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Implications of biodiesel production for enhancing thermostable T1.2RQ lipase secretion in *Pichia pastoris* via chaperone co-expression

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Abstract. *Puspitasari E, Rustam YH, Satya AA, Suwanto A, Wahyudi AT, Astuti RI. 2025. Implications of biodiesel production for enhancing thermostable T1.2RQ lipase secretion in* Pichia pastoris *via chaperone co-expression. Biodiversitas 26: 4557-4566.* The thermostable T1.2RQ lipase from *Geobacillus stearothermophilus* (isolated from Indonesian hot springs) was expressed in *Pichia pastoris* GS115 via multicopy integration (6X). Co-expression of chaperones *bmh2* (797 bp) and *sso2* (875 bp) was achieved using Gibson Assembly and pPICZAwbe. Transformants GS115/T1.2RQ(6X)_bmh2#3 showed 71% higher activity ($p = 0.017$) versus controls, while *sso2* reduced activity by 23% ($p = 0.028$). SDS-PAGE confirmed a 43 kDa band, and tributyrin assays verified extracellular secretion. In biodiesel trials, the lipase reduced Palm Fatty Acid Distillated Free Fatty Acid (PFAD FFA) to 4.9%, meeting SNI 7182:2015 standards. This study demonstrates *bmh2*'s role in alleviating Endoplasmic Reticulum (ER) stress and advancing bacterial lipase production in yeast, with applications in sustainable biodiesel. Gas Chromatography (GC) analysis showed methyl palmitate (C16:0) and methyl stearate (C18:0) as the dominant Fatty Acid Methyl Ester (FAME) species. This research demonstrates that *bmh*2 is the most effective chaperone for co-expression with a six-copy lipase, increasing extracellular activity. The enzyme produced by heterologous expression in *Pichia* can be used for lab-scale biodiesel production, comparable to that expressed in *Escherichia coli*. This is the first report of *G. stearothermophilus* T1.2RQ lipase, and these findings advance bacterial lipase gene expression research in the *P. pastoris* expression system. Future work should optimize tandem chaperone systems (*bmh*2 + PDI) and pilot-scale fermentation. This study underscores Indonesia's microbial biodiversity as a resource for novel biocatalysts, aligning with Biodiversitas's mission to bridge ecology and biotechnology.

Keywords: *bmh2*, *Geobacillus stearothermophilus*, lipase, multicopy, *Pichia pastoris*

INTRODUCTION

Lipases are enzymes that catalyze the hydrolysis of lipids at the oil-water interface, producing glycerol and long-chain fatty acids. They also facilitate various bioconversion reactions, such as esterification and inter-esterification, making them highly valuable in industries. Microbial lipases are particularly attractive due to their versatility, cost-effectiveness, and ease of genetic manipulation, which contribute to their increasing commercial demand (Kaczmarek et al. 2021; Lim et al. 2021; Verma et al. 2021).

The microbial lipase market is projected to reach USD 590.2 million by 2023, growing at a CAGR of 6.8% from 2018. Lipases of microbial origin also have a wide range of industrial applications, especially in the synthesis of modified molecules (Chandra et al. 2020; Adetunji and Olaniran 2021; Ali et al. 2023). Among microbial lipases, bacterial lipases, particularly extracellular ones, are preferred due to their high production efficiency and ease of purification (Mazhar et al. 2023).

The T1.2RQ lipase gene is a gene that encodes a thermostable lipase derived from the thermophilic bacterium *Geobacillus stearothermophilus* T1.2, was initially isolated from a hot spring on Seram Island, Maluku, Indonesia. The thermostable enzymes, such as lipase and protease of thermophilic bacteria, have advantages such as reducing contamination from mesophilic microorganisms, enhancing the reaction rate, and increasing the solubility of reactants and non-volatile products (Sabaria et al. 2024). The T1.2RQ lipase gene is well-expressed in *Escherichia coli*, but produces intracellular enzymes; this often results in protein misfolding and the formation of inclusion bodies. In contrast, *Pichia pastoris* offers an effective platform for recombinant protein production; however, the expression profile of each enzyme can vary significantly (Terol et al. 2021; Vittaladevaram 2021). The organism utilizes methanol as a sole carbon source and has the potential for whole biocatalysts with the *Pichia* surface display system (Yan et al. 2018; Hao et al. 2019; Mohammadzadeh et al. 2021).

Bacterial lipases often exhibit better activity in prokaryotic systems, yet secretion and correct folding in prokaryotes are challenging, especially for extracellular enzymes (Liu et al. 2017; Yao et al. 2021). Several strategies have been employed to enhance lipase production in *P. pastoris*, including codon optimization, increased gene dosage, and co-expression with molecular chaperones. Among these, chaperone co-expression has shown promise in improving protein folding and secretion, though it can also impact host

cell physiology (Cámara et al. 2017). In the metabolic pathway of *P. pastoris*, the secretion of heterologous proteins involves many chaperones, including the *bmh*2 and *sso*2 genes. These genes assist protein folding and secretion, which is related to the overexpression of multiple copies of the lipase gene, which is prone to misfolding and triggers Untranslated Protein Response (UPR) (Raschmanová et al. 2021). While *Pichia* chaperones like PDI and BiP are well-studied (Raschmanová et al. 2021), *bmh2*'s role in bacterial lipase secretion remains unexplored. This study bridges this gap, leveraging Indonesia's unique microbial resources to advance sustainable enzyme technology.

According to Puspitasari et al. (2024), the T1.2RQ lipase gene was successfully expressed in *P. pastoris* GS115 using a multicopy approach, and the strain containing six gene copies showed a 111% (two fold) increase in lipase activity compared to the single-copy strain and produced a translation product of 43 kDa. Similarly, Robert et al. (2019) demonstrated that a *Komagataella phaffii* strain containing three copies of the *Candida antarctica* lipase B (CalB) gene yielded 2.3 times more enzyme than the single-copy strain.

Biodiesel is typically produced via esterification reactions, either chemically or enzymatically. Lipases (E.C. 3.1.1.3) catalyze these reactions and have been used in immobilized forms such as Novozym 435, Lipozyme 435, Lipozyme RM IM, and Lipozyme TL IM (Sá et al. 2018; Salvi et al. 2018; Ortiz et al. 2019; Singh et al. 2022).

This study aims to investigate the co-overexpression effects of expression-related chaperone proteins on the production of the T1.2RQ lipase from *Geobacillus stearothermophilus* T1.2 using the *P. pastoris* expression system. We hypothesized that combining multicopy gene strategies with chaperone co-expression may enhance lipase activity and secretion efficiency, with potential applications in industrial biocatalysis, and that *bmh2* would outperform *sso2* by mitigating ER stress during multicopy expression.

MATERIALS AND METHODS

Strains and materials

This study used *Escherichia coli* DH5α (Novagen) and *Pichia pastoris* GS115 (Invitrogen) as host strains. *P. pastoris* GS115/T1.2RQ (6X) and the vector pPICZAwbe (a modified pPICZα-A lacking the α-factor) were collected from PT Wilmar Benih Indonesia. Growth media included LB (1% peptone, 0.5% yeast extract, 1% NaCl) and LSLB (1% peptone, 0.5% yeast extract, 0.5% NaCl) for growing *E. coli*. Zeocin was used for selection with pPICZα-A and ampicillin with pPIC9K. For *P. pastoris* cultivation, YPDB (1% yeast extract, 2% peptone, 2% dextrose) and YPDS agar (YPDB + 1 M sorbitol and 1.5% agar) were used. Lipase expression in *P. pastoris* was utilized with BMGY (1% yeast extract, 2% peptone, 0.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol, 100 mM potassium phosphate buffer, pH 6.0) for pre-induction, and BMMY (same as BMGY but with 1% methanol instead of glycerol) for induction. Plasmids pPICZα-A (Invitrogen) and pGEM-T Easy (Promega) were used in cloning. Enzymes, including T4 DNA ligase, Q5® High-Fidelity DNA polymerase, T4 polynucleotide kinase, and DNA polymerase, were from NEB (USA). GoTaq polymerase was obtained from Promega. DNA purification kits (miniprep, gel extraction, and PCR purification) were from Qiagen (USA). Yeast genomic DNA extraction kits were obtained from Thermo Scientific, and HEPES buffer was from Bio-World (USA).

Construction of recombinant plasmids: T1.2RQ lipase with chaperone genes

The *P. pastoris* GS115/T1.2RQ(6X) strain was used as the host for chaperone gene expression. The *bmh*2 and *sso*2 genes were PCR-amplified from *P. pastoris* GS115 genomic DNA. Primers were designed with added restriction sites. The *bmh*2 gene contains an internal *Eco*RI site at position 398. To eliminate this site for cloning purposes, site-directed mutagenesis was performed via Gibson Assembly. A nucleotide at position 402 was mutated (C→T). PCR annealing temperatures were 49-57°C for the upstream fragment and 54-57°C for the downstream fragment (30 cycles). These two PCR products were then mixed as overlap extension PCR templates, and the OE-PCR was conducted using the primer pair P15-AO-21_Pp-Bmh2-F-EcoRI and P15-AP-21_Pp-Bmh2-R-Not, generating the *bmh2* gene. PCR was performed with a pre-denaturation step at 98°C for 5 min, followed by 30 cycles of 98°C for 45 s, 43°C for 30 s, and 72°C for 50 s, with a final extension at 72°C for 5 min. The final amplicon was 797 bp with *Eco*RI and *Not*I sites at both ends (Figure 1).

The *sso*2 gene was amplified by overlap extension PCR (OE-PCR) as described for *bmh2*. The *sso*2 gene contains a *Sal*I site at position 336. Similar site-directed mutagenesis changed nucleotide 339 from A→T to remove the site. Upstream and downstream fragments were amplified using the primer pairs P15-AS-21_Pp-Sso2-F-EcoRI / P15-AV-21_Pp-Sso2-xSalI-R and P15-AU-21_Pp-Sso2-xSalI-F / P15-AT-21_Pp-Sso2-R-NotI, producing 335 bp and 548 bp fragments, respectively. Gibson Assembly was performed using the combined upstream and downstream fragments as templates with the outer primers, generating the *sso*2 gene. PCR was performed under the following conditions: 98°C for 5 min; 30 cycles of 98°C for 60 s, 45°C for 30 s, and 72°C for 60 s; with a final extension at 72°C for 5 min. The final product was 861 bp, with *Eco*RI and *Not*I restriction sites at both ends (Figure 2).

The *bmh*2 and *sso*2 genes were double-digested with *Eco*RI and *Not*I and then cloned into *Eco*RI/*Not*I-digested plasmid pPICZAwbe (map of pPICZAwbe shown in Figure 3.A), respectively, generating pPICZAwbe-Bmh2 and pPICZAwbe-Sso2 (Figure 3.B). These genes were verified by DNA sequencing using the ABI 3130 Genetic Analyzer (Applied Biosystems, USA). These constructs were transformed into competent *E. coli* DH5α, and positive clones were confirmed by PCR.

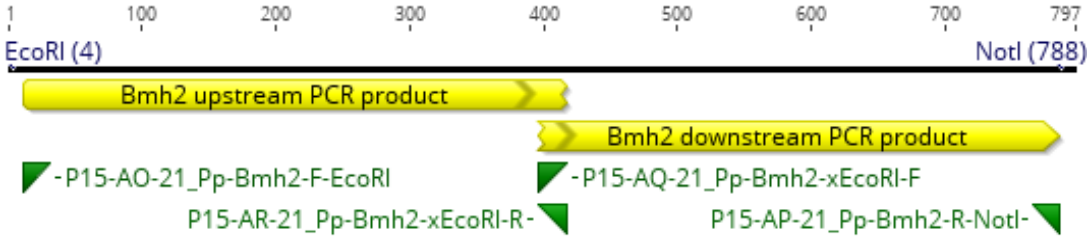

Figure 1. Gibson assembly product of the upstream and downstream *bmh2* amplicons

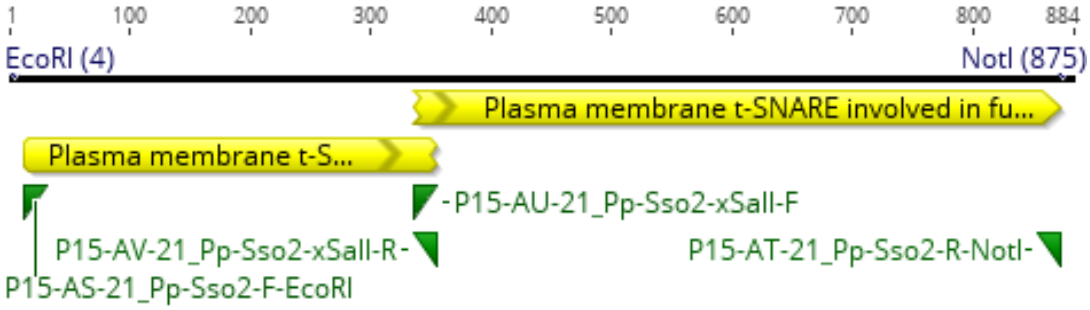

Figure 2. Gibson assembly product of the upstream and downstream *sso2* amplicons

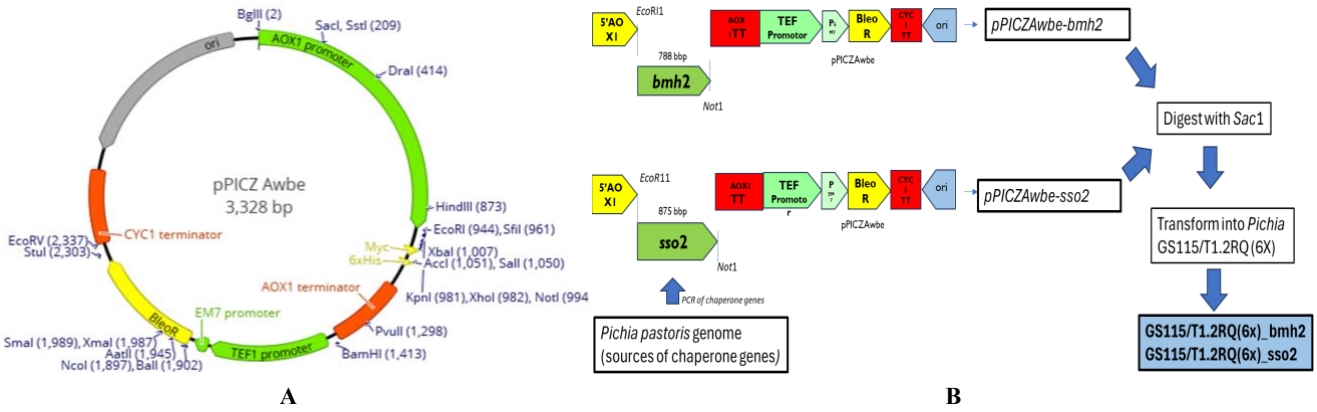

Figure 3. Map of: A. Plasmid pPICZAwbe and B. Illustration for the construct of GS115/T1.2RQ *bmh2* and GS115/T1.2RQ *sso2*

Transformation of *Escherichia coli* and *Pichia pastoris*

The ligated construct (20 µL) was transformed into 100 µL of $CaCl_2$-treated competent *E. coli* DH5α by heat shock (42°C, 1 min), recovered in 1 mL of Luria-Bertani medium, and shaken horizontally for 1 h at 37°C. Transformants were plated on LSLB agar containing 25 µg/mL Zeocin. The recombinant plasmid was linearized with *Sac*I before transformation.

To prepare *P. pastoris* competent cells, a patch (~2 × 1.5 cm) of *P. pastoris* GS115/T1.2RQ(6X) was grown on YPD agar at 30°C for 18-24 h. Cells were resuspended in 1 mL YPD containing 40 µL of 1 M DTT and 40 µL of 1 M HEPES-NaOH (pH 8.0), followed by gentle shaking at 30°C for 15 min. Cells were washed twice with sterile water (3,000×g, 3 min) and chilled on ice for 3-5 min.

Linearized plasmid DNA (2-5 µg) was added to 80 µL of competent cells and transferred into a pre-chilled 2 mm cuvette. After 5 min on ice, electroporation was performed using a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) at 1.5 kV, 200 Ω, 25 µF (~5 ms pulse). Immediately after pulsing, 1 mL of ice-cold 1 M sorbitol was added for recovery. When antibiotic selection was applied, cells were incubated at 30°C for 1-2 h before plating without shaking. Transformants were spread on YPDS plates containing 100 µg/mL Zeocin and incubated at 28-30°C for 2-3 days (Kumar 2019).

Enzyme production using recombinant *Pichia pastoris*

Enzyme production using recombinant *P. pastoris* seed culture preparation and enzyme expression followed the *Pichia* Expression Kit protocol (Invitrogen, USA). Confirmed

recombinant colonies were cultured on YPDS agar plates at 30°C for 2-3 days. A single colony was inoculated into 50 mL of BMGY (pH 6.0) and incubated at 30°C and 225 rpm for 18 h until OD_{600} reached 3-6. Cells were centrifuged at 4,000×g (22-25°C) for 10 min, and the pellet was resuspended in 50 mL BMMY (OD_{600} = 1.0). Cultures were maintained at 30°C and 225 rpm for 5 days, with 1% (v/v) methanol added daily to induce T1.2RQ expression. Cultures were harvested by centrifugation at 5,000×g and 4°C for 15 min. The supernatant was analyzed as a crude extracellular enzyme extract, and partially purified enzymes were obtained using Amicon filters for FAME and GC analysis.

Lipolytic assay

Lipase activity was measured using p-Nitrophenyl Palmitate (pNPP) as substrate with a SmartSpec™ Plus spectrophotometer (Bio-Rad, USA) at 405 nm. The reaction mixture (1 mL) consisted of: 10 µL of 10 mM pNPP (in isopropanol); 40 µL ethanol; 10 µL enzyme sample; 940 µL of 100 mM Tris-HCl buffer (pH 8.0). The mixture was incubated at 50°C for 5 min, and absorbance was measured immediately. Activity was determined under these assay conditions. Each experiment was independently repeated three times. Statistical significance was assessed by Student's T-Test (Welch's T-Test) using Minitab version 22. A P value<0.05 was considered significant. Blanks were prepared for each variable without the enzyme. Results are expressed as relative activity values for the effect of variables on enzyme activity. One unit (U) of enzyme activity was defined as the amount of enzyme releasing 1 µmol of p-nitrophenol per minute under assay conditions, and specific activity was expressed as U per mg of protein (Arifin et al. 2013; Gaol et al. 2020).

Protein analysis

Total protein concentration was quantified by the BCA assay (Thermo Scientific) with a VersaMax microplate reader at 562 nm. Bovine Serum Albumin (BSA) was used as standard from 0 to 250 µg/mL. Each experiment was repeated three times. Recombinant protein was detected by SDS-PAGE according to the modified Laemmli method, with a 6% stacking gel and a 12% separating gel. SDS-PAGE was run on a vertical mini-gel apparatus (Bio-Rad, Hercules, CA, USA). Gels were stained with Coomassie Brilliant Blue R-250 (Amresco, Solon, OH, USA). For qualitative detection, 20 µL of lysate was spotted onto filter paper on tributyrin-containing agar and incubated at 37°C for 24 h. Hac1 protein from previous work was used as a positive control (Satya et al. 2020).

Biodiesel production and FFA analysis

Biodiesel was produced by esterifying PFAD in 2 mL microtubes. The reaction mixture included 0.8 g PFAD, 1500 U partially purified lipase, and methanol added five times at 0.1 mL/hour. Stainless steel beads were used for mixing. The reaction was maintained for 5 h at 50°C and 225 rpm. After centrifugation (13,000 rpm, 10 min), the top layer (biodiesel) was collected. Free Fatty Acid (FFA) content was measured by titration. The biodiesel sample (200 µL) was dissolved in 5 mL ethanol, mixed with 250 µL of 0.2% phenolphthalein, and titrated with 0.05 N NaOH until a pink color persisted for 15 s (Adina et al. 2021).

FAME analysis by Gas chromatography

Chromatography FAME content was analyzed using a gas chromatograph with an HP-5 column (30 m × 0.25 mm × 0.25 µm). Oven program: initial 160°C (15 s hold); ramp 1: 20°C/min to 200°C; ramp 2: 8°C/min to 225°C (2.5 min hold); ramp 3: 50°C/min to 245°C (2.75 min hold); ramp 4: 10°C/min to 270°C (5 min hold). Hydrogen was the carrier gas at 40 cm/s. The FID detector was set to 280°C with air and N_2 flow at 400 mL/min and 25 mL/min, respectively. Samples (2 µL) were injected using an autosampler (Kumar et al. 2014). All data and strains are available upon request to ensure reproducibility.

RESULTS AND DISCUSSION

Construction of recombinant plasmids

The *bmh*2 gene isolated from the *P. pastoris* genome is 797 bp in size (Figure 4.A) and was then cloned into the pPICZAwbe plasmid. The resulting pPICZAwbe-Bmh2 recombinant plasmid is 4,062 bp (Figure 4.B). The recombinant plasmid was transformed into *E. coli* DH5α, and the transformants were verified by PCR using primers specific for *bmh*2 sequences (Figure 4.C), generating a 797 bp amplicon. PCR confirmed a 503 bp amplicon, indicating that *bmh*2 had integrated into the *Pichia* genome in the three transformants identified (Figure 4.D).

The *sso*2 gene isolated from the *P. pastoris* genome is 875 bp in size (Figure 5.A) and was then cloned into the pPICZAwbe plasmid, producing the pPICZAwbe-Sso2 recombinant plasmid at 4,149 bp (Figure 5.B). The recombinant plasmid was transformed into *E. coli* DH5α, and PCR verification of the 2 transformants identified the correct insert (Figure 5.C). The electrophoresis result of the 4 transformants showed a 980 bp band, confirming successful transformation (Figure 5.D).

Lipolytic assay

The transformant with the *bmh*2 chaperone gene showed the highest lipase activity, increasing it by 71%, while the *sso*2 chaperone gene decreased lipase activity by 23% compared to the initial activity of GS115/T1.2RQ(6X). The data is presented as relative specific activity (%) (Figure 6).

Protein analysis

Protein analysis for T1.2RQ (6X) with the chaperone gene was performed using SDS-PAGE (Figure 7.A), showing a band of approximately 43 kDa. Lanes 2 and 4 contain the chaperone protein, and lane 3 is a control (without chaperone protein). All lanes show lipase protein. This was confirmed by qualitative tests on Luria agar supplemented with tributyrin, resulting in clear zones (Figure 7.B). *Pichia* (P) served as a host control; T1.2RQ (6X) (T) as a multicopy lipase gene control; H as a positive control from previous work; and B and S as transformants containing *bmh*2 and *sso*2, respectively. All generated clear zones except *Pichia* (P).

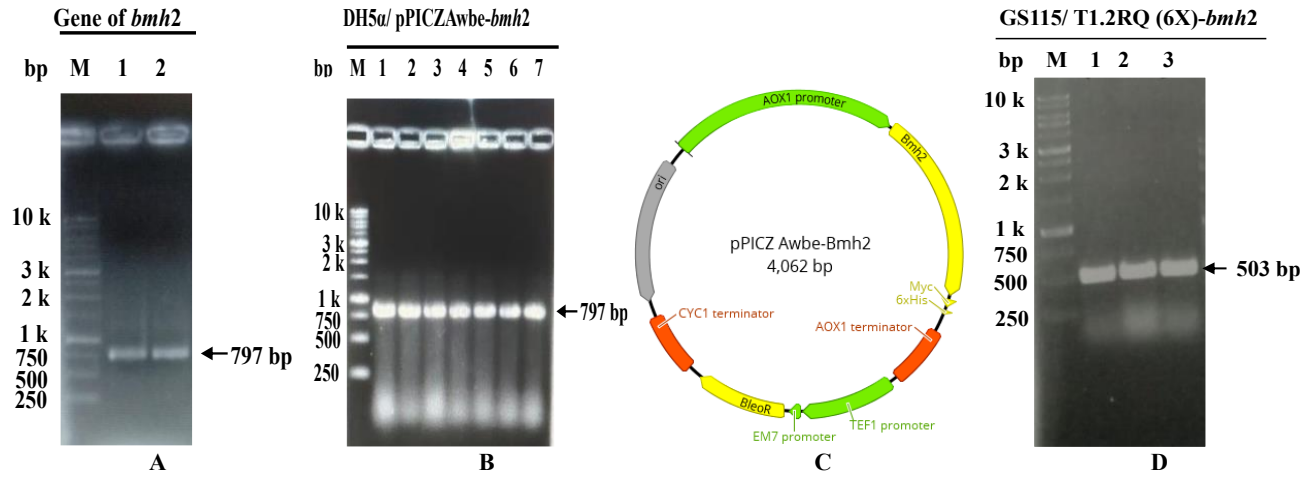

Figure 4. Transformants with *bmh*2 chaperone gene: 4.A. PCR result of *bmh*2 gene, 4.B. Map of recombinant plasmid pPICZAwbe-*bmh2*, 4.C. PCR result of *bmh*2 gene in transformant *E. coli* DH5α/pPICZAwbe-bmh2, 4.D. PCR result of *bmh*2 gene in *Pichia* GS115/T1.2RQ(×X)-bmh2 genome

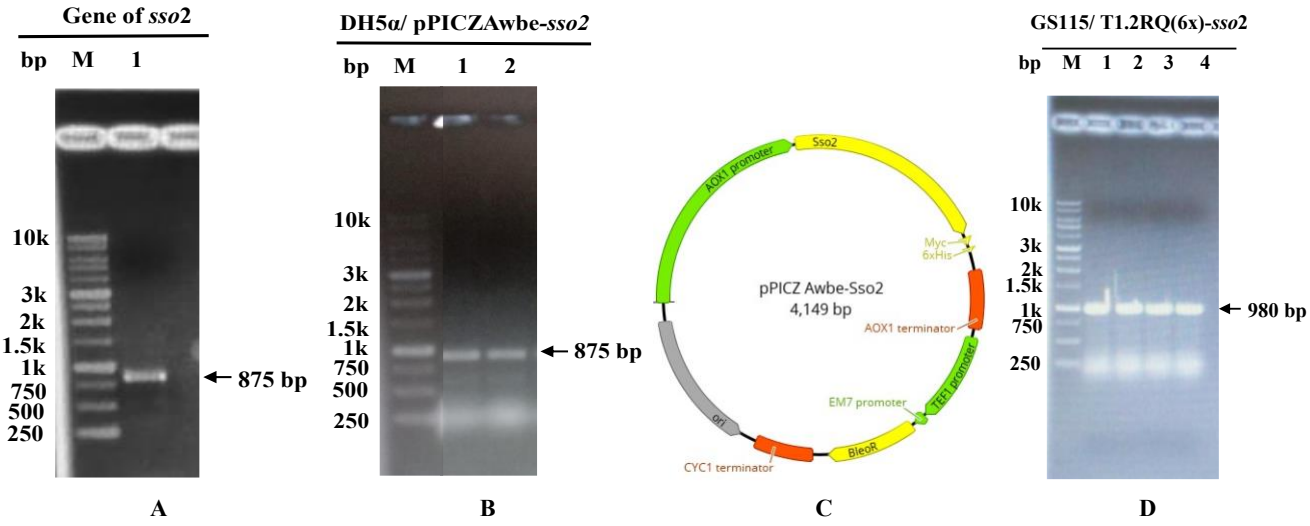

Figure 5. Transforman with *sso*2 chaperone gene: 5.A. PCR result of *sso*2 gene, 5.B. Map of recombinant plasmid pPICZAwbe-Sso2, 5.C. PCR result of *sso*2 gene in *E. coli* DH5α/pPICZAwbe-sso2, 5.D. PCR result of *Pichia* GS115/T1.2RQ(6X)-sso2 genome PCR

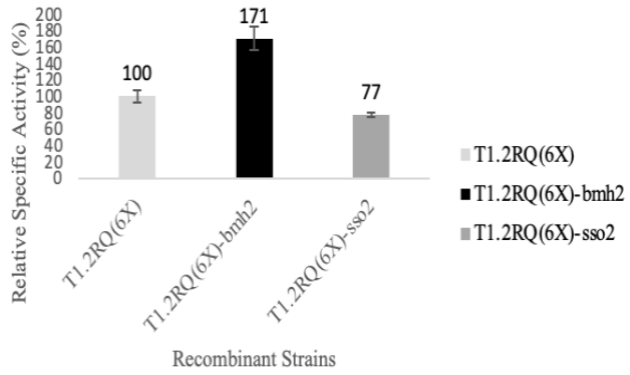

Figure 6. Effect of co-expressing chaperone proteins on T1.2RQ lipase production in the strain GS115/T1.2RQ (6X). All values are presented as the mean±standard deviation of three independent experiments

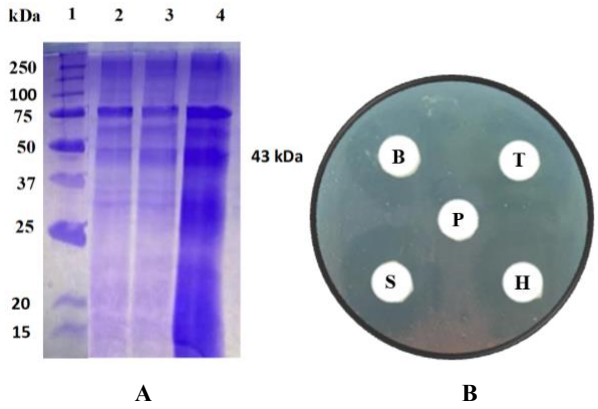

Figure 7. Protein analysis: 7.A. SDS-PAGE of T1.2RQ(6X)-Gen Chaperone protein: 1. Marker, 2. T1.2RQ (6X)_sso2, 3. T1.2RQ (6X), 4. T1.2RQ (6X)_bmh2; 7.B. Qualitative test on Luria Agar +TBN media; B: T1.2RQ (6X)_bmh2. P: *Pichia*; T: T1.2RQ (6X); S: T1.2RQ (6X)_sso2; H: positive control

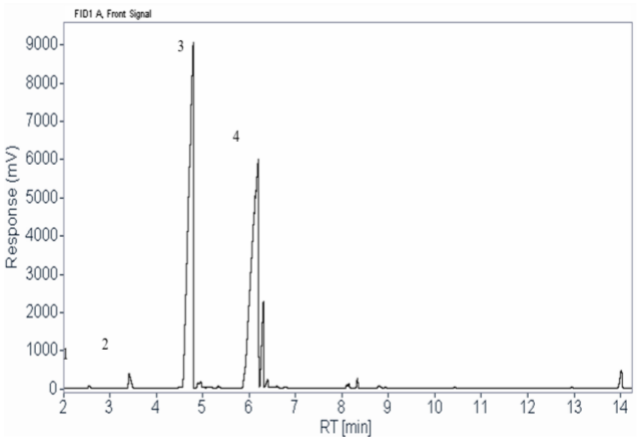

Figure 8. Chromatogram of FAME produced by PFAD with T1.2 -*E. coli* as a catalyst; Peak Identification: 1. C12:0, 2. C14:0, 3. C16:0, 4. C18:0

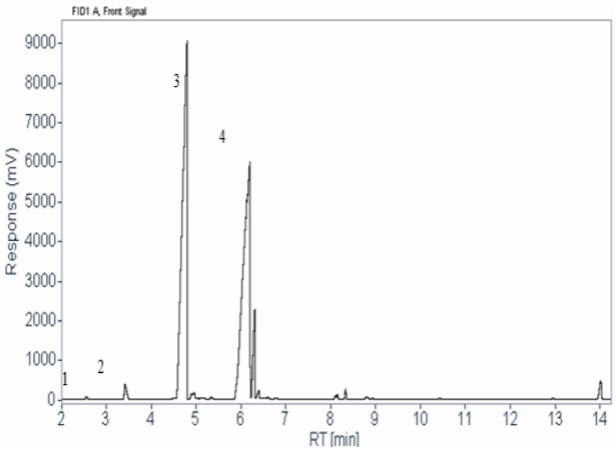

Figure 9. Chromatogram of FAME produced by PFAD with T1.2 - *Pichia* as a catalyst; Peak identification: 1. C12:0, 2. C14:0, 3. C16:0, 4. C18:0

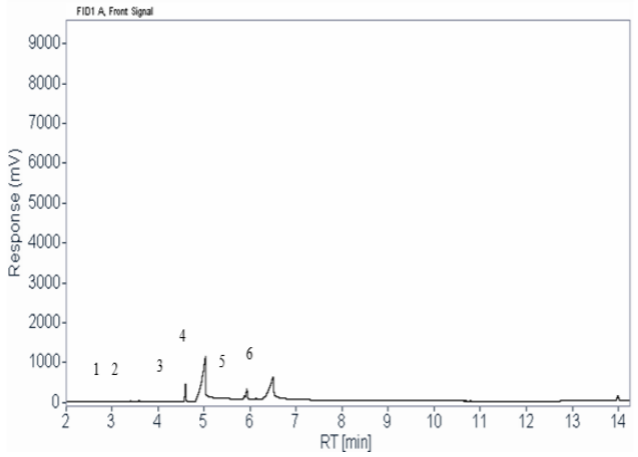

Figure 10. Chromatogram of FAME produced by PFAD without enzyme catalyst; Peak identification: 1. C12:0, 2. C14:0, 3. C16:0, 4. C18:2, 5. C18:1, 6. C18:0

Lipase application for biodiesel

Table 1 shows lipase application for biodiesel production with Free Fatty Acid (FFA) residue of less than 5% in both the positive control (T1.2 RQ_*E. coli*) and the sample (T1.2 RQ_*Pichia*). Methyl palmitate (C16:0) and methyl stearate (C18:0) were the dominant FAME contents (Table 2); the negative control produced very low levels. Figures 8 and 9 show chromatograms of FAME produced from PFAD using T1.2 RQ_*E. coli* lipase and T1.2 RQ_*Pichia* lipase as catalysts, which displayed similar patterns. The chromatogram of FAME produced from PFAD without an enzyme catalyst had a different pattern (Figure 10).

Discussion

Geobacillus stearothermophilus T1.2RQ lipase originates from thermophilic bacteria; it enriches microbial enzyme diversity, contributes to microbial biotechnology, and supports enzyme resource discovery. Previous research used the *Pichia* expression system to improve lipase activity with a multicopy lipase gene strategy. The transformant GS115/T1.2RQ(6X), harboring six copies of the lipase gene, showed the highest lipase activity. This study used GS115/T1.2RQ(6X) transformants to assess the effect of chaperone genes on lipase activity. Numerous chaperone genes have been identified. Jiao et al. (2018) showed that co-expression of *bmh*2 and *sso*2 positively affected *Rhizopus oryzae* lipase ROL production, with overexpression of *sso2* and *bmh2* enhancing ROL secretion by 39% and 32%, respectively. Notably, the GS115/5ROL-Ssa4-Sso2-Bmh2 4# strain achieved a maximum ROL activity of 41,700 U/mL, twice that of the GS115/pAOα-5ROL 11# strain. Li et al. (2016) reported that expressing one copy of chaperone proteins such as ERO1p and BiP with Lip1p produced 13,490 U/mL of lipase activity, significantly higher than expressing Lip1p alone or with a single chaperone. In another study using a combined strategy, ROL expression increased 15.8-fold, with the GS115/5ROL-Hrd1-Ubc1 1# strain reaching 33,900 U/mL via a sorbitol/methanol co-feeding strategy in a 3-L fermenter. These results suggest that heterologous overexpression of ROL in *P. pastoris* using this combined strategy is feasible for large-scale industrialization.

Table 1. Biodiesel application

Feed stock	Enzymes	Stock unit	Composition	FFA content
PFAD (0.8 g)	T1.2 RQ_*E. coli* (positive control)	13440.79 U/mL	111 µL enzyme + 160 µL H_2O	4.02%
PFAD (0.8 g)	T1.2 RQ_*Pichia* (sample)	3153.55 U/mL	476 µL enzyme + 160 µL H_2O	4.90%
PFAD (0.8 g)	No enzyme (negative control)	-	0 µL enzyme + 160 µL H_2O	63.25%

This study used *bmh*2 and *sso*2 as chaperone genes due to their direct involvement in heterologous protein secretion in *Pichia*. Protein secretion in *Pichia* involves many chaperones. Protein accumulates in the endoplasmic reticulum when multiple lipase gene copies are expressed, leading to misfolding and ER stress. Chaperones help export more protein from the ER, reducing unfolded protein and thereby increasing lipase activity. The *bmh*2 gene encodes a chaperone that mediates protein exit from the ER in the *Pichia pastoris* secretion pathway, and the *sso*2 gene facilitates vesicle membrane fusion with the cell wall (Raschmanová et al. 2021).

The *bmh*2 gene (797 bp) was successfully cloned into pPICZAwbe to produce a 4,062 bp pPICZAwbe-Bmh2 recombinant plasmid (Figures 4.A and 4.B). This plasmid lacked an α-factor secretion signal because *bmh2* is required intracellularly to help heterologous proteins exit the endoplasmic reticulum. The PCR product of the *sso*2 gene was 875 bp, and the pPICZAwbe-Sso2 recombinant plasmid measured 4,149 bp (Figures 5.A and 5.B). The *sso2* protein was also needed intracellularly, so it was cloned into a vector without a secretion signal. Figure 5D shows that PCR confirmed *sso*2 integration at the AOX locus, using an AOX forward primer in the *Pichia* genome and *sso*2 reverse primer in the plasmid. The ligated pPICZAwbe-Bmh2 and pPICZAwbe-Sso2 were transformed into *E. coli* DH5α to amplify recombinant plasmids because *Pichia* lacks an origin of replication.

The recombinant plasmid, linearized by *Sac*I, which cuts at the AOX locus, was then transformed into *Pichia pastoris* GS115 containing six copies of the lipase gene by homologous recombination. The T1.2RQ(6X) lipase genes were integrated into the *Pichia* genome at the HIS locus, while the *sso*2 and *bmh*2 genes were integrated at the AOX locus. The GS115/T1.2RQ(6X)-bmh2 transformant carries two *bmh*2 copies because *Pichia* naturally contains a *bmh*2 gene, as does the *sso*2 locus. Overexpression of *bmh*2 and *sso*2 was expected to enhance secreted protein levels; indeed, *bmh*2 overexpression increased lipase activity in GS115/T1.2RQ(6X)-bmh2 by 71%, whereas *sso*2 overexpression reduced activity by 23% (Figure 6).

Table 2. FAME content

FAME content				
Description	**Rt (minutes)**	**% Area**		
		Control (-)	**Control (+)**	**Sample**
Methyl laurate (C12:0)	2.542	0.036	0.153	0.154
Methyl myristate (C14:0)	3.407	0.198	0.153	1.058
Methyl palmitate (C16:0)	4.596	**4.589**	**44.45**	**44.66**
Methyl stearate (C18:0)	6.131	**0.34**	**45.49**	**45.16**
Methyl oleate (C18:1)	5.919	3.423	0	0
Methyl linoleate (C18:2)	5.875	1.071	0	0

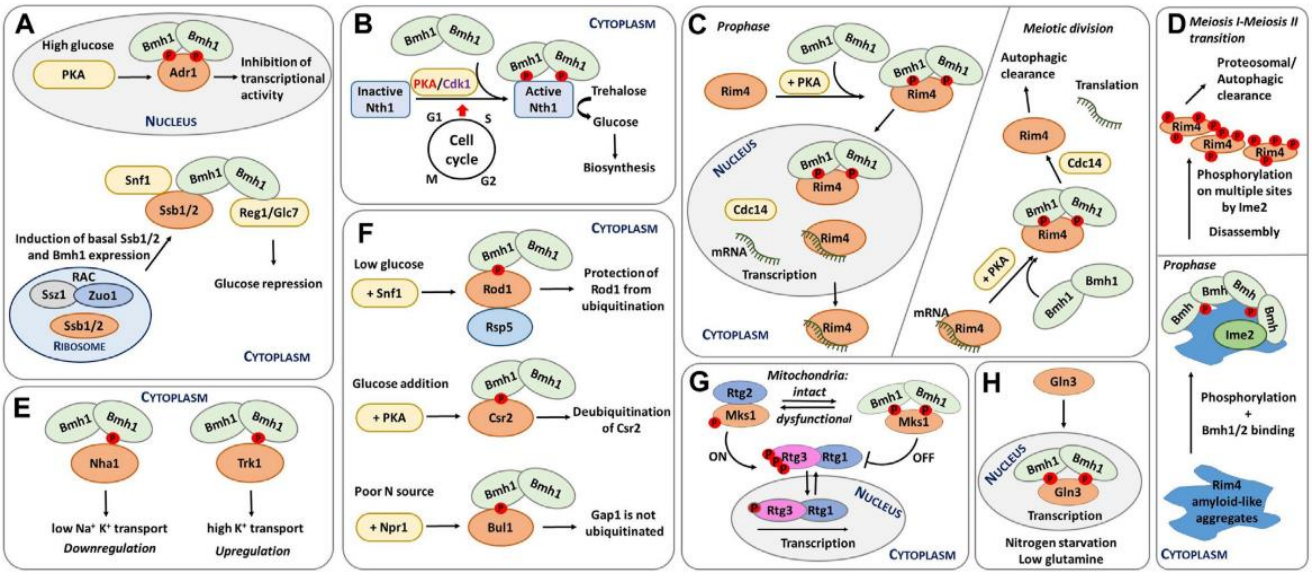

Figure 11. Schematic representation of selected functions of yeast 14-3-3 proteins: A. Transcriptional regulation of glucose-repressed genes, B. Regulation of neutral trehalase Nth1, C. Spatiotemporal control of meiosisby14-3-3proteins, D. Bmh participate in the phosphorylation-mediated clearance of Rim4 aggregates during meiosis, thereby contributing to global protein aggregate homeostasis. Bmh proteins bind to Rim4 amyloid-like aggregates and facilitate Rim4 phosphorylation by the meiosis-specific kinase Ime2. This phosphorylation subsequently leads to the disassembly of Rim4 aggregates. Modified from (Herod et al. 2022), E. Different mechanisms of regulation of yeast transporters by 14-3-3 proteins. 14-3- 3 binding downregulates the cation efflux activity of Nha1 but upregulates the activity of the potassium transporter Trk1 (Smidova et al. 2019; Masaryk et al. 2023), F. Endocytosis regulation, G. Dynamic regulation of mitochondrial retrograde signalling. When cells have functional mitochondria, the RTG pathway is inactive, phosphorylated Rtg3 is bound to Rtg1 in the cytoplasm, and phosphorylated Mks1 is bound to 14-3-3. Dysfunctional mitochondria trigger the activation of the RTG pathway. Under these conditions, Mks1 is dephosphorylated, binding to Rtg2 and, thus, prompting the translocation of the Rtg1-Rtg3 complex into the nucleus and the transcription of genes involved in the retrograde response, adapted from (Bui dan Labedzka-Dmoch 2024), H. Regulation of rapamycin-mediated cell signalling and nitrogen catabolite repression

The expression of various chaperone genes in *Pichia pastoris* significantly affected the specific activity of recombinant lipase. Statistical analysis using Welch's t-test confirmed these differences were significant. Co-expression of *bmh*2 yielded a considerable increase in lipase activity compared to the control (t = -7.35, df = 2.72, p = 0.017); in contrast, expression of *sso*2 significantly decreased activity (t = 5.45, df = 2.32, p = 0.028). The negative t-value for *bmh*2 indicates that mean activity in the treatment groups (with *bmh*2) was higher than in the control group (T1.2RQ(6X)), while the positive t-value for *sso*2 indicates lower activity.

The 71% activity boost with *bmh2* aligns with its role as a 14-3-3 scaffold protein, stabilizing lipase folding and ER export (Figure 11) (Obsilova and Obsil 2024). In contrast, *sso2*'s t-SNARE function may disrupt vesicle fusion stoichiometry, explaining the 23% drop (Peer et al. 2022). Overexpression of *sso2* can disturb the stoichiometric balance required for efficient SNARE complex formation between v-SNARE and t-SNARE proteins, a crucial step in exocytosis involving assembly of two t-SNAREs with a v-SNARE. This imbalance may lead to secretory vesicle buildup, impaired docking and fusion, ER overload, accumulation of unprocessed proteins, ER stress, and activation of the Unfolded Protein Response (UPR) pathway (Gurunathan et al. 2002; Liu et al. 2014). This stress may induce apoptosis or reduce overall protein synthesis in extreme cases. Therefore, although *sso2* is essential for secretion, its expression must be tightly controlled.

These results explain why *sso*2 overexpression did not enhance lipase activity and may have harmed recombinant protein secretion in *P. pastoris*. However, co-expression of *bmh*2 appeared more advantageous, likely because it stabilizes protein structure and trafficking, promoting secretion without inducing cellular stress. These findings underscore the importance of selecting appropriate chaperone systems to optimize recombinant enzyme production in *P. pastoris*. *bmh2* also mediates export of heterologous proteins from the ER, preventing lipase accumulation in the ER and promoting correct folding (Raschmanová et al. 2021).

The SDS-PAGE protein band at 43 kDa corresponds to the 1.2 kb T1.2RQ gene product, confirming that the *G. stearothermophilus* enzyme was expressed in *P. pastoris*. Supernatants from enzyme production were tested on Luria agar containing tributyrin (TBN) and produced clear zones, confirming that T1.2RQ lipase hydrolyses TBN and is secreted extracellularly. T1.2RQ lipase was applied to produce biodiesel (FAME) on a laboratory scale using T1.2RQ lipase expressed in *E. coli* as a control. Gas chromatography chromatograms showed identical patterns for both samples, in contrast to the negative control without enzyme. FAME analysis indicated that both the positive control and the *Pichia*-derived sample were dominated by methyl palmitate (C16:0) and methyl stearate (C18:0), unlike the negative control. In addition, FFA residue from the esterification reaction was <5%, meeting SNI biodiesel standards. Thus, *Pichia*-derived T1.2RQ lipase can be used for biodiesel production similarly to the *E. coli*-expressed enzyme.

This research shows that co-expressing *bmh*2 in *Pichia* significantly enhances T1.2RQ lipase secretion, offering a scalable platform for thermostable enzyme production. Future work should optimize tandem chaperone systems (*bmh*2 + PDI) and pilot-scale fermentation. This study underscores Indonesia's microbial biodiversity as a resource for novel biocatalysts.

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REFERENCES

Adetunji AI, Olaniran AO. 2021. Production strategies and biotechnological relevance of microbial lipases: A review. Braz J Microbiol 52 (3): 1257-1269. DOI: 10.1007/s42770-021-00503-5.

Adina SR, Suwanto A, Meryandini A, Puspitasari E. 2021. Expression of novel acidic lipase from *Micrococcus luteus* in *Pichia pastoris* and its application in transesterification. J Genet Eng Biotechnol 19 (1): 55. DOI: 10.1186/s43141-021-00155-w.

Ali S, Khan SA, Hamayun M, Lee I-J. 2023. The recent advances in the utility of microbial lipases: A review. Microorganisms 11 (2): 510. DOI: 10.3390/microorganisms11020510.

Arifin AR, Kim S-J, Yim JH, Suwanto A, Kim HK. 2013. Isolation and biochemical characterization of *Bacillus pumilus* lipases from the Antarctic. J Microbiol Biotechnol 23 (5): 661-667. DOI: 10.4014/jmb.1212.12040.

Bui THD, Labedzka-Dmoch K. 2024. RetroGREAT signaling: The lessons we learn from yeast. IUBMB Life 76 (1): 26-37. DOI: 10.1002/iub.2775.

Cámara E, Landes N, Albiol J, Gasser B, Mattanovich D, Ferrer P. 2017. Increased dosage of AOX1 promoter-regulated expression cassettes leads to transcription attenuation of the methanol metabolism in *Pichia pastoris*. Sci Rep 7: 44302. DOI: 10.1038/srep44302.

Chandra P, Enespa, Singh R, Arora PK. 2020. Microbial lipases and their industrial applications: A comprehensive review. Microb Cell Fact 19 (1): 169. DOI: 10.1186/s12934-020-01428-8.

Gaol MDFL, Satya AA, Puspitasari E, Mubarik NR, Suwanto A. 2020. Increasing hydrolytic activity of lipase on palm oil by PCR-based random mutagenesis. Intl J Oil Palm 3 (3): 78-87. DOI: 10.35876/ijop.v3i3.53.

Gurunathan S, David D, Gerst JE. 2002. Dynamin and clathrin are required for the biogenesis of a distinct class of secretory vesicles in yeast. EMBO J 21 (4): 602-614. DOI: 10.1093/emboj/21.4.602.

Hao Y, Zheng X, Zhang X, Zhang K, Lin Y, Liang S. 2019. Combined strategies for engineering a novel whole-cell biocatalyst of *Candida rugosa* lipase with improved characteristics. Biochem Eng J 151: 107337. DOI: 10.1016/j.bej.2019.107337.

Herod SG, Dyatel A, Hodapp S, Jovanovic M, Berchowitz LE. 2022. Clearance of an amyloid-like translational repressor is governed by 14-3-3 proteins. Cell Rep 39 (5): 110753. DOI: 10.1016/j.celrep.2022.110753.

Huang J, Zhao Q, Chen L, Zhang C, Bu W, Zhang X, Zhang K, Yang Z. 2020. Improved production of recombinant *Rhizomucor miehei* lipase by coexpressing protein folding chaperones in *Pichia pastoris*, which triggered ER stress. Bioengineered 11 (1): 375-385. DOI: 10.1080/21655979.2020.1738127.

Jiao L, Zhou Q, Su Z, Yan Y. 2018. Efficient heterologous production of *Rhizopus oryzae* lipase via optimization of multiple expression-related helper proteins. Intl J Mol Sci 19 (11): 3372. DOI: 10.3390/ijms19113372.

Jiao L, Li W, Li Y, Zhou Q, Zhu M, Zhao G, Zhang H, Yan Y. 2022. Employing engineered enolase promoter for efficient expression of *Thermomyces lanuginosus* lipase in *Yarrowia lipolytica* via a self-excisable vector. Intl J Mol Sci 24 (1): 719. DOI: 10.1016/j.enzmictec.2015.09.003.

Kaczmarek MB, Struszczyk-Swita K, Xiao M, Szczęsna-Antczak M, Antczak T, Gierszewska M, Steinbüchel A, Daroch M. 2021. Polycistronic expression system for *Pichia pastoris* composed of chitino- and chitosanolytic enzymes. Front Bioeng Biotechnol 9: 710922. DOI: 10.3389/fbioe.2021.710922.

Kumar A, Lindley MR, Mastana SS. 2014. A time efficient adaptation of GC-FID method for the analysis of PBMC lipid composition. J Biochem Technol 5 (3): 760-764.

Kumar R. 2019. Simplified protocol for faster transformation of (a large number of) *Pichia pastoris* strains. Yeast 36 (6): 399-410. DOI: 10.1002/yea.3383.

Li X, Zimin L, Guilong W, Dujie P, Liangcheng J, Yunjun Y. 2016. Overexpression of *Candida rugosa* lipase Lip1 via combined strategies in *Pichia pastoris*. Enz Microb Technol 82: 115-124. DOI: 10.1016/j.enzmictec.2015.09.003.

Lim HK, Han Y-J, Hahm M-S, Park SY, Hwang IT. 2021. Isolation and characterization of a novel triolein selective lipase from soil environmental genes. Microbiol Biotechnol Lett 48 (4): 480-490. DOI: 10.48022/MBL.2007.07005.

Liu W, Li M, Yan Y. 2017. Heterologous expression and characterization of a new lipase from *Pseudomonas fluorescens* Pf0-1 and used for biodiesel production. Sci Rep 7 (1): 15711. DOI: 10.1038/s41598-017-16036-7.

Liu Y, Xie W, Yu H. 2014. Enhanced activity of *Rhizomucor miehei* lipase by deglycosylation of its propeptide in *Pichia pastoris*. Curr Microbiol 68 (2): 186-191. DOI: 10.1007/s00284-013-0460-0.

Masaryk J, Kale D, Pohl P, Ruiz-Castilla FJ, Zimmermannová O, Obšilová V, Ramos J, Sychrová H. 2023. The second intracellular loop of the yeast Trk1 potassium transporter is involved in regulation of activity, and interaction with 14-3-3 proteins. Comput Struct Biotechnol J 21: 2705-2716. DOI: 10.1016/j.csbj.2023.04.019.

Mazhar H, Afzal A, Aman S, Khawar MB, Hamid SE, Ishaq S, Ali SS, Zhu H, Hussain Z. 2023. Purification and characterization of lipase produced from *Bacillus cereus* (PCSIR NL-37). Rev Bionatura 8 (1): 58. DOI: 10.21931/RB/2023.08.01.58.

Mohammadzadeh R, Karbalaei M, Soleimanpour S, Mosavat A, Rezaee SA, Ghazvini K, Farsiani H. 2021. Practical methods for expression of recombinant protein in the *Pichia pastoris* system. Curr Protoc 1 (6): e155. DOI: 10.1002/cpz1.155.

Obsilova V, Obsil T. 2024. The yeast 14-3-3 proteins Bmh1 and Bmh2 regulate key signaling pathways. Front Mol Biosci 11: 1327014. DOI: 10.3389/fmolb.2024.1327014.

Ortiz C, Ferreira ML, Barbosa O, dos Santos JCS, Rodrigues RC, Berenguer-Murcia Á, Briand LE, Fernandez-Lafuente R. 2019. Novozym 435: The "perfect" lipase immobilized biocatalyst? Catal Sci Technol 9 (10): 2380-2420. DOI: 10.1039/c9cy00415g.

Peer M, Yuan H, Zhang Y, Korbula K, Novick P, Dong G. 2022. Double NPY motifs at the N-terminus of the yeast t-SNARE Sso2 synergistically bind Sec3 to promote membrane fusion. Elife 11: e82041. DOI: 10.7554/eLife.82041.

Puspitasari E, Rustam YH, Satya AA, Suwanto A, Wahyudi AT, Astuti RI. 2024. Effect of multiple gene copy number of bacterial lipase to increase lipase production in *Pichia pastoris.* Hayati J Biosci 32 (2): 310-319. DOI: 10.4308/hjb.32.2.310-319.

Raschmanová H, Weninger A, Knejzlík Z, Melzoch K, Kovar K. 2021. Engineering of the unfolded protein response pathway in *Pichia pastoris*: Enhancing production of secreted recombinant proteins. Appl Microbiol Biotechnol 105 (11): 4397-4414. DOI: 10.1007/s00253-021-11336-5.

Robert J, Betancur M, Machado A, Arruda A, Reis V, Almeida R, Torres F, Alegre P, Valero F, Freire D. 2019. Increase of Candida antarctica lipase B production under PGK promoter in *Pichia pastoris*: Effect of multicopies. Brazilian J Microbiol 50 (2): 405-413. DOI: 10.1007/s42770-019-00056-8.

Sá AGA, de Meneses AC, Lerin LA, de Araújo PHH, Sayer C, de Oliveira D. 2018. Biocatalysis of aromatic benzyl-propionate ester by different immobilized lipases. Bioprocess Biosyst Eng 41 (5): 585-591. DOI: 10.1007/s00449-018-1893-4.

Sabaria E, Yasmin Y, Ismail YS, Bessania MA, Putri I, Fitri L. 2024. Characterization of thermophilic bacteria from Ie Seum Hot Springs, Aceh Besar, Indonesia as producers of protease enzyme. Biodiversitas 25 (5): 1867-1874. DOI: 10.13057/biodiv/d250502.

Salvi HM, Kamble MP, Yadav GD. 2018. Synthesis of geraniol esters in a continuous-flow packed-bed reactor of immobilized lipase: Optimization of process parameters and kinetic modeling. Appl Biochem Biotechnol 184 (2): 630-643. DOI: 10.1007/s12010-017-2572-7.

Satya AA, Hermosaningtyas AA, Puspitasari E, Suwanto A. 2020. Expression and characterization of a novel cold-adapted lipase from psychrotolerant bacteria *Psychrobacter* sp. S1B. Malays J Microbiol 16 (2): 79-87. DOI: 10.21161/mjm.180177.

Singh R, Dien BS, Singh V. 2022. Solvent-free enzymatic esterification of free fatty acids with glycerol for biodiesel application: Optimized using the Taguchi experimental method. J Am Oil Chem Soc 99 (9): 781-790. DOI: 10.1002/aocs.12633.

Smidova A, Stankova K, Petrvalska O, Lazar J, Sychrova H, Obsil T, Zimmermannova O, Obsilova V. 2019. The activity of *Saccharomyces cerevisiae* Na+, K+/H+ antiporter Nha1 is negatively regulated by 14-3-3 protein binding at serine 481. Biochim Biophys Acta Mol Cell Res 1866 (12): 118534. DOI: 10.1016/j.bbamcr.2019.118534.

Terol GL, Gallego-Jara J, Martínez RAS, Vivancos AM, Díaz MC, de Diego Puente T. 2021. Impact of the expression system on recombinant protein production in *Escherichia coli* BL21. Front Microbiol 12: 682001. DOI: 10.3389/fmicb.2021.682001.

Verma S, Meghwanshi GK, Kumar R. 2021. Current perspectives for microbial lipases from extremophiles and metagenomics. Biochimie 182: 23-36. DOI: 10.1016/j.biochi.2020.12.027.

Vittaladevaram V. 2021. A short communication on *Pichia pastoris* vs. *E. coli*: effcient expression system. Ann Proteomics Bioinform 5 (1): 49-50. DOI: 10.29328/journal.apb.1001016.

Yan J, Han B, Gui X, Wang G, Xu L, Yan Y, Madzak C, Pan D, Wang Y, Zha G, Jiao L. 2018. Engineering *Yarrowia lipolytica* to simultaneously produce lipase and single cell protein from agro-industrial wastes for feed. Sci Rep 8 (1): 758. DOI: 10.1038/s41598-018-19238-9.

Yao W, Liu K, Liu H, Jiang Y, Wang R, Wang W, Wang T. 2021. A valuable product of microbial cell factories: Microbial lipase. Front Microbiol 12: 743377. DOI: 10.3389/fmicb.2021.743377.