteins across the endoplasmic reticulum membrane. *Science.* 258: 931-936.
- Rodrigues RC, Lafuente RF. 2010. Lipase from *Rhizomucor miehei* as an industrial biocatalyst in chemical process. *J Mol Catal B: Enzym.* 64: 1-22. doi: 10.1016/j.molcatb.2010.02.003.
- Sambrook J, Russel DW. 2001. *Molecular cloning: Laboratory manual vol. 1.* 3rd ed. Cold Spring Harbour. New York: xxvii+1.1-7.94 page.
- Sarma R, Chisti Y, Banerjee UC. 2001. Production, purification, characterization, and application of lipases. *Biotechnol Adv.* 19: 627-662. doi: 10.1016/S0734-9750(01)00086-6.
- Scorer CA, Clare JJ, McCombie WR, Romanos MA, Sreekrishna K. 1994. Rapid selection using G418 of high copy number transformants of *Pichia Pastoris* for high-level foreign gene expression. *Nat Biotechnol.* 12:181-184. doi: 10.1038/Nbt0294-181.
- Shen Q, Wu M, Wang HB, Naranmandura H, Chen SQ. 2012. The effect of gene copy number and co-expression of chaperone on production of albumin fusion proteins in *Pichia pastoris*. *Appl Microbiol Biotechnol.* 96:763-772. doi: 10.1007/s00253-012-4337-0.
- Soccol CR, Vandenberghe LPS. 2003. Overview of applied solid-state fermentation in Brazil. *Biochem Eng J.* 13:

- 205-218. doi:10.1016/S1369-703X(02)00133-X.
- Sreekrishna, Koti, Brankamp RG, Kropp KE, Blankenship DT, Jiu-Tsair Tsay, Smith PL, Wierschke JD, Subramaniam A, Birkenberger LA. 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* 190: 55-62. doi: 10.1016/S0378-1119(96)00672-5.
- Vakhlu J, Kour A. 2006. Yeast lipase: enzyme purification, biochemical properties and gene cloning. *Electronic J Biotechnol.* 9: 69-85. doi: 10.2225/vol9-issue1-fulltext-9.
- Wang Z, Pengmei Lv, Luo W, Yuan Z, He D. 2016. Expression in *Pichia pastoris* and characterization of *Rhizomucor miehei* lipase containing a new propeptide region. *J Gen Appl Microbiol.* 62: 25-30. doi: 10.2323/jgam.62.25.
- Wu S, Letchworth GJ. 2004. High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. *Biotechniques.* 36: 152-154.
- Wu XY, Jääskeläinen S., Linko WY. 1996. Purification and partial characterization of *Rhizomucor miehei* lipase for ester synthesis. *Appl Biochem Biotechnol.* 59:145-158.