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Research Article

Effect of Multiple Gene Copy Number of Bacterial Lipase to Increase Lipase Production in *Pichia pastoris*

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ABSTRACT

Bacterial lipase poses potential challenges when expressed in eukaryotic protein expression systems such as *Pichia pastoris*. This research aims to increase extracellular T1.2RQ lipase secretion (free lipase) with multiple gene copy number strategies in *Pichia pastoris* and it was first performed on lipase from *Geobacillus stearothermophilus* T1.2. In this study, the T1.2RQ lipase gene from *Geobacillus stearothermophilus* T1.2 was expressed in *Pichia pastoris* GS115 through a strategy involving multiple copies of lipase, resulting in increased lipase activity. Three copies of the lipase gene in pPIC9K_T1.2RQ(3×) recombinant plasmid were integrated into the genome of *Pichia pastoris* GS115, and quantitative analysis using qPCR technique confirmed that the GS115 transformant strain contained six copies of T1.2RQ gene, indicating two integration events. Lipase activity measurement showed that the GS115/T1.2RQ(6×) strain exhibited a 111% increase compared to that containing a copy of the T1.2RQ gene. SDS-PAGE and Zymogram results showed a protein band with a size of 43kDa. Qualitative analysis in LA+TBN media of all strains containing the T1.2RQ gene showed clear zones. Lipase production in flask fermentation took at least 120 hours to produce the best lipase activity. Thus, strategies with multiple copy numbers of gene lipase have significantly increased the expression of the bacterial lipase gene in *Pichia pastoris* GS115.

1. Introduction

Enzymes are biocatalysts that function to accelerate chemical reactions. The growth of enzyme industries looks constant, reaching about 7% annually, and is projected to reach $10.5 billion by 2024 (Cavalcante *et al*. 2021). Lipase enzyme (EC 3.1.1.3) is a crucial biocatalyst capable of catalyzing hydrolysis and synthesis of long-chain acyl glycerol. It has been used in various industries such as food, pharmaceutical, organic, skin, cosmetic, detergent additives, animal feed, and biodiesel industries (Lim *et al*. 2021; Verma *et al*. 2021; Yang *et al*. 2021; Najm & Walsh 2022). Most of the biochemical industry is in a stage of rapid development, the leading cause of which is biocatalysis based on new technologies that attract markets to new areas (Angajala *et al*. 2016). This enzyme has received significant attention, especially from organic chemists, because of its general ease of handling, wide substrate tolerance, high stability to temperature and solvents, and convenient commercial availability (Kumar *et al*. 2016).

Lipase is a versatile and relatively static enzyme obtained from animals, plants, and microorganisms. However, the industry used only microbial-originated

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lipases significantly (Patel & Parikh 2022). Enzyme production from lipase bacteria easier and faster, beside that genetic engineering of bacteria has been studied a lot. Effective lipase production is facilitated by first screening the habitat of lipase-producing bacteria and optimizing production parameters afterward (Ilesanmi *et al*. 2020). T1.2RQ lipase gene initially isolated from a thermopile *Geobacillus stearothermophilus* T1.2 bacterium exhibited great potential for industrial applications and was expressed in the *E. coli* expression system (collected by R&D Biotechnology, PT WBE).

Biotechnology is essential in developing lipase enzymes suitable for industrial applications, particularly strain development. High-throughput experimental techniques with computational modeling could be combined with systems biotechnology to accelerate strain improvement for industrial purposes. Metabolic footprinting represents a holistic approach to gathering large-scale metabolomic information about a given biological system and is a driving force for systems biology and bioprocess development (Reiter *et al*. 2022). A viable bioprocess will be achieved if it has the concept of resilience in strain design (Olsson *et al*. 2022).

High enzyme prices constrain the use of lipase enzymes in the industry as they are usually purchased from overseas markets. The demand for microbial lipase is increasing, with manufacturers operating globally using lipase enzymes (Chandra *et al*. 2020). Sources of natural lipase-producing microorganisms are accessible to explore but usually exhibit low lipase activity. Researchers have isolated lipase-producing bacteria from various sources, such as wastewater, and optimized conditions for enhanced lipase production (Storz 2020).

Bacterial lipases are often complex to be expressed in their active form in the eukaryote expression system, such as *Pichia pastoris*. The resulting lipase catalytic activities were usually lower than those of *Escherichia coli* (Liu *et al*. 2017) due to differences in protein processing and folding mechanisms in both systems. Various ways have been implemented to overcome this challenge, but bacterial lipases generally perform better when expressed in prokaryotic than in eukaryotic systems (Yao *et al*. 2021).

However, prokaryotic expression systems such as *Escherichia coli* have limitations in expressing complex proteins and has problems with correct protein folding (Viswanath 2021). For example, the inability to perform post-translational modifications would affect protein functions and enzyme biological activity, especially for those requiring complex modifications. In addition, the lack of disulfide bond formation capability would result in incorrect protein folding, leading to the formation of insoluble aggregates in the cytoplasm. These aggregates (i.e., inclusion bodies) make protein purification more difficult (Terol *et al*. 2021). *Pichia pastoris* expression system can overcome these limitations. However, the optimal production conditions of recombinant proteins in the *Pichia pastoris* expression system differ according to the target protein. The uniqueness of each enzyme is a research gap that must be filled to obtain information about the expression of bacterial lipase, especially *Geobacillus stearothermophilus* lipase in the *Pichia* expression system.

Some strains of *Escherichia coli* are not considered as generally recognized as safe (GRAS) as they produce toxins or are resistant to some antibiotics, making them unsafe for consumption and posing potential harm to human health (Gras *et al*. 2019; Ramos *et al*. 2020).That this is what causes the need to conduct exploration to look for prokaryotic candidates other than *E.coli* as lipase bacteria producers that can be implemented in industry.

Gene expression in *Pichia pastoris* is enzyme-type dependent. The yield and folding of the protein may vary depending on the enzyme produced, even if they used a similar approach (Kaczmarek *et al*. 2021). Optimal enzyme production needs improvement, such as developing an efficient enzyme production strain, determining optimal enzyme expression conditions (including methanol consumption), and understanding the effects of the producing enzymes on the overall energetics, productivity, and substrate absorption rates in *P. pastoris* strains (Karbalaei *et al*. 2020; Sinzinger *et al*. 2024). Moreover, optimizing methanol utilization pathways in *P. pastoris* strains through a metabolic engineering pathway would increase recombinant proteins' specific growth rate and overall productivity. This strategy, combined with developing efficient expression strains and utilizing advanced genetic editing systems, contributes to overcoming challenges and increasing enzyme production in *Pichia pastoris* (Wu *et al*. 2023; Zha *et al*. 2023). According to Robert *et al*. (2019) and Che *et al*.(2020), to improve the protein production of *Pichia pastoris*, several studies have focused on a combination strategy of codon optimization, gene dosage, and process optimization

of the desired gene. A genetic engineering approach with a multicopy of lipase-encoding genes is expected to increase mRNA and proteins.

This research is necessary because it will show how lipase-encoding genes from bacteria, specifically *Geobacillus stearothermophilus* T1.2, are expressed in the expression system of eukaryotes *Pichia pastoris*. Previous study have shown that prokaryotic lipases are rarely expressed in expression system of eukaryot *Pichia pastoris* with multicopy genes strategy. This research aims to increase extracellular T1.2RQ lipase secretion (free lipase) with multiple gene copy number strategies in *Pichia pastoris*.

2. Materials and Methods

2.1. Strains and Materials

In this study, pGEMT-easy was purchased from Promega (USA), while pICZα-A and pPIC9K were purchased from Invitrogen (USA). *E. coli* DH5α (Novagen) and *P. pastoris* GS115 (Invitrogen) were used as hosts. All restriction enzymes, Q5® High Fidelity DNA polymerase, T4 DNA ligase, and T4 polynucleotide kinase DNA polymerase, were purchased from NEB (USA). Go-Taq polymerase was purchased from Promega. The purification kit uses QIAprep Spin Miniprep, QIAquick PCR Purification, and QIAquick Gel Extraction from Qiagen (USA). The extraction kit for yeast DNA was purchased from Thermo Scientific (USA). HEPES Buffer was purchased from Bio-World (USA). LB (1% peptone, 0.5% yeast extract, 1% NaCl) and LSLB (1% peptone, 0.5% yeast extract, 0.5% NaCl) were used for *E. coli* growth with appropriate antibiotics. YPDB (1% yeast extract, 2% peptone, 2% dextrose) was used for the *P. pastoris* growth. BMGY (1% yeast extract, 2% peptone, 0.34% YNB, 4 × 10-5% biotin, 1% glycerol, and 100 mM potassium phosphate buffer pH 6.0) was used for the *P. pastoris* pre-induction growth medium. BMMY (1% yeast extract, 2% peptone, 0.34% YNB, 4 × 10-5% biotin, methanol, and 100 mM potassium phosphate buffer pH 6.0) was used for the *P. pastoris* induction medium. RDB was used for screening His transformant.

2.2. Construction of Recombinant Plasmids T1.2RQ Lipase and Vector Modification

T1.2RQL.Pp gene, a lipase gene with a codon optimized for heterologous expression in *Pichia pastoris*, was synthesized and delivered in the pMG plasmid (kanR) by Macrogen, namely pMG_T1.2RQL.Pp with a size of 3,838 bp.The T1.2RQL.Pp gene fragment was cloned into the pPICZαA plasmid by digestion-ligation method using *Kpn*I and *Eco*RI restriction enzymes and T4 DNA Ligase, respectively, resulting in the pPICZα A_T1.2RQL.Pp recombinant plasmid. The plasmid was transformed into competent *E.coli* DH5α cells to produce *E. coli* DH5α (pPICZα A_T1.2RQL.Pp) transformant.

To facilitate the construction of plasmid containing multicopy of lipase gene, modification of pPIC9K was performed by removing the fraction of AOX promoter and terminator, resulting in pPIC9K_ΔAOXOp. The pPIC9K recombinant plasmid contains a single copy of T1.2RQL.Pp gene was performed by ligation of T1.2RQL.Pp gene fragment from pPICZα A_T1.2RQL. Pp into pPIC9K_ΔAOXOp backbone previously cleaved with SacI and BamHI restriction enzymes. The size of the recombinant plasmid, namely pPIC9K_T1.2RQL. Pp(SB)(1x) was 10,502 bp.

To generate recombinant plasmids harboring two copies of the lipase gene, the pPIC9K_T1.2RQL. Pp(SB)(1x) was used as a backbone and insert sources. Parallel double digestion of the plasmid with BamHI-NcoI and BglII-NcoI produced an 8.4 kb vector backbone containing a copy of the lipase gene and a 5.5 kb T1.2RQL gene insert, respectively. Since BamHI and BglII restriction enzymes produce compatible overhangs, ligation of the backbone and insert resulted in a recombinant plasmid pPIC9K_T1.2RQL.Pp(2x). In the same way, the recombinant plasmid pPIC9K_ T1.2RQL.Pp(3x) was created from pPIC9K_T1.2RQL. Pp(1x) and pPIC9K_T1.2RQL.Pp(2x). The recombinant plasmid pPIC9K_T1.2RQL.Pp(4x) was created from a recombinant plasmid pPIC9K_T1.2RQL.Pp(2x) as sources of backbond and insert.

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

Transformations of plasmids into *Pichia pastoris* cells were carried out through two stages. Firstly, a ligated construct was transformed into *E.coli* using a heat-shock method DH5α competent cells. Secondly, the plasmid DNA was isolated and then linearized using restriction enzymes corresponding to the *Pichia* genome's insertion locus target. SacI was used for insertion at the AOX locus, while NcoI was used for the HIS locus. Electroporation transformed the linearized recombinant plasmid into previously treated HEPES buffer *Pichia pastoris* GS115 competent cells (Kumar 2019). The transformants were spread on selective RDB for HIS transformant screening or YPDS plates supplemented with antibiotics.

2.4. Enzyme Production

Enzyme production was conducted using the *Pichia* Expression Kit protocol (Invitrogen, USA). The transformant was inoculated into 50 ml BMGY medium (pH 6.0) at 30°C and 225 rpm for 18 h. The cells were collected by centrifugation at 4000 × g at room temperature for 10 min. The cell pellet was resuspended in 50 ml BMMY medium (pH 6.0). The culture was maintained at 30°C, 225 rpm for 120 h, and methanol (1% v/v) was added to the culture to induce protein expression every 24 h. The culture was harvested by centrifugation at 5000 × g at 4°C for 15 min. Lipase crude enzyme was collected from the supernatant.

2.5. Lipase Assay

Lipase activity was determined spectrophotometrically using *p*-nitrophenyl palmitate (pNPP) as a substrate (Arifin 2013). The reaction mixture consisted of 40 µL of ethanol, 10 µL of 10 mM pNPP in isopropanol, and 940 µL of 100 mM Tris-HCl buffer (pH 8.0) that contained 10 µL of the enzyme. The mixture was then incubated at 50°C for five minutes. Absorbances were measured at 405 nm using a SmartSpecTM Plus spectrophotometer (Bio-Rad, USA). One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol of *p*-nitrophenol in one minute under assay conditions through the hydrolysis of p-nitrophenyl substrate.

2.6. Protein Analysis

Quantitative analysis of crude extract enzymes was measured using bicinchoninic acid (BCA) kits (Thermoscientific) with bovine serum albumin (BSA) as standard proteins with concentrations of 250, 125, 50, 25, 5, and 0 µg/ml. Protein concentrations were measured using a Versa-Max microplate reader (Molecular Devices, USA) at a wavelength of 562 nm. The molecular weight characteristics of lipase proteins were obtained using qualitative analysis of the SDS-PAGE and zymograms methods. Protein samples were migrated on polyacrylamide gel (12%) and stained with Coomassie Brilliant Blue R-250. In addition, tributyrin (TBN) was used as a lipase substrate in the zymogram and YPD media.

2.7. Determination of Gene Copy Number

The described method uses MyGo Pro ESR Real-time PCR (Promega) to quantify the integrated expression cassettes. The thermal profile was initiated with 95°C to 55°C for 45 cycles by 2-step Amplification, Held at 96°C for 180s, Melting 60°C to 97°C at 0.1°C/s. PCR mixture consisted of 5 µL of SensiFAST HRM mix (2x), 1 µL of forward primer, 1 µL of reverse primer, 1 µL of DNA sample, and 2 µL distilled water to reach 10 µL of total volume. The standard curve was generated by amplifying pPIC9K_T1.2RQ_GAPDH recombinant plasmid as a DNA template. The genomic DNA of GS115/T1.2RQ transformants harboring 1 to 4 copies of the lipase gene were used as samples. The GAPDH gene is a housekeeping gene with one copy number in the *Pichia pastoris* genome. The copy number is calculated by absolute quantification, according to Abad (2010).

3. Results

3.1. Construction of Recombinant Plasmids

The pPICZαA-T1.2RQL.Pp recombinant plasmid was the first construct to harbor T1.2RQL. Pp lipase gene. The gene was then transferred to the pPIC9K expression vector as they have compatible restriction enzyme sites. Recombinant plasmids harboring multiple copies of the T1.2RQ.Pp gene was constructed by utilizing the pPIC9K_T1.2RQL_Pp_ (1x)_SB#11 recombinant plasmid containing one copy of the T1.2RQL lipase gene in pPIC9K as the template (Figure 1A).

Verification of *E.coli* DH5α/pPIC9K-T1.2RQ.Pp (1x) lipase transformant was conducted by PCR using primers flanking a region spanning AOX promoter, T1.2RQ.Pp lipase gene and AOX terminator regions. The gel electrophoresis result (Figure 1B) shows DNA bands measuring 3,070 bp, indicating correct plasmid constructions of transformants 1 to 4 (lanes b to e). Similarly, PCR using P06_AI and P06_AJ primers flanking a full T1.2RQ.Pp lipase gene was utilized to verify the integration of the lipase gene into the *Pichia pastoris* GS115 genome. (Figure 1C) shows gel electrophoresis of PCR products of *Pichia* GS115/pPIC9K-T1.2RQ (1x) transformants. DNA band measuring 1,164 bp indicated that one copy of the lipase gene was successfully inserted into the *Pichia* chromosome. The size of recombinant plasmids harboring 1 to 4 copies of the lipase gene was confirmed by electrophoresis, as shown in Figure 2A. Figures 2B-D show the maps of recombinant plasmids containing 2 to 4 copies of T1.2RQ.Pp gene. PCR-based verification was also performed using primers P04_U5_HIS and P04_U4_HIS on the transformants. PCR products

502 bp (Figure 2E) confirmed successfully inserting the target gene T1.2RQ.Pp in HIS locus of the *Pichia* chromosome. Table 1 shows the various genes and recombinant plasmids used or designed in this study.

3.2. Enzyme Production of T1.2RQ Lipase

Enzyme lipase production of T1.2RQ.Pp was carried out for 120 hours with 1% (v/v) methanol induction. Figure 3A shows the lipase relative activities of transformants harboring 1 to 4 copies of the gene at various time points. Lipase activity of GS115/T1.2RQ(3x) was 111% higher than GS115/T1.2RQ(1x). Protein analysis for T1.2RQ (3x) lipase was performed using SDS-PAGE and Zymogram. T1.2RQ lipase was indicated by a protein band of about 43 kDa on the gel (Figure 3B). The activity was

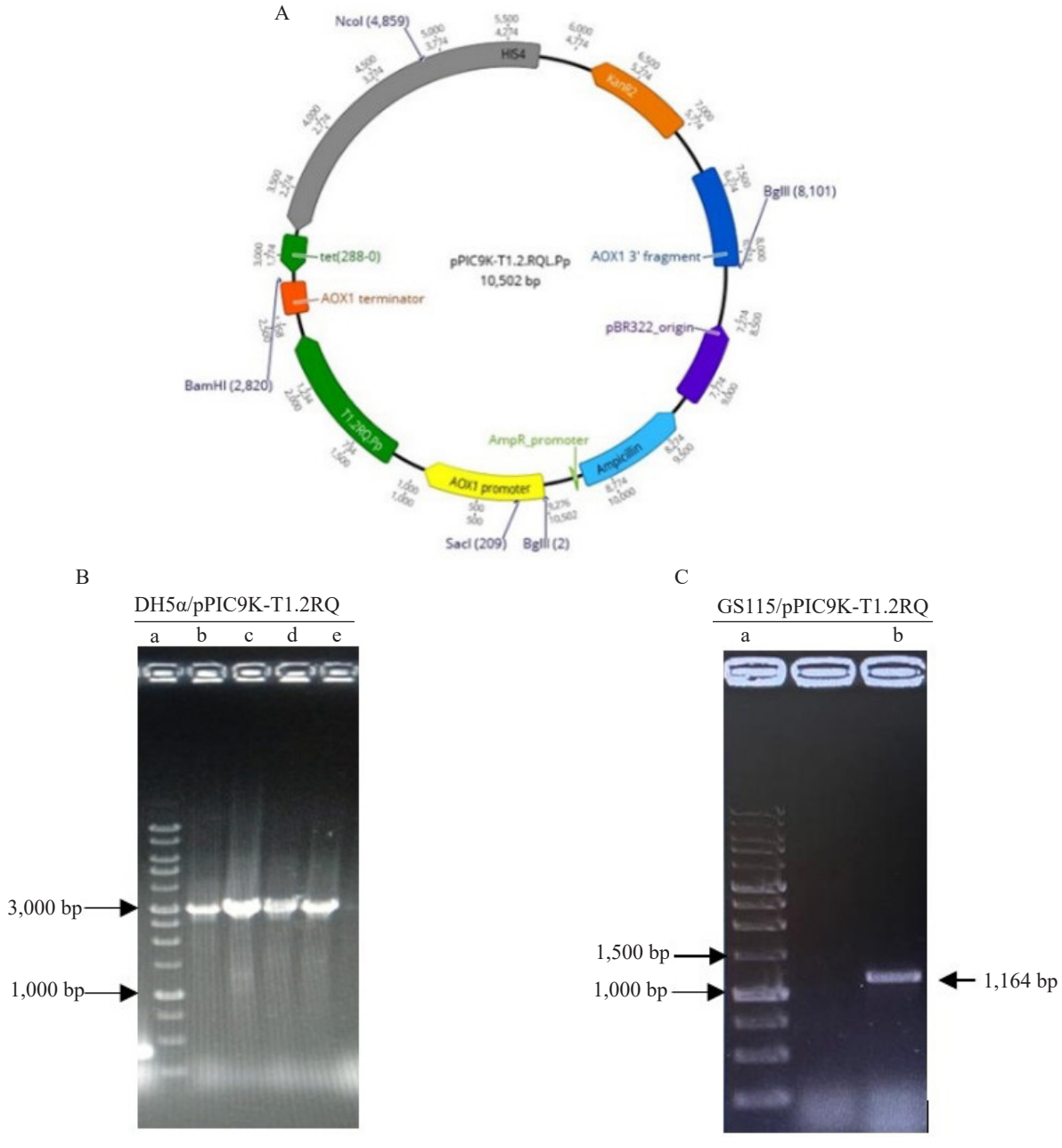


Figure 1. Genetic map of recombinant plasmid and electrophoresis result; (A) Genetic map of recombinant Plasmid pPIC9K_T1.2RQL_Pp_(1x)_SB#11 (10.502 bp). (B) Colony PCR of *E. coli* DH5α/pPIC9K-T1.2RQ# 1-4 and (C) verification of *P. pastoris* GS115/pPIC9K-T1.2RQ using PCR genome

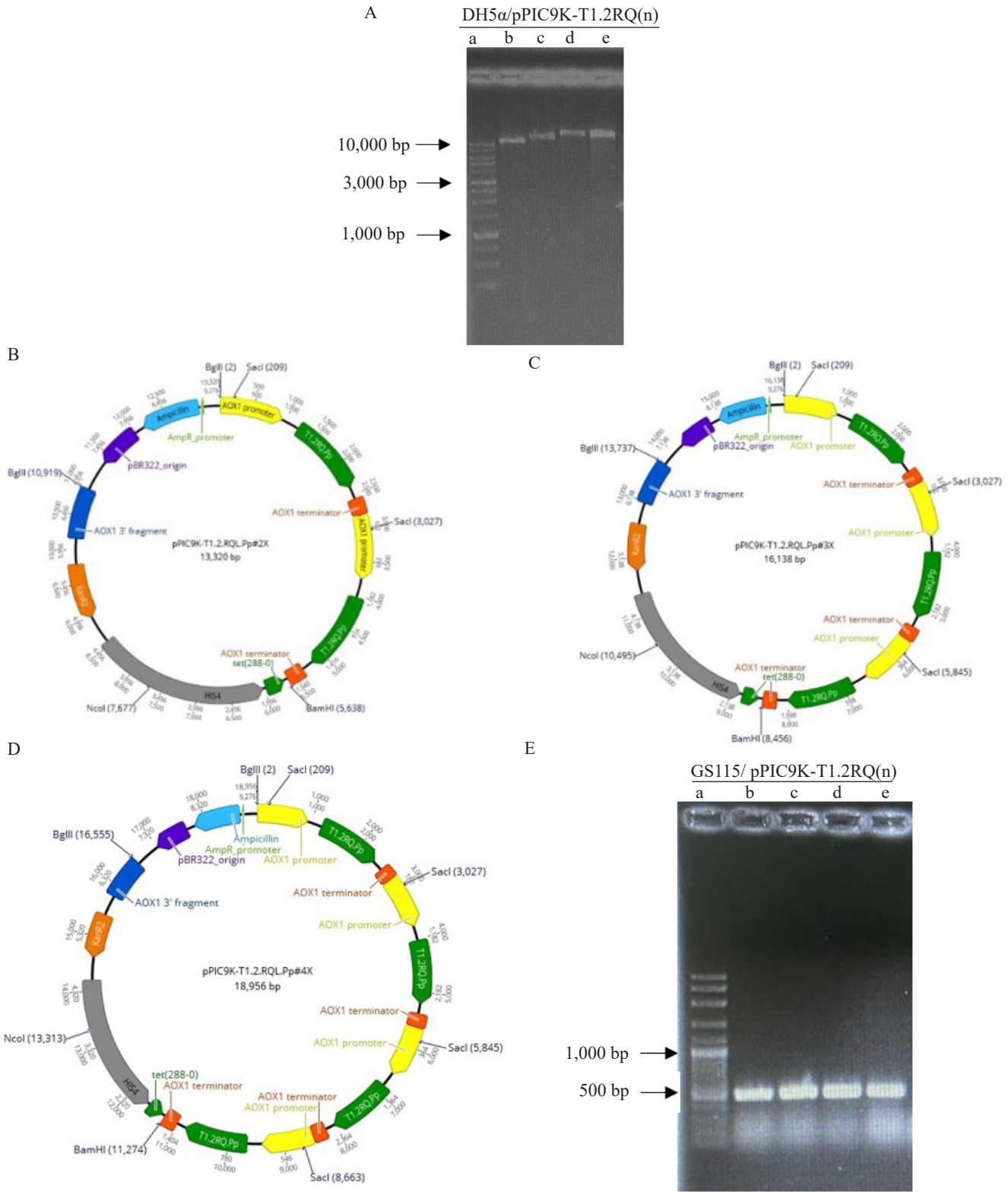
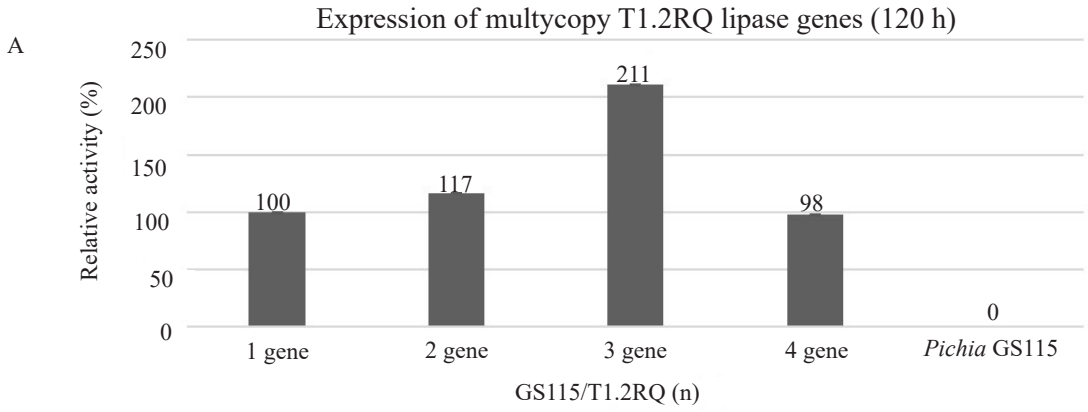
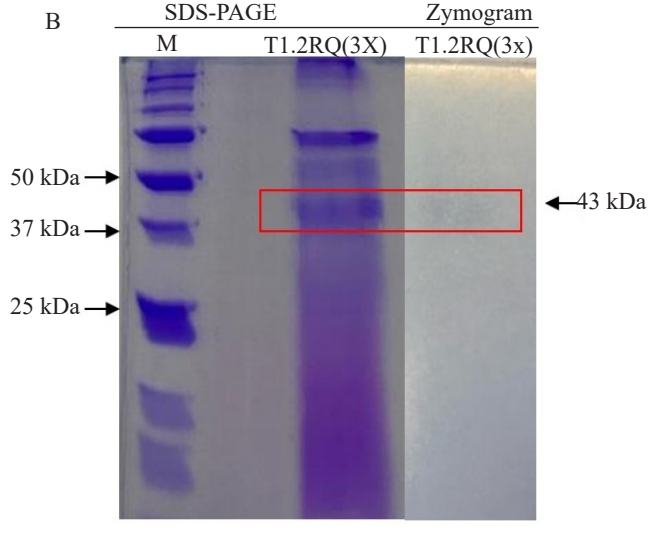


Figure 2. Transforman verification of multicopy T1.2RQ(n) and map of recombinant plasmid (A). Electrophoresis result of Sac1-digested of recombinant plasmid; (a) Marker 1 kb Ladder, (b) DH5α/pPIC9K-T1.2RQ (1x) has a size of 10,502 bp, (c) DH5α/pPIC9K-T1.2RQ (2x) has a size of 13,320 bp, (d) DH5α/pPIC9K-T1.2RQ (3x) has a size of 16,138 bp, (e) DH5α/pPIC9K-T1.2RQ (4x) has a size of 18,956 bp. (B) Map of recombinant plasmid DH5α/pPIC9K-T1.2RQ (2x). (C). Map of recombinant plasmid DH5α/pPIC9K-T1.2RQ (3x). (D) Map of recombinant plasmid DH5α/pPIC9K-T1.2RQ (4x). (E) Transforman verification of GS115/pPIC9K-T1.2RQ(n); (a) Marker 100 bp Ladder, (b) GS115/pPIC9K-T1.2RQ (1x), (c) GS115/pPIC9K-T1.2RQ (2x), (d) GS115/pPIC9K-T1.2RQ (3x), (e) GS115/pPIC9K-T1.2RQ (4x)

Table 1. List of genes and plasmids used or designed in this study

Name	Type	Description
T1.2RQL.Pp	Gene	*G. stearothermophilus* T1.2 lipase gene with the codon optimized for expression in *P. pastoris* host cells
GAPDH	Gene	*GAPDH* gene isolated from *Pichia pastoris* GS115
pMG (T1.2RQL.Pp)	Recombinant Plasmid	Recombinant plasmid harboring one copy of T1.2RQL.Pp lipase gene cassette in pMG vector (R&D collection plasmid)
pPICZαA-T1.2RQL.Pp (n)	Recombinant Plasmid	Recombinant plasmid harboring T1.2RQL.Pp with 1-4 copy number gene in pPICZαA vector
pPIC9K-T1.2RQL.Pp(1x)	Recombinant Plasmid	Recombinant plasmid harboring one copy of T1.2RQL.Pp lipase gene cassette in pPIC9K vector
pPIC9K-T1.2RQL.Pp(2x)	Recombinant Plasmid	Recombinant plasmid harboring two copies of T1.2RQL.Pp lipase gene cassette in pPIC9K vector
pPIC9K-T1.2RQL.Pp(3x)	Recombinant Plasmid	Recombinant plasmid harboring three copies of T1.2RQL.Pp lipase gene cassette in pPIC9K vector
pPIC9K-T1.2RQL.Pp(4x)	Recombinant Plasmid	Recombinant plasmid harboring four copies of T1.2RQL.Pp lipase gene cassette in pPIC9K vector
pPIC9K-T1.2RQL.Pp(1x)_GAPDH	Recombinant Plasmid	Recombinant plasmid harboring one copy of T1.2RQL.Pp lipase gene cassette and one copy GAPDH gene in pPIC9K vector





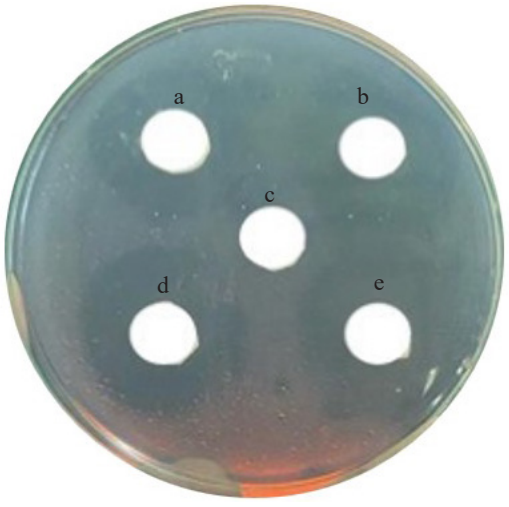


Figure 3. Transformant GS115/pPIC9K_T1.2RQ.Pp(n). (A) Lipase activity of transformant GS115/pPIC9K_T1.2RQ.Pp(n), (B) SDS-PAGE and Zymogram of transformant GS115/pPIC9K_T1.2RQ.Pp(3x), (C) lipase activity of GS115/pPIC9K_T1.2RQ.Pp(n) on LBA+TBN medium; (a) GS115/pPIC9K_T1.2RQ.Pp(1x), (b) GS115/pPIC9K_T1.2RQ.Pp(2x), (c) *Pichia pastoris* GS115 (control), (d) GS115/pPIC9K_T1.2RQ.Pp(3x), (e) GS115/pPIC9K_T1.2RQ.Pp(4x)

further confirmed by clear zones formed on zymogram and qualitative tests on LA+TBN media(Figure 3C). These results suggested that the T1.2RQ lipase gene was expressed in *Pichia pastoris* GS115.

3.3. Determination of Gene Copy Number

The copy number of the T1.2RQ.Pp lipase gene inserted in *Pichia pastoris* GS115 was confirmed by real-time PCR (SYBR Green Absolute Quantification) analysis, as shown in Table 2. The results indicated that T1.2RQ.Pp containing recombinant plasmids were integrated into the *Pichia* genome as expected, except for T1.2RQ.Pp (3x). Absolute quantification suggested that the GS115/T1.2RQ(3x) transformant harbored six copies of the lipase gene, probably resulting from two integration events. The verification results prove that the gene copy number significantly increases lipase activity, with the GS115/T1.2RQ(3x) transformant having the highest lipase activity.

4. Discussion

Lipase enzymes as industrial components are still imported, so exploring lipase enzyme-producing organisms is essential. High enzyme prices constrain the use of lipase enzymes in the industry as they are usually purchased from overseas markets. The demand for microbial lipase is increasing, with manufacturers operating globally using lipase enzymes (Chandra *et al*. 2020).

Lipases expressed in natural microorganisms such as *Geobacillus stearothermophilus* T1.2 often exhibit low lipase activity (Apituley 2012). Getting microorganisms with high lipase activity is challenging due to several factors. The main challenges are optimizing conditions to increase lipase production, including pH, temperature, nitrogen and carbon sources, and agitation (Storz 2020).

In this study, overexpression of the T1.2RQ gene in *Pichia pastoris* was performed to overcome some *E. coli* expression system drawbacks. Especially not being able to produce extracellular enzymes. *Pichia pastoris* GS115 enables an extracellular expression of lipase, which streamlines the harvesting process with no breakdown of host cells required. *Pichia pastoris* is a commonly used expression system for producing recombinant proteins; it offers significant advantages over the *E. coli* expression system, such as avoiding problems with proper protein folding (Angajala *et al*. 2016).

Recently, lipase genes expressed in *Pichia* with a gene multicopy approach and helper genes have shown that lipase is derived from fungi and rarely from bacteria (Li *et al*. 2015; Jiao *et al*. 2018; Huang *et al*. 2020). The recombinant plasmid pPIC9K-T1.2RQ (1x) (Figure 1A) was utilized as a template to create a multicopy recombinant plasmid of 2-4 copies of the T1.2RQ gene. Each T1.2RQ gene cassette has one promoter until the terminator, producing one protein. In case there is no fusion protein. The expression systems of prokaryotes and eukaryotes are different, so there is a need for genetic engineering or modification of the organism according to purpose. Currently, research focuses heavily on screening various lipase strains, modification of existing lipase genes, analysis of modified lipase function, and broader application of lipase in industrial settings (Yao *et al*. 2021). In addition, applying *Pichia pastoris* GS115 as host can increase methanol consumption to improve the expression system (Gupta *et al*. 2021).

This approach, conducting genetic engineering in the cell nucleus area by multicopying the gene target, is one of the efforts to increase the number of mRNA and target proteins. However, the protein produced will impact the next secretory organelle system; in this case, it will affect the endoplasmic reticulum and the downstream of other secretion systems organelle in that pathway. Comprehensive research is required to get perfect results (Raschmanová *et al*. 2021).

Pichia pastoris GS115 needs to be conditioned according to the target enzyme. The development of lipase-producing strains with gene dose showed a positive impact, which can increase lipase activity. Figure 3A shows that GS115/T1.2RQ(3X) transformants with six copies of the lipase gene have

Table 2. Copy number of T1.2RQ gene

Sample	Copy number T1.2RQ and GAPDH	Copy number	
		Absolute quantification (T1.2RQ/GAPDH)	Expected copy number
GS115/T1.2RQ(1×)	6,310±0.46	0.85	1
GAPDH	7,455±1.31		
GS115/T1.2RQ(2×)	27,818±3.59	1.52	2
GAPDH	18,352±0.58		
GS115/T1.2RQ(3×)	668,473±12.60	6.17	3
GAPDH	108,324±0.82		
GS115/T1.2RQ(4×)	102,324±13.82	4.19	4
GAPDH	24,430±0.50		

*Deviation standard n = 3

the highest lipase activity, meaning that gene multicopy positively increases lipase activity. Extracellular lipase of GS115/T1.2RQ(3X) is confirmed by the results of SDS, with a protein band of about 43 kDa and a zymogram that produces a clear zone (Figure 3B). The results of qualitative tests in LBA TBN media showed that all transformant lipase secreted extracellular lipase enzymes, forming a clear zone in the media.

This study showed that the strategy of the multiple copy number of the T1.2RQ gene increased extracellular lipase production in *Pichia pastoris* and, for the first time, reported for *Geobacillus stearothermophilus* lipase. Gen T1.2RQ in as many as six copies increased lipase production by 111%. Qualitative tests showed protein bands with a size of 43kDA in both SDS-PAGE and Zymogram results. In addition, LA + TBN selective media produced a clear zone, and its lipase could degrade the substrate. This study's results positively impacted the expression of bacterial lipase genes in the expression system of *Pichia pastoris*.

The limitation of this research is that it must be supported by helper protein for optimal extracellular secretion. Further research must be done using the optimal number of copies of genes and co-expression with helper genes.

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