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Research Article

Enhanced Lipase Production in *Pichia pastoris* via Multiple Copies of Bacterial Lipase Genes and Co-expression of the HAC1 Gene

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ABSTRACT

The *hac*1 gene, a key regulator of the untranslated protein response (UPR), was co-expressed in *Pichia pastoris* GS115 to enhance the production of a lipase from *Geobacillus stearothermophilus*. Multicopy lipase constructs (1X and 4X) were transformed with pPICZAwbe_*hac*1, generating GS115/T1.2RQ(1X)_*hac*1 and GS115/T1.2RQ(4X)_*hac*1 strains. The GS115/T1.2RQ(1X)_*hac*1 strain showed an 186% lipase activity after 120 hours versus the control (100%), while the GS115/T1.2RQ(4X)_*hac*1 strain showed a faster initial increase (38% at 48 hours) and 28% at 120 hours, which was beneficial for efficient enzyme production. Overexpression of the *hac*1 gene enhances lipase production because it activates UPR genes when the endoplasmic reticulum is stressed due to a large number of recombinant proteins and forms proteins that are not appropriately folded. SDS-PAGE and tributyrin plate assays confirmed extracellular lipase expression (~43 kDa). These results demonstrate that *hac*1 co-expression significantly ($p = 0.01$) enhances lipase production in *Pichia pastoris*, especially in lower-copy constructs. This is the first report of co-expressing *hac*1 with *Geobacillus stearothermophilus* lipase genes in yeast. The findings are expected to contribute to developing more efficient microbial cell factories for producing industrial enzymes.

1. Introduction

Lipase (EC 3.1.1.3) is a versatile hydrolase enzyme that catalyzes triacylglycerol breakdown into free fatty acids and glycerol. Lipase is widely used in various industrial sectors, including food processing, pharmaceuticals, detergents, and biofuel production, due to its broad substrate specificity and ability to function under mild reaction conditions (Sharma & Kanwar 2014; Mahyon *et al.* 2018; Chandra *et al.* 2020; Sumarsih *et al.* 2024). Bacterial lipase is one of the most interesting sources of lipase because of its high catalytic activity, thermostability, and ease of genetic manipulation (Ramnath *et al.* 2017; Syah *et al.* 2023).

The T1.2RQ lipase gene is a thermostable lipase-encoding gene derived from the thermophilic bacterium *Geobacillus stearothermophilus*, identified as a promising producer of robust lipases suitable for high-temperature industrial processes, along with a collection of PT WBE bacterial cultures. However, naturally occurring enzyme production often presents limitations such as low yields and complex purification steps. Thus, heterologous expressions have been widely adopted to increase enzyme production and simplify downstream processing. This bacterium was initially isolated from a hot spring on Seram Island, Maluku, Indonesia. This bacterium is a natural resource contributing of the diversity of bacterial lipase sources. The T1.2RQ lipase gene is well expressed in the *E. coli* expression system, which produces intracellular enzymes. The limitation of this expression system is that it requires an inefficient

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post-harvest process because inclusion bodies and proteins are often formed incorrectly. Thus, another expression system is required to produce extracellular enzymes. In addition, *E. coli* is not GRAS (Generally Recognised As Safe), which limits the application of the enzyme. *Pichia pastoris* offers an effective platform for producing extracellular recombinant proteins; however, each enzyme has a different expression profile, which can vary significantly (Lozano Terol *et al.* 2021; Viswanath 2021).

The methylotrophic yeast *Pichia pastoris* has emerged as a preferred host for recombinant protein expression due to its ability to grow to high cell densities, the presence of strong promoters (e.g., AOX1), and efficient secretion mechanisms (Liu *et al.* 2018). Moreover, *P. pastoris* can post-translational modifications and secretes relatively low levels of endogenous proteins, making it ideal for producing and purifying recombinant enzymes, including lipases, and has the potential to be used as a whole biocatalyst with the *Pichia* surface display system (Jiao *et al.* 2018a; Hao *et al.* 2019; Gupta *et al.* 2021; Mohammadzadeh *et al.* 2021). Bacterial lipase will be more easily expressed in the prokaryotic system, but achieving proper secretion and folding of extracellular enzymes will be a challenge (Liu *et al.* 2017; Yao *et al.* 2021). The *Pichia pastoris* system offers a solution to this problem. Many strategies have already been implemented. However, the experimental approach that combines the bacterial lipase multicopy gene with a co-expression chaperone gene in the expression of Pichia pastoris primarily results in a fungal lipase. Thus, the T1.2RQ bacterial lipase (*Geobacillus stearothermophilus*) with this approach is a novel finding of this study.

The T1.2RQ lipase gene has been successfully cloned into pPIC9K_ΔAOXOp at the HIS locus, forming a recombinant plasmid with up to six copies of the lipase gene integrated into the *Pichia* genome. It may produce more proteins than a single copy of the lipase gene (Puspitasari *et al.* 2025). Overexpression of recombinant proteins can result in inhibition of production and secretion because they exceed the secretory ability of the *Pichia pastoris* (*Komagataella* sp.), which causes the protein to misfold and accumulate in the endoplasmic reticulum (ER). This is naturally responded to by pathways (UPRs), which regulate the expression of genes encoding chaperones and helper proteins (e.g., Kar2p, Pdi1, Ero1p) through Hac1p transcription activators. Irreparably unfolded/misfolded proteins degrade through the ER-associated degradation pathway (ERAD), which decreases productivity. In this case, many approaches are carried out by expressing the UPR gene (untranslated protein response) together with the recombinant gene (Raschmanová *et al.* 2021). Despite these advantages, secretion bottlenecks can occur during complex or thermostable bacterial protein expression, often resulting from improper protein folding or overload in the endoplasmic reticulum. Recent strategies to address this include the co-expression of chaperone proteins and SNARE components (e.g., *hac*1, *bmh*2, *sso*2), which assist in protein folding and vesicle trafficking, thereby enhancing secretion efficiency (Zhou *et al.* 2020; Korpys-Woźniak & Celińska 2023). The *hac*1gene serves as a regulator of the unfolded protein response center (UPR). HAC1 plays an essential role in ER (endoplasmic reticulum) stress by promoting the transcription of companion proteins and other components of the ER quality control. The mechanism of the open protein response pathway is induced by the presence of stress on the endoplasmic reticulum (ER), which then activates an extensive transcription program to increase the folding capacity of the ER. The Ire1 endonuclease ejects introns from the HAC1 mRNA, allowing exons to be translated into proteins and inducing hundreds of stress response genes (Cherry *et al.* 2019). According to Jiang (2023), five different molecular chaperones (PDI, CPR5, ERO1, HAC1, and Bip) were used to improve the expression level of FumDM in Pichia pastoris (also known as *Komagataella phaffii*) GS115. The co-expression of different chaperones caused varying degrees of improvement in FumDM activity for FB1. IRE1 kinase/endoribonuclease, which senses the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum, plays an essential role in yeast and catalyses a committed step in the unconventional HAC1 mRNA splicing that encodes the transcription factor bZIP (Maldonado-Bonilla 2020). Ire1p is present in the ER membrane under normal conditions as a monomer related to Kar2/Bip (Guerfal *et al.* 2010). When ER stress occurs first, Kar2 dissociates from Ire1p, leading to the incorporation of Ire1p into the ER membrane, which results in trans autophosphorylation of the kinase domain (K) and simultaneous activation of endoribonuclease (R) activity. The activation of Ire1p initiates an unconventional mRNA splicing reaction, which removes introns from the HAC1 mRNA species, resulting in Hac1p. This transcription factor activates the target gene that codes for the companion, folding, and lipid synthesis, among other processes. This pathway is conserved throughout eukaryotes and is closely

associated with ER-related degradation (ERAD) and protein folding pathways. Each stage of gene expression is naturally monitored by a mechanism to maintain cellular homeostasis. In the UPR pathway, the mRNA encoding HAC1 is the target of the Isw1 RNA. Direct binding of Isw1 to the untranslated 3' region of HAC1 mRNA is required for accurate UPR reduction. Thus, the inactivation of ISW1 makes the cells sensitive to endoplasmic reticulum (ER) stress. ER's voltage-induced Isw1 binding to HAC1 mRNA provides a feedback loop that enhances UPR attenuation to guarantee homeostatic adaptation to ER stress (Matabishi-Bibi *et al.* 2022).

This study aims to investigate the expression of a thermostable lipase gene from *Geobacillus stearothermophilus* in *Pichia pastoris* and evaluate the effect of co-expressing the *hac*1 gene on enzyme activity and secretion. The findings are expected to contribute to developing more efficient microbial cell factories for producing industrial enzymes.

2. Materials and Methods

2.1. Strains and Materials

This research used *E. coli* DH5α (Novagen), *P. pastoris* GS115/T1.2RQ (1X), and *P. pastoris* GS115/T1.2RQ (4X) as hosts (WBE's collection). pPICZα-A and pPIC9K were purchased from Invitrogen (USA). NEB (USA) supplied all restriction enzymes, including Q5® High Fidelity DNA polymerase, T4 DNA ligase, and T4 polynucleotide kinase DNA polymerase. Go-Taq polymerase was purchased from Promega. The purification kit uses QIAprep Spin Miniprep, QIAquick PCR Purification, and QIAquick Gel Extraction from Qiagen (USA). Thermo Scientific (USA) provided the yeast DNA extraction kit. We bought HEPES Buffer from Bio-World (USA). With the Zeosin 25 µg/ml antibiotics (for pPICZα-A plasmid), *E. coli* was grown in LSLB (1% peptone, 0.5% yeast extract, 0.5% NaCl). For the growth of *P. pastoris*, YPDB (1% yeast extract, 2% peptone, and 2% dextrose) containing Geneticin 100 µg/ml antibiotics (for pPIC9K plasmid) was utilised. The pre-induction growing media for *P. pastoris* was BMGY (1% yeast extract, 2% peptone, 0.34% YNB, 4x10−5 % biotin, 1% glycerol, and 100 mM potassium phosphate buffer pH 6.0). For the *P. pastoris* induction media, BMMY (1% yeast extract, 2% peptone, 0.34% YNB, 4 × 10−5% biotin, methanol, and 100 mM potassium phosphate buffer pH 6.0) was utilised. Transformant GS115/T1.2RQ (4X)_*Bmh*2 from previous research as a positive control for lipase activity.

2.2. Vector Modification

The pPICZαA plasmid, measuring 3600 bp, was eliminated with α-factor signalling by the PCR overlapping extension method, producing the pPICZAwbe plasmid. Primers P15-AE-21_pPICZAwbe-F (forward) and P15-AF-21_pPICZAwbe-R (reverse) are used to amplify the plasmid region by excluding the α-factor signal peptide coding sequence, as illustrated in Figure 1. The PCR produced a plasmid without the α-factor signal peptide coding sequence. A 17-bp homologous sequence is present at both ends of the product, allowing it to form a circular plasmid. The parental plasmid is then degraded using the *Dpn*I enzyme, leaving it in the form of a vector that no longer has an α-factor signal. The reaction PCR mixture consists of primer forward (10 pmol) 1 µL, primer reverse (10 pmol) 1 µL, plasmid (300 ng/µL) 0.5 µL, Q5 Polymerase (2X) 25 µL, and deionised water 22.5 µL. PCR was conducted for 17 cycles with conditions predenaturation at 98°C for 5 minutes, denaturation at 98°C for 10 seconds, annealing at 50°C for 50 seconds, extension at 72°C for 30 seconds, and an additional extension at 72°C for 5 minutes.

2.3. Construction of Recombinant Plasmids pPICZAwbe_*hac*1

The *hac*1 gene was isolated from the *Pichia pastoris* GS115 genome using the PCR (Polymerase Chain Reaction) method. The PCR reaction was performed with pre-denaturation at 98°C for 5 min, followed by 30 cycles of 98°C for 45 s, 50°C for 30 s, and 72°C for 50 s, and a final extension at 72°C for 5 min. The final product will be 1200 bp. The *hac*1 gene was employed as an insert, and pPICZAwbe as a vector. The *hac*1 gene was double-digested with EcoR1 and Not1 and then cloned into EcoR1 and Not1-digested pPICZAwbe to produce pPICZAwbe_*hac*1 and then transformed into *E. coli* competent cells treated with $CaCl_2$. PCR confirmed the positive transformants.

2.4. Transformation into *E. coli* and *Pichia pastoris* GS115

There were two steps involved in transforming plasmids into *Pichia pastoris* cells. Initially, a ligated construct pPICZAwbe_*hac*1 (20 µL) was transformed into 100 µL of $CaCl_2$-treated competent cells *E. coli* DH5α using a heat–shock method (42°C, 1 min), recovered in 1 mL Luria Bertani, then shaken horizontally for 1 hour at 37°C. Transformants were spread on LSLB agar with 25 µg/mL Zeocin. Second, the recombinant plasmid was isolated and linearised using SacI, then

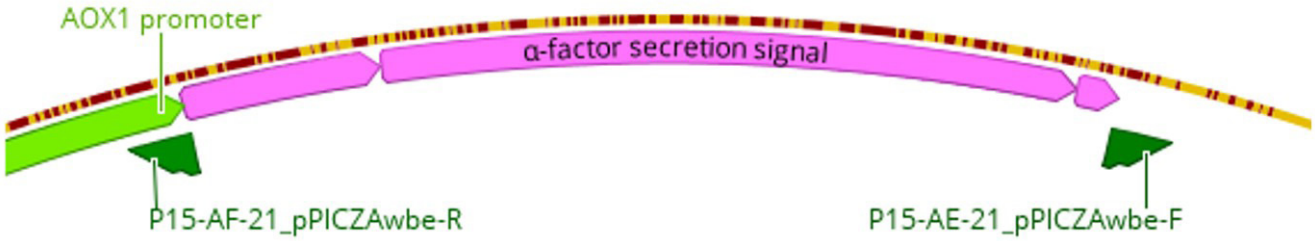

Figure 1. Positions of P15-AE-21_pPICZAwbe-F and P15-AF-21_pPICZAwbe-R in pPICZα A

transformed into GS115/T1.2RQ(1X) and GS115/T1.2RQ(4X) competent cells. To generate competent *P. pastoris* cells, a colony patch of approximately 2 × 1.5 cm was cultivated on YPD agar at 30°C for 18 to 24 hours. The harvested cells were suspended in 1 mL of YPD medium supplemented with 40 µL of 1 M DTT and 40 µL of 1 M HEPES-NaOH buffer (pH 8.0), then incubated with gentle agitation at 30°C for 15 minutes. Subsequently, the cells were washed twice with sterile water (centrifuged at 3000×g for 3 minutes each) and placed on ice for 3 to 5 minutes (Gasser & Mattanovich 2019; Kumar 2019).

Linearised plasmid DNA of 2–5 µg was mixed with 80 µL of competent *P. pastoris* cells and transferred into a pre-chilled 2 mm electroporation cuvette. After resting on ice for 5 minutes, electroporation was carried out using a Gene Pulser system (Bio-Rad, Hercules, CA, USA) with settings of 1.5 kV, 200 Ω, and 25 µF, resulting in a pulse of approximately 5 milliseconds. Immediately following the pulse, 1 mL of ice-cold 1 M sorbitol was added to facilitate recovery. When antibiotic selection was applied, the cells were incubated at 30°C for 1–2 hours without shaking before plating. The transformed cells were then spread onto YPDS agar plates containing 100 µg/mL Zeocin and incubated at 28–30°C for 2–3 days to allow colony development.

2.5. Enzyme Production

A single colony of *Pichia* transformant was inoculated into 50 mL of BMGY medium (pH 6.0) and incubated for 18 hours at 30°C and 225 rpm (OD_{600} = 3-6) following the protocol provided in the Pichia Expression Kit, Invitrogen-USA. Centrifugation at 4000xg using the centrifuge (Thermal Scientific) for 10 minutes at 22-25°C to collect the cells. Fifty millilitres of BMMY media resuspended the cell pellet (pH 6.0, OD_{600} = 1). Fermentation was conducted for 5 days, and the culture was maintained at 30°C and 225 rpm. Every 24 hours, methanol (1% v/v) was added to the culture to promote protein expression. Centrifugation was used to harvest the culture at 5000×g for 15 minutes at 4°C; the supernatant was a crude enzyme lipase.

2.6. Lipolytic Assay

Spectrophotometric measurement of lipase activity was performed using p-nitrophenyl palmitate (pNPP) as a substrate. Absorbances were determined at 405 nm using a SmartSpec™ Plus spectrophotometer (Bio-Rad, USA). The reaction mixture consisted of 40 µL of ethanol, 1 mM pNPP (10 µL of 10 mM pNPP in isopropanol), 940 µL of 100 mM Tris-HCl buffer (pH 8.0), and 10 µL of the enzyme. The mixture was then incubated at 50°C for five minutes, and its absorbance was measured. The analysis used Minitab version 22, with a significance threshold set at a P value below 0.05. Blanks were prepared based on the specific measurement conditions for each variable, excluding enzyme addition. The outcomes were presented as relative activity values to illustrate the effects of different variables on enzyme activity. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses the p-nitrophenyl substrate to release 1 µmol of p-nitrophenol in a minute under test conditions (Arifin 2013; Gaol *et al.* 2020).

2.7. Protein Analysis

Protein concentrations were measured using a Versa-Max microplate reader (Molecular Devices, USA) at 562 nm. Thermoscientific bicinchoninic acid (BCA) kits were utilised for analysis, and bovine serum albumin (BSA) was used as a reference protein at concentrations of 250, 125, 50, 25, 5, and 0 µg/mL. Each experiment was repeated three times. SDS-PAGE was utilised to determine the molecular weight of the lipase proteins, following a modified Laemmli protocol. The electrophoresis system included a 6% stacking gel and a 12% resolving gel. The analysis was carried out using a vertical mini-gel system (Bio-Rad, Hercules, CA, USA), and protein bands were visualised using Coomassie

Brilliant Blue R-250 staining (Amresco, Solon, OH, USA). For qualitative assessment of lipase activity, 20 µL of cell lysate was applied onto filter paper placed on agar containing tributyrin, followed by incubation at 37°C for 24 hours. A previously characterised *Bmh*2 protein served as the positive control (Satya *et al.* 2020).

3. Results

3.1. Construction of Recombinant Plasmids

The gene of *hac*1 isolated from the *Pichia pastoris* genome has a size of 1200 bp (Figure 2A) and was then cloned into the pPICZAwbe plasmid (Figure 3A),

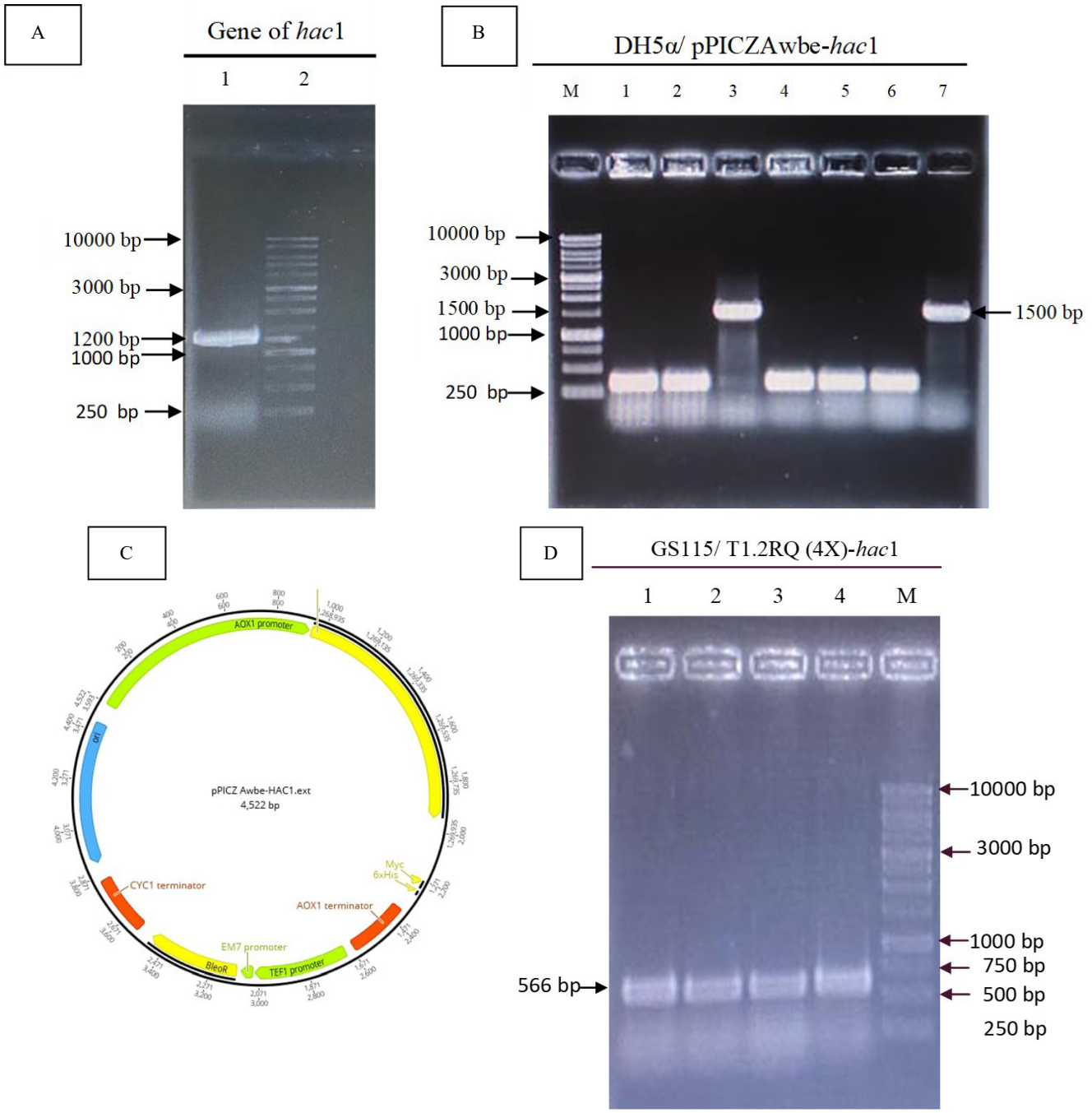

Figure 2. Transformants with the hac1 helper gene. (A) PCR result of the hac1 gene, (B) PCR result of *E. coli* DH5α/ pPICZAwbe-*hac*1 (colony 1-7), (C) map of recombinant plasmid pPICZA-*hac*1, (D) PCR result of Pichia GS115/ T1.2RQ (4X)-hac1 genome PCR (colony 1-4)

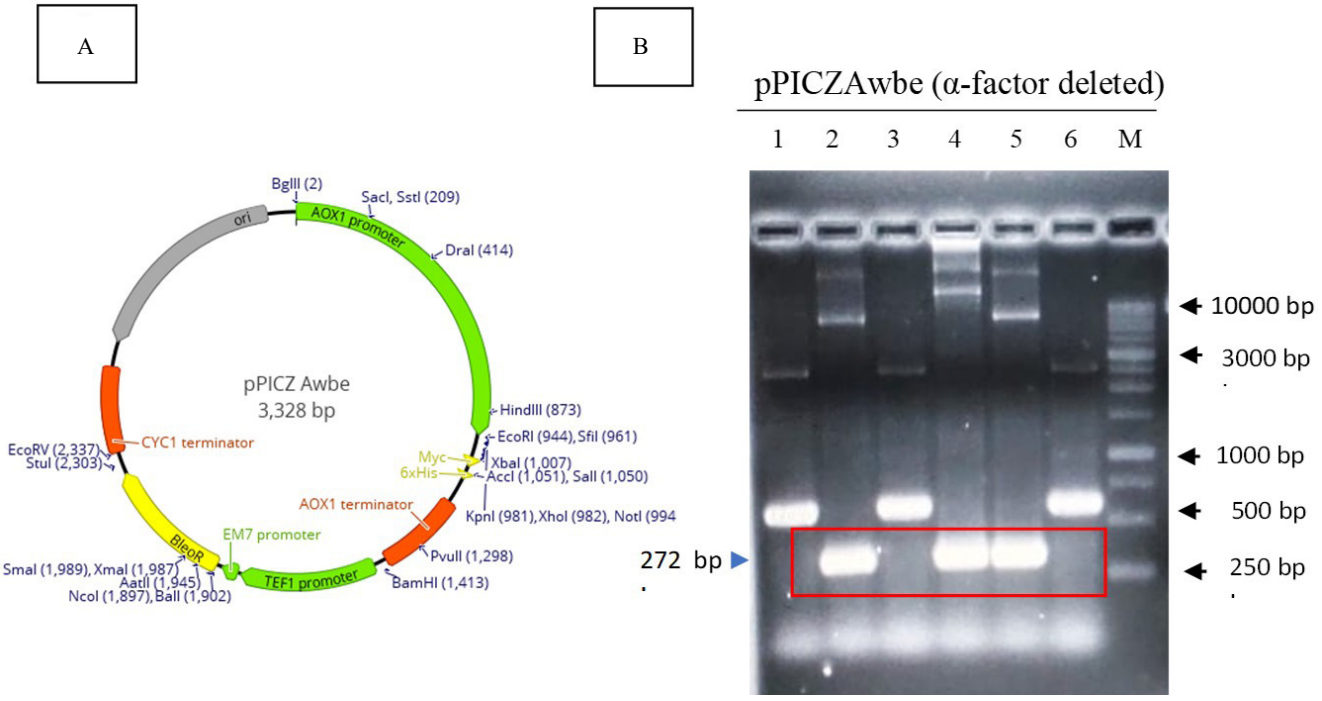

Figure 3. Vector modification. (A) Map of pPICZAwbe, (B) pPICZAwbe verification (α-factor deleted)

producing a pPICZA-*hac*1 recombinant plasmid with a size of 4,552 bp (Figure 2C). Figure 3B represented verification of the pPICZAwbe without the α-secretion signal factor, a size of 276 bp. The recombinant plasmid was transformed into *E. coli* DH5α, and the transformant was verified by PCR results (Figure 2B). The linearised recombinant plasmid was integrated into the Pichia genome, and PCR results confirmed the successful transformation (Figure 2D).

3.2. Lipolytic Activity

Figure 4 represents the relative activity of lipase enzymes from each transformant. Transformant GS115/T1.2RQ(1X)_*hac*1 showed a significant increase in lipase activity by 86% compared with transformant GS115/T1.2RQ(1X) at 120 hours of production time. Pichia pastoris GS115 as a control of host and transformant GS115/T1.2RQ(1X)Δα-factor as a control for the vector without α signal factor; both have no lipase activity. Transformant GS115/T1.2RQ(4X)_*hac*1 represented lipase activity increased by 38% achieved at 48 hours of fermentation, and tends to decrease until 120 h of fermentation.

3.2. Protein Analysis

Protein analysis for T1.2RQ (4X) with helper gene was performed on SDS-PAGE (Figure 5A). The band was about 43 kDa, confirmed by the size of the T1.2RQ gene (1200 bp). Qualitative tests of lysate on LA+TBN media formed a clear zone (Figure 5B).

4. Discussion

The *hac*1 gene is naturally present in the *Pichia* genome and acts as a transcriptional activator. Overexpression of the *hac*1 gene in the *Pichia* transformant is thought to increase lipase activity because it activates UPR genes when the endoplasmic reticulum is stressed by a large number of recombinant proteins that are not appropriately folded (Cámara *et al.* 2017; Schwarzhans *et al.* 2017; Jiao *et al.* 2018b; Gasser & Mattanovich 2019; Wang *et al.* 2019; Gasset *et al.* 2022).

In this study, the *hac*1 gene was isolated from the Genome of *Pichia* GS115 by the PCR method produced a size 1200 bp (Figure 2A) and then pPICZAα already was removed α secretion signal with confirmed with PCR product a size 276 bp using primers P04-57-5AOX1 and P04-11-10 3end AOX1 different with plasmid containings α secretion signal a size more than 500 bp (Figure 3A). We used transformant GS115/T1.2RQ(1X) Δα-factor as a control vector without a secretion signal to ensure construction compliance for intracellular protein; the result showed that no lipase activity was secreted from the cell (Figure 4). Hence, pPICZAwbe could be used as a vector for expressing

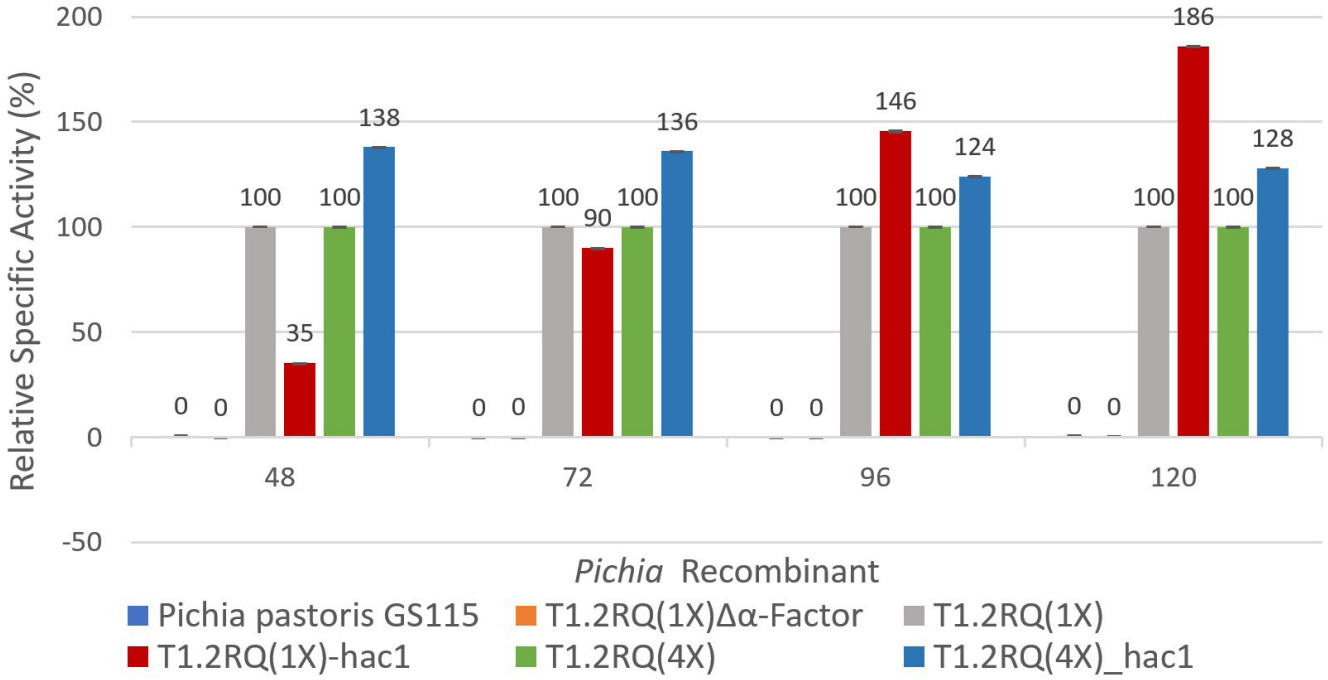

Figure 4. Effect *hac*1 gene on T1.2RQ lipase production (During Fermentation 120 h)

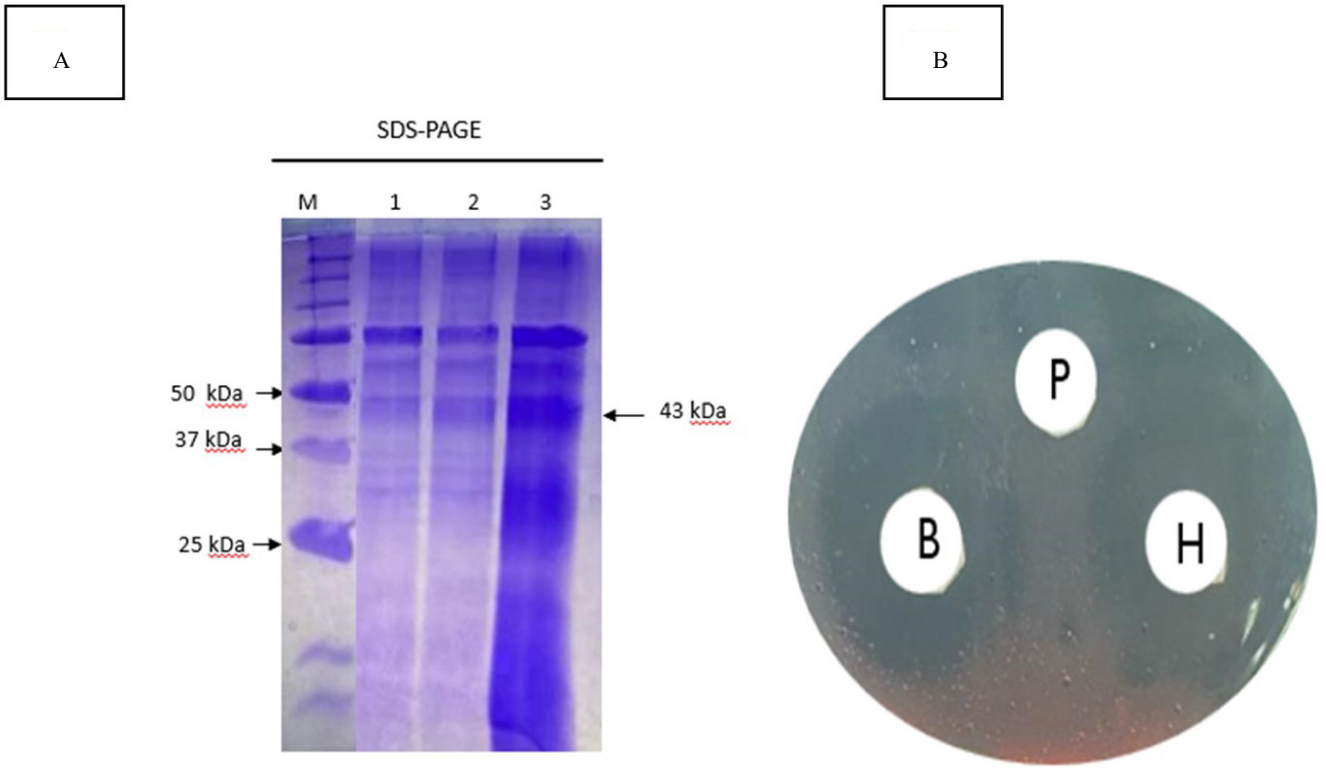

Figure 5. SDS-PAGE analysis and qualitative test in LBA +TBN Medium of *Pichia* recombinant. (A) M = Marker, 1) T1.2RQ (4X), 2) T1.2RQ (4X)_*hac*1, 3) T1.2RQ (4X)_*bmh*2), (B) B = T1.2RQ (4X)-*bmh*2, P = *Pichia pastoris* GS115, H = T1.2RQ (4X)-*hac*1.

the *hac*1 gene intracellularly (Figure 3B). The *hac*1 gene was cloned into pPICZAwbe, which does not have a secretion signal, and produced the recombinant plasmid pPICZAwbe-*hac*1, with a size of 4522 bp (Figure 2C).

pPICZAwbe-*hac*1 replicated in *E. coli* DH5α because the plasmid does not have an original replication (ori) in *Pichia pastoris*. Transformant verification using primer AOX forward and AOX1 reverse showed that two transformants have a size of 1500 bp (1200 bp was

the *hac*1 gene and a 300 bp fragment from the vector) in Figure 2B, representing the *hac*1 gene inserted in pPICZAwbe. The recombinant plasmid was linearised by *Sac*1 and transformed into *Pichia pastoris* GS115/T1.2RQ(4X) and *Pichia pastoris* GS115/T1.2RQ(1X), which have 4 and 1 lipase genes. Figure 2D shows the PCR product of a size 566 bp using primers AOX forward and P15-BW-23-HAC1-472 R that represented the *hac*1 gene inserted into the AOX locus in the *Pichia* genome.

Processing and folding proteins in the endoplasmic reticulum (ER) are significant impediments to the production and secretion of proteins from *Pichia pastoris* (*Komagataella* sp.). The transcription activator Hac1p contributes to restoring proper protein folding. The cell naturally triggers an unfolded protein response (UPR) pathway, which upregulates the expression of genes coding for chaperones and other folding-assisting proteins (e.g., Kar2p, Pdi1, Ero1p). Effect of *hac*1 gene in *Pichia* GS115/T1.2RQ(4X)-*hac*1 different with *Pichia* GS115/T1.2RQ(1X)-*hac*1 during 120 hours fermentation (Figure 4). Lipase specific activity of *Pichia* GS115/T1.2RQ(1X)-*hac*1 significantly increased by 86% during 120 h of fermentation. The increase in lipase-specific activity is a result of genetic engineering conducted in this research. Previous research has conducted multiple copies of the lipase gene. Statistical analysis using the Welch's t-test revealed these differences were statistically significant (P value <0,05). The co-expression of *hac*1 resulted in a considerable increase in lipase activity compared to the control (t-test, p = 0.01). However, in contrast with Pichia GS115/T1.2RQ(4X)-*hac*1, the lipase activity increases from the control but remains relatively constant during fermentation and even tends to decrease. This indicates a limitation of the Hac1 protein in supporting heterologous recombinant protein within the multicopy gene of lipase. Need another helper protein to process that protein. Irreparably unfolded/misfolded proteins degrade through the ER-associated degradation pathway (ERAD), which decreases productivity (Raschmanová *et al.* 2021). On the other hand, *Pichia* GS115/T1.2RQ(4X)-*hac*1 achieved an increase of lipase activity in a short time, which was beneficial for efficient enzyme production. According to Guerfal *et al.* (2010), co-expression of Hac1p increased, decreased, or did not affect the expression level depending on the heterologous protein. Their data represented that for the strains overexpressing the inducible Hac1p, the expression level was either lower or higher, as follows: a) in the strain dis- playing mouse interferon-γ, the expression level was 1.8- fold lower; b) in the strain displaying human interferon-β, the low initial expression level was completely abolished in the strain displaying human thrombomodulin, the expression level increased 1.9-fold compared to the refer- ence strain, and d) in the strain displaying human eryth- ropoietin, expression was 1.3-fold lower.

Protein analysis revealed that a protein of 43 kDa was the translation product from the T1.2RQ gene (1200 bp) as expected. Qualitative tests confirmed that the lysate from the transformant was a lipase, as it could degrade the TBN medium, forming a clear zone. The lysate was the supernatant from the enzyme production step without breaking cells, confirming that lipase was secreted extracellularly.

This study demonstrates that the co-expression of a thermostable lipase gene from *Geobacillus stearothermophilus* T1.2 (multicopy gene) in *Pichia pastoris* with the *hac*1 gene significantly enhances ($p<0.05$) enzyme activity and secretion, especially in lower-copy constructs (increased 86%). The *hac*1 gene was co-expressed under the regulation of the AOX promoter (inducible promoter). Future research was necessary to co-expression tandem with the other chaperons (e.g., *hac*1+*bmh*2, PDI, sso2). The findings are expected to contribute to developing more efficient microbial cell factories for producing industrial enzymes.

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