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Research Article

Extracellular expression in *Bacillus subtilis* of a thermostable *Geobacillus stearothermophilus* lipase

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ABSTRACT

Background: The extracellular expression of enzymes in a secretion host such as *Bacillus subtilis* is a useful strategy in reducing the cost of downstream processing of industrial enzymes. Here, we present the first report of the successful extracellular expression in *Bacillus subtilis* WB800 of *Geobacillus stearothermophilus* lipase (T1.2RQ), a novel industriallydesirable thermostable lipolytic enzyme which has an excellent hydrolytic and transesterification activity. Signal peptides of α-amylase, extracellular protease, and lipase A, as well as two different promoters, were used in the secretion and expression of lipase T1.2RQ.
Results: Lipase activity assay using p-nitrophenyl laurate showed that all three signal peptides directed the secretion of lipase T1.2RQ into the extracellular medium. The signal peptide of lipase A, resulted in the highest extracellular yield of 5.6 U/ml, which corresponds to a 6-fold increase over the parent *Bacillus subtilis* WB800 strain. SDS-PAGE and zymogram analysis confirmed that lipase T1.2RQ was correctly processed and secreted in its original size of 44 kDa. A comparison of the expression levels of lipase T1.2RQ in rich medium and minimal media showed that the enzyme was better expressed in rich media, with up to an 8-fold higher yield over minimal media. An attempt to further increase the lipase expression level by promoter optimization showed that, contrary to expectation, the optimized promoter exhibited similar expression levels as the original one, suggesting the need for the optimization of downstream factors.
Conclusions: The successful extracellular secretion of lipase T1.2RQ in *Bacillus subtilis* represents a remarkable feat in the industrial-scale production of this enzyme.
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1. Introduction

Lipases are enzymes of high industrial significance which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids. Lipases also catalyze the transesterification and synthesis of esters. They can perform very specific biotransformation reactions and are widely used in food, detergent, cosmetic, organic synthesis, and pharmaceutical industries [1].

Extremophilic lipases with features such as thermostability, psychrophilic tendency, and organic solvent-tolerance are even more desirable in industrial applications due to their ability to carry out catalysis under unique conditions [2]. The novel thermostable *Geobacillus stearothermophilus* lipase T1.2RQ, isolated from the hot springs of Maluku, Indonesia is one of such lipases. Lipase T1.2RQ is an α/β hydrolase which, in addition to its high thermal stability, has an excellent hydrolytic and transesterification activity and is very promising for application in food and biodiesel industries – owing to its ability to catalyze reactions at elevated temperatures. To achieve industrial applicability of lipase T1.2RQ, an efficient system for affordable large-scale production is required.

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Various heterologous expression systems exist for the production of enzymes of industrial significance, each one with its advantages and disadvantages. The *Escherichia coli* expression systems are the most widely used and are very versatile in the expression of heterologous enzymes due to their ease of culture, short doubling time, and ease of genetic manipulation [3]. However, *E. coli* expression systems are characterized by obstacles such as the formation of inclusion bodies, accumulation of target protein in the cytoplasmic or periplasmic space, and contamination by endotoxin LPS – which limits the application of the produced protein in the food and medical sector [4].

More recently, *Bacillus subtilis* has attracted much attention as an alternative host for the production of recombinant proteins. *Bacillus subtilis* expression systems, in addition to the advantage of the *E. coli* system, have a remarkably efficient protein secretion capacity and are generally recognized as safe (GRAS) [5,6] i.e., when a protein of interest is paired with a suitable *Bacillus subtilis* signal peptide, the protein can be effectively secreted into the extracellular medium. The extracellular secretion simplifies the process of downstream purification by eliminating the laborious process of cell disruption, as the recombinant protein is harvested from the spent medium [7]. Likewise, the secreted protein will be endotoxin-free and safe for use in the food and medical industry. *B. subtilis* WB800 is an eight protease-deficient (nprE aprE epr bpr mpr::ble nprB::bsr vpr wprA::hyg) strain widely used in the secretory expression of foreign proteins [8].

In this study, the secretion capability of *Bacillus subtilis* is exploited to express the thermostable *Geobacillus stearothermophilus* lipase (T1.2RQ) extracellularly with the expression plasmid PHT43, using signal peptides – AmyQ, Epr & LipA – selected based on high signal peptide prediction score (D-score) and reports of high efficiency. The expression of recombinant proteins using vector PHT43 is controlled by the strong IPTG-inducible Pgrac promoter, native to the *groESL* operon of *Bacillus subtilis*. Phan et al. [9] also constructed an improved version of the Pgrac promoter, named Pgrac100, by introducing mutations in the consensus sequences of the Pgrac01 promoter, as well as the addition of mRNA controllable stabilizing elements (CoSE). These changes have been proven to enhance the amount of accumulating recombinant proteins to reach up to 30 % of the total cellular protein of *B. subtilis* [9].

Therefore, here we report the cloning of the *Geobacillus stearothermophilus* thermostable lipase T1.2RQ gene into *B. subtilis*, as well as an OFAT signal peptide, production media, and promoter optimization of lipase T1.2RQ expression. To the best of our knowledge, this is the first-ever report on the heterologous expression of a thermostable *Geobacillus* lipase in *Bacillus subtilis.*

2. Materials and methods

2.1. Bacterial strains, plasmids, and kits

E. coli BL21 (DE3) pLyss-PET28a-T1.2RQ RQ strain, carrying T.12 lipase gene, was the donor host in this experiment, while *E. coli* strains DH5α and *B. subtilis* WB800 (Mobitech GmbH) were used as the cloning and expression hosts, respectively. The shuttle plasmid vector, PHT43, bearing the AmyQ signal peptide was used as the expression vector. The signal peptides, Lip A and Epr signal peptides, and other primers were obtained as synthetic oligonucleotides with restriction site overhangs (Macrogen). DNA polymerases, Phusion (NEB) and Go-Taq (Promega), restriction enzymes (*Kpn*I, *BamHI*, *XbaI, XmaI)* polynucleotide kinase, T4 ligase (NEB), Qiaquick plasmid extraction kit, Qiaprep Spin-column gel extraction kit were also used in this research. Chemicals were obtained from Merck Millipore.

2.2. Construction of lipase expression vector

E. coli BL21 (DE3) pLyss-PET28a-T1.2RQ R230Q strain was grown in 10 mL nutrient broth (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract) at 37°C and 200 rpm for 24 h. The plasmid vector, PET28a-T1.2RQ R230Q, was isolated using Qiaquick plasmid isolation kit and amplified with primers, P06U3-BamHI and P06IC-XmaI to obtain T1.2RQ gene flanked by the *BamHI* and *XmaI* restriction sites. The vector PHT43, bearing the Pgrac promoter and AmyQ signal peptide, and the T1.2RQ amplicon was digested using *BamHI* and *XmaI*. The T1.2RQ gene was then ligated to the predigested PHT43 with T4 ligase at 16°C overnight. The resulting plasmid was named PHT43-Pgrac01-AmyQSp-T.12. In the construction of vectors with a different signal peptide, new recombinant plasmids named PHT43-Pgrac01-Epr-T.12 and PHT43-Pgrac01-LipA-T.12 was prepared as follows: PHT43-Pgrac01-AmyQ-T.12 was digested with XbaI and BamHI and ligated to LipA and Epr signal peptide oligonucleotides to form PHT43-Pgrac01-LipA-T.12 and PHT43-Pgrac01-Epr -T.12, respectively. Furthermore, a vector without signal peptides, PHT43-Pgrac01-T1.2RQ, was constructed by mutagenesis PCR of PHT43-Pgrac01-AmyQ-T.12 using primers P04-04 & P06-FL to eliminate the signal peptide. Finally, the Pgrac100 bearing vector was constructed using primers Pgrac100-F & Pgrac100-R, to change the -35, -10, and -1 region to generate PHT43-Pgrac100-T.12RQ.

2.3. Transformation into Bacillus subtilis WB800

Electro-competent *Bacillus subtilis* WB800 were prepared as follows: Cryopreserved *Bacillus subtilis* WB800 was streaked on freshly prepared LA medium and cultured overnight at 37°C. A single colony of the freshly grown *Bacillus subtilis* WB800 was inoculated in a 10 mL LB liquid and cultured overnight at 37°C. 1 mL of the overnight culture was transferred into a 250 mL Erlenmeyer flask containing 40 mL LB + 0.5 M sorbitol (1:1) and incubated at 37°C for 3 h till the optical density reached 0.7–0.8. The culture was transferred into a 50 mL falcon tube, and cooled on ice, and centrifuged at 5000× g and 4°C. The pellet was washed 4 times with ice-cold electroporation media (0.5 M sorbitol, 0.5 M mannitol, 0.5 M trehalose, 10% glycerol). The pellet was then suspended in 2 mL electroporation media, and aliquots of 60 μL were made. Cryopreserved 60 μL aliquots' competent cells were thawed on ice, after which 50 ng of plasmid DNA was added. The cell-DNA mixture was transferred into a pre-cooled cuvette and incubated on ice for 10 min. Subsequently, the mixture was pulsed with a pulser at 1500 Volts, 200 Ω, 25 μF and 5 ms. 1 mL recovery medium (2% tryptone (w/v), 1% yeast extract (w/v), 1% NaCl (w/v), 0.38 M mannitol, 0.5 M sorbitol) was added immediately after shocking the mixture. The bacterial was allowed to recover for 3 h, spun at 2000 rpm, resuspended in 200 μL 2YT/LB medium and subsequently spread on LA agar containing 5 μg/mL chloramphenicol.

2.4. Culture conditions and induction of T.12 lipase expression

All recombinant *Bacillus subtilis* WB800 were cultured as follows: Cryopreserved recombinant *Bacillus subtilis* WB800 were streaked on LA agar and cultured overnight. A single loop of the freshly cultivated bacteria was transferred into 10 mL 2YT/LB liquid medium containing 5 μg/mL chloramphenicol at 37°C and 200 rpm for 8–10 h as the seed culture. 5% (v/v)/ 2.5 mL of the seed culture was transferred into a 50 mL terrific broth (TB) medium (45 ml TB media + 5 mL TB salt), supplemented with 0.05% $CaCl_2$, 0.04% $MgSO_4$ and 5 μg/mL chloramphenicol. When the optical density (OD 600 nm) reaches 0.9, 1.0 mM IPTG was added to induce T.12 lipase expression, and the temperature was decreased to 33°C. The culture period varied between 24 h to 48 h depending

on the data required. The culture medium of the engineered *Bacillus subtilis* WB800 cells was centrifuged at 5000× g for 10 min at 4°C. The pellet was suspended in 5 mL Tris-HCl (pH 8.0) and subjected to sonication on ice for 15 min in the presence of 120 μL of 25 mg/ml lysozyme. The supernatant and pellet samples were independently concentrated by ultrafiltration using a 30 kDa cut-off membrane (Amicon). The filtrate was used for lipase assay and other subsequent analyses.

2.5. Determination of the hydrolytic activity of lipase

Lipase activity was determined using the following reaction mixture: 940 μL of 0.1 M Tris-HCl (pH 8.0), 40 μL of cold absolute ethanol, 10 μL of enzyme solution, and 10 μL of 10 mM para-nitrophenyl-laurate solution (suspended in isopropanol). The reaction mixture was incubated at 50°C for 5 min, and absorbance at 405 nm was measured immediately using a spectrophotometer (Biorad Smart spec^{Tm} plus). 1 unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmoL of p-nitrophenol from p-nitrophenyl-laurate in 1 min. The data obtained for lipase yield from 3 independent cultures for T.12 lipase were run and expressed as mean ± SEM of 3 independent determinations. The means were compared by a one-way ANOVA followed by the Tukey post hoc test. A value of P less than 5% ($P < 0.05$) was considered statistically significant.

2.6. Protein quantitation and SDS PAGE-zymogram

Protein concentrations were measured using the BCA assay as follows: a mix of 200 μL of BCA solution and 4 μL of $CuSO_4$ was prepared in an Eppendorf tube. 200 μL of the mix was transferred into another tube, to which 25 μL was then added. This mixture was incubated at 60°C for 15 min and allowed to cool for 5 min. The mixture was subsequently transferred into a microplate reader and the absorbance at 562 nm was recorded. Bovine serum albumin (BSA) was used as the standard protein solution to produce a standard curve. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide separation and 5% stacking gels on a vertical mini-gel apparatus at 100 V for 2 h. The gel was stained with Coomassie brilliant blue to detect protein and analyzed. For the zymogram, unstained migrated gel was washed twice with 1% Triton-X100, and twice with 0.1 M Tris-HCl, and subsequently with ddH_2O. The gel was then plated on tributyrin-agar with and without phenol red pH indicator.

3. Results and discussions

3.1. Construction of the extracellular expression vector

The open reading frame (ORF) of the lipase T1.2RQ gene was amplified using the primers P06U3-BamHI and P06IC-XmaI, flanked by the restriction sites BamHI-XmaI, to obtain a 1.3 kb DNA fragment (rectangle in Fig. 1). To create sticky end fragments, the shuttle vector, PHT43, and purified amplicon were independently subjected to BamHI-XmaI overnight double digest to generate a 7.9 kb vector and 1.3 kb insert linear sticky end DNA fragments respectively. The vector (7.9 kb linearized vector) and insert (1.3 kb PCR product) sticky ends fragments were joined together using T4 DNA ligase with a 3:1 insert-vector ratio.

The 9.2 kb ligation product was transformed into the cloning host, *E. coli DH5α* by heat shock method and spread onto Luria agar plate containing 100 μg/ml ampicillin to produce recombinant colonies. Recombinant colonies produced about 1.3 kb band PCR products after colony PCR, which corresponds to the correct size of the target lipase T1.2RQ, indicating a successful transformation of the recombinant plasmid (PHT43-T1.2RQ). Digestion of isolated plasmids using BamHI-XmaI produced two fragments of 7.9 kb and 1.3 kb, corresponding to the correct sizes of the original vector and insert before ligation. To substitute the signal peptides, PHT43-T1.2RQ was digested with XbaI-BamHI, the digest reaction produced an approximately 9.1 kb DNA fragment -with no signal peptide- which was subsequently ligated to Epr and LipA oligonucleotides. Sequencing results obtained after the transformation of the ligation products, PHT43-T1.2RQlip_Epr and PHT43-T1.2RQlip_LipA, showed that the signal peptides were accurately substituted and in the correct reading frame.

3.2. Qualitative expression of lipase T1.2RQ on tributyrin agar

Recombinants vectors, PHT43 (plasmid control), PHT43-T1.2RQ, PHT43-T1.2RQ_AmyQ, PHT43-T1.2RQ_Epr, and PHT43-T1.2RQ_LipA, were transformed by electroporation into *Bacillus subtilis* WB800. The electroporation was successful with the optimum condition of 2500 V, 200 ohms, 25 μF, and 5 ms.

Subsequently, recombinant *B. subtilis* PHT43 (negative control) and PHT43-T1.2RQlip + signal peptides were streaked on the lipidic agar, LA + TBN (containing 1 mM IPTG). All four recombinants, including the negative control (*B. subtilis* WB800-PHT43), produced clear zones on the TBN agar plate after overnight incubation (Fig. 2), indicating the presence of a lipolytic enzyme in all of the recombinant *B. subtilis* cells. Clear zone on the negative control (Fig. 2) suggests that *B. subtilis* WB800 possess native lipolytic enzyme (s) capable of hydrolyzing a tri-ester such as the tributyrin substrate into fatty acids. A review of *B. subtilis* WB800 genome revealed the presence of two native extracellular lipases, a 19 kDa lipase A (LipA) and a 22 kDa lipase B (LipB). Reports also showed that these lipases are differentially expressed depending on the nutrient and PH conditions of the medium [10].

Meanwhile, Fig. 2 also shows that PHT43-T1.2RQlip + signal peptides (AmyQ, Epr, and LipA) have clear zones with significantly higher diameters than *B. subtilis WB800*-PHT43 (negative control). This phenomenon suggests that the recombinant thermostable lipase T1.2RQ introduced into these cells was successfully expressed, thereby increasing the lipolytic capability of its host cells [11,12]. Similarly, variation in the lipolytic quotient, based on the observable clear zone, can also be seen among recombinants bearing different signal peptides. This implies that the type of signal peptide affects either the expression or the transport of the extracellular protein to which they are attached i.e., some signal peptides are more effective than others in the extracellular expression of proteins in *B. subtilis* [13].

3.3. Quantitative expression of lipase T1.2RQ and signal peptide optimization

To determine the yield from the recombinant *B. subtilis* and its variation due to the attached signal peptides, lipase T1.2RQ was expressed in 50 mL terrific broth media, under 1 mM IPTG induction for 24 h. Fig. 3 summarizes the lipolytic activity of the recombinants based on p-nitrophenyl-laurate hydrolysis by the cell-free supernatant. Recombinant *B. subtilis* WB800 bearing lipase T1.2RQ, with and without signal peptides, demonstrated a higher lipase activity than *B. subtilis* WB00-PHT43 (the negative control). Similarly, recombinant *B. subtilis* WB00_T1.2RQ with signal peptides (*Amy, Epr, and LipA)* exhibits a significantly higher extracellular lipase activity than without signal peptide ($p < 0.05$). This supports the hypothesis that the presence of signal peptide is necessary for extracellular protein export.

Among the three signal peptides used in this study, *LipA* signal demonstrated the highest extracellular activity (5.6 IU/ml), and

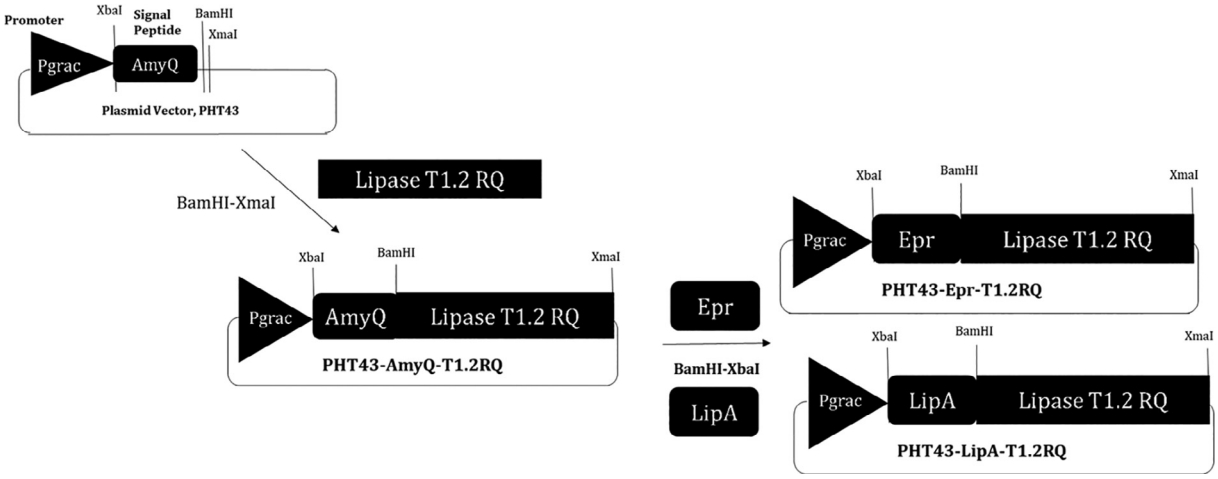

Fig. 1. Demonstration of the cloning procedure of lipase T1.2RQ Gene into plasmid PHT43. The open reading frame (ORF) of the lipase T1.2RQ gene was amplified using the primers P06U3-BamHI and P06IC-XmaI, flanked by the restriction sites BamHI-XmaI, to obtain a 1.3 kb DNA fragment. The 1.3 kb PCR product was cloned into the empty vector by BamHI-XmaI restriction cloning to generate PHT43-T1.2RQ. Recombinant vector, PHT43 + T1.2RQ was subsequently subjected to BamHI-XbaI restriction cloning to substitute the signal peptides. Pgrac = Promoter Pgrac, AmyQ = α-amylase signal peptide, Epr = extracellular protease signal peptide, LipA = lipase A signal peptide.

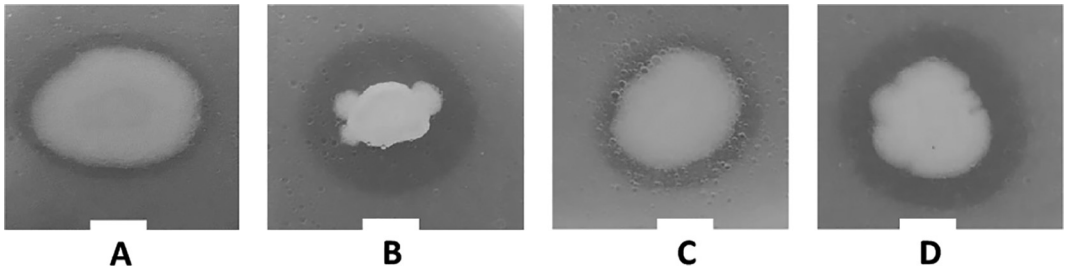

Fig. 2. Qualitative detection of lipolytic activity of recombinant *Bacillus subtilis* WB800. Recombinant *Bacillus subtilis* were streaked on Luria agar containing 1% tributyrin (TBN) + 1 mM IPTG. The recombinant cells produced halo zones of different sizes, relative to their lipolytic capabilities, on LA + TBN agar after 16 h culture period at 37°C. (A) *B. subtilis* WB800-PHT43 (negative control) (B) *B. subtilis* WB800-T1.2RQ_AmyQ (C) *B. subtilis* WB800-T1.2RQ_Epr (D) *B. subtilis* WB800-T1.2RQ_LipA.

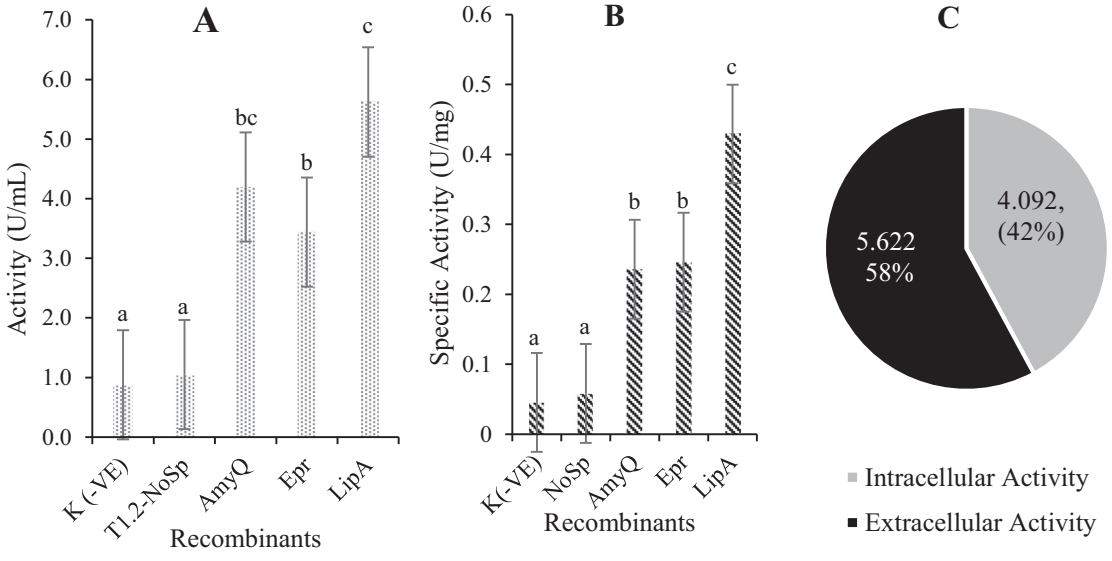

Fig. 3. Expression of lipase T1.2RQ in recombinant *B. subtilis* WB800. Values are lipase activity of 90× supernatant ultrafiltrates expressed as mean ± SEM ($n = 3$). (K (−VE) = *B. subtilis* WB800-PHT43; 01-NoSp = *B. subtilis* WB800-T1.2RQ; AmyQ = *B. subtilis* WB800-T1.2RQ_AmyQ; Epr = *B. subtilis* WB800-T1.2RQ_Epr; LipA = *B. subtilis* WB800-T1.2RQ_LipA). (A) Extracellular lipase activity of recombinant *Bacillus subtilis* (B) Specific Extracellular lipase activity of recombinant *Bacillus subtilis.* (C) Secretion efficiency of LipA signal peptide. The intracellular activity was determined using ultrafiltrates of the crude lysate.

specific activity of 0.655 IU/mg, which is about 2.5-fold higher in expression than either AmyQ (0.273 IU/mg) and Epr (0.262 IU/mg) signal peptides. Further analysis shows that with LipA signal peptide, roughly 60% of the expressed protein was successfully

expressed extracellularly in an active state. So far, only very few reports exist on the heterologous extracellular expression of lipases in *Bacillus subtilis*. Nevertheless, our findings are in accord with those reported in previous studies. For instance, an extracellular lipase yield of 3.5 U/ml and 5.0 U/ml was obtained in the heterologous expression of Antarctic *Bacillus pulmulus* lipases BPL1 and BPL2, using their respective signal peptides, in *B. subtilis* DB104 [12]. Similarly, the extracellular lipolytic activity yield recorded when *Fusarium solani* cutinase was heterologously expressed in *B. subtilis* TEB1030, was 4.5 U/ml, 2.79 U/ml, and 0.67 U/ml using Epr, LipA, and AmyE signal peptides, respectively [13]. A yield of 356.8 U/mL was obtained when *Proteus Vulgaris* PVL lipase was extracellularly expressed using SacB signal peptide in *B. subtilis* WB800 [12].

Since we considered the signalP prediction score (D-score) in the selection of the signal peptides used in this study, the extracellular lipase activity of these signal peptides was compared to their D-score. Surprisingly, the Epr signal peptide, despite having the highest prediction score (D score = 0.702), produced the least extracellular activity of all three signal peptides, while AmyQ with a D score of 0.488, produced a moderately high activity. LipA which has a D-score of 0.690 produced the highest extracellular enzyme yield. This means that a high D-score does not necessarily translate into a high secretion efficiency [6,13]. In this case, our result is in line with the findings of Brockmeier et al. [13] and Zanen et al. [14] which claims that, although the D-score is a useful tool in the prediction of signal peptide efficiency, the efficiency of a signal peptide depends on the protein-signal peptide interaction, and has a weak correlation with the D-score.

Nevertheless, the high lipase activity and export efficiency (58%) of *B. subtilis* WB800-T1.2RQ_LipA indicates that the *LipA* signal peptide can be beneficial in the industrial scale extracellular expression of lipase T1.2RQ in *Bacillus subtilis* (Fig. 3).

3.4. Time-course expression profile of B. subtilis WB800-T1.2RQ_LipA

The time-course expression profile of *B. subtilis* WB800-T1.2RQ_LipA in terrific broth was determined by periodic sampling every 6 h. The result obtained showed that lipase activity was detected in the supernatant as early as two and a half hours into the culture period – before induction. This lipase activity detected prior to induction is likely due to leaky expression typical of inducible promoters such as the PGrac promoter used in this study. After induction with IPTG at OD600nm = 0.9, the lipase activity increased sharply and continued to increase concurrently with the biomass (Fig. 4), reaching the highest activity level of 5.7 U/ml after 20 h. However, 6 h after this period, a decline in activity was recorded. Meanwhile, *B. subtilis* WB800-PHT43 (negative control) maintained very low extracellular lipase activity throughout the culture period. Its highest activity was 0.47 U/ml, which was only detected 20 h into the culture period, before plummeting afterward.

The decline in the lipase activity of *B. subtilis* WB800-PHT43 and *B. subtilis* WB800-T1.2RQ_LipA after 24 h of culture is accompanied by a slight decrease in the cell density (OD600). The pellet obtained at this point was characterized by reddish pigmentation which is a characteristic of *Bacillus* species at stationary phase [15]. Therefore, considering the decrease in cell density, the decline in lipase activity may be attributed to the possible release of endogenous proteolytic enzymes by autolysis, since reports have shown that *Bacillus subtilis* strains, such as WB800, deficient in the extracellular proteases, AprE and NprE, are prone to autolysis following transition to early stationary phase [16]. These proteases, AprE and NprE, are said to be involved in the modulation of autolytic enzymes. Their absence results in unchecked autolytic activity [16]. This is claimed to have implications on the use of protease-deficient strains of *Bacillus subtilis* for the production of heterologous proteins since the recombinant protein can either be degraded by the released intracellular proteases or a decrease in yield might result due to a reduced number of actively producing cells [8,17]. Nevertheless, it can be deduced from Fig. 4 that the expression of lipase T1.2RQ by *B. subtilis* WB800-T1.2RQ_LipA is growth-associated since an increase in cell density is concomitant with an increase in lipase activity. Also, as seen in Fig. 4, the expression of lipase T1.2RQ does not result in any detrimental effect on the growth of *B. subtilis* WB800-T1.2RQ_LipA.

3.5. Induction efficiency and comparison of T1.2RQ lipase expression pattern in minimal vs rich media

To ensure that the presence of IPTG induces the expression of lipase T1.2RQ, the effect of induction was studied through the direct assay of cell-free culture supernatant at intervals for 36 h. We observed that IPTG-induced lipase T1.2RQ expression has about 60% higher lipase yield than uninduced expression (Fig. 5A). Similarly, the presence or absence of IPTG-induction did not result in a significant variation in cell density (Fig. 5B), indicating that IPTG does not exhibit toxic effects towards the growth of *B.*

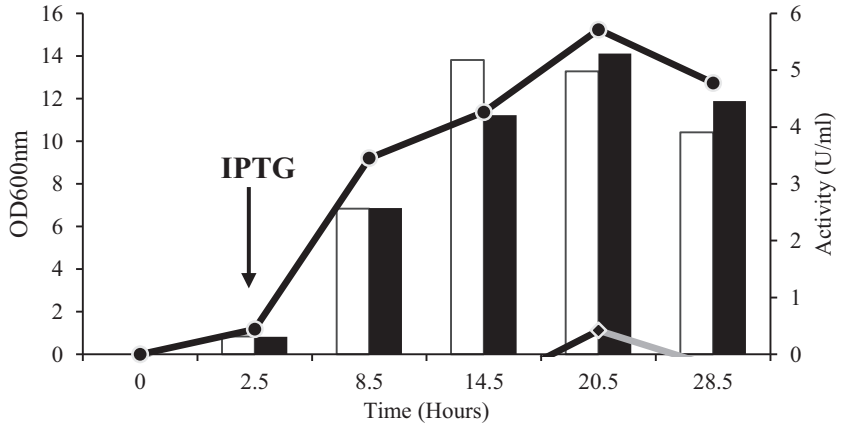

Fig. 4. Time course expression profile of *Bacillus subtilis WB800-* PHT43-T1.2RQ_LipA. Lipase T1.2RQ was expressed in 50 ml terrific broth medium and induced with 1 mM IPTG at OD600nm = 0.9. Sampling was done every 6 h. Values are lipase activity of 90× supernatant ultrafiltrates expressed as mean ± SEM (n = 3), and the optical density of the cell culture at 600 nm wavelength. (◆) Lipase activity of *B. subtilis* WB800-PHT43, (●) Lipase activity of *B. subtilis* WB800-T1.2RQ_LipA, (□) OD600nm of *B. subtilis* WB800-PHT43, (■) OD600nm of *B. subtilis* WB800-T1.2RQ_LipA.

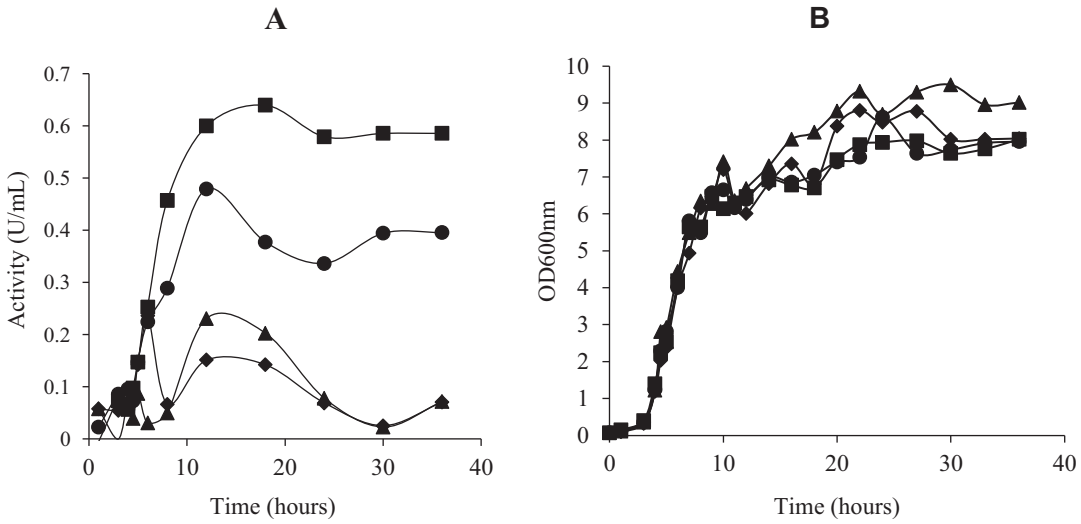

Fig. 5. 36 hours culture of recombinant *B. subtilis* WB800-PHT43 and *B. subtilis* WB800-T1.2RQ_LipA with and without IPTG induction (A) Time course activity profile by direct assay of cell-free supernatant. Lipase T1.2RQ was expressed in 50 ml terrific broth medium and induced with 1 mM IPTG at OD600nm = 0.9. Sampling was done at intervals. Values are lipase activity of supernatants and the optical density of the cell culture at 600 nm wavelength. (B) Growth curve showing the OD600nm throughout the culture period. (▲) *B. subtilis* WB800-PHT43 (◆) *B. subtilis* WB800-PHT43 + IPTG (●) *B. subtilis* WB800-T1.2RQ_LipA, (■) *B. subtilis* WB800-T1.2RQ_LipA + IPTG.

subtilis WB800-T1.2RQ_LipA and *B. subtilis* WB800-PHT43 [17]. Although the OD600nm was slightly lower for *B. subtilis* WB800-T1.2RQ_LipA, we attributed this to the additional metabolic burden resulting from the expression of the recombinant lipase T1.2RQ.

Subsequently, we expressed lipase T1.2RQ in defined minimal media (M9) and compared the expression and cell growth pattern to when the enzyme was expressed in terrific broth (Fig. 5). We observed that the extracellular lipase activity of *B. subtilis* WB800-PHT43 and *B. subtilis* WB800-T1.2RQ_LipA in M9 minimal media was only detectable at a low level, 0.05 U/ml for the former and 0.08 U/ml for the latter, within the first 12 h of the culture period, after which the lipase activity plummeted and remained low afterward throughout the culture period (Fig. 5A). However, when *B. subtilis* WB800-T1.2RQ_LipA was cultured in terrific broth (TB), lipase activity was detected in the supernatant within 30 min of induction (0.09 U/ml), and continued to increase, reaching its highest activity, 0.64 U/ml, within the first 24 h (Fig. 6A). This shows that TB medium is more favorable for the extracellular expression of lipase T1.2RQ in *Bacillus subtilis* WB800.

The difference between lipase T1.2RQ expression pattern in M9 and TB media is similar to that reported for *B. subtilis* lipase B (Lip B) in which case, LipB can only be expressed in rich media (LB + glucose), in contrast to lipase A (LipA) which can be expressed in both rich and minimal media [10,18]. Also, the high and readily available amino acid content of TB media possibly supported the expression of lipase T1.2RQ, similar to the case where the lipB gene was not expressed when *B. subtilis* was grown in minimal medium, but appreciable activity was recorded after the addition of 1 mg/ml casamino acids to the medium [10]. Eggert et al. [10] attributed this phenomenon to a regulation mechanism that controls enzyme expression using the level of available free amino acid and external pH. Meanwhile, further investigation is required to elucidate the metabolic processes responsible for the differential expression of lipase genes in *Bacillus subtilis* with reference to growth media.

Furthermore, in terrific broth, the cell density quickly attained the OD600 = 0.9, necessary for induction, within 2.5 h. The growth rate in this media was fast and consistent, reaching a maximum OD600nm of 9.5 and 7.9 for *B. subtilis* WB800-PHT43 and *B. subtilis*

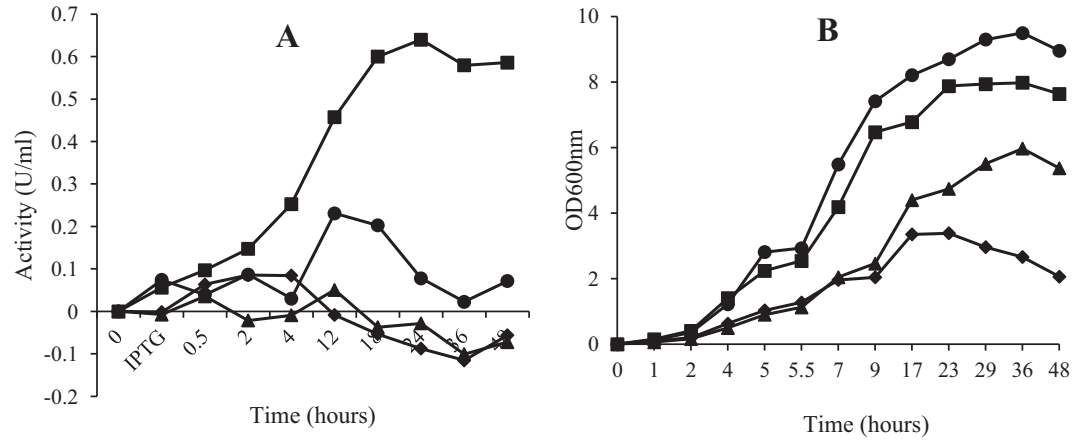

Fig. 6. Expression of lipase T1.2RQ in rich and minimal media. (A) Activity profile of *B. subtilis* WB800-PHT43 and *B. subtilis* WB800-T1.2RQ_LipA in terrific broth medium and M9 minimal medium. (B) 48 h growth dynamics of *B. subtilis* WB800-PHT43 and *B. subtilis* WB800-T1.2RQ_LipA in terrific broth medium and M9 minimal medium. Values are lipase activity of supernatants, and the optical density of the cell culture at 600 nm wavelength. (●) *B. subtilis* WB800-PHT43 + TB medium, (▲) *B. subtilis* WB800-PHT43 + M9 minimal medium, (■) *B. subtilis* WB800-T1.2RQ_LipA + TB medium, and (◆) *B. subtilis* WB800-T1.2RQ_LipA + M9 minimal media.

WB800-T1.2RQ_LipA respectively (Fig. 6B). However, *B. subtilis* WB800-PHT43 and *B. subtilis* WB800-T1. 2RQ_LipA exhibited a slow growth rate in M9 media, requiring up to 5 h to reach OD600 = 0.9. Also, contrary to TB media in which cell density remained high throughout the culture period, in M9 media, the OD600nm of *B. subtilis* WB800-T1.2RQ_LipA began to plummet 6 h after induction. The highest OD600nm in M9 minimal media was 6.0 and 3.3 for *B. subtilis* WB800-PHT43 and *B. subtilis* WB800-T1.2RQ_LipA, respectively, which dropped to 5.7 and 2.0, respectively at 48 h culture time (Fig. 6B). The higher biomass yield of the cells grown in TB suggests that the growth of *Bacillus subtilis* cells is favorably supported in TB media than M9 media. The metabolic burden resulting from the expression of lipase T1.2RQ in *B. subtilis* WB800-T1.2RQ_LipA further dampens the growth of the latter in M9 media. Evidently, such metabolic burden was surmounted by the use of rich media, as shown in Fig. 6B. The low biomass yield in M9 minimal media likely contributed to the low lipase yield in this media since Fig. 5 showed that lipase T1.2RQ expression in *B. subtilis* is a growth-associated process.

3.6. SDS-PAGE and zymogram analysis

The protein concentration of the supernatant ultrafiltrate was determined by the BCA method. 20 μg of the ultrafiltrate was used for 12% denaturing SDS-PAGE. Zymogram was done using TBN agar plate, with and without phenol red indicator. Prior Bioinformatic analysis has shown that lipase T1.2RQ has a molecular weight of about 44 kDa. SDS-PAGE analysis in this study shows that a band near 44 kDa is present in both *B. subtilis* WB800-T1.2RQ_LipA and *B. subtilis* WB800-PHT43 (negative control) (Fig. 7).

However, when zymogram analysis was performed using 90 × ultrafiltrate of cell-free supernatants, only samples of *B. subtilis* WB800-T1.2RQ_LipA demonstrated lipase activity - halo zone was observed after the overnight incubation of the renatured agar gel on TBN agar (Fig. 7). Samples of *B. subtilis* WB800-PHT43 produced no clear zone, indicating the absence of lipase activity. Similarly, when the renatured agar gel was plated on TBN + phenol red agar. *B. subtilis* WB800-T1.2RQ_LipA produced a yellow zone on a pink background. Supernatant samples of *B. subtilis* WB800-PHT43 which should still contain LipA (19 kDa) and LipB (22 kDa) did not produce a halo zone. This is because proteins with molecular weight less than 30 kDa have been removed by the preceding step of supernatant ultrafiltration using a 30 kDa cut-off amicon filter. This formation of a lipolytic zone by the supernatant ultrafiltrate of *B. subtilis* WB800-T1.2RQ_LipA shows that lipase T1.2RQ, which has a molecular weight of 44 kDa, was successfully expressed extracellularly in *B. subtilis* in an active state.

3.7. PCR mutagenesis of promoter Pgrac01 to Pgrac100

For the expression of lipase T1.2RQ to be industrially feasible, a higher enzyme yield is required. We attempted to improve lipase T1.2RQ expression in *Bacillus subtilis* by promoter engineering of the Pgrac01 promoter to Pgrac100. Pgrac100 is based on Pgrac01 but possesses an optimized −35, −15, −10, and +1 region, which has been proven to produce a 9-fold increase in promoter activity in the expression *Geobacillus stearothermophilus* β-galactosidase gene [9]. To eliminate the effect of extracellular export on the yield of the original and optimized promoter, Pgrac01, and Pgrac100 expression vector without signal peptide was constructed. To create promoter Pgrac100, expression vector PHT43-T1.2RQ (with no signal peptide) was amplified using primers PGRAC100-F and PGRAC100-R to produce a 9.2 kb DNA product, which was then self-ligated (Fig. 8) and transformed into *E. coli* DH5α. Transformants were selected by PCR colony, and verified by DNA sequencing. Sequence verified vectors were transformed into *B. subtilis* WB800

3.8. Qualitative comparison of Pgrac01 to Pgrac100

B. subtilis WB800 (PHT43, pGrac01-T1.2RQ & pGrac100-T1.2RQ) was cultured in 50 ml TB medium containing 5 μg/ml chloramphenicol, under 1 mM IPTG induction. It was observed that, as expected, the intracellular and total lipase activity of *B. subtilis* WB800 pGrac01-T1.2RQ and *B. subtilis* WB800-pGrac100-T1.2RQ was significantly higher than *B. subtilis* WB800-PHT43 ($p < 0.05$). However, contrary to our expectation and the report of Phan et al. [10] that pGrac100 produced 9-fold β-galactosidase activity and 27-fold GFP expression levels over PGrac01, the lipase activity of both *B. subtilis* WB800 pGrac01-T1.2R Q and *B. subtilis* WB800-pGrac100-T1.2RQ were not significantly different ($p < 0.05$) Table 1 and Table 2), indicating that the improved promoter did not increase lipase T1.2RQ expression.

In support of our findings in Table 1 and Table 2, it has been reported that the use of a high-performing promoter does not necessarily result in a higher expression. This is because efficient

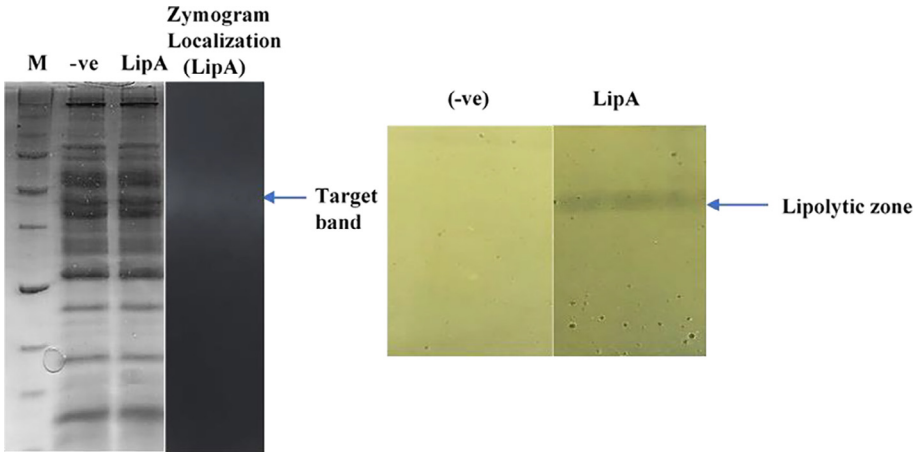

Fig. 7. 12% SDS-PAGE and Zymogram of the cell-free supernatants. Supernatant samples were processed by 30 kDa cut-off ultrafiltration to concentrate the sample. Sample volumes equivalent to 20 μg protein was loaded into each well for SDS-page. 200ug was used for zymogram analysis. M = Biorad Precision Unstained Marker, (−VE) = *B. subtilis* WB800-PHT43; LipA = *B. subtilis* WB800-T1.2RQ_LipA. The SDS-PAGE analysis showed the presence of a band corresponding to the size of the target protein. Zymogram localization also confirmed the position of the protein, based on migration distance Rf calculation (Data not shown).

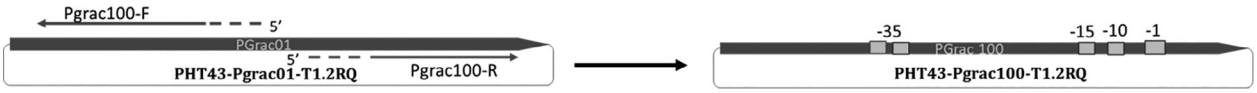

Fig. 8. Construction of pGrac100 vector by Mutagenesis PCR. Vector, PHT43-T1.2 (without signal peptide) was used as a template. 18 cycles of mutagenesis PCR was carried out using Phusion high-fidelity DNA polymerase with primers, Pgrac100-F, and Pgrac100-R. The PCR reaction produced a 9.2 kb linear DNA. Contaminating template DNA was removed by DpnI digest. The 9.2 kb was subsequently self-ligated to produce a circular plasmid. pGrac01-T1.2RQ = Vector PHT43_Pgrac01 + lipase T1.2RQ gene, pGrac100-T1.2RQ = Vector PHT43_Pgrac100 + lipase T1.2RQ gene, (- - -) = region of primers containing mutations of interest, (□) = regions of mutated sequences.

Table 1
Lipase Activity of Recombinant *Bacillus subtilis* pGrac01-T1.2RQ and *B. subtilis* pGrac100-T1.2RQ.

Recombinant *Bacillus subtilis* WB800	PNPL Hydrolytic Activity			
	Extracellular		Intracellular	
	Miller Unit (U/mL)	Specific Activity (U/mg protein)	Miller Unit (U/mL)	Specific Activity (U/mg protein)
Control (−ve)	0.876 ± 0.310[a]	0.056 ± 0.018[a]	0.103 ± 0.162[a]	0.020 ± 0.025[a]
Pgrac01-T1.2RQ	1.048 ± 0.090[a]	0.055 ± 0.005[ab]	2.351 ± 0.297[b]	0.259 ± 0.029[b]
Pgrac100-T1.2RQ	1.592 ± 0.165[b]	0.086 ± 0.009[b]	2.582 ± 0.366[b]	0.260 ± 0.018[b]

Data were obtained from three (3) independent cultures, followed by three (3) independent pNPL assays. Lipase activity values are expressed as mean ± SEM (n = 3). [a]Values in the same column bearing the same letters are not significantly different at a 5% confidence interval (Tukey HSD's posthoc Test).

Table 2
Total Lipase Activity of Recombinant *Bacillus subtilis* WB800 pGrac01-T1.2RQ and *B. subtilis* WB800 Pgrac100-T1.2RQ.

Recombinants *Bacillus subtilis* WB800	PNPL Hydrolytic Activity	
	Total Activity	
	Miller Unit (U/mL)	Specific Activity (U/mg protein)
Control (−ve)	0.979 ± 0.471[a]	0.0759 ± 0.041[a]
Pgrac01-T1.2RQ	3.398 ± 0.233[b]	0.317 ± 0.016[b]
Pgrac100-T1.2RQ	4.174 ± 0.207[b]	0.346 ± 0.011[b]

Data were obtained from three (3) independent cultures, followed by three (3) independent pNPL assays. Lipase activity values are expressed as mean ± SEM (n = 3). [a]Values in the same column bearing the same letters are not significantly different at a 5% confidence interval (Tukey HSD's post hoc Test).

transcription does not guarantee a concomitant increase in translation and correct folding of proteins [5,19], as many regulatory checkpoints exist in the process of translation, folding, and beyond. In the past, the optimization of downstream regulatory factors such as RBS, codon usage, and chaperone expression has resulted in an increased yield of recombinant protein [5,20]. For example, when *hrcA*, the regulatory element in the expression of the GroE and the DnaK series of *B. subtilis* intracellular molecular chaperones, was inactivated, the expression of antidigoxin scFv and fibrin-specific ScFv increased significantly [21,22]. Similarly, the overexpression of the chaperone/foldase, PrsA, bound to the outer surface of the cell membrane which mediates protein folding at the late stage of secretion, alone produced a 7-fold increase in *Bacillus stearothermophilus* α-amylase (AmyS) [23] and led to an increase in the total amount of single-chain antibody fragment, scFv [20,24]. Likewise, Wang et al. [25] also obtained a 67.5% increase in microbial transglutaminase activity expressed in *B. subtilis* SCK6 after codon optimization of the gene to its preferred codon usage in *B. subtilis*. They argued that the low level of transfer RNA molecules prevented protein expression when rare codon charged-tRNA molecules are much lower than the abundant codons. Therefore, these downstream regulatory factors can be candidates for optimization in future attempts to increase the yield of lipase T1.2RQ and attain industrial feasibility.

It is also worthy of note that reports have also shown that lipase expression in *B. subtilis* is subject to strict metabolic regulations which prevent *Bacillus subtilis* from exorbitantly producing lipase [12]. Kennedy and Lennarz [26] and Kent and Lennarz [27] also demonstrated the presence of a lipase inhibitor protein produced by *B. subtilis* to prevents the hydrolysis of its membrane lipids and maintain osmotic-stability. Similarly, the genus *Bacillus* has been reported to produce lipopeptides such as iturin, fengycin, surfactin, and plipastatins that act as inhibitors of lipase and phospholipase activity [28,29].

4. Conclusions

Thermostable *Geobacillus stearothermophilus* lipase T1.2RQ was successfully expressed extracellularly in *Bacillus subtilis* WB800 using different signal peptides. Lipase A (LipA) signal peptide produced the highest extracellular lipase activity, a 6-fold higher yield over the parent *Bacillus subtilis* WB800. We hope that the findings of this study will provide a basis for the improvement and future industrial application of lipase T1.2RQ.

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Conflict of interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2021.07.003.

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