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Research Article

Molecular cloning of a dual-promoter system for the expression of extracellular α-amylase and neutral protease in *Bacillus subtilis* PY79

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ABSTRACT

The genus *Bacillus* has extensively been exploited for the production of biopharmaceutical proteins and industrial enzymes as naturally secreted products. Recent studies in *Bacillus* suggested that combining two strong promoters could potentially enhance protein yields. In this study we aimed to construct *Bacillus subtilis* PY79 that secretes α-amylase and neutral protease under the control of either one or two promoters and evaluate their secreted enzyme's activities. First, plasmids pDGM1 and pDGM2 containing P_{spoVG} and P_{amyL}–P_{spoVG} promoter system, respectively, were constructed. Genes encoding α-amylase (*amy*E) from *B. subtilis* PY79 and neutral protease (*npr*E) from *Bacillus amyloliquefaciens* were then inserted to the constructed plasmids, after which they were transformed into *B. subtilis* PY79. Effects of promoter systems on the expression of these two genes were evaluated through the activity of enzymes secreted in the growth medium. As a result, after 48 hours, the recombinant carrying the P_{amyL}–P_{spoVG} dual promoter provided the highest enzyme activity, 2.6-fold higher than the control *B. subtilis* PY79 for the expression of both extracellular enzymes. At the same time, the folds increased in enzyme activity of *B. subtilis* PY79 strain bearing the P_{spoVG} single promoter was 1.3 and 1.9 for α-amylase and neutral protease, respectively. The result of this study consolidated the rational design of dual-promoter systems to enhance the gene expression in *B. subtilis*. In particular, the recombinant *B. subtilis* strain carrying the P_{amyL}–P_{spoVG} dual promoter holds promising potentials for larger scale enzyme production applications in pharmaceutical biotechnology.

Keywords:
α-Amylase; *Bacillus subtilis*; Dual promoter; Neutral protease, Self-cloning

1. INTRODUCTION

Enzymes are biocatalysts playing crucial roles in biotechnology for the synthesis of valuable compounds in pharmaceutical, agricultural and food industries. Microbial technology has long been exploited as a handy work hourse for enzyme production with outstanding advantages, e.g., low cost, high yield of biomass, simple purification procedure, and environmental safety. *Escherichia coli* and *Bacillus* species (*Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*) are among the most popular microbes used for the manufacture of recombinant proteins in industry. For instance, α-amylase (in starch hydrolysis) and proteases (as laundry agents) currently used are largely supplied by *Bacillus* fermentation where the enzymes are naturally secreted into the medium during their growth[1]. For pharmaceutical proteins, *E. coli* is more commonly used as an expression host since the genetic toolbox has been intensively studied and well accessible for most laboratories. Nevertheless, as a Gram-negative bacterium, *E. coli* contains lipopolysaccharides (LPS, generally referred to as endotoxins) as a constituent of its outer cell membrane that can complicate the downstream purification to ensure a pyrogen-free medicinal or food product.

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In constrast to *E. coli*, the Gram-positive *B. subtilis* represents an attractive host for protein expression for pharmaceutical biotechnology as it is generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA). *B. subtilis*is also among the best characterized Gram-positive bacterium in terms of genomic and proteomic studies which allows a high amenability for metabolic engineering[2-3]. The capability of *B. subtilis* to directly export target proteins into the extracellular medium also naturally simplifies the downstream purification, thus reduces the process costs, especially in large-scale applications.

Despite the clear advantages favoring the use of *B. subtilis* as a secretion host, its applications in the pharmaceutical industry remain less widespread than that of *E. coli*. Such a reservation to use *Bacillus* in general may partly due to the lack of efficient vectors to obtain high yields of proteins[3-4]. This drawback can be addressed by the optimization of transcription and translation process by fine-tuned design of the expression cassette with suitable components. A pivotal element of a strong expression vector is the promoter which regulates the transcription process: a highly powerful and controllable promoter is prerequisite to result in an elevated amount of protein expressed. Interestingly, recent studies in *B. subtilis* have suggested that the rational combination of strong promoters for the control of one gene could give rise to a higher level of expression than when a single promoter was used. Examples of efficient tandem (dual- or triple-) promoter systems that were successfully developed are P_{SpoVG}–P_{amyQ}, P_{gsiB}–P_{HpaII}, P_{HpaII}–P_{amyQ}, P_{HpaII}–P_{HpaII}–P_{P43}, to name but a few[5-9]. In particular, the P_{SpoVG} promoter is activated during the late exponential and stationary phases of bacterial growth, triggering the production of spore coat proteins in response to adverse conditions. Leveraging this promoter for recombinant protein production in *Bacillus* has shown promise in enhancing protein yields, particularly enzymes[5,6]. Liu (2018) demonstrated that *B. subtilis* containing P_{SpoVG} produced the highest extracellular pullulanase activity among the single promoter systems examined, achieving 412.9 U/mL. Combining P_{SpoVG} with P_{AmyL} significantly increased pullulanase activity, reaching 625.5 U/mL[5]. Similarly, Ferrando (2024) reported that the dual-promoter system P_{SpoVG}–P_{amyQ} in strain BSQ1a yielded substantial enzyme activity, while the triple-promoter system P_{SpoVG}–P_{amyQ}–P_{cry3A} resulted in a 4.1-fold increase compared to the single-promoter strain[6].

Advances in recombinant technology have allowed the generation of engineered strains that can sustain harsh artificial growth conditions while being able to express higher levels of target proteins/enzymes. Nevertheless, as with other genetic modified organisms, the safety of such recombinant microbes have remained a world-wilde concern when they are released to the environment[10]. With this regard, self-cloning has emerged as an attractive strategy where the recombinant elements are derived from bacteria of the same species or of phylogenetically closely related ones. The application of self-cloning has been widely applied in brewing yeasts for the fermented beverage industries[11], and more recently in *Aspergillus oryzae*[12].

Motivated by these positive results, in this study we aimed to make use of both approaches in *B. subtilis* PY79, *i.e.*, designing an expression cassette bearing highly active dual-promoter systems and with self-cloned genetic components. The generated recombinant *B. subtilis* PY79 could serve as an effcient protein cell factory while remaining environmentally safe. Genes encoding two extracellular enzymes, α-amylase and neutral protease, were cloned and their expression levels from the single- and dual-promoter vectors were compared.

2. MATERIALS AND METHODS

2.1. Materials

Escherichia coli DH5α and *B. subtilis* PY79 were used as cloning and expression host, respectively. Plasmid pDP carrying the expression cassette for secretion was designed and synthesized by Phusa Genomics (Can Tho, Vietnam). Plasmid pDG364 (Invitrogen) was used as a background vector to insert the target genes together with the locus *amy*E for integrating into *B. subtilis* genome. Restriction enzymes, DNA T4 ligase were purchased from New Englands Biolabs. DNA SiZer™-1000 ladder was a product of iNtRON Biotechnology (Korea).

2.2. Methods

2.2.1. Constructing plasmid pDP carrying the expression cassette for secretion with strong promoters and transformation into E. coli *DH5α*

The expression cassette for secretion was designed and synthesized with the following order: *Bam*HI – UP-hag – P_{SpoVG} – *Bgl*II – P_{AmyL} – RBS – SP_{amyQ} – MCS – T_{amyE} – *Eco*RI, which was transferred to plasmid pUC19 and provided as plasmid pDP. Plasmid pDP was then digested with either pair of restriction enzymes *Bgl*II/*Eco*RI or *Bam*HI/*Eco*RI to receive the insert M1 (cassette with one promoter), or the insert M2 (cassette with two promoters). *Bam*HI/*Eco*RI treated plasmid pDG364 was joined with inserts M1, M2 by T4 DNA ligase to generate plasmids pDGM1 and pDGM2, respectively **(Figures 1 and 2)**. The two recombinant vectors were transformed into chemically competent *E. coli* DH5α cells by heat shock. The transformants were selected based on colony PCR; the size of the extracted vector was examined by linearizing cleavage with *Xho*I.

As the expression system used the export signal of *amy*Q, the primers to amplify the targeted genes were designed to anneal right after the secretion region together with the recognition sequence of *Bsa*I for cloning into the cassette. The export signal was identified by SignalIP 5.0 Server[13].

The genes *amy*E (Gene ID: 938356) and *npr*E (Gene ID: CP003332.1) was amplified from the genomic DNA of *B. subtilis* PY79 and *Bacillus amyloliquefaciens*, respectively, using the corresponding pairs of primers listed in Table 1. After being cut with *Bsa*I, the PCR products were connected to either vector pDGM1 or pDGM2 by T4 DNA ligase to yield the corresponding vectors pDGM1-*amy*E, pDGM2-*amy*E, pDGM1-*npr*E, and pDGM2-*npr*E. The four recombinant vectors were transformed into *E. coli* DH5α; the transformants were analyzed using colony PCR and by linearizing the isolated vectors with *Xho*I.

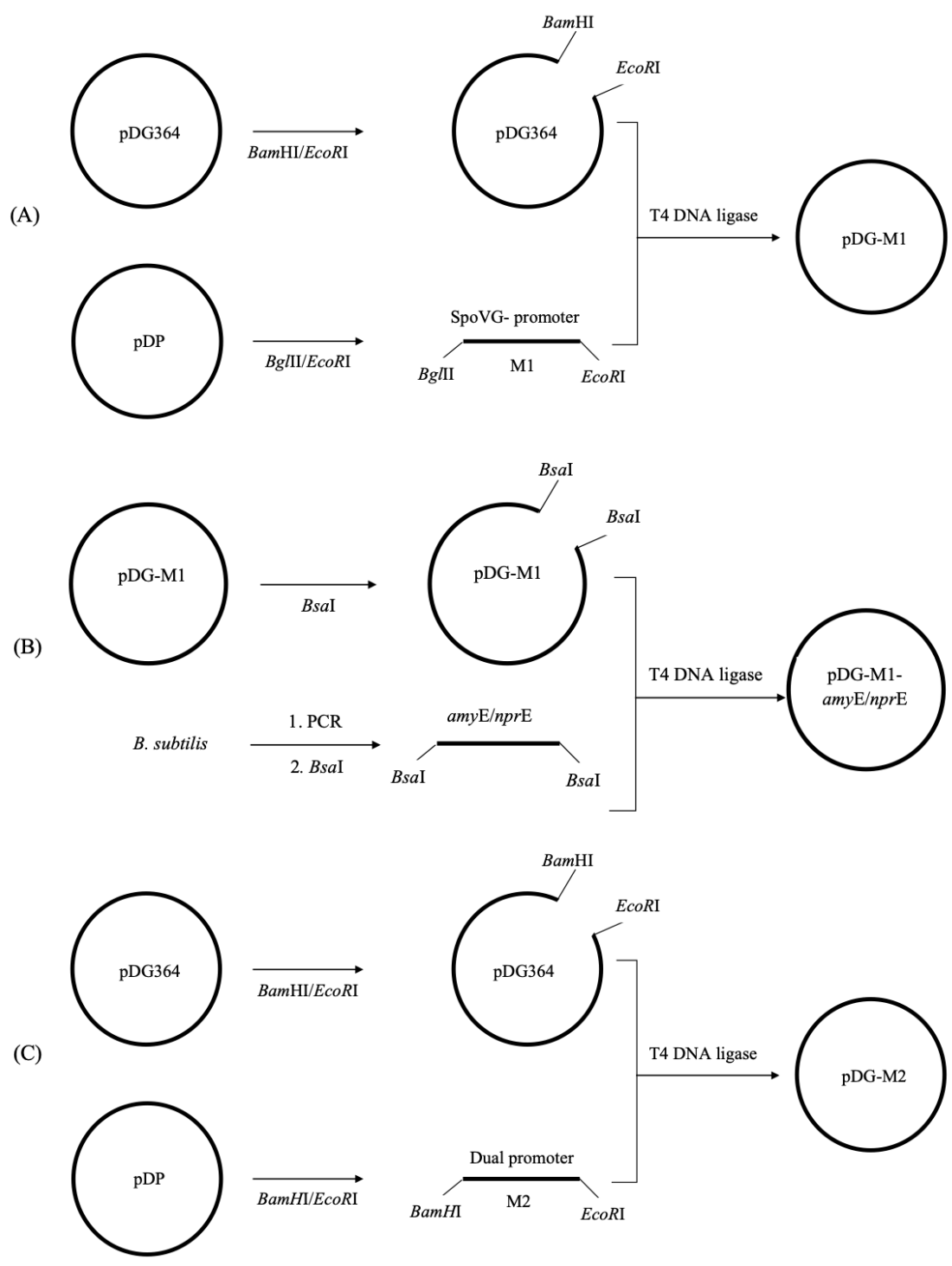

Figure 1. Schematic diagram of the construction process of recombinant plasmids pDG-M1 **(A)**, pDG-M1-*amy*E/*npr*E **(B)**, and pDG-M2 **(C)**.

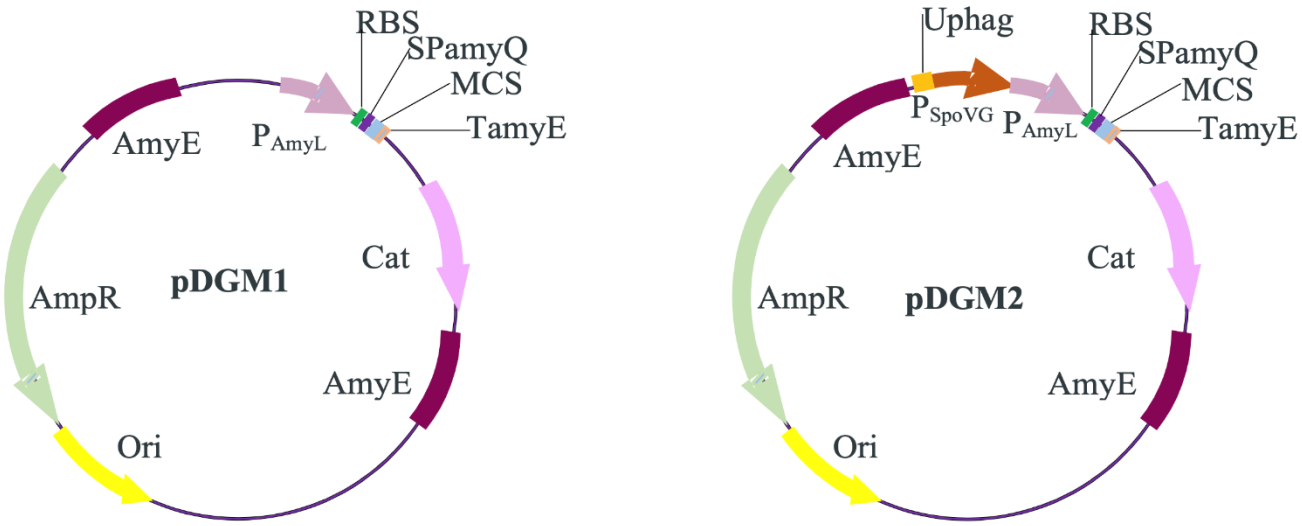

Figure 2. Schematic illustration of plasmids pDGM1 and pDGM2. P, promoter; RBS, ribosome binding site; MCS, multiple cloning site; AmyE, the gene encoding α-amylase from *B. subtilis* PY79; AmpR, the gene encoding β-lactamase; Cat, the gene encoding chloramphenicol acetyl transferase; SPamyQ, secreted peptide sequence from *Bacillus amyloliquefaciens*; TamyE, transcription termination sequence from *Bacillus subtilis*

2.2.2. Generating B. subtilis *PY79 carrying promoters and genes encoding extracellular enzymes*

Vectors pDGM1-*amy*E, pDGM2-*amy*E, pDGM1-*npr*E and pDGM2-*npr*E were first linearized by *Pst*I and then transformed into competent *B. subtilis* PY79 cells using double crossover method with locus *amy*E[14] to generate four corresponding transformants: PY-M1-*amy*E, PY-M2-*amy*E, PY-M1-*npr*E and PY-M2-*npr*E. The transformed bacteria were first selected from Luria–Bertani agar plates containing 5 µg/mL chloramphenicol (LBA_{C5}). PY-M1-*amy*E and PY-M2-*amy*E were screened by PCR using the genomic DNA and the pair of primers AmyE_DPF/catR_phag. For PY-M1-*npr*E and PY-M2-*npr*E, the same analysis were applied using the pair of primers nprE_F/nprE_R **(Table 1)**.

Table 1. Primers used in this study[(1)]

Purpose	Primer	Sequence 5′–3′
Amplification of *amy*E	AmyE_DPF	GGTCTCTAGTCGGgaaacggcgaacaaatcgaatg
	AmyE_DPR	GGTCTCTACTGtcaatggggaagagaaccgc
Amplification of *npr*E	nprE_F	GGTCTCTAGTcaggccgctgagaatcctc
	nprE_R	GGTCTCTACTGttacaatccgactgcattccagg
Analysis of transformants	Amy_CF	ccgctgaagaatatggcataaagg
	Amy_CR	gaatttaggaggcttacttgtctgc
	AmyQ_F	GGTCTCTAACAatgattcaaaaacgaaagcg
	TamyEDP_R	CCGgaattctctttttgtttgtgaag
	catR_pHag	GGTCTCTCAgctctcccttatgcgactcc

[(1)] Additional sequence for restriction cleavage are marked with nucletides in upper case, of which the *Bsa*I restriction sites are underlined.

2.2.3. Evaluation of gene amy*E expression via α-amylase activity by Heinkel method*

Starch hydrolysis ability of PY-M1-*amy*E, PY-M2-*amy*E was preliminarily judged by the diameter of the hydrolysis halos surrounding bacterial growth on nutrient agar (NA) plates supplemented with 1% (w/v) starch and 5 µg/mL chloramphenicol. After that, they were grown in nutrient broth (NB) with the same supplemements at 37 ℃, 200 rpm. The growth medium which contained the secreted α-amylase was collected at 24, 36 and 48 hours and the enzyme activity was determined by Heinkel method[15]. In particular, the amount of starch degraded was measured based on the reduction in color intensity of the reaction mixture with iodine solution. Briefly, 0.1 ml of the sample, 0.3 ml of phosphate buffer pH 6 and 0.1 ml of 1% starch were incubated for 15 minutes at 30 °C. Then, 0.5 ml of 20% sulfosalicylic acid was added, and 0.1 ml of the solution was transferred into a new tube. Next, 0.9 mL of iodine was added, and the absorbance at 560 nm was measured. One activity unit is defined as the amount of enzyme capable of hydrolizing 1 mg of starch after 15 minutes at 30 ℃.

2.2.4. Evaluation of gene npr*E expression via neutral protease activity by Anson method*

Proteolysis activity of PY-M1-*npr*E, PY-M2-*npr*E strains was preliminarily checked by the diameter of the hydrolysis halos surrounding bacterial growth on NA plates supplemented with 1% (w/v) gelatin and 5 µg/mL chloramphenicol upon addition of 50% trichloroacetic acid (TCA). After that, they were grown in NB with the same supplements at 37 °C, 200 rpm. The growth medium which contained the secreted neutral protease was collected at 24, 36 and 48 hours and the enyzme activity was determined by Anson method[16]. Briefly, 0.1 mL of the medium was added to 0.5 mL of 0.65% casein and incubated for 30 minutes at 37 °C. Then, 0.5 mL of 20% TCA was added, incubated for 30 minutes at 37 °C, and centrifuged at 10,000 × *g* for 10 minutes. Next, 0.2 mL of the supernatant was transferred to a new tube where 0.5 mL of 500 mM Na_2CO_3, and 0.1 mL of Folin's reagent were added. Finally, the absorbance was measured at 660 nm. One activity unit is defined as the amount of enzyme catalyzing the formation of product soluble in TCA reacting with Folin's reagent that is equivalent to 1 µM tyrosine.

2.2.5. Statistics

The results were reported as means and standard deviations of measured values (n = 3). Statistical analysis was performed using multiple paired t-test with GraphPad Prism version 9.1.0 for Windows (GraphPad Software, Boston, Massachusetts USA). A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Generating *E. coli* DH5α bearing promoters and genes encoding extracellular enzymes

The two inserts M1 (320 bp) and M2 (424 bp) were obtained from the synthesized plasmid pDG by cleaving with the different pairs of restriction enzymes. After ligating the inserts and the *Bam*HI/*Eco*RI-treated pDG364 vector, the resulting plasmids were transformed into *E. coli* DH5α. Colony PCR (data not shown) and digestion of the isolated plasmids showed bands with the predicted size on the agarose gel: pDGM1 (6.7 kb), pDGM2 (6.8 kb) (Figure S1A).

Gene *amy*E (1906 bp, from *B. subtilis* PY79) and genes *npr*E (1513 bp, from *B. amyloliquefaciens*) were sucessfully amplified with the designed pairs of primers that contains the *Bsa*I restriction site. The two genes were cloned into the either plasmid pDGM1 or pDGM2 (previously treated with *Bsa*I) using *E. coli* DH5α as a host. Colony PCR (data not shown) and digestion of the extracted plasmids showed the bands having the calculated size upon electrophoeresis: pDGM1-*amy*E (8.6 kb) and pDGM1-*npr*E (8.1 kb) (Figure S1B), pDGM2-*amy*E (8.7 kb) and pDGM2-*npr*E (8.2 kb) (Figure S1C).

3.2. Generating *B. subtilis* PY79 carrying promoters and genes encoding extracellular enzymes

Plasmids with either one promoter (pDGM1-*amy*E and pDGM1-*npr*E) and two promoters (pDGM2-*amy*E and pDGM2-*npr*E) were linearized with *Pst*I and transformed into *B. subtilis* by double-crossover method and selected using LBA containing 5 µg/mL chloramphenicol. Three colonies for each transformation were selected and grown for genomic DNA extraction to identify the presence of the genes inserted by PCR. As a result, for transformed *B. subtilis* carrying gene *amy*E (using vector pDGM1-*amy*E and pDGM2-*amy*E), amplified DNA product had a size of ~3.3 kb as predicted (colonies 1–3 in Figure S2A, 1 and 3 in Figure S2B). The obtained recombinant strains were named PY-M1-*amy*E and PY-M2-*amy*E. Similarly, transformants with gene *npr*E (using vectors pDGM1-*npr*E and pDGM2-*npr*E) were confirmed as the PCR products having the expected size of 1.5 kb (colonies 1 and 2 in Figure S2C, 2 and 3 in Figure S2D). The recombinant *B. subtilis* were stored as PY-M1-*npr*E and PY-M2-*npr*E.

3.3. Expression of gene *amy*E within the recombinant promoters in *B. subtilis* transformants

The two recombinant *B. subtilis* strains PY-M1-*amy*E, PY-M2-*amy*E could grow on NA plates containing chloramphenicol 5 µg/mL, in contrast to the negative control *B. subtilis* PY79 **(Figure 3A, B)**. They could also hydrolyze starch as indicated by the clear halo zone formed around the colonies visualized by adding the Lugol solution. Based on the diameters of the halos, it can be observed that the PY-M2-*amy*E strain with the dual promoter P_{amyL}–P_{spoVG} manifested the highest starch hydrolysis activity in compared with that of PY-M1-*amy*E carrying a single promoter P_{amyL} and the *B. subtilis* PY79 control (Table S1 and **Figure 3C**).

Activity of secreted α-amylase for the transformants were measured from the growth medium by time using Heinkel method (Table S2). The PY-M2-*amy*E with the dual promoter P_{amyL}–P_{spoVG} consistently displayed the highest α-amylase activity among the three strains. After 24 h, the PY-M1-*amy*E and PY-M2-*amy*E showed 1.3- and 1.9-fold α-amylase activity in compared with the original PY79 strain, respectively. In particular, the fold differences at 36 h and 48 h were even more noticeable for the PY-M2-*amy*E strain, being correspondingly 2.2 and 2.6 (**Figure 4A** and Table S2). The highest secreted α-amylase activity was achieved at 48 h for all three evaluated strains.

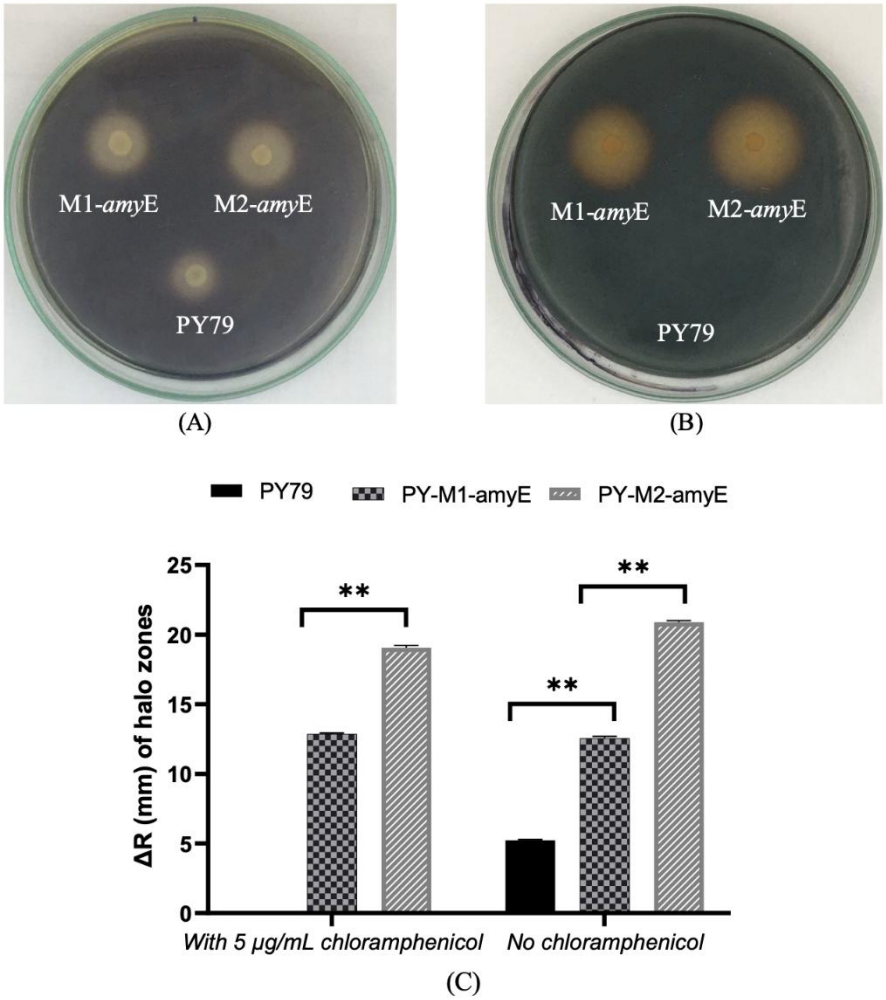

Figure 3. Halo zone formation by starch hydrolysis test for *B. subtilis* transformants on 1% (w/v) starch NA plates without **(A)** and with 5 μg/mL chloramphenicol **(B)**. The diameters of the halo zones from the two recombinant strains were compared with that of the PY79 control **(C)**. (**: $p < 0.01$)

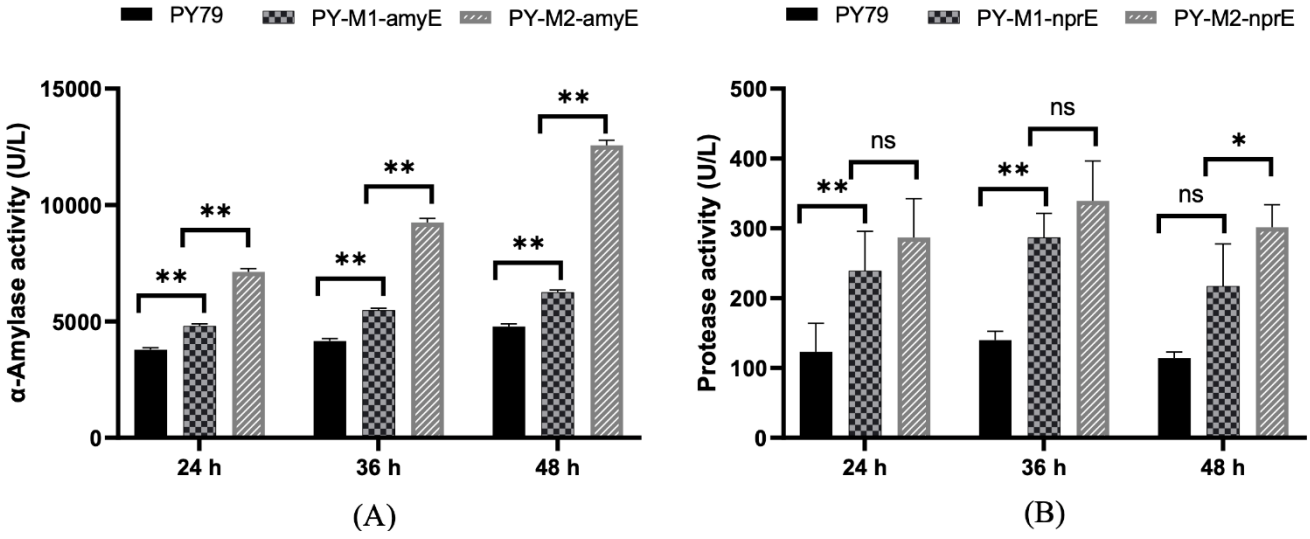

Figure 4. Secreted α-amylase (**A**) and protease (**B**) activities of *B. subtilis* strains at various time points. (*: $p < 0.05$, **: $p < 0.01$, ns: not significant)

3.4. Expression of gene *npr*E within the recombinant promoters in *B. subtilis* transformants

The two transformants PY-M1-*npr*E and PY-M2-*npr*E were first confirmed by their growth on NA plates containing chloramphenicol 5 µg/mL as compared with the negative control *B. subtilis* PY79 (**Figure 5A,B and Table S3)**. The two recombinants could also hydrolyze gelatin as indicated by the clear halos remained upon addition of 50% TCA. Measuring the diameters of the gelatin hydrolyzing halos showed that PY-M2-*npr*E with the dual promoter P_{amyL}–P_{spoVG} displayed a similar hydrolyzing halo with that of the PY-M1-*npr*E carrying the single promoter P_{amyL}. Both of them had slighly larger hydrolyzing halos than did the control *B. subtilis* PY79 strain (**Figure 5C and Table S3)**.

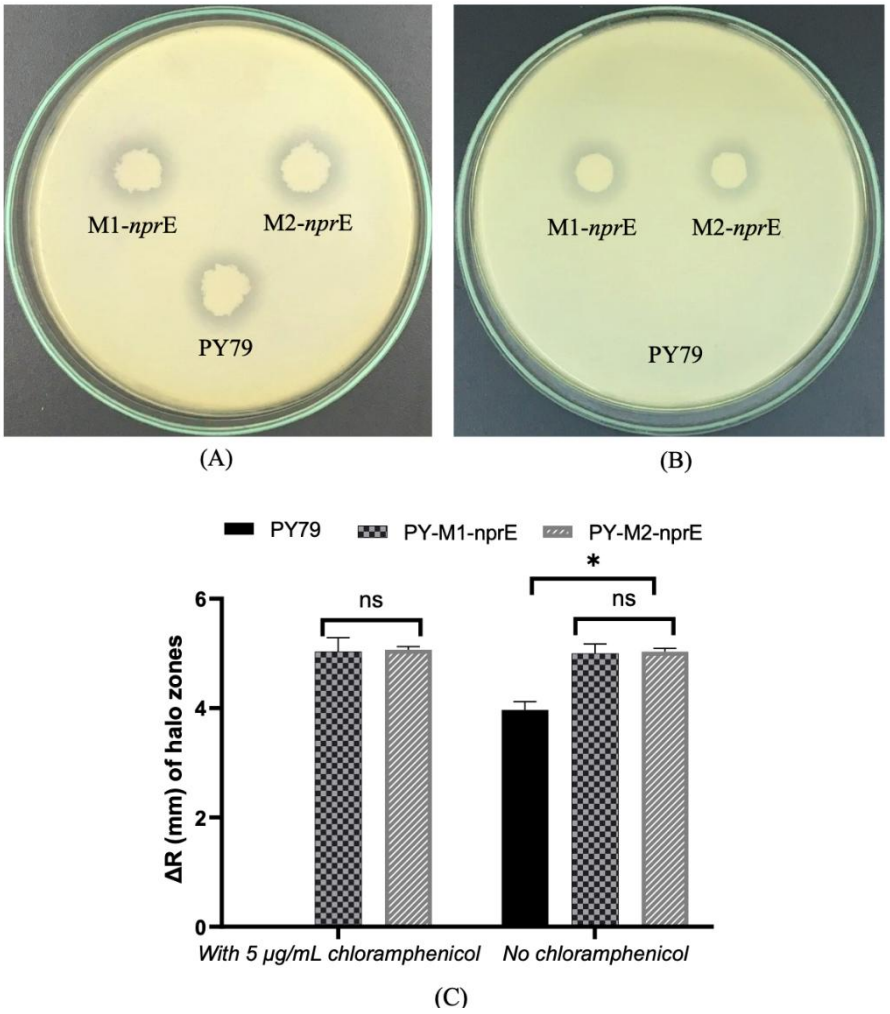

Figure 5. Halo zone formation by gelatin hydrolysis test for *B. subtilis* transformants on 1% (w/v) gelatin NA plates without **(A)** and with 5 µg/mL chloramphenicol **(B)**. The diameters of the halo zones from the two recombinant strains were compared with that of the PY79 control **(C)**. (*: $p < 0.05$, ns: not significant)

The expression of gene *npr*E were further analyzed by protease activity determination for the growth medium using Anson method. As a result, the PY-M2-*npr*E strain with two promoters P_{amyL}–P_{spoVG} displayed highest protease activity compared with the single promoter P_{amyL} containing strain (PY-M1-*npr*E) and the PY79 control. At 24 h, the PY-M1-*amy*E and PY-M2-*amy*E strains showed 1.9- and 2.3-fold protease activity compared with the original PY79, respectively. Consistent with the expression of α-amylase, the folds increased in protease activity of the PY-M2-*amy*E strain compared with PY79 after 36 h and 48 h were even more pronounced, being correspondingly 2.4 and 2.6 **(Figure 4B and Table S4)**. Nevertheless, the highest secreted protease activity was observed at 36 h for all three strains examined, which was at 48 h for α-amylase. The tendency of the protease activity to decrease after 36 hours can be attributed to several reasons, *i.e.*, nutrient depletion and stress response, the instability of the secreted enzyme due to proteolytic degradation and environmental factors such as temperature and changes in pH of the medium[17].

3. DISCUSSION

In this study, we have successfully generated *B. subtilis* PY79 transformants carrying powerful promoters to express the genes of interest. In particular, recombinant cells with the dual promoter P_{amyL}–P_{spoVG} displayed the highest secreted enzyme activity for both genes examined. After two days, α-amylase and neutral protease activities measured for two dual-promoter borne strains were both ~2.6-fold higher than that of the original *B. subtilis* PY79. The single strong promoter P_{spoVG} also conveyed better enzyme expression levels in compared with that of *B. subtilis* PY179: 1.3- and 1.9-fold higher enzymatic activity for α-amylase and protease, respectively, was observed at 48 hours. These increased secreting enzyme levels could be largely contributed by the rational design of the gene expression cassette.

A crucial component of an expression cassette that controls the transcription process is promoter, our main focus of this work. Two promoters were selected for examination, namely P_{amyL} and P_{spoVG}, both of which are considered as strong promoters without sigma factors involved or being affected by *B. subtilis* sporulation. Moreover, being auto-inducible promoters, P_{amyL} and P_{spoVG} are active from late log phase to stationary phase where proteins are synthesized in *B. subtilis*[5]. In addition, the two promoters appear to have two distinct sigma factor recognition regions: P_{amyL} is likely dependent on σ^A which is active during the stationary phase[5,18–19] whereas P_{spoVG} is σ^H-dependent, exhibiting high activity during both exponential and stationary phases[20]; thus combining these two promoters could potentially optimize the transcription time[21]. It was also postulated that, when two promoters are present within an expression cassette, one of them could play a role of an enhancer that helps increase the transcriptional rate[22]. In fact, in a previous study where 16 pairs of dual promoters were examined, highest pullulanase activities in *B. subtilis* ATCC6051 were obtained with the dual-promoter system P_{amyL}–P_{spoVG}, approximately twice as much as when either the single promoter P_{amyL} or P_{spoVG} was used[5]. the results presented in this study further consolidate the beneficial role of using the dual promoters for the enhancement of transcriptional efficiency.

In addition to promoter selection, the design of the gene expression cassette also involves various components derived from other species in the *Bacillus* genus to optimize the amount of secreted enzyme. They include the UP-hag element region for increased promoter activity[23], ribosomal binding site (RBS) sequences within the *spoVG* gene system of *B. subtilis* for higher translation efficiency[24], a strong export signal of the *amy*Q gene from *B. amyloliquefaciens*[25], a cloning region to insert the desired genes, and the T_{amyE} transcription termination sequence[26]. The genes examined for the expression was selected from species well known for high capability of secreting enzymes: *amy*E from *B. subtilis* and *npr*E from *B. amyloliquefaciens*[27-28]. All components of the expression cassette are originate from *Bacillus* genus, satisfying the self-cloning criteria and can thus be considered environmentally friendly for large-scale applications.

With this ecological safety and high expression levels of secreting proteins, the PY-M2 strains bearing two promoters hold high potential in pharmaceutical biotechnology for the production of therapeutic proteins/enzymes or biocatalysts. The dual-promoter strategy can be further investigated by varying the combination of known strong promoters. Furthermore, optimization of the expression cassette components can also be tailored for each gene of interest to obtain a desired protein yield.

4. CONCLUSION

In this work, we have demonstrated the self-cloning construction of the cassette bearing one and two strong promoters for the expression of genes encoding extracellular enzymes in *B. subtilis* PY79. The resulted recombinant strains could secrete high levels of α-amylase and neutral protease. In particular, the PY-M2 strain could serve as a host to express proteins/enzymes of high biological value.

5. ACKNOWLEDGEMENTS

Author contribution
TTV and QTN conceived the study and designed the experiments. PXTL and BTT performed the experiments. TTV and QTN drafted the manuscript. All authors contributed to analyzing the data and writing the paper.

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