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Lipolytic activity of Itb1.1 and Lk3 thermostable lipases expressed in *Escherichia coli* and *Pichia pastoris*

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ABSTRACT

Pichia pastoris has been reported as a better expression host compared to *Escherichia coli* for producing various heterologous proteins. Gene encoding thermostable lipases Itb1.1 and Lk3 were previously expressed in *E. coli* with low specific activity. In this study, the genes were subcloned successfully into *Pichia* expression vector pPICZαA and integrated into the genome of *P. pastoris* GS115. The yeast transformants were grown in buffered methanol-complex medium with methanol induction to express the proteins. Meanwhile, *E. coli* BL21 (DE3) recombinants were regenerated in Luria Bertani with isopropyl β-d-1-thiogalactopyranoside induction to express the proteins. Purified proteins were obtained through nickel-nitrilotriacetic acid affinity chromatography. The lipolytic activity assay was conducted at pH 8 and 70°C for Itb1.1 and pH 8 and 50°C for Lk3. The specific activity of Itb1.1 expressed in *E. coli* cells was 1.2064 U/mg, whereas that of the enzyme expressed in *P. pastoris* was 7.6836 U/mg. Moreover, the specific activity of Lk3 expressed in *E. coli* was 0.3523 U/mg, whereas that in *P. pastoris* was 4.8508 U/mg. Therefore, the specific activity of Itb1.1 and Lk3 expressed in *P. pastoris* was 6 and 14 times higher than that expressed in *E. coli*, respectively. The data suggested that *P. pastoris* is a better host for the expression of Itb1.1 and Lk3 proteins.

INTRODUCTION

Lipases are one of biocatalysts that act in various ranges of bioconversion, including hydrolysis, esterification, interesterification, alcoholysis, acidolysis, and aminolysis (Soliman *et al.,* 2007). It works at the hydrophilic-lipophilic interfaces and is tolerant to the presence of organic solvents in a reaction mixture (Hasan *et al.,* 2006). Furthermore, lipases show potential applications in industries. Thermostable lipase from thermophilic microbes was used commercially such as on modification of fats, flavor enrichment in food processing, organic synthesis, hydrolysis of fats and oils, resolution of racemic mixtures, and other chemical analysis industries (Contesini *et al.,* 2020; Li and Zhang, 2005).

Thermophilic microorganisms are able to produce thermozymes or also known as thermostable enzymes such as thermostable lipases exhibiting extreme stability at elevated temperatures and in organic solvents (Bornscheuer *et al.,* 1994). Thermal denaturation in most enzymes will negatively affect the activity (Maciver *et al.,* 1994). The properties of thermostable lipases are essential in industrial applications and biotechnology, especially in converting mixtures at a higher temperature.

Most thermostable lipases are generally expressed at low levels by wild-type hosts. In recent years, they have been produced using bacteria and yeast expression systems. Studies on the interesting enzyme have been extensively explored to express in *Escherichia coli*; however, some of the enzymes show low levels of lipolytic activity. A local thermophilic microorganism from Manuk hot spring, Kamojang, West Java, has been isolated and cultivated and the gene encoding thermostable lipase Itb1.1 was cloned and expressed into *E. coli* expression vector [pET-30a(+)] with 1,248 base pairs (bp) coding for 416 amino acids (Widhiastuty *et al.,* 2011). The enzyme showed low specific activity under the condition at pH 8.0 and 70°C using *p*-nitrophenyl palmitate (pNPP) as substrate (Brilliantoro *et al.,* 2015). Another thermostable lipase Lk3

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was obtained from compost based on metagenome exploration. The highest homology of thermostable lipase Lk3 was closest to *Pseudomonas stutzeri*. Gene encoding thermostable lipase Lk3 was cloned and expressed into *E. coli* with 843 bp coding for 280 amino acids (Nurhasanah *et al.,* 2015). The enzyme showed a low specific activity under the condition at pH 8.0 and 50°C using *p*-nitrophenyl laurate (pNPL) as substrate (Nurhasanah *et al.,* 2017).

Nowadays, other expression systems such as yeast expression systems have been widely used to establish more efficient production of thermostable recombinant lipases. *Pichia pastoris* is one of the yeast expression systems that have the potential as a host to express typical and thermostable lipases (Furqan and Akhmaloka, 2020; Lan *et al.,* 2016; Sabri *et al.,* 2009). The methylotrophic yeast, *P. pastoris*, was an attractive host for producing heterologous protein growing on methanol as the sole carbon and energy source, and the proteins involved in methanol metabolism are strongly induced (Gasser *et al.,* 2013; Yurimoto *et al.,* 2011).

Pichia pastoris was introduced 40 years ago by Philips Petroleum to produce single-cell protein commercially as animal food additive ingredients because of the fermentation process in high-density cells (Ahmad *et al.,* 2014). *Pichia pastoris* genome contains four chromosomes with a total size of about 9.43 Mbp. Genome sequences of yeast strain GS115 have been successfully determined in recent years (Fickers, 2014). The strain was the most valuable for industrial and research development used to study the gene expression and regulation through methanol. The alcohol oxidase (*AO*) promotor was the first methanol metabolism pathway regulated tightly. When the yeast was grown in medium containing glucose or ethanol, *AO* was not detected in cells, but when the cell was grown in medium-containing methanol, *AO* highly detected up to 35% of cellular protein. In addition, *P. pastoris* has many advantages such as being able to grow to very high cell densities and produce large amounts of protein, posttranslational modification, strong methanol-inducible *AOX1* promoter, extracellular secretion for novel protein, and stable integration events in host chromosomal DNA (Cereghino and Cregg, 2000; Korona *et al.,* 2006; Olędzka *et al.,* 2003; Yamada *et al.,* 2016).

Some studies have reported using *P. pastoris* expression systems such as thermostable lipase from *Bacillus thermocatenulatus* (Quyen *et al.,* 2003), recombinant thermostable lipase B from *Candida antarctica* (Robert *et al.,* 2017), highly Ca^{2+}-activated thermostable L_2 lipase (Sabri *et al.,* 2009), thermostable lipase from *Pseudomonas fluorescens* 26-2 (Yang *et al.,* 2009), thermostable T_1 lipase from *Geobacillus zalihae* (Oslan *et al.,* 2013), and thermostable lipase Lk1 from bacterial compost using metagenome technique (Furqan and Akhmaloka, 2020).

This paper reports the heterologous expression of thermostable recombinant lipases Itb1.1 and Lk3 in *E. coli* BL21 (DE3) under the control of T_7 promoter and in *P. pastoris* GS115 under the control of *AOX1* promoter. Following purification of the enzyme using Immobilized Affinity Chromatography (IMAC) nickel-nitrilotriacetic acid (Ni-NTA) the activity of both proteins expressed in *E. coli* and in *P. pastoris* was compared.

MATERIALS AND METHODS

Host strain and plasmids

The host yeast strain used was *P. pastoris* GS115; cloning plasmid pJET1.2/blunt and expression plasmid pPICZαA were purchased from Invitrogen, USA. *E. coli* TOP10F' was used for subcloning and plasmid propagation. The gene encoding thermostable lipase Itb1.1 was amplified from the recombinant plasmids pET30a(+)-Itb1.1 (pITBLip1.2) and Lk3 from the recombinant plasmids pET30a(+)-lk3 (pITBLip2.1) in *E. coli* BL21 (DE3) cells.

Recombinant plasmid construction in pPICZαA

Recombinant plasmids pITBlip1.2 and pITBLip2.1 in *E. coli* BL21 (DE3) cells first analyzed the restriction sites using *Eco*RI and *Hind*III to confirm the existence of gene encoding thermostable lipases Itb1.1 and Lk3. Then mature Itb1.1 and Lk3 lipases were subcloned into pJET1.2/blunt (pJET1.2-itb1.1 or pITBLip1.3 and pJET1.2-lk3 or pITBLip2.2), and increasing of plasmid was conducted in *E. coli* TOP10F'. Furthermore, mature Itb1.1 lipase was amplified by using a forward primer incorporated with restriction sites. *Eco*RI was for ITB1.1 (5′-CTTAGAATTCGCATCCCCACG-3′), and reverse primer incorporated with *Xba*I was revITB1.1 (5′-GAAGTCTAGATAAGGCCGCAAAC-3′).

Moreover, mature Lk3 lipase was amplified using forward primer forLK3 (5′-CGTCGAATTCATGAACAA GAACAAAACCTTGC-3′) and reverse primer revLK3 (5′-GAA GTCTAGATAGAGCCCCGCGTTCTTC-3′) with restriction endonucleases *Eco*RI and *Xba*I being underlined.

The purified amplicon and pPICZαA plasmids were digested with *Eco*RI and *Xba*I. The insert and plasmid were ligated and transformed in *E. coli* TOP10F'. The recombinant plasmid construction for *Itb1.1* gene was designed as pITBLip1.4 (pPICZαA-itb1.1) and for *lk3* gene was designed as pITBLip2.3 (pPICZαA-lk3). The amplified polymerase chain reaction (PCR) product encoded 404 amino acids for mature Itb1.1 lipase, and 296 amino acids for Lk3 lipase have been fused with poly histidine-tag at C-terminal for further purification steps and fused with α-factor secretion signal at N-terminal. Recombinant plasmids pITBLip1.2 and pITBLip1.4 were then confirmed by sequencing through First Base Inc., Singapore.

Yeast transformation

Transformation of *P. pastoris* GS115 was performed by electroporation method prepared according to EasySelect™ *Pichia* Expression Kit User Manual, Cat. No. K1740-01 (Invitrogen, USA) (Invitrogen Life Technologies, 2010). *Sac*I linearized the recombinant plasmid pITBLip1.4 and pITBLip2.3 before gene integration into the yeast genome. To confirm positive colonies, colony PCR was conducted using *AOX1* forward-reverse primers and size screening. All positive transformants were selected in yeast peptone dextrose sorbitol agar medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar) at 30°C with the addition of 100 µg/ml zeocin and further selection on higher zeocin concentration 1,000–2,000 µg/ml for analysis multicopy of cassette expression (Fig. 1).

Heterologous expression in *E. coli* and *P. pastoris*

For the production of thermostable lipase Itb1.1 and Lk3 through *E. coli*, an overnight culture of *E. coli* BL21 (DE3) harboring pITBlip1.2 and pITBLip2.1 was inoculated in Luria Bertani liquid medium, which contains antibiotic of 0.1% kanamycin until it reached the optical density of 0.6; then, it was induced by 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) within 4 hours and shaken at 180 rpm with temperature 37°C. After 4 hours, the culture was cultivated by centrifugation at 6,000 *g* for 20–30 minutes. All supernatants were removed, and all pellets were collected and then mixed

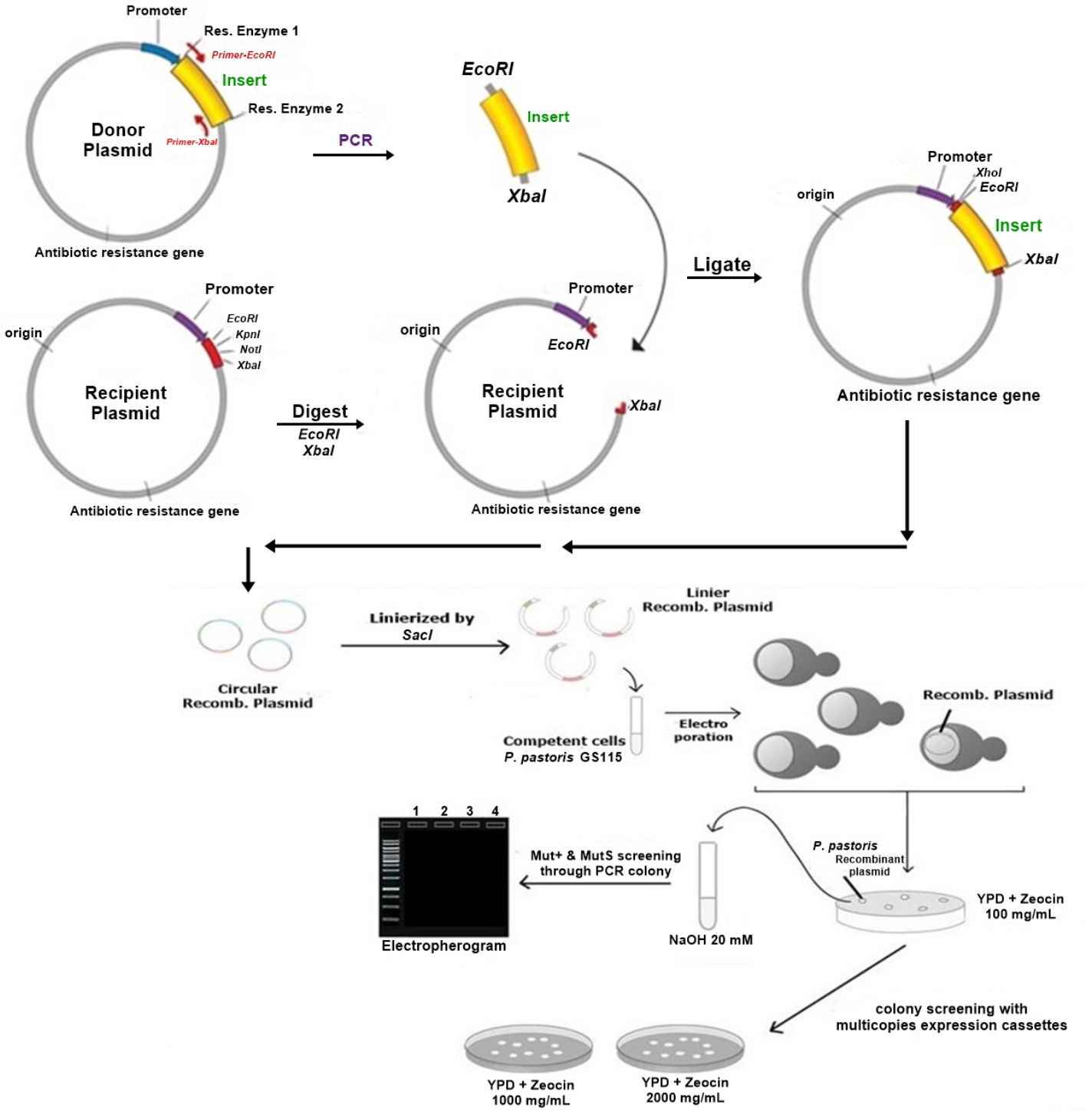

Figure 1. Illustration for construction of recombinant vector and yeast transformation.

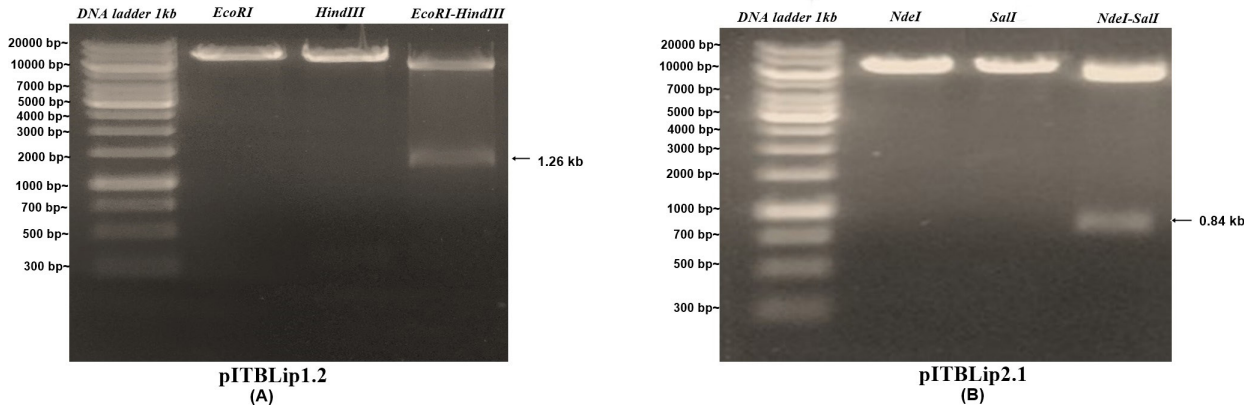

Figure 2. Electropherogram of single and double digest from recombinant *E. coli* expression vectors.

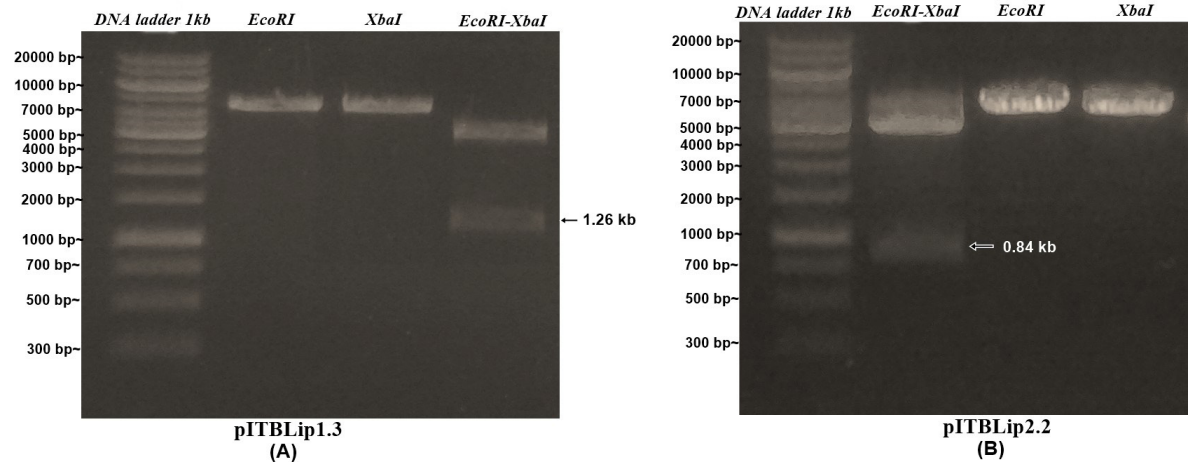

Figure 3. Electropherogram of single and double digest from recombinant *E. coli* cloning vectors.

with lysis buffer [50 mM phosphate buffer saline (PBS) buffer at pH 8 with 0.1% sodium dodecyl sulfate (SDS)]. The cell pellets containing Itb1.1 lipase were incubated for 30 minutes at 70°C and lipase Lk3 at 50°C. Furthermore, all supernatants as crude extract of Itb1.1 and Lk3 thermostable lipases were collected by centrifugation at 8,000 *g* for 30 minutes and then concentrated through Amicon diafiltration with 30 and 10 kDa cutoff (Permana *et al.,* 2017).

To produce Itb1.1 and Lk3 thermostable lipases through *P. pastoris*, one positive transformant from each isolate was used for recombinant thermostable lipase expression. A single colony from YPDz (1% yeast extract, 2% peptone, and 2% dextrose) agar supplemented with 100 µg/ml zeocin was inoculated into 10 ml YPDz broth for 24 hours at 30°C and agitation rate 250 rpm. Then, 500 µl of culture was transferred into 50 ml buffered glycerol-complex medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base without amino acid, and 1% glycerol) in 500 ml conical flask and agitation with 250 rpm at 30°C until the culture reached an OD_{600} = 4–6 (approximately 16–20 hours). The cells were harvested by centrifuging 3,000 *g* for 10–15 minutes at 4°C and then resuspended the cells to an OD_{600} of 1.0 in 50 ml buffered methanol-complex medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer with pH 6.0, 1.34% yeast nitrogen base without amino acid, and 0.5% methanol) to induce expression. The cultures were induced with 0.5% methanol at 24 hours for 6 days. Afterward, the cultures were cultivated by centrifugation at 8,000 *g* for 30 minutes and then concentrated through Amicon diafiltration with 30 and 10 kDa cutoff (Invitrogen Life Technologies, 2010). To get the optimum methanol concentration for the inducer, the same procedure was carried out as before but using various methanol concentrations from 1% to 5% (v/v).

The lipolytic test was performed three times according to the colorimetric method (Govindappa *et al.,* 2014). One unit (U) of thermostable lipase activity was defined as the rate of *p*-nitrophenol from *p*-NPP for Itb1.1 lipase and *p*-NPL for Lk3 lipase as substrate in µmole per minutes.

Protein purification

Lipase purification was carried out by using Ni-NTA affinity chromatography in cold conditions (4°C). As much as 2

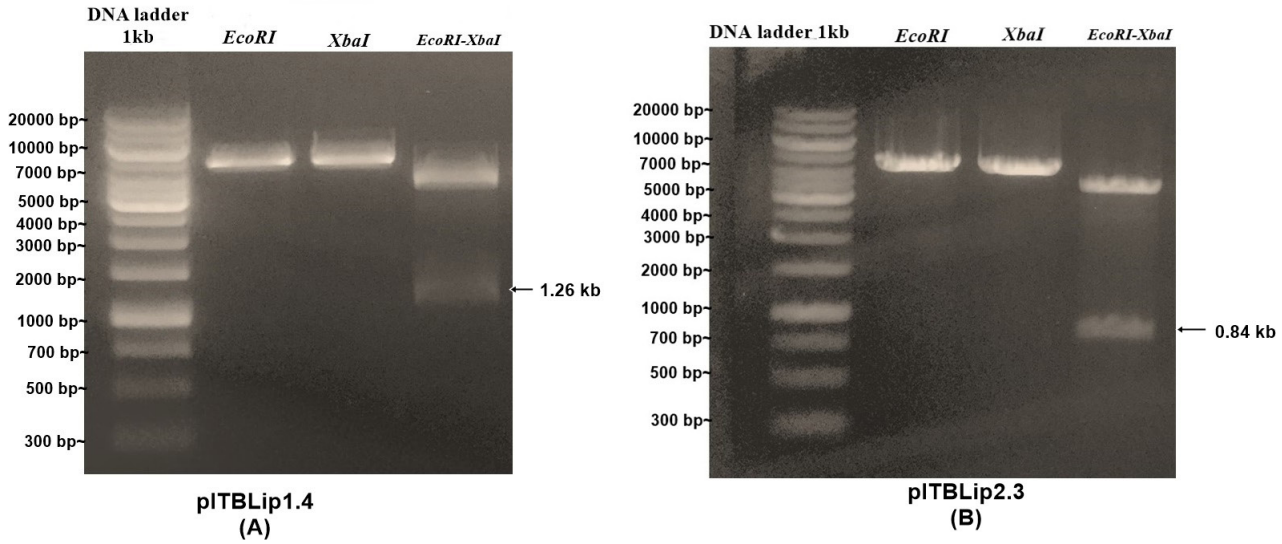

Figure 4. Electropherogram of single and double digest from recombinant *P. pastoris* expression vectors.

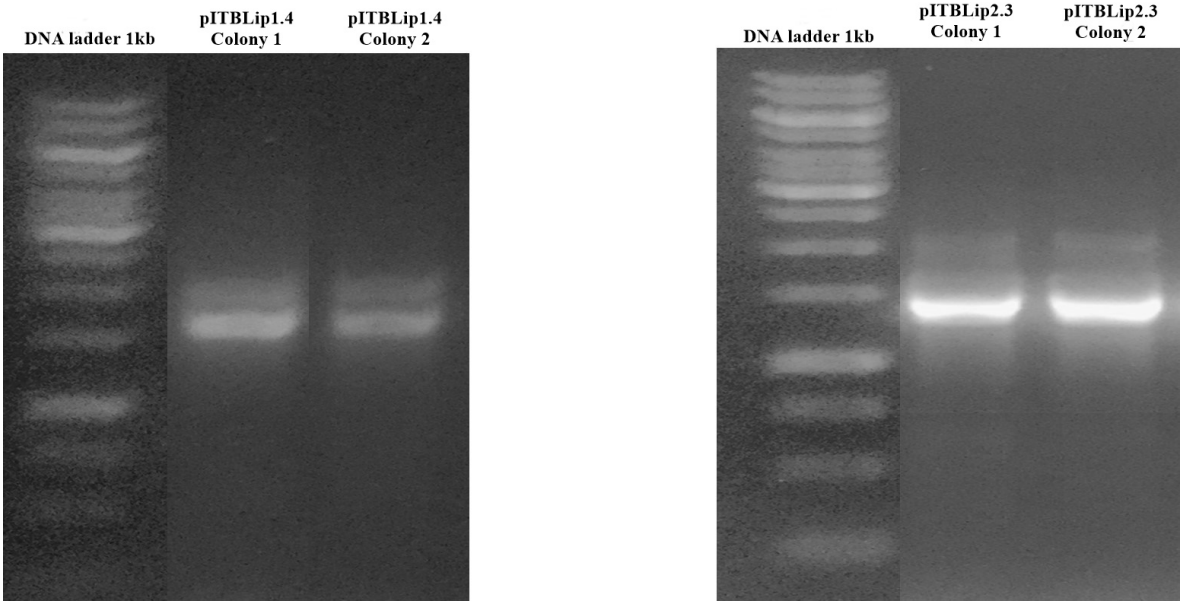

Figure 5. Electropherogram of PCR colony *P. pastoris* GS115.

ml of Ni-NTA agarose resin was put into a 10 ml chromatography column, and then we waited until all resin had been settled and separated from the solvent. The resin was washed with 3 × 6 ml sterile mili-Q aqua. Next, the resin was irrigated with 4 × 6 ml binding buffer solution (50 mM PBS buffer pH 8, 100 M NaCl, and 0.1% (v/v) Triton-X 100) frequently. Furthermore, a 5 ml protein solution (concentrated crude extract of ITB1.1 lipase) was put into the column and let pass through the resin continuously. Then, the resin was washed with 2 × 10 ml buffer solution (50 mM PBS buffer pH 8, 0.1 M NaCl, and 10 mM Imidazole). The protein already bound with resin was eluted with 5 ml elution buffer (50 mM PBS buffer pH 8, 300 mM NaCl, and 100 mM Imidazole). After that, the resin was rinsed with the final buffer (50 mM PBS buffer pH 8, 300 mM NaCl, and 250 mM Imidazole). Dialysis filtration using Amicon with 30 and 10 kDa cutoff was performed by centrifugation 4,000 *g* for 30 minutes to remove impurities solutions, and pure protein was analyzed using SDS-poly-acrylamide gel electrophoresis (PAGE) electrophoresis (Furqan and Akhmaloka, 2020).

RESULTS AND DISCUSSION

Construction of recombinant vector and transformation

Recombinant yeast expression vectors were constructed using the pPICZαA vector through *Eco*RI and *Xba*I restriction sites. The vectors were linearized, followed by yeast

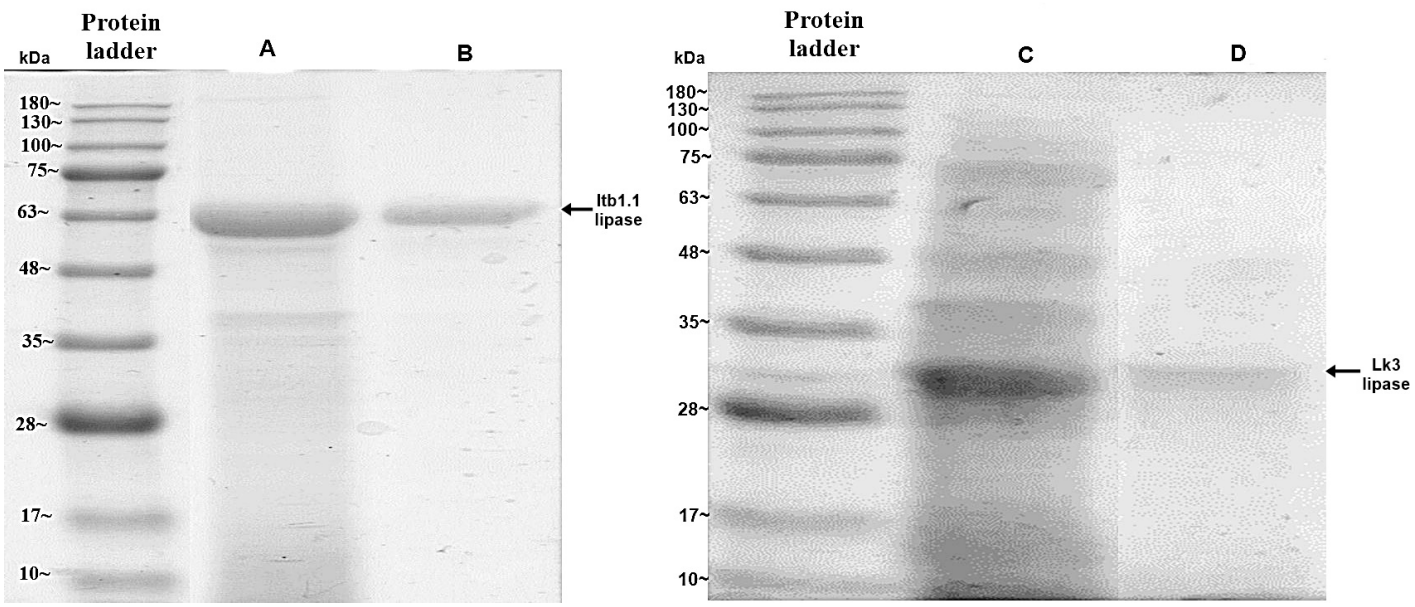

Figure 6. Electropherogram of IMAC Ni-NTA chromatography for Itb1.1 and Lk3 expressed in *E. coli* (line A for Itb1.1 crude extract, line B for purified Itb1.1, line C for Lk3 crude extract, and line D for purified Lk3)

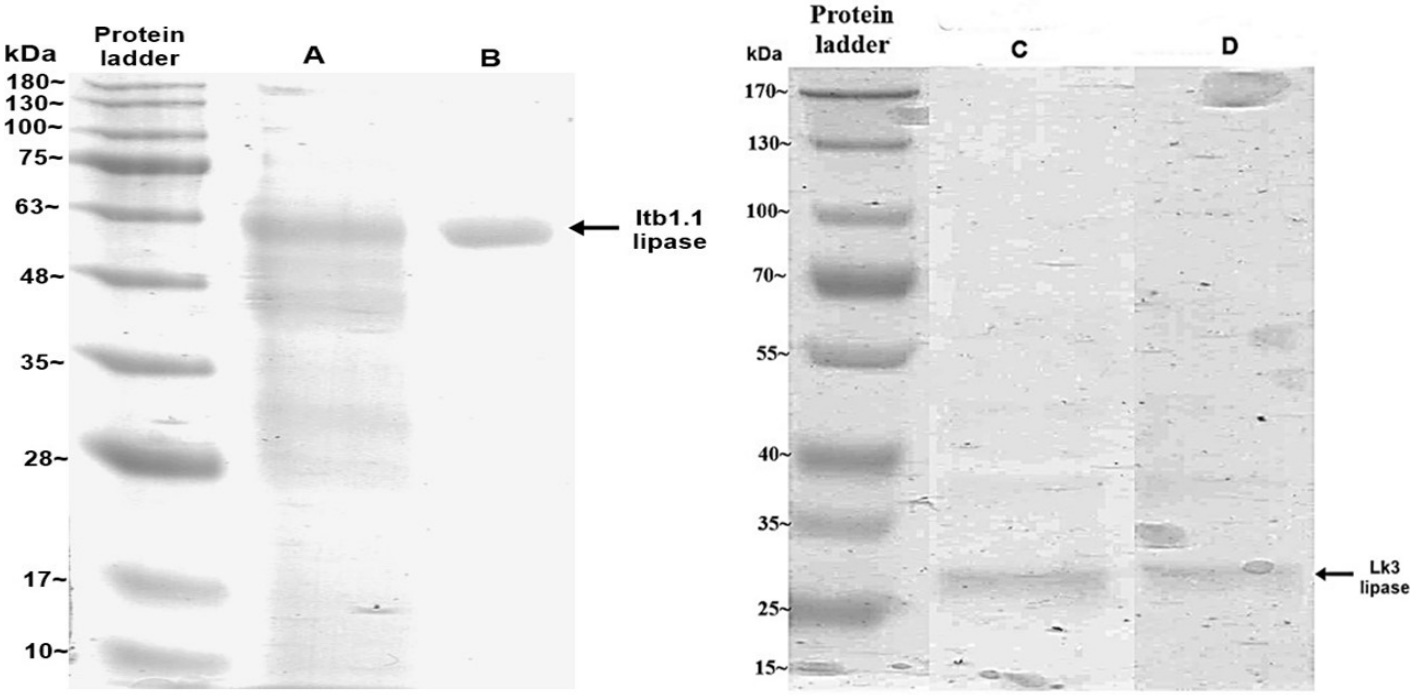

Figure 7. Electropherogram of IMAC Ni-NTA chromatography for Itb1.1 and Lk3 expressed in *P. pastoris* (line A for Itb1.1 crude extract, line B for purified Itb1.1, line C for Lk3 crude extract, and line D for purified Lk3).

transformation (Fig. 1). For *Itb1.1* gene, pITBlip1.2 plasmid was digested with *Eco*RI and *Hind*III resulting in fragment gene at around 1.26 kb (Fig. 2A); meanwhile, for *lk3* gene, pITBlip2.1 plasmid was digested with *Nde*I and *Sal*I resulting in *lk3* fragment at around 0.84 kb (Fig. 2B). The fragments were then ligated at the pJET1.2/blunt cloning vector resulting in pITBlip1.3 and pITBlip2.2 plasmids. The recombinant plasmids, pITBlip1.3 and pITBlip2.2, were then digested by *Eco*RI and *Xba*I resulting in 1.26 and 0.84 kb fragments for pITBlip1.3 pITBlip2.2, respectively (Fig. 3). The fragments were then

Table 1. Lipolytic test of thermostable lipase crude extract.

Sample	Total activity (U/ml)	Protein level (mg/ml)	Spesific activity (U/mg)
	Escherichia coli host cell		
Itb1.1	0.1022 ± 0.0011	0.1996 ± 0.0012	0.5121 ± 0.0069
Lk3	0.0121 ± 0.0017	0.4814 ± 0.0017	0.0251 ± 0.0034
	Pichia pastoris host cell		
Itb1.1	0.1925 ± 0.0049	0.0663 ± 0.0205	2.9025 ± 0.3023
Lk3	0.0917 ± 0.0052	0.2777 ± 0.0016	0.3301 ± 0.0167

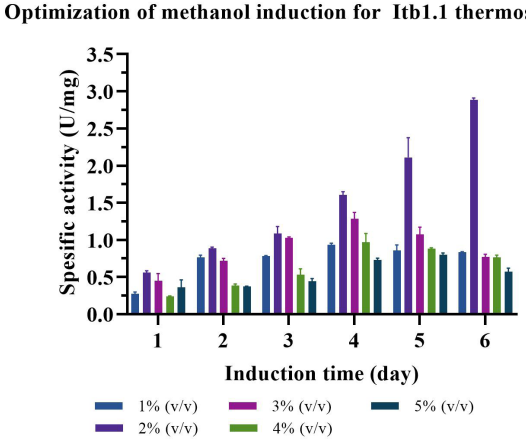

Figure 8. Optimization of methanol concentration to express Itb1.1 thermostable lipase.

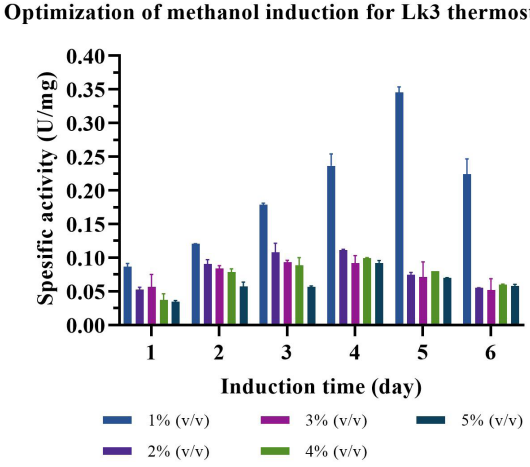

Figure 9. Optimization of methanol concentration to express Lk3 thermostable lipase.

inserted into the yeast expression vector (pPICZαA) through *Eco*RI and *Xba*I restriction sited resulting in pITBlip1.4 and pITBlip2.3 expression vectors, respectively. To confirm that the lipase gene was inserted on pPICZαA, the vectors were digested and sequenced. Following digestion by *Eco*RI and *Xba*I, agarose gel electrophoresis showed that the genes were correctly inserted (Fig. 4). Furthermore, the sequencing results showed that the genes were correctly fused with an α-factor signal sequence and 6x His-tag. The α-factor has a role in the secretion of the protein in the media (Furqan and Akhmaloka, 2020)

Linear plasmids of pITBLip1.4 and pITBlip2.3 were used to transform *P. pastoris* GS115. Positive colonies were confirmed through PCR colony. Two bands appeared on agarose electropherogram (Fig. 5), indicating that Mut$^+$ phenotypic cells or methanol utilization plus means that the *AOX1* gene was not deleted from the genome. Positive colony transformants were chosen for further analysis.

Expression of thermostable lipase in *E. coli* and *P. pastoris*

Thermostable lipases of Itb1.1 and Lk3 were successfully expressed in *E. coli* and *P. pastoris.* Expression of Itb1.1 and Lk3 lipases in *E. coli* was induced by 1 mM IPTG. The enzymes were expressed as intracellular proteins. The crude extracts of the enzymes were electrophorized on SDS-PAGE following lysis cells. Dominant bands on SDS-PAGE with the size at around 50 kDa for Itb1.1 lipase and 32 kDa for Lk3 lipase, respectively, appeared, showing that the proteins were overexpressed (Fig. 6). Meanwhile, the expression of the proteins on *P. pastoris* was induced by 0.5% (v/v) methanol and expressed as extracellular proteins. The cell-free supernatants were electrophorized on SDS-PAGE. The protein size was around 50 kDa for Itb1.1 lipase and 32 kDa for Lk3 lipase, respectively (Fig. 7). All the enzymes expressed in *E. coli* and *P. pastoris* showed lipolytic activity (Table 1).

Methanol concentration and incubation time varied to probe the best expression of Itb1.1 and Lk3 lipases in *P. pastoris*. The result showed that 2% (v/v) methanol concentration and 6-day incubation were the best conditions to express Itb1.1 lipase (Fig. 8); meanwhile, Lk3 lipase appeared at 1% (v/v) methanol concentration for 5-day incubation (Fig. 9).

Purification and lipolytic activity of Itb1.1 and Lk3 lipases

Itb1.1 and Lk3 thermostable lipases were produced at their best condition using *E. coli* and *P.* pastoris host cells. *pastoris*. The crude extract (*E. coli*) and the cell-free supernatant (*P. pastoris*) were purified using IMAC Ni-NTA chromatography. For crude extract enzymes from *E. coli*, the samples were directly purified by IMAC Ni-NTA. Meanwhile, cell-free supernatant from *P. pastoris* was needed to pretreat the samples by washing with 50 mM PBS pH 8 using Amicon diafiltration with 30 kDa cutoff. The samples were then centrifuged until yellow color brownish became yellow.

The purified proteins were electrophorized by SDS-PAGE to determine the molecular weights. The result showed that

Table 2. Lipolytic test of purified thermostable lipase.

Sample	Total activity (U/ml)	Protein level (mg/ml)	Spesific activity (U/mg)
	Escherichia coli host cell		
Itb1.1	0.1415 ± 0.0112	0.1172 ± 0.0017	1.2064 ± 0.1129
Lk3	0.0196 ± 0.0014	0.0559 ± 0.0199	0.3523 ± 0.0505
	Pichia pastoris host cell		
Itb1.1	0.2295 ± 0.0075	0.0299 ± 0.0011	7.6836 ± 0.0314
Lk3	0.0684 ± 0.0007	0.0141 ± 0.0007	4.8508 ± 0.1749

the molecular weight of Itb1.1 and Lk3 lipases is 50 and 32 kDa, respectively (Figs. 6 and 7). A sharp single band on the SDS-PAGE electropherogram showed that each protein was successfully purified.

The lipolytic activity of the purified protein was carried out using the colorimetric method (Govindappa *et al.,* 2014). The specific activity of Itb1.1 lipase expressed in *E. coli* was two times higher than that in the crude extract. Meanwhile, the specific activity of the enzyme expressed in *P. pastoris* was three times higher than that in the cell-free supernatant (Table 2). The specific activity of purified Itb1.1 lipase expressed in *E. coli* was six times higher than that expressed in *P. pastoris*. Furthermore, the specific activity of purified Lk3 lipase expressed in *E. coli* was 14 times higher than that in the crude extract. Meanwhile, the specific activity of the purified enzyme expressed in *P. pastoris* was 15 times higher than that in the cell-free supernatant (Table 2). The specific activity of purified Lk3 lipase expressed in *E. coli* was 14 times higher than that expressed in *P. pastoris*. Therefore, the specific activity of Itb1.1 and Lk3 thermostable lipases expressed in *P. pastoris* host was higher than expressed in *E. coli*. Thus, it suggests that *P. pastoris* was a better host to express Itb1.1 and Lk3 thermostable lipase.

CONCLUSION

In conclusion, the specific activity of purified Itb1.1 lipase expressed in *P. pastoris* host cell was six times higher than expressed in *E. coli*. Additionally, the specific activity of purified Lk3 lipase expressed in *P. pastoris* host cell was 14 times higher than that expressed in *E. coli*. Finally, it could be concluded that the secretory expression of Itb1.1 and Lk3 thermostable lipases expressed in *P. pastoris* system was significantly better than that expressed in *E. coli*.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

CONFLICT OF INTERESTS

The authors report no financial or any other conflicts of interest in this work.

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ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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