AsPac J. Mol. Biol. Biotechnol. 2024
Vol. 32 (3) : 113-127

Plasmid-based and genome-based expression of recombinant T1 lipase in sucrose-utilizing *E. coli* strain W

Siti Hajar Yusof[a], Adam Thean Chor Leow[a,b], Raja Noor Zaliha Raja Abd Rahman[a,c], Mohamad Syazwan Ngalimat[a], Si Jie Lim[a], and Suriana Sabri[a,c*]

[a]*Enzyme and Microbial Technology (EMTech) Research Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.*
[b]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.*
[c]*Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.*

Received 15th April 2024 / Accepted 25th June 2024 / Published 30th July 2024

Abstract. Given its thermoalkaliphilic properties, T1 lipase holds significant potential for diverse industrial applications. However, traditional expression methods in *Escherichia coli*, specifically the plasmid-based system, present challenges of exerting metabolic burden on host cells and elevated costs due to antibiotic usage. This study addresses these issues by pioneering the expression of recombinant T1 lipase in a sucrose-utilizing *E. coli* strain W, using molasses as an economical carbon source. The gene cassette (KIKO plasmid), containing the T1 lipase gene regulated by *tac* and *trc* promoters, was integrated into the *E. coli* genome *via* the λ Red recombinase system. T1 lipase was optimally expressed in shake flasks at 16°C and a 3% molasses concentration in M9 medium with 0.8 mM IPTG as inducer, yielding 0.44 U/mL activity in the genome-based system compared to 0.94 U/mL in the plasmid-based system. This study not only underscores the potential of employing sucrose-utilizing *E. coli* strain for industrial recombinant protein production but also highlights the need for further optimization of genome-based expression systems. It offers an alternative to reduce costs and enhance sustainability in the stable production of industrially relevant enzymes like T1 lipase, without the need for antibiotic supplementation, and has broader implications for leveraging inexpensive carbon sources like molasses in biotechnological applications.

Keywords: *E. coli* W, genome-based expression, molasses, plasmid-based expression, recombination, thermostable lipase

INTRODUCTION

Lipase can be applied in various industries including detergents, food, paper, and cosmetics, and is highly valued for its thermostability (Cheng & Nian, 2023; Hamdan *et al.*, 2021). Although most thermostable lipases are found in thermophilic bacteria, their native lipase production is often low due to lower growth rates and less compatible physiological properties during fermentation (Contesini *et al.*, 2020). Therefore, recombinant thermostable lipase production in heterologous expression hosts such as bacteria and yeasts are crucial to address such caveat. *Escherichia coli* serves as a prominent bacterial host for producing a diverse range of proteins, from pure, fine-quantity pharmaceuticals to commercial enzymes in bulk. In *E. coli* fermentation, securing cost-effective carbon sources is vital for maximizing production yields. Glucose, as a simple monosaccharide that accounts for 47% of the raw material cost, has been suggested to be replaced by glycerol which is the by-product of biodiesel production (Ferreira *et*

*Author for correspondence: Suriana Sabri, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
Email – suriana@upm.edu.my

al., 2018). However, glycerol still incurs comparable core costs (>30%) with nitrogen sources in autoinduction media such as complex and defined media with lactose as the inducer (Cardoso *et al.*, 2020). In non-autoinduction-defined media, the inducer emerges as the most significant cost component, followed by carbon (glycerol) (Cardoso *et al.*, 2020).

Molasses, a byproduct of sugar production from sugarcane and sugar beet, emerges as a promising candidate for biotechnological production through microbial fermentation due to its economic advantages (Erian *et al.*, 2018). With its richness in sucrose content, molasses has been effectively utilized as the carbon source for producing heterologous compounds (Feng *et al.*, 2023; Kingsly *et al.*, 2022), recombinant proteins (Akdağ & Çalık, 2015; Yaman & Çalık, 2017), and microbial biomass (Chin *et al.*, 2023). However, most *E. coli* strains capable of utilizing sucrose, a major component of molasses, are enteropathogenic, posing safety concerns. *E. coli* W, categorized as non-enteropathogenic and uniquely capable of metabolizing sucrose, presents an attractive option for industrial recombinant protein production using molasses as a carbon source (Arifin *et al.*, 2014; Lee & Chang, 1993).

Plasmid-based expression systems commonly employed for industrial protein production face challenges such as plasmid instability, high antibiotic costs, and environmental risks associated with antibiotic resistance dissemination (Lan *et al.*, 2021; Mittal *et al.*, 2018; Tan & Ng, 2020; Wang *et al.*, 2019; Xie, 2022). Additionally, plasmids can impose metabolic burdens on host cells, diminishing cell growth and recombinant protein productivity (Li & Rinas, 2020; Sun *et al.*, 2021; Weber *et al.*, 2021). Alternatively, genome-based expression systems offer a promising avenue for recombinant protein expression in *E. coli*. Various strategies exist for integrating target genes into the *E. coli* genome, including homologous recombination, lambda (*λ*) site-specific recombination, the recET system, CRISPR recombineering technique, and *λ* Red recombination technique facilitated by pKIKO plasmids (Egger *et al.*, 2020; Kim *et al.*, 2014; Sabri *et al.*, 2013; Sawitzke *et* al., 2022; Thomason *et al.*, 2016; Weber *et al.*, 2021; Yang *et al.*, 2014).

Considering the substantial capital required for recombinant protein and heterologous compounds production, minimizing costs through low-cost carbon sources has become increasingly attractive in *E. coli* fermentation (Carranza-Saavedra *et al.*, 2023; Lozano Terol *et al.*, 2019; Puetz & Wurm, 2019). Molasses, with its affordability and abundance, presents a compelling alternative to traditional carbon sources. Efforts to utilize molasses in *E. coli* fermentation have been hindered by the inability of most *E. coli* strains to metabolize sucrose and safety concerns associated with enteropathogenic strains (Jahreis *et al.*, 2002). However, *E. coli* W, distinguished by its non-enteropathogenic nature and sucrose utilization capabilities, demonstrates rapid, efficient sucrose metabolism with minimal acetate production, making it an ideal host for recombinant protein production using molasses (Arifin *et al.*, 2014). Such advantages relieve the stacking disadvantages of metabolic burdens (by-product secretion) caused by recombinant protein production and carbon overfeeding (Li & Rinas, 2020).

T1 lipase, a well-characterized thermostable lipolytic enzyme, has historically been expressed using plasmid-based systems in *E. coli*. Previous research has demonstrated high expression levels of T1 lipase under various promoters in plasmid-based systems, indicating its industrial potential (Leow *et al.*, 2007). However, this study marks the first report of T1 lipase expression using a genome-based expression system in sucrose-utilizing *E. coli* W. By leveraging the advantages of genome-based expression systems and sucrose-rich molasses as a cost-effective carbon source, this study paves the way for sustainable, economical production of industrially relevant enzymes like T1 lipase, with implications for various biotechnological applications.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are shown in Table 1 and Table 2, respectively. *E. coli* strains were grown in Luria Bertani (LB) medium (Sigma, USA) at 37°C for general cloning

and maintenance. For expression studies, *E. coli* strains were grown in M9 minimal medium (M9 salt [0.04 M Na_2HPO_4, 0.02 M KH_2PO_4, 9 mM NaCl, 0.02 M NH_4Cl], 4 μM thiamine, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with sucrose or molasses. The molasses used were pretreated fifth-grade molasses provided by Central Sugar Refinery, Malaysia. Ampicillin (100 μg/mL) or chloramphenicol (25 μg/mL) was included in the agar for post-transformation and post-marker removal screenings. Sucrose utilization experiments were performed using M9 minimal agar and MacConkey agar (Sigma, USA) supplemented with 2% (w/v) of sucrose.

Table 1. Bacterial strains used in this study

Bacterial strains	Reference/source
E. coli W	ATCC 9637[a]
E. coli TOP10	Invitrogen, USA
E. coli BW25141	(Haldimann & Wanner 2001)[b]
E. coli MG1655 harbouring pKD46	CGSC[1]
E. coli TOP10 harbouring pCP20	Invitrogen, USA
E. coli TOP10 harbouring pGEX_*T1*	This study
E. coli TOP10 harbouring pTrcHis_*T1*	This study
E. coli W/pGEX_*T1*	This study
E. coli W/pTrcHis_*T1*	This study
E. coli BW25141 harbouring pKIKO*arsBCm_tacT1*	This study
E. coli BW25141 harbouring pKIKO*arsBCm_trcT1*	This study
E. coli W/pKD46	This study
E. coli WΔ*arsB::tac_T1_FRT*-cat-*FRT*	This study
E. coli WΔ*arsB::trc_T1_FRT*-cat-*FRT*	This study
E. coli WΔ*arsB::tac_T1*	This study
E. coli WΔ*arsB::trc_T1*	This study

[a] Purchased from the American Type Culture Collection.
[b] Purchased from the *Coli* Genetic Stock Centre (CGSC).

Table 2. Plasmids used in this study

Plasmids	Purpose / Description	Reference/source
pGEX_*T1*	- Expression vector for T1 lipase gene under *tac* promoter - Template for amplification of T1 lipase gene cassette under *tac* promoter (GST tag)	(Leow *et al.*, 2007)
pTrcHis_*T1*	- Expression vector for T1 lipase gene under *trc* promoter - Template for amplification of T1 lipase gene cassette under *trc* promoter (His tag)	(Leow *et al.*, 2007)
pKIKO*arsBCm*	- A helper plasmid for easy knock-in of gene of interest (T1 lipase gene cassette) and knock-out of *arsB* gene using homologous recombination	(Sabri *et al.,* 2013)
pKD46	- Expression vector for λ Red recombination system	(Cherepanov & Wackernagel 1995)
pCP20	- Expression vector for flippase gene (*FLP-FRT* recombination)	(Datsenko & Wanner 2000)
pKIKO*arsBCm_tacT1*	- Recombinant plasmid pKIKO*arsBCm* carrying T1 lipase gene cassette (with *tac* promoter)	This study
pKIKO*arsBCm_trcT1*	- Recombinant plasmid pKIKO*arsBCm* carrying T1 lipase gene cassette (with *trc* promoter)	This study

Molecular techniques

Standard molecular cloning procedures (Sambrook & Russell, 2006) were employed. Primers used in this study are shown in Table 3. The plasmid DNA extraction was carried out using the PureYield™ Plasmid Miniprep System (Promega, USA) following the manufacturer's instruction. DNA fragments and polymerase chain reaction (PCR) products were electrophoresed and excised from a 1% agarose gel. Sequencing was conducted by MyTACG Bioscience Sdn. Bhd., Malaysia.

Integration of pGEX_T1 and pTrcHis_T1 into E. coli W

E. coli TOP10 harboring pGEX_*T1* and *E. coli* TOP10 harboring pTrcHis_*T1* were subjected for plasmid extraction after growing overnight in LB broth supplemented with 100 μg/mL ampicillin. Plasmids carrying T1 lipase were then transformed into the competent cells of *E. coli* W that was prepared using the calcium chloride method as previously described (Tu *et al.*, 2005), through the heat shock method (Froger & Hall 2007) before plating on LB agar supplemented with 100 μg/mL ampicillin. To verify the positive transformants, colony PCR was performed in 10 μL reaction mixture containing 5 μL 2× PCR Biotaq Mix (PCR Biosystem, London, UK), 0.2 mM p*TacT1*/F-*Spe*I or p*TrcT1*/F-*Spe*I forward primer, 0.2 mM *T1*/R-*Eco*RI reverse primer, 1.5 μL template DNA of *E. coli* W/pGEX_*T1* and *E. coli* W/pTrcHis_*T1*, respectively. The thermal cycler (Thermal Cycler S1000, Bio-Rad, USA) was set up as follow: initial denaturation step at 95°C for 4 min, 29 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec and extension at 72°C for 30 sec. A final extension step consisting of 72°C for 3 min was included. To further confirm the positive transformants, extracted plasmids from *E. coli* W/pGEX_*T1* and *E. coli* W/pTrcHis_*T1* were digested using restriction enzyme (RE), *Pvu*I.

Table 3. Primers used in this study

Primer name	Sequence (5'-3')[a]	Application
cscA-F2	ATGACGCAATCTCGATTGCATG	Amplify *cscA* gene
cscA-R2	TTAACCCAGTAGCCAGAGTGC	Amplify *cscA* gene
cscK-F2	ATGTCAGCCAAAGTATGGGTTTTAGG	Amplify *cscK* gene
cscK-R2	GATAAGAGCGACTTCGCCGTT	Amplify *cscK* gene
cscB-F2	ATGGCACTGAATATTCCATTCAGAA	Amplify *cscB* gene
cscB-R3	GTTTACGTCTATATTGCTGAAGGTAC	Amplify *cscB* gene
cscR-F2	ATGGCTTCATTAAAGGATGTCG	Amplify *cscR* gene
cscR-R2	TCAGGTGGAACAACGGATC	Amplify *cscR* gene
JSP22	TTCTGCGAAGTGATCTTCCG	Screening oligo for KIKO vector inserts
JSP123	CAACCTGGCTCGACAAAACT	Confirmation of *arsB* genomic insertion
JSP124	GTGTCACAAACAGCACAGGC	Confirmation of *arsB* genomic insertion
p*TacT1*/F-*Spe*I	CGCACTAGTTTGACAATTAATCATCGGC	Amplify T1 lipase gene from pGEX_*T1* (*Spe*I)
p*TrcT1*/F-*Spe*I	CGCACTAGTCTGTTGACAATTAATC	Amplify T1 lipase gene from pTrcHis_*T1* (*Spe*I)
T1/R-*Eco*RI	GCTGAATTCTTAAGGCTGCAAGCTC	Amplify T1 lipase gene from pGEX_*T1* and pTrcHis_*T1* (*Eco*R1)
pKD46(F)	GATTAGCGGATCCTACCTGACGC	Confirmation for the insertion of pKD46 plasmid
pKD46(R)	CCGGATATTATCGTGAGGATGCG	Confirmation for the insertion of pKD46 plasmid

[a] The restriction enzyme (RE) sites are underlined.

Integration of T1 lipase gene cassette into E. coli W genome

The T1 lipase gene cassettes (amplified from pGEX_*T1* and pTrcHis_*T1* for T1 lipase with *tac* and *trc* promoters, respectively) were incorporated into the *E. coli* W genome using homologous recombination aided by pKIKO vector for easy and rapid knock-in and knock-out of genes (Datsenko & Wanner, 2000; Sabri *et al*., 2013). T1 lipase gene cassettes with *tac* and *trc* promoters were integrated with restriction sites, *Spe*I and *Eco*RI, using the PCR technique for subsequent cloning. The gene cassettes were cloned into pKIKO vector at the *arsB* gene site. The PCR products (T1 lipase gene with *tac* and *trc* promoters integrated with restriction sites) and pKIKO*arsBCm* plasmid were ligated using T4 DNA ligase (Thermo Fisher, USA). The ligated product was transformed into *E. coli* BW25141 and later plated on LB agar supplemented with 25 µg/mL chloramphenicol. The positive transformant of *E. coli* BW25141 harboring pKIKO*arsBCm_tacT1* and pKIKO*arsBCm_trcT1* plasmids, respectively, were verified using colony PCR. The primers used were p*TacT1*/F-*Spe*I and *T1*/R-*Eco*RI for pKIKO*arsBCm_tacT1* and pTrcHis*T1*/F-*Spe*I and *T1*/R-*Eco*RI for pKIKO*arsBCm_trcT1*. To further confirm, recombinant plasmids (sequences provided as Supplementary Data under Supplementary Material) were verified through sequencing and restriction digestion using REs, *Spe*I and *Eco*RI.

To prepare pKIKO*arsBCm_tacT1* and pKIKO*arsBCm_trcT1* for further recombination, first, plasmids were linearized using a single cutter RE, *Nde*I. The linearized plasmids were treated with calf intestinal phosphatase (New England BioLabs, USA). The plasmids were then purified using Wizard SV Gel (Promega, USA) and PCR Clean-Up System (Promega, USA) according to the manufacturer's instruction. The linearized pKIKO*arsBCm_tacT1* and pKIKO*arsBCm_trcT1* were transformed into electrocompetent cells of *E. coli* W with *λ* Red helper plasmid (*E. coli* W/pKD46) according to the *λ* Red recombination technique as previously described (Sabri *et al*., 2013). The transformed cells of *E. coli* W were screened on LB agar supplemented with 100 µg/mL ampicillin and 25 µg/mL chloramphenicol. This transformation leads to the production of *E. coli* WΔ*arsB::tac_T1_FRT*-cat-*FRT* and *E. coli* WΔ*arsB::trc_T1_FRT*-cat-*FRT* strains. Colony PCR was performed to verify the successful transformation using JSP123 and JSP124 primers for both strains (*E. coli* WΔ*arsB::tac_T1_FRT*-cat-*FRT* and *E. coli* WΔ*arsB::trc_T1_FRT*-cat-*FRT*). To delete the chloramphenicol resistance gene, *E. coli* WΔ*arsB::tac_T1_FRT*-cat-*FRT* and *E. coli* WΔ*arsB::trc_T1_FRT*-cat-*FRT* strains were transformed with *FLP*-consisting pCP20 plasmid by electroporation couple with *FLP-FRT* recombination technique (Datsenko & Wanner 2000; Cherepanov & Wackernagel 1995). The positive transformants (*E. coli* WΔ*arsB::tac_T1* and *E. coli* WΔ*arsB::trc_T1*) that were screened on LB agar supplemented with 100 µg/mL ampicillin were verified by colony PCR using primer pair, JSP123 and JSP124, and further confirmed by sequencing (MyTACG Bioscience Sdn. Bhd., Malaysia). The PCR amplicons were run on 1% agarose gel at 100 V and 30 min. The gel was then post-stained with 5% Redsafe (iNtRON, Korea) solution for 30 min and bands were viewed using G:Box F3 Gel Documentation System (Sysgene, UK).

Expression of T1 lipase in E. coli W and its extraction

Expression of T1 lipase using plasmid-based and genome-based expression systems was carried out in LB broth. For the expression, the cells (OD_{600nm}=0.5) were induced with 0.05 mM isopropyl-β-d-thiogalactopyranoside (IPTG; Gold Biotechnology, USA) and incubated at 37°C with 200 rpm shaking. A final A_{600nm} of IPTG-induced cells (50 mL) after 15 h incubation was subjected for centrifugation at 12, 000 × *g*, 4°C for 10 min. The pellet was mixed with 2.5 mL of 50 mM glycine-NaOH buffer, pH 9 and sonicated at amplitude 25, duty cycle 30 and 4 min. It was then centrifuged at 12, 000 × *g*, 4°C for 20 min. The supernatant was collected for enzyme assays. All assays were performed at three technical replicates using three independent samples.

Determination of lipase activity

The colorimetric lipase activity assay was carried out according to Kwon & Rhee (1986). The mixture (2.5 mL olive oil emulsion, 20 µL 0.02 M $CaCl_2$ and 1 mL enzyme) was incubated at 70°C with 200 rpm shaking for 30 min (Leow et al.,

2007). Olive oil emulsion was prepared by 1:1 ratio (v/v) of olive oil (Bertolli, Italy) and 50 mM Glycine-NaOH buffer, pH 9 followed by stirring using a magnetic stirrer for 10 min. The reaction was terminated by adding 1 mL of 6 N HCl (Qrec, Malaysia) and 5 mL of isooctane (Qrec, Malaysia), following by agitating for 30 sec by using a vortex mixer. Copper acetate-pyridine reagent (1 mL) was added to the separated fatty acid's isooctane layer (4 mL). After vortexed for 30 sec, the mixture was left to stand for 30 min. Absorbance at 715 nm was taken and lipase activity was estimated by evaluating the amount of free fatty acids produced from the standard curve. One unit of lipase activity was described as the amount of enzyme giving out 1 μmole of fatty acid in 1 minute at 70°C. All measurements were carried out in triplicate, and from values, the average value was taken.

Optimization of T1 lipase expression in E. coli W

The optimization of T1 lipase production using a genome-based expression system was carried out in three different media: 1) LB broth; 2) M9 + 2% (w/v) of sucrose; and 3) M9 + molasses. The cell culture was induced with various IPTG concentrations (without, 0.2, 0.4, 0.6, 0.8, and 1.0 mM) when OD_{600nm} reached approximately 0.5. To determine the effect of temperature on lipase activity, the flasks containing induced cells were incubated at various temperatures (16, 23, 30, and 37°C). The optimization of molasses concentration was determined at 1, 3, 5, 7, and 9 % (w/v) of molasses. All flasks containing the induced cells were incubated in an incubator shaker at 200 rpm for 15 h throughout the optimization study. The supernatant from sonicated cells was subjected to enzyme assay.

Statistical analysis

Lipase activity of different *E. coli* W strains in LB broth and the effects of molasses concentration on lipase activity in *E. coli* WΔ*arsB::trc_T1* cultured in M9 medium were analyzed statistically using One-Way ANOVA. The effects of IPTG concentration and temperature in different media on lipase activity were analyzed using Two-Way ANOVA. All ANOVAs were followed with Tukey multiple comparisons. The lipase activity between *E. coli* W/pTrcHis_*T1* and *E. coli* WΔ*arsB::trc_T1* in the optimized medium was statistically compared using t-test (unpaired, non-parametric). All statistical analyses were performed using GraphPad Prism 9.2.0 at $p<0.05$, unless otherwise specified.

RESULTS

Transformation of E. coli W with recombinant plasmid carrying T1 lipase gene

In this study, *E. coli* W with its unique ability to utilize sucrose was confirmed using both enriched (M9 + 2% sucrose) and differential (MacConkey + 2% sucrose) media on agar plates (Figure 1A). The bacterium was then subjected to transformation with T1 lipase-containing plasmids. Two recombinant plasmids (pGEX_*T1* and pTrcHis_*T1*) carrying the mature T1 lipase gene (without signal peptide) were transformed into *E. coli* W. The results for successful transformation were confirmed using colony PCR. This resulted in the amplification of 1938 bp and 1513 bp of DNA fragments from the recombinant *E. coli* W/pGEX_*T1* and *E. coli* W/pTrcHis_*T1*, respectively (Data not shown). To further confirm the results, extracted plasmids from *E. coli* W/pGEX_*T1* and *E. coli* W/pTrcHis_*T1* were digested using *Pvu*I. The digestion of pGEX_*T1* plasmid using *Pvu*I led to the production of four fragments with sizes of 2865 bp, 1778 bp, 1022 bp, and 468 bp (Figure 1B), whereas digestion of pTrcHis_*T1* resulted in three fragments with sizes of 3963 bp, 1126 bp, and 468 bp (Figure 1C). The RE profile corresponded to the number of *Pvu*I sites, as confirmed by *in silico* analysis using Clone Manager software. Thus, the successful transformation of T1 lipase-harbouring plasmids into *E. coli* W was confirmed.

Integration of T1 lipase gene cassette into E. coli W genome

The integration of T1 lipase gene into the *E. coli* W genome was conducted using pKIKO plasmid with homologous recombination technique, while, the *FLP-FRT* recombination technique was used for removal of chloramphenicol resistance gene. At the molecular level, the successful transformants of *E. coli* WΔ*arsB::tac_T1* (with *tac*

promoter) and *E. coli* WΔ*arsB::trc_T1* (with *trc* promoter) were verified using colony PCR with primer pair, JSP123 and JSP124. The size of *E. coli* W wild-type amplicon was 1357 bp. After integration with the T1 lipase gene cassette, the size of the amplicon increased to 4441 bp (with *tac* promoter) and 4016 bp (with *trc* promoter). After the removal of chloramphenicol resistance gene, the size of the amplicon reduced to 3518 bp (with *tac* promoter) and 3093 bp (with *trc* promoter) (Supplementary Figure 1A). The *in silico* results of T1 lipase gene cassette integrated with *tac* and *trc* promoters into *E. coli* W genome is shown in Supplementary Figure 1B.

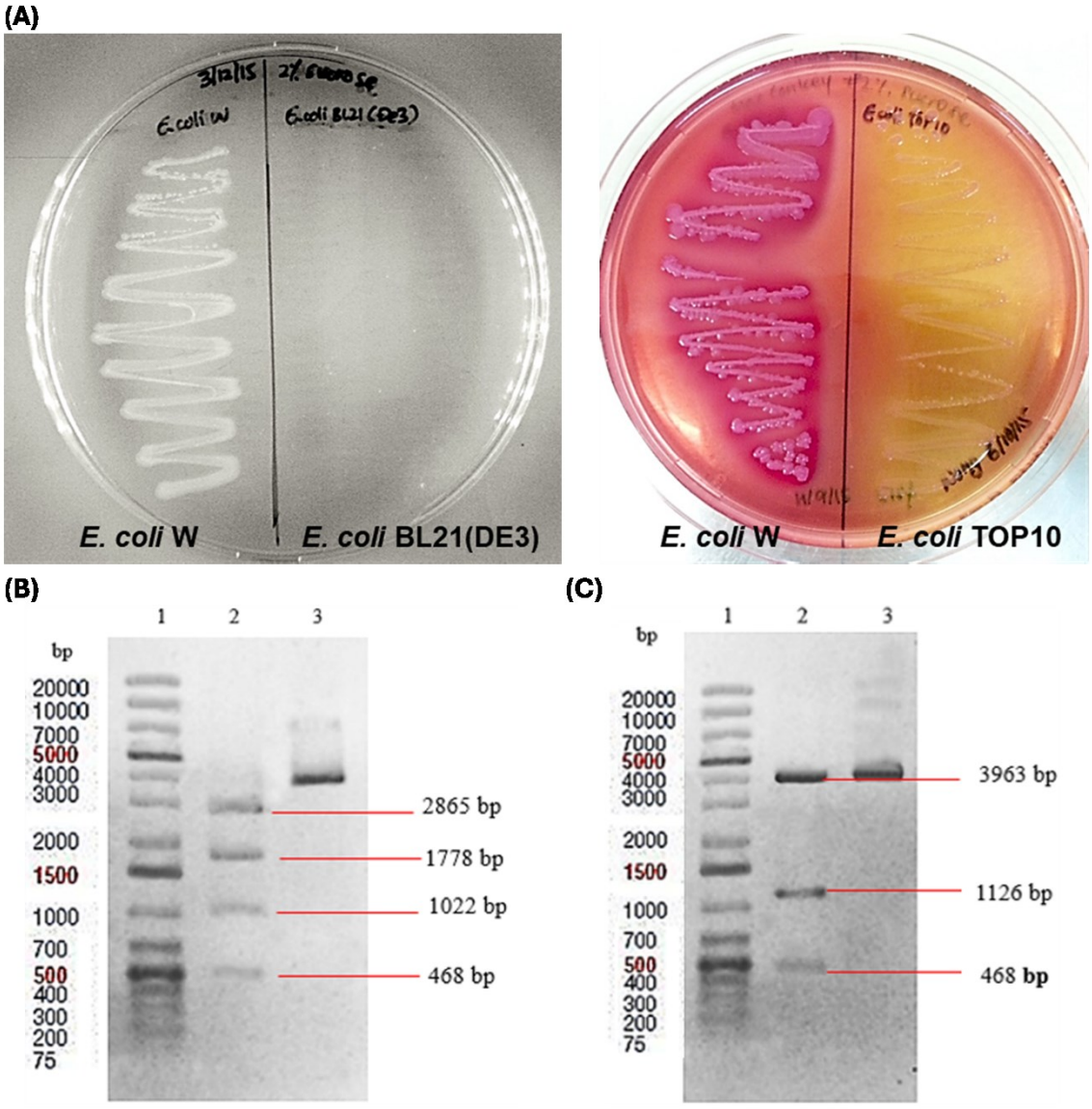

Figure 1. Confirmation of sucrose-utilizing ability of *E. coli* W and validation of vectors containing T1 lipase gene. **(A)** M9 + 2% sucrose (left panel) and MacConkey + 2% sucrose (right panel). *E. coli* W was streaked on the left half of the agars while *E. coli* BL21(DE3) and TOP10 were the negative controls for each agar. *E. coli* W exhibited positive sucrose-utilizing ability was shown by the pink zones around the colonies. RE profile for confirmation of recombinant plasmids pGEX_*T1* **(B)** and pTrcHis_*T1* **(C)** into *E. coli* W. Lane 1: GeneRuler 1 kb Plus DNA Ladder, Lane 2: pGEX_*T1* or pTrcHis_*T1* digested with *Pvu*I and Lane 3: undigested pGEX_*T1* or pTrcHis_*T1*. The plasmids were electrophoresed on 1 % (w/v) agarose gel and post-stained with RedSafe stain.

Expression of T1 lipase

The expression of T1 lipase using a plasmid-based and genome-based expression system under different promoters (*tac* and *trc*) was compared. The plasmid-based expression system strains, *E. coli* W/pGEX_*T1* (with *tac* promoter) and *E. coli* W/pTrcHi_*T1* (with *trc* promoter), and the genome-based expression system strains, *E. coli* WΔ*arsB::tac_T1* (with *tac* promoter) and *E. coli* WΔ*arsB::trc_T1* (with *trc* promoter), were used. The wild-type, *E. coli* W was used as the negative control. Overall, plasmid-based expression gave a higher T1 lipase activity as compared to the genome-based expression system (Figure 2). The lipase activities of the recombinant *E. coli* strains harboring recombinant plasmids were 0.94 U/mL (*E. coli* W/pGEX_*T1*) and 2.91 U/mL (*E. coli* W/pTrcHis_*T1*). The lipase activities from the recombinant strains carrying the *T1 lipase* gene in the genome were 0.21 U/mL (*E. coli* WΔ*arsB::tac_T1*) and 0.29 U/mL (*E. coli* WΔ*arsB::trc_T1*).

Optimization of T1 lipase expression in recombinant strains

After confirmation of successful T1 lipase expression, optimization was performed to improve the lipase production using *E. coli* WΔ*arsB::trc_T1* because *trc* promoter showed higher T1 lipase expression than *tac* promoter. The lipase expression by the recombinant strain was analyzed in three media: 1) LB broth; 2) M9 + 2% sucrose; and 3) M9 + molasses, with three parameters tested for the optimization including IPTG concentration, temperature, and molasses concentration.

To optimize the IPTG concentration, *E. coli* WΔ*arsB::trc_T1* was grown in the three media and induced with different IPTG concentrations (Figure 3). For LB medium, the highest lipase activity was recorded at 0.8 mM IPTG. The difference between 0.8 mM IPTG was noticeably higher, 0.67 U/mL, as compared with the other IPTG concentrations. Similarly, for M9 + molasses medium, the highest lipase activity was detected at 0.8 mM IPTG, 0.36 U/mL, as compared with the other concentrations. The lipase activity showed an increasing trend with the increment of IPTG concentration. However, higher than 0.8 mM IPTG induction in M9 + molasses medium resulted in a decrement of the lipase activity. On the other note, for M9 + 2% sucrose medium, lipase activity showed similar results despite a change in IPTG concentrations. Thus, the difference in lipase activity for optimization of IPTG in M9 + 2% sucrose was not statistically significant as compared to in LB and M9 + molasses media (Supplementary Table 1).

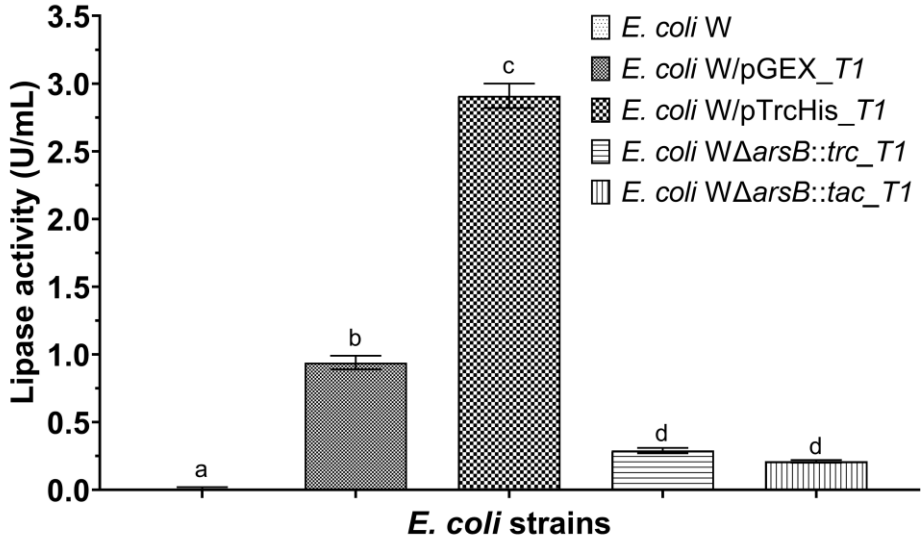

Figure 2. Lipase activity of recombinant strains grown in LB broth with 0.05 mM IPTG induction at 37°C. The wild-type *E. coli* W was used as the negative control. Data are presented as the mean ± standard deviation of three biological replications with three technical replicates (n=3). Statistical difference of lipase activity between *E. coli* strains ($p<0.05$) is stated as different alphabets above the bars.

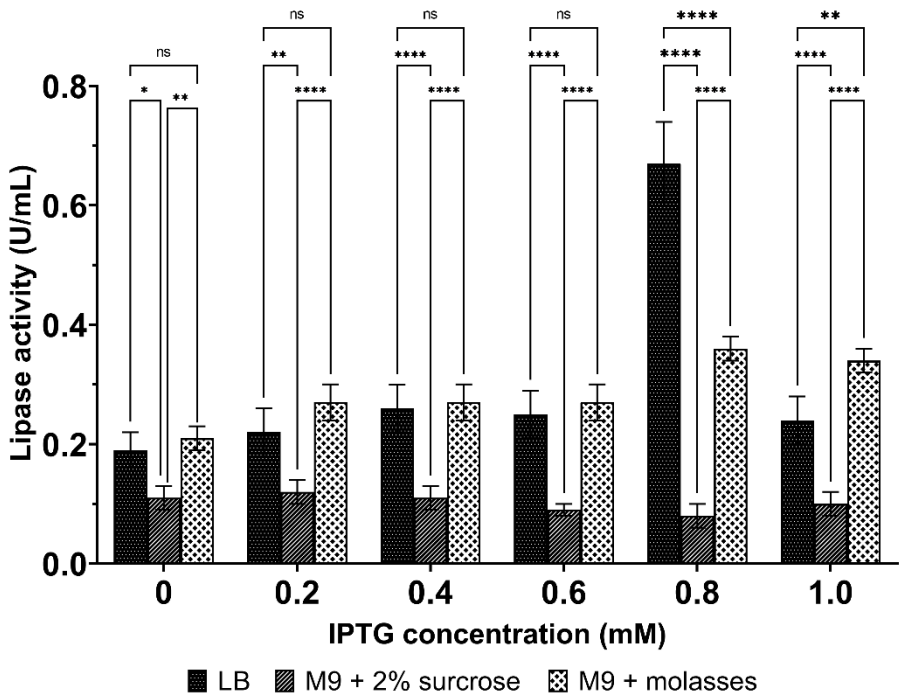

Figure 3. Optimization of T1 lipase expression in *E. coli* WΔ*arsB::trc_T1* using three culture media (LB, M9 + 2% sucrose and M9 + molasses) at different IPTG concentrations. Data are presented as the mean ± standard deviation of three biological replications with three technical replicates (n=3). Statistical difference of lipase activity between different media of the same IPTG concentration is stated as ns (non-significant; $p>0.05$), * ($p<0.05$), ** ($p<0.01$), and **** ($p<0.001$).

The expression of T1 lipase in *E. coli* WΔ*arsB::trc_T1* has been further optimized at different temperatures in the three media (Figure 4). The expression was induced with optimum IPTG concentration, 0.8 mM. For LB medium, the highest lipase activity was recorded at 16°C, 0.23 U/mL, as compared to other temperatures. In M9 + molasses medium, lipase activity showed a distinct trend of decreasing lipase activity with the increasing temperature. The highest lipase activity in M9 + molasses medium was detected at 16°C, 0.21 U/mL. For M9 + 2% sucrose medium, lipase activity showed similar results despite a change in temperatures. Thus, the difference in lipase activity for optimization of temperature in M9 + 2% sucrose is not noticeable as compared to in LB and M9 + molasses media (Supplementary Table 2).

To optimize molasses concentration, *E. coli* WΔ*arsB::trc_T1* was incubated in M9 medium supplemented with different concentrations of molasses (Figure 5). The cells were grown at optimum IPTG concentration (0.8 mM) and temperature (16°C). The highest T1 lipase expression was detected at 3% molasses (0.45 U/mL) as compared to other concentrations (< 0.30 U/mL).

Comparison of T1 lipase expression between plasmid-based and genome-based expression system in molasses

A comparison of T1 lipase expression between plasmid-based and genome-based expression systems in molasses was conducted under optimized conditions (Figure 6). The plasmid-based expression strain, *E. coli* W/pTrcHis_*T1*, and genome-based expression strain, *E. coli* WΔ*arsB::trc_T1*, were grown in M9 medium supplemented with 3% of molasses and induced with 0.8 mM IPTG at 16°C. As a result, the plasmid-based expression system (0.94 U/mL) gave a higher level of T1 lipase expression as compared to the genome-based expression system (0.44 U/mL).

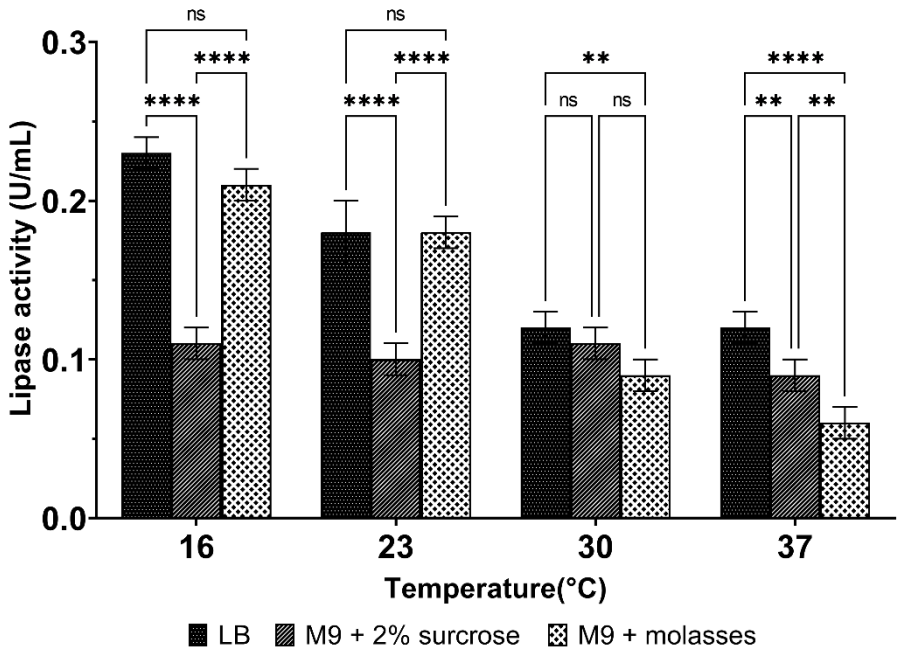

Figure 4. Optimization of T1 lipase expression in *E. coli* WΔ*arsB::trc_T1* using three culture media (LB media, M9 + 2% sucrose and M9 + molasses) at different temperatures. Data are presented as the mean ± standard deviation of three biological replications with three technical replicates (n=3). Statistical difference of lipase activity between different media at same temperature is stated as ns (non-significant; $p>0.05$), * ($p<0.05$), *** ($p<0.005$), and **** ($p<0.001$).

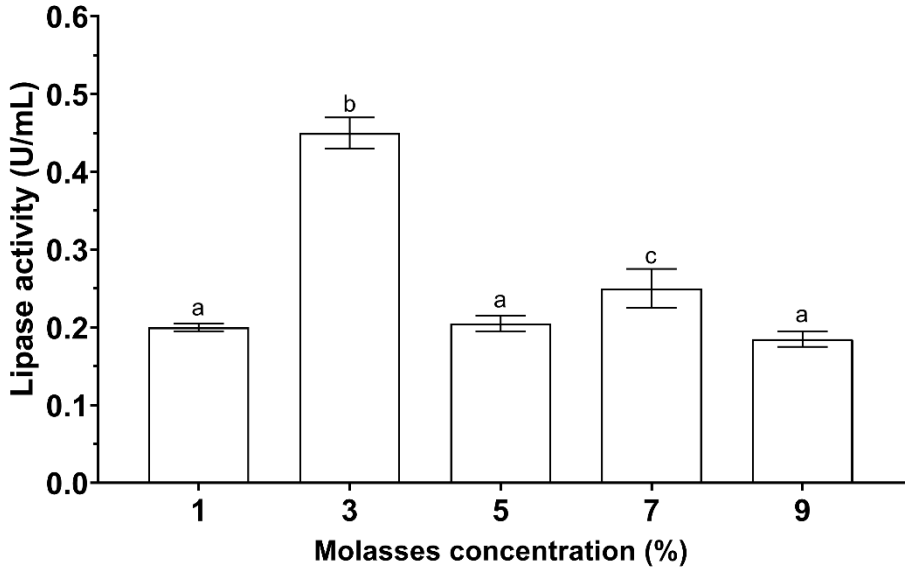

Figure 5. Optimization of T1 lipase expression in *E. coli* WΔ*arsB::trc_T1* using M9 + molasses of different concentrations. Data are presented as the mean ± standard deviation of three biological replications with three technical replicates (n=3). Statistical difference of lipase activity between different molasses concentration in the M9 medium ($p<0.05$) is stated as different alphabets above the bars.

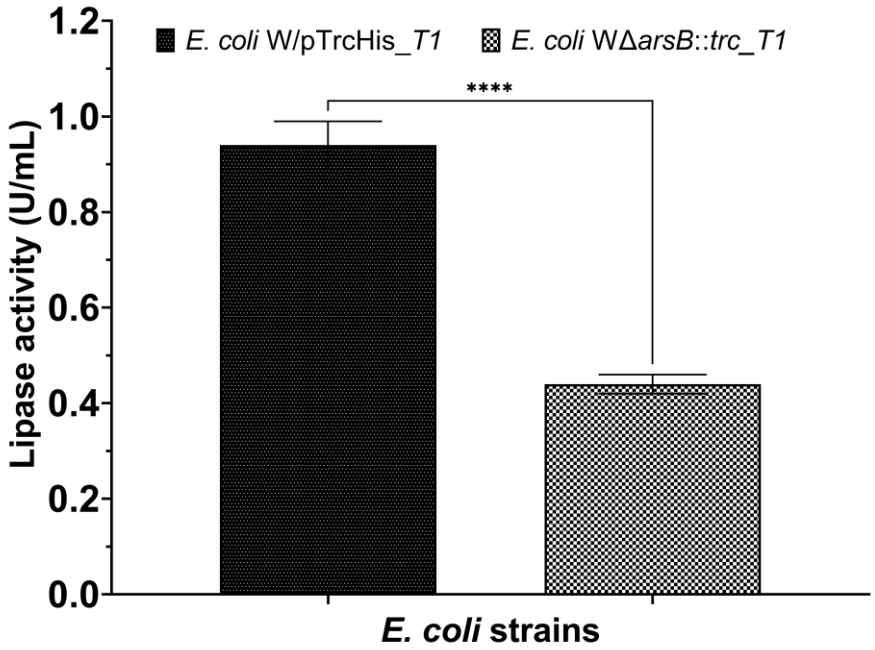

Figure 6. T1 lipase expression between plasmid-based and genome-based expression systems under optimized conditions in molasses (0.8 mM IPTG, 16°C and 3% molasses). Data are presented as the mean ± standard deviation of three biological replications with three technical replicates (n=3). Statistical difference of lipase activity between *E. coli* W strains in the optimized medium is stated as **** (p<0.001).

DISCUSSION

T1 lipase was successfully expressed in *E. coli* W by both plasmid-based and genome-based expression systems. The integration of T1 lipase into the *E. coli* W genome was conducted with the aid of pKIKO plasmids using the *λ* Red recombination technique (Datsenko & Wanner, 2000; Sabri et al., 2013). In this study, pKIKO*arsBCm* plasmid was chosen because the integration at the *arsB* region gave the highest expression of a gene of interest as previously suggested (Sabri *et al.*, 2013). To support the replication, *E. coli* BW25141 has been used as the cloning host as it is a *λ pir* strain that can support the replication of pKIKO plasmid due to the presence of R6K origin of replication (Philippe *et al.*, 2004). An advantage of having R6K origin of replication is the expression host cannot replicate the plasmid in the events that circular plasmid was transformed into the host (Donnenberg & Kaper, 1991). This is important to avoid false-positive results.

For further recombination, the linearized pKIKO plasmids integrated with the gene of interest need to be transformed into the electrocompetent cells harboring the *λ* Red helper plasmid, pKD46. The pKD46 plasmid was essential because it carries the *λ* Red recombination system that is responsible for enabling homologous recombination (Datsenko & Wanner, 2000). To complete the integration of the gene of interest into the *E. coli* genome, the cells were transformed with pCP20 plasmid according to the *FLP-FRT* recombination technique (Cherepanov & Wackernagel, 1995). This step is important to remove the antibiotic resistance gene at the *arsB* site. Therein, the pCP20 plasmid contained *FLP* gene that encodes for the flippase enzyme. This enzyme recognized the flippase recognition target site (*FRT*) that functions to cleave the fragments (antibiotic resistance gene) in between the *FRT* regions (Swings *et al.*, 2018). By removing the antibiotic resistance gene, the metabolic burden on the expression host is reduced, thus, increasing the efficiency of recombination protein expression (He *et al.*, 2017; Wu *et al.*, 2016). In addition, the antibiotic resistance gene removal also reduces the risk of horizontal gene transfer within bacteria (Laghari *et al.*, 2022).

In this study, the expression of the T1 lipase was higher in the plasmid-based expression system as compared to the genome-based expression system. This is due to the higher copy number of the recombinant T1 lipase gene in the *E. coli* cells. The high copy number of the recombinant gene in the plasmid-based expression system will subsequently give higher expression levels compared to the genome-based expression system (single expression system for each cell) (Mairhofer *et al*., 2013). In addition, it has been found that *trc* promoter gave higher T1 lipase activity in both plasmid-based and genome-based expression systems. The result suggested that *trc* promoter might be a better promoter in the expression of T1 lipase in *E. coli* W. Of note, the *trc* promoter is derived from *tac* promoter that has an addition of spacing consensus designed for productive and effective expression of recombinant proteins (Brosius et al., 1985; Hawley & McClure, 1983; Stefano & Gralla, 1982). The *trc* promoter has been reported to provide a high level of basal transcription (Terpe, 2006).

To further optimize the expression of T1 lipase using a genome-based expression system, three parameters (IPTG concentration, temperature, and molasses concentration) have been investigated. The highest T1 lipase expression was detected under these conditions; 16°C induction temperature with 0.8 mM IPTG at 3% molasses in the M9 medium. The reduced expression at higher than 3% molasses could be due to the overfeeding effects that result in the metabolic burden of the bacterial cells, by which the rate of anabolism superseded that of sucrose catabolism (Li & Rinas, 2020). Notably, the lowest T1 lipase expression was found in the cells grown in M9 + 2% sucrose for both expression systems. This might be due to the culture conditions of M9 + 2% sucrose medium itself, since it is the only defined medium, unlike LB medium (rich medium) and M9 + molasses medium (complex medium). Rich and complex media provide a higher amount of nutrients or other additives that help in cell growth and maintenance while decreasing the limiting factors for higher expression of recombinant protein (Rosano & Ceccarelli, 2014).

Overall, a low level of T1 lipase expression using the genome-based expression system in *E. coli* W has become the main limitation in this study. Therefore, extensive investigations on multiple integration sites (higher gene copies) (Goormans *et al*., 2020; Gu *et al*., 2015) and molecular tools that allow rapid integration of multiple genes without antibiotic selection (Egger *et al*., 2020; Riley *et al*., 2023), are required to increase the expression of recombinant protein in this genetically modified *E. coli* W. This, without a doubt, could improve the regulation of recombinant product expression, mRNA stability and protein expression (Kucharova *et al*., 2013; Mahalik *et al*., 2014). In contrast, *E. coli* W could also be genetically engineered to increase its disulfide bond-forming frequency in its cytoplasm for the expression of a recombinant protein that requires disulfide bond formation for proper folding (Berkmen, 2012). The genetically modified *E. coli* W is a promising candidate for the bioconversion of low-cost starting materials (sucrose-molasses) to produce industrially important recombinant proteins.

CONCLUSION

The study explored genome-based expression systems as an alternative to plasmid-based ones for producing recombinant proteins and successfully eliminated the reliance on expensive, non-environmentally friendly antibiotics. However, the plasmid-based system outperformed in T1 lipase expression compared to the genome-based expression system due to higher gene copy numbers. Recombinant protein production in the safe, sucrose-utilizing *E. coli* W using the cost-effective molasses as a carbon source undeniably reduces the production cost incurred by other carbon sources (glycerol and glucose) and is crucial at bioreactor scales. Despite that, both systems are highlighted for their potential to produce industrially significant recombinant proteins. Future research could enhance genome-based systems by integrating multiple genes at optimal chromosomal sites to surpass the performance of plasmid-based expression systems. Thus, this study sheds light on optimizing recombinant protein production in *E. coli* W, emphasizing the importance of carbon sources and the potential for further

advancements in genome-based expression systems.

ACKNOWLEDGEMENTS

The author acknowledges that the language and sentence structures in this article were partially improved by ChatGPT (powered by OpenAI's language model, GPT-3.5; http://openai.com). The initial write-up and final editing were manually performed by the human authors. This study was funded by Putra Grant - Putra Young Initiative (GP–IPM/2014/9437800) and Putra Grant - Putra Graduate Initiative (GP-IPS/2016/9482200) from Universiti Putra Malaysia that were awarded to the corresponding author (SS).

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

REFERENCES

Akdağ, B. & Çalık, P. 2015. Recombinant protein production by sucrose-utilizing *Escherichia coli* W: untreated beet molasses-based feeding strategy development. *Journal of Chemical Technology & Biotechnology* 90(6): 1070–1076. https://doi.org/https://doi.org/10.1002/jctb.4411

Arifin, Y., Archer, C., Lim, S., Quek, L.-E., Sugiarto, H., Marcellin, E., Vickers, C. E., Krömer, J. O. & Nielsen, L. K. 2014. *Escherichia coli* W shows fast, highly oxidative sucrose metabolism and low acetate formation. *Applied Microbiology and Biotechnology* 98(21): 9033–9044. https://doi.org/10.1007/s00253-014-5956-4

Berkmen, M. 2012. Production of disulfide-bonded proteins in *Escherichia coli*. *Protein Expression and Purification* 82(1): 240–251. https://doi.org/10.1016/j.pep.2011.10.009

Brosius, J., Erfle, M. & Storella, J. 1985. Spacing of the -10 and -35 regions in the *tac* promoter. Effect on its *in vivo* activity. *Journal of Biological Chemistry* 260(6): 3539–3541. https://doi.org/10.1016/S0021-9258(19)83655-4

Cardoso, V. M., Campani, G., Santos, M. P., Silva, G. G., Pires, M. C., Gonçalves, V. M., de C. Giordano, R., Sargo, C. R., Horta, A. C. L. & Zangirolami, T. C. 2020. Cost analysis based on bioreactor cultivation conditions: Production of a soluble recombinant protein using *Escherichia coli* BL21(DE3). *Biotechnology Reports* 26: e00441. https://doi.org/https://doi.org/10.1016/j.btre.2020.e00441

Carranza-Saavedra, D., Torres-Bacete, J., Blázquez, B., Sánchez Henao, C. P., Zapata Montoya, J. E. & Nogales, J. 2023. System metabolic engineering of *Escherichia coli* W for the production of 2-ketoisovalerate using unconventional feedstock. *Frontiers in Bioengineering and Biotechnology* 11: 1176445. https://doi.org/10.3389/fbioe.2023.1176445

Cheng, W. & Nian, B. 2023. Computer-aided lipase engineering for improving their stability and activity in the food industry: State of the art. *Molecules (Basel, Switzerland)* 28(15). https://doi.org/10.3390/molecules28155848

Cherepanov, P. P. & Wackernagel, W. 1995. Gene disruption in Escherichia coli: *Tc*R and *Km*R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158(1): 9–14. https://doi.org/10.1016/0378-1119(95)00193-a

Chin, W. L., Kee, P. E., Ng, H. S., Lan, J. C.-W. & Tan, J. S. 2023. Selective screening *Lactobacillu*s spp. against *Escherichia coli* and *Salmonella* Typhimurium and optimizing a cane molasses-based medium with *Lactobacillus acidophilus* as a guiding model. *Journal of the Taiwan Institute of Chemical Engineers* 105197. https://doi.org/10.1016/j.jtice.2023.105197

Contesini, F. J., Davanço, M. G., Borin, G. P., Vanegas, K. G., Cirino, J. P., Melo, R. R., Mortensen, U. H., Hildén, K., Campos, D. R. & Carvalho, P. D. 2020. Advances in recombinant lipases: Production, engineering, immobilization and application in the pharmaceutical industry. In *Catalysts* (Vol. 10, Issue 9). https://doi.org/10.3390/catal10091032

Datsenko, K. A. & Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America* 97(12): 6640–6645. https://doi.org/10.1073/pnas.120163297

Donnenberg, M. S. & Kaper, J. B. 1991. Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infection and Immunity* 59(12): 4310–4317. https://doi.org/10.1128/iai.59.12.4310-4317.1991

Egger, E., Tauer, C., Cserjan-Puschmann, M., Grabherr, R. & Striedner, G. 2020. Fast and antibiotic free genome integration into *Escherichia coli* chromosome. *Scientific Reports* 10(1): 16510. https://doi.org/10.1038/s41598-020-73348-x

Erian, A. M., Gibisch, M. & Pflügl, S. 2018. Engineered *E. coli* W enables efficient 2,3-butanediol production from glucose and sugar beet molasses using defined minimal medium as economic basis. *Microbial Cell Factories* 17(1): 190. https://doi.org/10.1186/s12934-018-1038-0

Feng, T., Wang, Z., Li, H., Li, Q., Guo, Y., Zhao, J. & Liu, J. 2023. Whole-cell biotransformation for simultaneous synthesis of allitol and D-gluconic acid in recombinant *Escherichia coli*. *Journal of Bioscience and Bioengineering* 135(6): 433–439. https://doi.org/10.1016/j.jbiosc.2023.03.004

Ferreira, R. da G., Azzoni, A. R. & Freitas, S. 2018. Techno-economic analysis of the industrial production of a low-cost enzyme using *E. coli*: The case of recombinant β-glucosidase. *Biotechnology for Biofuels* 11(1): 81. https://doi.org/10.1186/s13068-018-1077-0

Goormans, A. R., Snoeck, N., Decadt, H., Vermeulen, K., Peters, G., Coussement, P., Van Herpe, D., Beauprez, J. J., De Maeseneire, S. L. & Soetaert, W. K. 2020. Comprehensive study on *Escherichia coli* genomic expression: Does position really matter? *Metabolic Engineering* 62: 10–19. https://doi.org/10.1016/j.ymben.2020.07.007

Gu, P., Yang, F., Su, T., Wang, Q., Liang, Q. & Qi, Q. 2015. A rapid and reliable strategy for chromosomal integration of gene(s) with multiple copies. *Scientific Reports* 5(1): 9684. https://doi.org/10.1038/srep09684

Hamdan, S. H., Maiangwa, J., Ali, M. S. M., Normi, Y. M., Sabri,

S. & Leow, T. C. 2021. Thermostable lipases and their dynamics of improved enzymatic properties. *Applied Microbiology and Biotechnology* 105(19): 7069–7094. https://doi.org/10.1007/s00253-021-11520-7

Hawley, D. K. & McClure, W. R. 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Research* 11(8): 2237–2255. https://doi.org/10.1093/nar/11.8.2237

He, L., Xiu, Y., Jones, J. A., Baidoo, E. E. K., Keasling, J. D., Tang, Y. J. & Koffas, M. A. G. 2017. Deciphering flux adjustments of engineered *E. coli* cells during fermentation with changing growth conditions. *Metabolic Engineering* 39: 247–256. https://doi.org/10.1016/j.ymben.2016.12.008

Jahreis, K., Bentler, L., Bockmann, J., Hans, S., Meyer, A., Siepelmeyer, J. & Lengeler, J. W. 2002. Adaptation of sucrose metabolism in the *Escherichia coli* wild-type strain EC3132. *Journal of Bacteriology* 184(19): 5307–5316. https://doi.org/10.1128/JB.184.19.5307-5316.2002

Kim, J., Webb, A. M., Kershner, J. P., Blaskowski, S. & Copley, S. D. 2014. A versatile and highly efficient method for scarless genome editing in *Escherichia coli* and *Salmonella enterica*. *BMC Biotechnology* 14(1): 84. https://doi.org/10.1186/1472-6750-14-84

Kingsly, J. S., Chathalingath, N., Parthiban, S. A., Sivakumar, D., Sabtharishi, S., Senniyappan, V., Duraisamy, V. S., H, A. J. & Gunasekar, A. 2022. Utilization of sugarcane molasses as the main carbon source for the production of polyhydroxyalkanoates from *Enterobacter cloacae*. *Energy Nexus* 6: 100071. https://doi.org/10.1016/j.nexus.2022.100071

Kucharova, V., Strand, T. A., Almaas, E., Naas, A. E., Brautaset, T. & Valla, S. 2013. Non-Invasive Analysis of Recombinant mRNA Stability in *Escherichia coli* by a Combination of Transcriptional Inducer Wash-Out and qRT-PCR. *PloS One* 8(6): e66429. https://doi.org/10.1371/journal.pone.0066429

Kwon, D. Y., & Rhee, J. S. 1986. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *Journal of the American Oil Chemists' Society* 63: 89–92. https://doi.org/10.1007/BF02676129

Laghari, A. A., Liu, L., Kalhoro, D. H., Chen, H. & Wang, C. 2022. Mechanism for Reducing the Horizontal Transfer Risk of the Airborne Antibiotic-Resistant Genes of *Escherichia coli* Species through Microwave or UV Irradiation. *International Journal of Environmental Research and Public Health* 19(7). https://doi.org/10.3390/ijerph19074332

Lan, L., Xie, Y., Kong, X., Li, C. & Liu, D. 2021. Investigation of reduction in risk from antibiotic resistance genes in laboratory wastewater by using O(3), ultrasound, and autoclaving. *Water Environment Research : A Research Publication of the Water Environment Federation* 93(3): 479–486. https://doi.org/10.1002/wer.1451

Lee, S. Y., & Chang, H. N. 1993. High cell density cultivation of *Escherichia coli* W using sucrose as a carbon source. *Biotechnology Letters* 15(9): 971–974. https://doi.org/10.1007/BF00131766

Leow, T. C., Rahman, R. N. Z. R. A., Basri, M. & Salleh, A. B. 2007. A thermoalkaliphilic lipase of *Geobacillus* sp. T1. *Extremophiles* 11(3): 527–535. https://doi.org/10.1007/s00792-007-0069-y

Li, Z., & Rinas, U. 2020. Recombinant protein production associated growth inhibition results mainly from transcription and not from translation. *Microbial Cell Factories* 19(1): 83. https://doi.org/10.1186/s12934-020-01343-y

Lozano Terol, G., Gallego-Jara, J., Sola Martínez, R. A., Cánovas Díaz, M., & de Diego Puente, T. 2019. Engineering protein production by rationally choosing a carbon and nitrogen source using *E. coli* BL21 acetate metabolism knockout strains. *Microbial Cell Factories* 18(1): 151. https://doi.org/10.1186/s12934-019-1202-1

Mahalik, S., Sharma, A. K. & Mukherjee, K. J. 2014. Genome engineering for improved recombinant protein expression in *Escherichia coli*. *Microbial Cell Factories* 13: 177. https://doi.org/10.1186/s12934-014-0177-1

Mairhofer, J., Scharl, T., Marisch, K., Cserjan-Puschmann, M. & Striedner, G. 2013. Comparative transcription profiling and in-depth characterization of plasmid-based and plasmid-free *Escherichia coli* expression systems under production conditions. *Applied and Environmental Microbiology* 79(12): 3802–3812. https://doi.org/10.1128/AEM.00365-13

Mittal, P., Brindle, J., Stephen, J., Plotkin, J. B. & Kudla, G. 2018. Codon usage influences fitness through RNA toxicity. *Proceedings of the National Academy of Sciences of the United States of America*, 115(34): 8639–8644. https://doi.org/10.1073/pnas.1810022115

Philippe, N., Alcaraz, J.-P., Coursange, E., Geiselmann, J. & Schneider, D. 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. *Plasmid* 51(3): 246–255. https://doi.org/10.1016/j.plasmid.2004.02.003

Puetz, J. & Wurm, F. M. 2019. Recombinant Proteins for Industrial versus Pharmaceutical Purposes: A Review of Process and Pricing. *Processes* 7(8). https://doi.org/10.3390/pr7080476

Riley, L. A., Payne, I. C., Tumen-Velasquez, M. & Guss, A. M. 2023. Simple and Rapid Site-Specific Integration of Multiple Heterologous DNAs into the *Escherichia coli* Chromosome. *Journal of Bacteriology* 205(2): e0033822. https://doi.org/10.1128/jb.00338-22

Rosano, G. L. & Ceccarelli, E. A. 2014. Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology* 5: 172. https://doi.org/10.3389/fmicb.2014.00172

Sabri, S., Steen, J. A., Bongers, M., Nielsen, L. K. & Vickers, C. E. 2013. Knock-in/Knock-out (KIKO) vectors for rapid integration of large DNA sequences, including whole metabolic pathways, onto the *Escherichia coli* chromosome at well-characterised loci. *Microbial Cell Factories* 12(1): 60. https://doi.org/10.1186/1475-2859-12-60

Sambrook, J. & Russell, D. W. 2006. Preparation and transformation of competent *E. coli* using calcium chloride. *CSH Protocols* 2006(1). https://doi.org/10.1101/pdb.prot3932

Sawitzke, J. A., Costantino, N., Hutchinson, E., Thomason, L. C. & Court, D. L. 2022. Enhancement of RecET-mediated *in vivo* linear DNA assembly by a *xonA* mutation. *BioRxiv* 2022.01.13.476211. https://doi.org/10.1101/2022.01.13.476211

Stefano, J. E. & Gralla, J. D. 1982. Spacer mutations in the *lac ps* promoter. *Proceedings of the National Academy of Sciences of the United States of America* 79(4): 1069–1072. https://doi.org/10.1073/pnas.79.4.1069

Sun, X.-M., Zhang, Z.-X., Wang, L.-R., Wang, J.-G., Liang, Y., Yang, H.-F., Tao, R.-S., Jiang, Y., Yang, J.-J. & Yang, S. 2021. Downregulation of T7 RNA polymerase transcription enhances pET-based recombinant protein production in *Escherichia coli* BL21 (DE3) by suppressing autolysis. *Biotechnology and Bioengineering* 118(1): 153–163. https://doi.org/10.1002/bit.27558

Swings, T., Marciano, D. C., Atri, B., Bosserman, R. E., Wang, C., Leysen, M., Bonte, C., Schalck, T., Furey, I., Van den Bergh, B., Verstraeten, N., Christie, P. J., Herman, C., Lichtarge, O. & Michiels, J. 2018. CRISPR-*FRT* targets shared sites in a knock-out collection for off-the-shelf genome editing. *Nature Communications* 9(1): 2231. https://doi.org/10.1038/s41467-018-04651-5

Tan, S.-I. & Ng, I.-S. 2020. New Insight into Plasmid-Driven T7 RNA Polymerase in *Escherichia coli* and Use as a Genetic Amplifier for a Biosensor. *ACS Synthetic Biology* 9(3): 613–622. https://doi.org/10.1021/acssynbio.9b00466

Terpe, K. 2006. Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology* 72(2): 211–222. https://doi.org/10.1007/s00253-006-0465-8

Thomason, L. C., Costantino, N. & Court, D. L. 2016. Examining a DNA Replication Requirement for Bacteriophage λ Red- and Rac Prophage RecET-Promoted Recombination in *Escherichia coli*. *MBio* 7(5). https://doi.org/10.1128/mBio.01443-16

Wang, H., Wang, J., Li, S., Li, J. & Jing, C. 2019. Prevalence of antibiotic resistance genes in cell culture liquid waste and the virulence assess for isolated resistant strains. *Environmental Science and Pollution Research International* 26(31): 32040–32049. https://doi.org/10.1007/s11356-019-06299-0

Weber, J., Li, Z. & Rinas, U. 2021. Recombinant protein production provoked accumulation of ATP, fructose-1,6-bisphosphate and pyruvate in *E. coli* K12 strain TG1. *Microbial Cell Factories* 20(1): 169. https://doi.org/10.1186/s12934-021-01661-9

Wu, G., Yan, Q., Jones, J. A., Tang, Y. J., Fong, S. S. & Koffas, M. A. G. 2016. Metabolic Burden: Cornerstones in Synthetic Biology and Metabolic Engineering Applications. *Trends in Biotechnology* 34(8): 652–664. https://doi.org/10.1016/j.tibtech.2016.02.010

Xie, D. 2022. Continuous biomanufacturing with microbes — upstream progresses and challenges. *Current Opinion in Biotechnology* 78: 102793. https://doi.org/10.1016/j.copbio.2022.102793

Yaman, S. & Çalık, P. 2017. Beet molasses-based feeding strategy enhances recombinant thermostable glucose isomerase production by *Escherichia coli* BL21 (DE3). *Biotechnology and Applied Biochemistry* 64(6): 944–954. https://doi.org/10.1002/bab.1549

Yang, J., Sun, B., Huang, H., Jiang, Y., Diao, L., Chen, B., Xu, C., Wang, X., Liu, J., Jiang, W. & Yang, S. 2014. High-efficiency scarless genetic modification in *Escherichia coli* by using lambda red recombination and I-*Sce*I cleavage. *Applied and Environmental Microbiology* 80(13): 3826–3834. https://doi.org/10.1128/AEM.00313-14