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Cloning of Synthetic Lipase Gene from *Thermomyces lanuginosus* into *Pichia pastoris* with its Original Signal Peptide

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Lipase is one of the most important industrial enzymes, which is widely used in the preparation of food additives, cosmetics, and pharmaceutical industries. In the previous study, we have cloned synthetic *Thermomyces lanuginosus* lipase gene into *Bacillus subtilis* and *Escherichia coli* and resulting low expression for enzyme activity. The aim of this research was to construct the *T. lanuginosus* lipase (TLL) gene into *Pichia pastoris* vector expression with TLL original signal peptide. TLL gene was amplified by PCR and contained original signal peptide and then inserted into pPICZα A between *Xho*I and *Xba*I site, and transformed into competent cell *E.coli* DH5α. From the transformant, two of positive recombinants were analyzed by sequencing analysis. As the result, both of two recombinant have a positive target gene which has lipase gene. The correct plasmid was linearized and then was transformed in *P. pastoris* X-33 by electroporation method. *Thermomyces lanuginosus* synthetic lipase gene successfully integrated into chromosome of *P. pastoris* X-33, which revealed by clear zones around the colony on Yeast Extract Peptone Dextrose Tributyrin (YPD.TB) plate with zeocin. The *T. lanuginosus* lipase had an open reading frame of 942 bp encoding TLL of 314 amino acids with theoretical molecular mass of 35 kDa. The recombinant enzyme, *T. lanuginosus* lipase had optimal temperature at 80 °C and optimal pH at pH 8.0.

Key words: original signal peptide, *Pichia pastoris*, pPICZα A, synthetic lipase gene, *Thermomyces lanuginosus*

Lipase merupakan salah satu enzim industri yang penting, dan banyak digunakan dalam pembuatan zat aditif makanan, kosmetik, dan industri farmasi. Pada penelitan sebelumnya, telah dilakukan kloning gen sintetik *Thermomyces lanuginosus* lipase ke dalam *Bacillus subtilis* dan *Eschericihia coli* dan menghasilkan aktivitas enzim lipase yang rendah. Penelitian ini bertujuan untuk mengkloning gen sintetik *T. lanuginosus* lipase (TLL) ke dalam vektor ekspresi *Pichia pastoris* menggunakan sinyal peptida alami TTL. Gen TLL yang mengandung sinyal peptida alami diamplifikasi dengan PCR, dan disisipkan ke dalam pPICZα A diantara situs *Xho*I dan *Xba*I, kemudian ditransformasikan ke dalam sel kompeten *E. coli* DH5α. Dari hasil transformasi dipilih dua rekombinan positif untuk dilakukan analisa sekuensing. Hasil sekuensing, kedua rekombinan mengandung gen target lipase. Plasmid yang telah dikonfirmasi kemudian dilinearisasi dan ditransformasikan ke dalam *Pichia pastoris* X-33 dengan menggunakan metoda elektroporasi. Gen *T. lanuginosus* lipase berhasil diintegrasi ke dalam kromosom *P. pastoris* X-33, yang ditunjukkan dengan terbentuknya zona bening pada media *Yeast Extract Peptone Dextrose Tributyrin* (YPD.TB) agar yang mengandung zeocin. *T. lanuginosus* lipase memiliki daerah *open reading frame* (ORF) 942 bp yang mengkode 314 asam amino dengan massa molekul teoritis 35 kDa. Enzim rekombinan *T. lanuginosus* memiliki suhu optimum 80˚C dan pH optimum 8.0.

Kata kunci: *Pichia pastoris*, sinyal peptide alami, PPICZα A, gen sintetik lipase, *Thermomyces lanuginosus*

Lipases are abundant in the nature, including in plants, animals, and microorganisms. Microbes are a major source of many enzymes including lipase for industrial application (Vakhlu and Kour 2006). Lipases had more than 60% usage in the industries, such as detergent, food, and starch industry. Most of the industries use recombinant enzymes for their product (Cowan 1996). Yeast has been used in many industrial enzymes, because it's considered to be easily handled and has high cell-density than bacteria (Cregg *et al*. 1993).

Gene cloning and protein expression of lipase gene require expression vectors and suitable host. Synthetic *Thermomyces lanuginosus* lipase in previous studies has been cloned and expressed in *Escherichia coli* using pUC57 and pET plasmid vector, and *Bacillus subtilis* using vector pSKE194 (Haniyya 2016). The result of studies showed that expression levels of lipase was low. Because of that, it needed another alternative to increase expression of lipase gene. Researchers are looking for alternative hosts for increasing the expression of lipase such as methylotrophic yeast *Pichia pastoris.*

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Recombinant enzymes in eukaryotic systems are a good alternative to expression of enzymes extracellularly (Yang *et al*. 2009). Recombinant lipase enzyme can be expressed and secreted in the methylotrophic yeast *P. pastoris.* It can be used as the host to expression of lipase gene in high quantities (Fang *et al*. 2014). One of the common strategies to increase the expression level of a specific gene is to screen and select a strong promoter. Expression system *P. pastoris* can be used several promoter, including AOX1, GAP, FLD1, PEX8, and YPT1. The pAOX1-regulated systems are the most common (Cregg *et al*. 2003). *T. lanuginosus* lipase (Zheng *et al*. 2011), *Rhizopus chinensis* lipase (Yu*et al*. 2009), *Pseudomonas fluorescens* (Yang *et al*. 2009) were expressed under the alcohol oxidase promoter. Many researchers used AOX1 promoter to control expression of heterologous protein, because the promoter was able to control protein expression in large number with methanol as sole carbon source (Cereghino and Cregg 2000).

Pichia protein expression system can use a different signal peptide. Researchers can clone a foreign gene in frame with sequence encoding either the native signal, the *Saccharomyces cereviceae* α-factor signal peptide, or the *P. pastoris* acid phosphatase (PHO1) signal. Although several different secretion signal sequences, including the native secretion signal present on heterologous proteins, have been used successfully, results have been variable (Cereghino and Cregg 2000). In some case, many studies using α-factor signal peptide in *Pichia* protein expression system including, *Rhizopus chinensis* lipase Yu *et al.* (2009); *Beauveria bassiana* lipase Vici *et al.*(2015); or *Rhizopus oryzae* lipase (Minning *et al*. 1998).

This paper describes cloning of a synthetic lipase gene from *T. lanuginosus* into *Pichia* vector expression with its original signal peptide and under promoter AOXI for expression of protein recombinant using *P. pastoris* strain X33 as a host. It also reports partial characterization of the recombinant lipase produced by the recombinant *P. Pastoris.*

MATERIALS AND METHODS

Strains, Plasmids, and Media. *E.coli* DH5α was used for the cloning and propagation of plasmid. *P. pastoris* X-33 were used as hosts for lipase expression. Plasmid pSKE 194 containing *T. lanuginosus* lipase gene (TLL) is used as template for amplification of lipase gene. The vector pPICZα A was supplied from Invitrogen. *E.coli* was grown at 37 °C in Luria-Bertani (LB), or in LB medium containing 25 μg mL^{-1} zeocin when used for selection. *P. pastoris* recombinant was grown in YPD plate or YPD Tributyrinplate at 30 °C, in medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar, 2% Tributyrin, and 100 μg mL^{-1} zeocin. *P. pastoris* recombinants were grown in Buffered Glycerol-complex (BMGY)/Buffered Methanol-complex (BMMY) medium1% (w/v) yeast extract, 2% peptone for expression of lipase recombinant.

Recombinant DNA Techniques and Construction of the Plasmid pPICZα A-TLL. Standard recombinant DNA methods were carried out according to the methods described in Sambroock *et al*. (1989).The plasmid vector pSKE 194 containing the TLL gene served as a template for polymerase chain reaction (PCR). The open reading form of the TLL gene was amplified from this template DNA using a pair of primers, sequence-natSP-TLlip-*Xho*I-F1 (5'GCATCCTCGAGAAAAGAGAGGCTGAAGCTATGAGGAGCTCCCTTGTGCTGTTC-3') and His-TLlip-*Xba*I-R2 (5'-GCATCTCTAGAGCAAGACATGTCCCAATTAACCCGAAGTAC-3'), by High Fidelity Phusion DNA polymerase (Thermo Scientific). The restriction sites *Xho*I and *Xba*I were incorporated into the forward and reverse primer sequence respectively. The PCR fragment was ligated into the respective sites of pPICZα A resulting in pPICZα A-TLL.natSP under the control of the methanol inducible alcohol oxsidase 1 promoter (AOX1). The mixture of ligation then was transformed into *E.coli* DH5α in LB low salt containing 25 μg mL^{-1} zeocin. Transformants were analyzed by PCR with specific primer,and analyzed with restriction enzymes digestion (*Xho*I, *Xba*I, and *Pst*I) and sequenced to confirm the correct frame for expression.

DNA-sequencing. For the sequence determination, samples of plasmid recombinant were sent to PT. Genetika Science for sequencing. Furthermore, the result of sequencing was analyzed by SnapGene™ 1.1.3 software.

Transformation of *Pichia pastoris.* The recombinant plasmids pPICZα A-TLL.natSP confirmed with sequencing method then was linearized with *Pme*I, resolved on a 1% agarose gel, and purified. 6.5 μL linearized pPICZα A-TLL.natSP and 100 μL *P. pastoris* competent cells X33 was incubated on ice for 30 min and electroporated into *P. pastoris* X33 competent cellswith Gene pulser, BioRad electroporator under 1,5 kV, 25 Mf, 200 Ω, using a 0.2 cm cuvette (Invitrogen). One hundred μL of sample was spread on YPD plates containing 100 μg mL^{-1} zeocin and incubated for 2-3 d

at 30 °C until colonies with appropriate size appeared.

Confirmation of Recombinant Lipase Expression. After 2-3 d, all recombinants grown on YPD plate containing zeocin were transformed on YPD Tributyrin plate. The colonies grew on YPD Tributyrin plates have clear zones were picked up for further analyzes. All of these colonies were picked and screened by colony PCR, and the PCR products weredigested with enzyme restriction *Pst*I. Several clones were obtained but only 2 were chosen for further use, respectively.The recombinant colonies then were analyzed by PCR with specific primer, and the PCR product were digested with restriction enzymes (*Xho*I, *Xba*I, and *Pst*I) and sequenced to confirm the presence of TLL gene.

Cultivation of Recombinant *P. pastoris* Producing *T. lanuginosus* Lipase in Shaking Flask. Colonies of recombinant *P. Pastoris* grown on YPD plate containing zeocin, were picked and inoculated into a 125 mL Erlenmeyer flask containing 25 mL of BMGY medium at 30 °C and 250 rpm for culture starter. After 16-18 h incubation, the optical density (OD) of culture cells were measured by spectrophotometer, untill optical density reached OD_{600} 0.2, the cells were harvested (3800 rpm for 15 min), washed and resuspended in 200 mL BMGY medium. Then cultivation continued until optical density reached OD between 4.0 and 6.0, the cells were harvested (3800 rpm for 15 min), washed and resuspended in 50 mL BMMY medium. The cultures were grown at 30 °C and 250 rpm in 250 mL shake flasks for 120 h the cultures were supplemented daily with 3% (v/v) methanol, and sampled every 24 h. The sample was collected by separating the supernatant from the cells by centrifugation (4 °C, 3800 rpm for 15 min).

SDS-PAGE Analysis. The recombinant lipase in the supernatant was analyzed by SDS-PAGE, which was conducted using a 6% stacking gel and a 12% separating gel on a vertical mini gel apparatus (Bio-Rad, USA), as described by Laemmli (1993). Protein molecular weight marker was purchased from Fermentas (Burlington, Canada). Samples were mixed equally with 2× loading buffer and heated at 100 °C for 5 min before electrophoresis. Proteins were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, USA).

Partial Characterization of Lipase. The experiment of the effect of temperature on lipase activity was conducted at different temperatures (40-80 °C) for 20 min under pH 8.0. Each measurement was performed three times under pH 8.0. The effect of pH on lipase activity was measured at different pH (5-10) using the following buffers Na-citrate (pH 5), Na-phosphate (pH 6-8), Tris/HCl (pH 8-9), dan Glycine/NaOH (pH 9-10).

Lipase Activity Assay. The lipase activity of supernatants was determined by the alkaline titration method, using olive oil as substrate. The reaction was conducted in a mixture of 5 mM Tris-HCL (pH 8.0), 4 mL of emulsion of olive oil [25% (v/v) olive oil emulsified with 1.5% (w/v) polivinyl alcohol solution and 1 mL of proper dilute enzyme solution at 70 °C for 20 min in shaking incubator. Finally, a 5 mL methanol absolute 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.

RESULT

Construction of Recombinant Plasmid pPICZ α A Containing *T. lanuginosus* Lipase Gene (TLL). The fragment DNA encoding recombinant lipase gene with natural signal peptide was amplified by PCR using the primer pair natSP-TLlip-Xho-F1/ His-TLlip-Xba-R2 with pSKE194-TLL as the template. The total size of TLL was 942 bp. After digested with *Xho*I and *Xba*I the fragment was inserted into the same restriction enzymes site of the vector pPICZα A (3568 bp after cutting). The ligation mixture then used to transform competence cells *E. coli* DH5α. Transformants were grown in media low salt LB + zeocin that plasmids extracted and authenticated using restriction enzymes *Xho*I, *Xba*I, and *Pst*I. Positive results were confirmed using restriction enzymes (Fig 1), restriction enzyme XhoI and *Xba*I have one restriction site, the results seen in agarose gel that was one band in 4405 bp. Meanwhile, restriction *Pst*I has two restriction site, as shown on agarose gel has two bands in 4137 bp and 268 bp. Further sequencing to confirm the recombinant plasmid was conducted. The sequencing result show that the fragment DNA was inserted correctly in frame with the alpha factor in the pPICZ alpha vector. Based on the sequence analyses the deduced amino acid were homologous to the lipases from *T. lanuginosus* lipase (LGY, 100% similarity, GenBank accession no. EU022703.1), (MGY, 99% similarity, GenBank accession no. EU370914.1) and (lip, 99% similarity, GenBank accession no. AF054513.1) (Fig 2). Sequence analyses confirmed that there was a start codon (Methionine) in the 5' fragment, six histidine

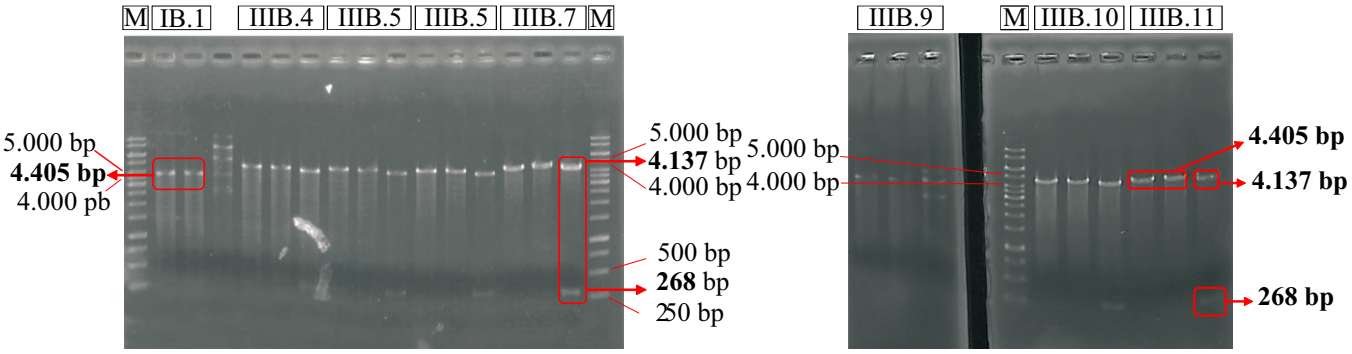

Fig 1 Confirmation result of recombinant plasmid with restriction enzymes (*Xho*I, *Xba*I, and *Pst*I).

residue, and the stop codon.The predicted mature lipase consisted of 314 amino acid (Fig 2) with a molecular weight of approximately 35 kDa.

Transformation of *P. pastoris* Plasmid pPICZα A.TLL.NatSp (clone IIIB7) extracted and linearized with restriction enzyme *Pme*I. Then the results of linearized transformed into competent cells *P. pastoris* strain X33 using electroporation methods. Colonies growth in YPD Tributyrin medium, eight colonies of transformantsn *P. Pastoris* X-33 picked up for screening positive recombinant. Transformant was verified by PCR technique, in order to screen positive recombinant (Fig 3). Colonies showed positive results, subsequently grown on YPD Tributyrin plates containing zeocin. All transformants X33 were grown in YPD Tributyrin showed clear zones that was indicated expression of lipase activity (Fig 4).

SDS-PAGE Electrophoresis. Supernatant from shaking flask cultivication culture was running on 12% SDS-PAGE, and the target lipase recombinant migrated as a 35 kDa band, as expected (Fig 5). There were contaminating proteins present in the supernatant from shaking flask cultivation (Fig 5). Nevertheless, the molecular weight of protein contained in the range of 35 kDa, which constantly appears in every addition of methanol per 24 h.

Partial Characterization of Recombinant Lipase Cultivated in Shaking Flask. To determine the optimal temperature and pH of the lipase recombinant, the enzymes were incubated at pH 8.0 for

CTCGAGAAAAGAGAGGCTGAAGCTATGAGGAGCTCCCTTGTGCTGTTCTTTGTCTCTGCGTGGACGGCCTTGGCCAGTCC
TATTCGTCGAGAGGTCTCGCAGGATCTGTTTAACCAGTTCAATCTCTTTGCACAGTATTCTGCAGCCGCATACTGCGGAAAAA
ACAATGATGCCCCAGCTGGTACAAACATTACGTGCACGGGAAATGCCTGCCCCGAGGTAGAGAAGGCGGATGCAACGTTTCT
CTACTCGTTTGAAGACTCTGGAGTGGGCGATGTCACCGGCTTCCTTGCTCTCGACAACACGAACAAATTGATCGTCCTCTCTTT
CCGTGGCTCTCGTTCCATAGAGAACTGGATCGGGAATCTTAACTTCGACTTGAAAGAAATAAATGACATTTGCTCCGGCTGCA
GGGGACATGACGGCTTCACTTCGTCCTGGAGGTCTGTAGCCGATACGTTAAGGCAGAAGGTGGAGGATGCTGTGAGGGAGCA
TCCCGACTATCGCGTGGTGTTTACCGGACATAGCTTGGGTGGTGCATTGGCAACTGTTGCCGGAGC AGACCTGCGTGGAAATG
GGTATGATATCGACGTGTTTTCATATGGCGCCCCCCGAGTCGGAAACAGGGCTTTTGCAGAATTCCTGACCGTACAGACCGGC
GGAACACTCTACCGCATTACCCACACCAATGATATTGTCCCTAGACTCCCGCCGCGCGAATTCGGTTACAGCCATTCTAGCCC
AGAGTACTGGATCAAATCTGGAACCCTTGTCCCCGTCACCCGAAACGATATCGTGAAGATAGAAGGCATCGAT GCCACCGGC
GGCAATAACCAGCCTAACATTCCGGATATCCCTGCGCACCTATG **GTACTTCGGGTTAATTGGGACATGTCTTGCTCTAGA**

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

Fig 2 DNA sequence from *Thermomyces lanuginosus* lipase (TLL.natSp) and the deduced amino acid sequence TLL.natSp.

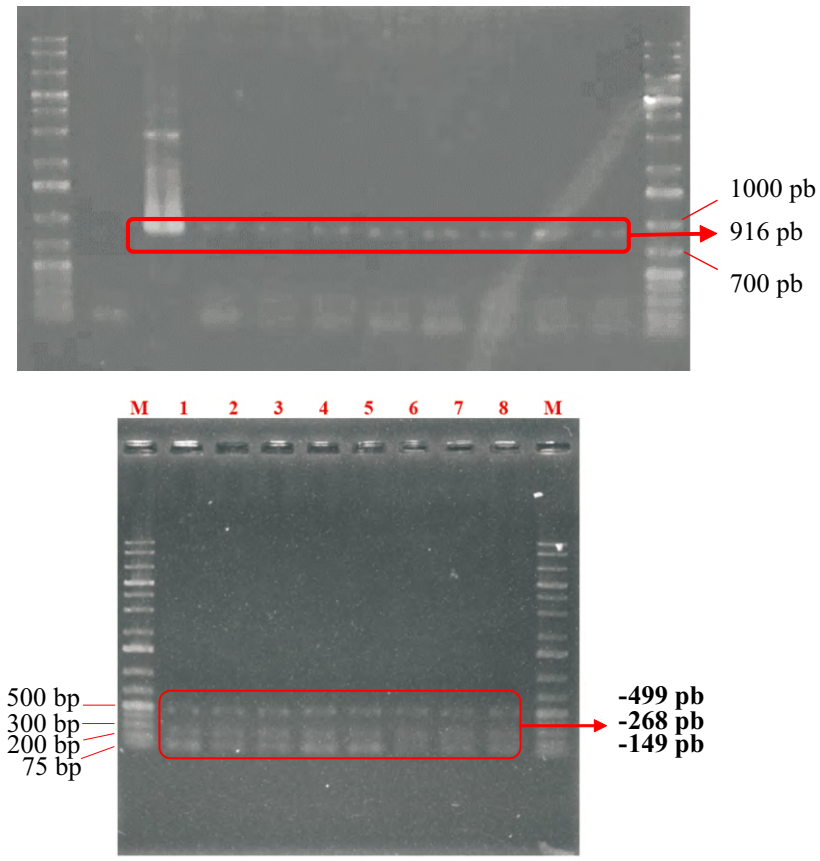

Fig 3 The verification of colony PCR *Pichia pastoris* X-33 recombinant by colony PCR (A) the restriction analysis of PCR colony product *Pichia pastoris* X-33 with *Pst*I (B).

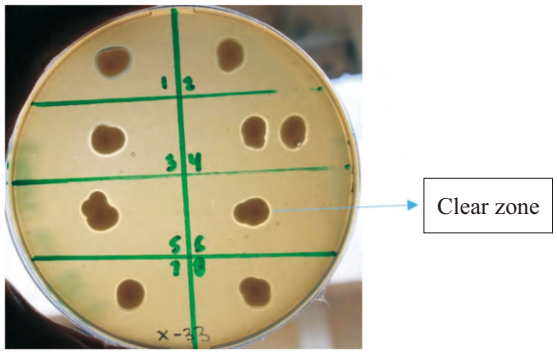

Fig 4. *Pichia pastoris* X33 recombinant expressing lipase in YPD TB plate.

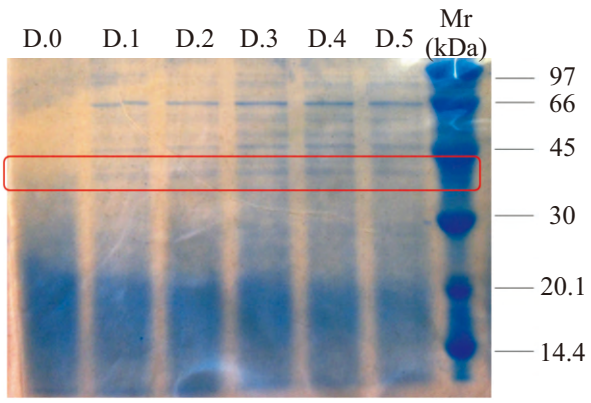

Fig 5 SDS-PAGE analysis of the recombinant expression lipase from *Pichia pastoris* strain X33 (line 1-6: day 0-5; line 7: marker LMW).

20 min at different temperatures (40-80 °C) or at 70 °C at different pH, respectively. It was found that the enzyme had an optimal temperature at 80 °C and an optimal pH at 8.0 (Fig 6).

DISCUSSION

Protein expression system is commonly used in cell-based, which is a package consisting of expression vector, cloned DNA, and a host cell, so that the foreign gene can be expressed by the host cell and is produced in large quantities. For example, a commonly used host is bacteria (such as *E. coli* (Li *et al.* 2014), yeast (such as *S. cereviceae*, *P. pastoris* (Yu*et al*. 2009; Minning *et al*. 1998; Zheng *et al*. 2011; Kademi *et al*. 2003). While commonly used vectors are viruses, plasmids, artificial chromosomes, and bacteriophage (such as lambda).

Synthetic *T. lanuginosus* lipase gene has been successfully constructed into plasmid pPICZαA using natural peptide signal and integrated into the *P. pastoris* X-33 chromosome under the control of AOX1 promoter. The *P. pastoris* expression system can be used several promoters, including AOX1, GAP, FLD1, PEX8, and YPT1. The AOX1 promoter was used in several the study, Zheng *et al.* (2011), Yan *et al.* (2014), Yu *et al.* (2009). Under the control of AOX1 promoter for expression protein using methanol as a major carbon source. In addition AOX1 promoter, the use of multiple promoters were also used to increase the expression of heterologous proteins (Fang *et al*. 2014). The use of the lipase gene original signal peptide naturally rare, researchers generally used a signal peptide derived from the host expression system. In *P. pastoris* expression systems commonly used α factor signal peptide available on plasmid vector (Zheng *et al.* 2011; Yan *et al*. 2014; Yu *et al*. 2009).

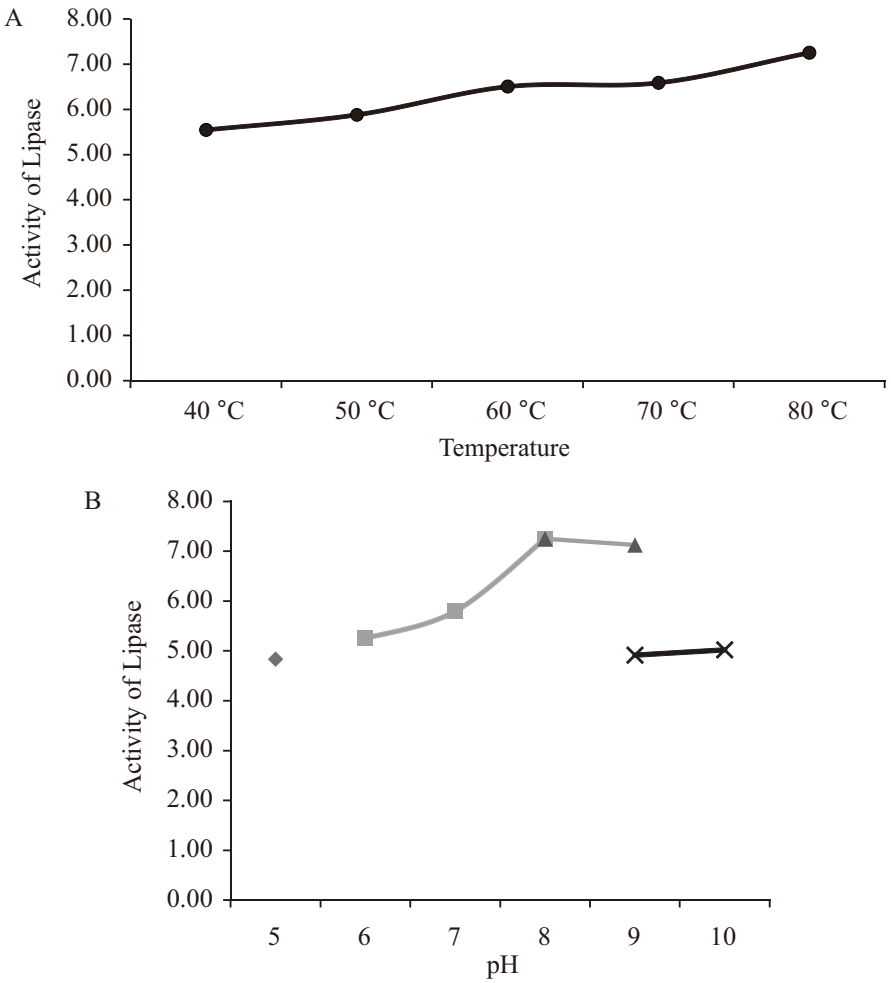

Fig 6 The optimum reaction temperature of expression lipase (A) The optimum reaction PH of expression lipase (buffers Na/citrate (pH 5), Na-phosphate (pH 6-8), Tris/HCl (pH 8-9), dan Glycine/NaOH (pH 9-10)) (B). —◆—:Na-citrate, —■— : Na-Phospate, —▲— :Tris-Cl, and —×— :Glycine-NaOH.

In this study, the optimum temperature and pH on lipase activity reached 7.25 ± 0.26 U mL^{-1} and 7.25 ± 0.12 U mL^{-1}. Zheng *et al.* (2011) used the signal peptide from expression vector for expression of lipase, the

activity of lipase reached 61 U mL^{-1}. Whereas, Fang *et al*. (2014) inserted lipase gene into several expression vector loci to increase expression of lipase, the activity of lipase reached 4350 U mL^{-1}.

Methanol concentration used as a major source of carbon in heterologous protein expression varied. *Pichia* strains that used affects the concentration of methanol induction. In this study, 2.5% methanol used for *P. pastoris* X-33. It is also found varies in others study, 1.2% (Fang *et al.* 2014), 1% (Zheng *et al.* 2011) methanol for induction in the host *P. pastoris* GS115, 1% methanol for induction in the host *P. pastoris* X-33 (Yan *et al.* 2014).

In conclusion, this study reported the cloning, expression, and partial characterization of recombinant *T. lanuginosus* lipase. *T. lanuginosus* synthetic gene lipase has been successfully integrated into chromosome of *P. pastoris*, which have clear zones in YPD. TBA agar with zeocin. The optimum temperature of activity recombinant lipase at 80 °C and optimum pH at 8.0.

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