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Enhanced expression of L-asparaginase II by fusion of *pSSPM3* synthetic promoter into pET-28a+ expression vector for alternative targeted therapy of acute lymphoblastic leukaemia

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ABSTRACT

The use of commercial L-asparaginase II from *E. coli* and *Erwinia chrysanthemi* for acute lymphoblastic leukemia (ALL) therapy causes adverse effects (allergic reactions, neurotoxicity), necessitating safer alternatives. *Serratia plymuthica* UBCF_13, harboring the ansB gene, offers a promising source. This study constructed the recombinant plasmid pET-28a+:*pSSPM3:ansB* to enhance L-asparaginase II expression *in E. coli* using the synthetic promoter *pSSPM3*. Molecular verification confirmed successful steps: promoter fusion (674 bp band), *ansB* digestion (1,057 bp), gene insertion (1,682 bp), and BL21 transformation. Critically, enzyme activity assays revealed that pSSPM3 did not enhance expression in BL21 (0.519 U/mL), showing significantly lower activity ($p<0.05$) than native controls (0.621 U/mL) and DH10B transformants (0.636 U/mL). While the functional plasmid establishes a platform for novel enzyme production, the unexpected activity reduction in BL21 and higher yield in DH10B highlight host-promoter compatibility challenges. Further optimization of expression systems, purification protocols, and preclinical validation (cytotoxicity, allergenicity) are essential to advance this recombinant enzyme toward therapeutic and scalable industrial applications for ALL in resource-limited settings.

Keywords: *AnsB* gene; promoter fusion; *Serratia plymuthica* UBCF_13; neurotoxicity; enzyme activity

1. INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is one of the leading causes of death in children and is characterised by abnormal lymphoblast production that impairs blood cell function. The incidence of ALL continues to rise globally, with the number of cases increasing from 49.1 thousand in 1990 to 64.2 thousand in 2017 (Dong et al., 2020). In the United States, 38,136 cases of ALL were recorded between 2001 and 2014, with an increase of approximately 6,000 cases per year (Siegel et al., 2017, 2020). In Indonesia, in 2018, the incidence rate of ALL reached 4.32 per 100,000 children, with an estimated 3,434 new cases (Garniasih et al., 2022). This significant increase in cases has created an urgent clinical and scientific need to find new and more effective therapeutic strategies. Furthermore, the high incidence in Indonesia exacerbates healthcare challenges such as restricted access to treatment, treatment-related complications or side effects, and post-therapy disease relapse. Therefore, ALL has become a major focus of research in the development of cancer therapies.

The current main therapies for ALL are chemotherapy and radiotherapy, which are able to achieve long-term remission but have significant side effects such as DNA mutations, genomic instability and the risk of secondary cancers (van den Boogaard et al., 2022). Due to limited resources and varying health infrastructure, managing the long-term complications of chemotherapy and radiotherapy is a particular challenge in Indonesia, with the

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potential to reduce patients' quality of life and life expectancy. To overcome these limitations, targeted therapy using the enzyme L-asparaginase II was developed. This enzyme hydrolyses asparagine into aspartic acid and ammonia, inhibiting the growth of asparagine-dependent cancer cells (Pavlova and Thompson, 2016).

The currently used L-asparaginase II is derived from *Escherichia coli* and *Erwinia chrysanthemi*, but it often causes side effects such as allergic reactions and neurotoxicity due to L-glutaminase activity (Batool et al., 2016; Tsegaye et al., 2024). Treatment problems in Indonesia arise due to allergic reactions and neurotoxicity that often occur as a result of L-asparaginase II, which can cause treatment interruptions or discontinuation of therapy, ultimately affecting the success rate of ALL treatment. Therefore, safer alternative sources are needed for ALL therapy.

Serratia plymuthica UBCF_13 has been identified as a potential source of L-asparaginase II based on its genomic data (Fatiah et al., 2021). To increase the production of this enzyme, the recombinant DNA technique is used because it is able to increase productivity, purity, and enzyme activity (Mahajan et al., 2012). This technique has been successfully applied in various studies, such as the expression of L-asparaginase II from *Lactobacillus casei* subsp. casei ATCC 393 and *Bacillus subtilis* CH11 using *E. coli* as the expression host (Aishwarya et al., 2019; Arredondo-Nuñez, et al., 2023). However, recombinant protein expression in *E. coli* faces challenges such as inclusion formation, endotoxins, and low solubility (Baeshen et al., 2014), which can be overcome by vector selection and expression promoter optimization.

Optimization of recombinant production can be done by using the pET-28a+ vector that has a T7 promoter and other regulatory elements that enhance protein expression (G2P, 2021; Li et al., 2022). In addition, the use of synthetic promoters offers a new approach in enhancing protein expression. Promoter engineering allows increasing the effectiveness of gene transcription with strategies such as modifying the core region and upstream activating sequences (Deng et al., 2021; Chen et al., 2023). Although the use of the pET-28a+ vector and synthetic promoters has shown potential in optimizing protein expression, no studies have explicitly explored the fusion of the *ansB* gene with the synthetic promoter *pSSPM3* specific to the pET-28a+ vector system for the production of L-asparaginase II.

Therefore, this study contributes to the field of biotechnology by providing further understanding of this specific genetic engineering strategy. The fusion of the *ansB* gene with the synthetic promoter pSSPM3 in the pET-28a+ vector is expected to substantially optimize the production of L-asparaginase II, an aspect that has not been fully explored in previous studies. This increase in production efficiency has the potential not only to significantly reduce the production costs of L-asparaginase II enzymes but also to support the development of more effective and affordable ALL therapies, particularly in developing countries. Additionally, this research will expand the industrial applications of this enzyme by providing more efficient and economical production methods.

2. MATERIAL AND METHODS

2.1 Plasmid DNA preparation

The DNA of pET-28a+ Expression Vector were prepared using the Presto™ Mini Plasmid Kit, controlled by 1% agarose gel electrophoresis using 0.5X TBE, 100 V for 40 minutes. Similar step was also done for pGEM T Easy:*ansB* Recombinant Plasmid in *E. coli* DH10B. Following plasmid preparation, plasmid DNA and the *pSSPM3* Synthetic Promoter digestion and was performed using *Bgl*II and *Nhe*I restriction Enzymes. Reaction was done in Double digestion reaction using Tango+BSA buffer at 37°C for 2 hours. The digestion products were separated on a 1% agarose gel, and the target bands (pET-28a+ fragment and pSSPM3) were extracted using the GeneJET Gel Extraction Kit enzymes (Thermo Scientific-USA).

2.2 Ligation and transformation of *pSSPM3* synthetic promoter with pET-28a+ plasmid into *E. coli*

The purified *pSSPM3* promoter (5 ng/µL) was ligated to the digested pET-28a+ vector (11 ng/µL) using T4 DNA Ligase (Promega-USA) at 4°C for 16 hours. The ligation mixture (5 µL) was then transformed into competent *E. coli* DH10B cells using heat shock approach for 30 minutes at 42°C (1 minute) and transferred into ice directly (for 2 minutes). The cells were recovered in SOC medium (37°C, 220 rpm, 75 minutes) and propagated on LB agar + kanamycin (50 µg/ml) at 37°C for overnight. Similar methods were applied for

cloning procedures of ansB gene into pET-28a+:*pSSPM3* and subsequently into *E. coli* strain BL21. Identification of transformant were detected using kanamycin antibiotic and PCR colony.

2.3 L-asparagine II enzyme activity

Enzyme activity was measured in the culture supernatant: *S. plymuthica* UBCF_13, wild-type *E. coli* BL21, pET-28a+:ansB transformants, and pET-28a+:pSSPM3:ansB transformants. The supernatant (100 µL) was reacted with Tris-HCl buffer pH 8.6 (50 µL) and L-asparagine substrate 80 mM (50 µL) (37°C, 30 minutes). The reaction was stopped with 20% TCA (30 µL), diluted with distilled water (750 µL), added with Nessler's reagent (100 µL), and incubated (15 minutes, room temperature). Absorbance was measured at 425 nm. Enzyme activity (U/mL) was calculated based on ammonia content (determined from the ammonia standard curve, y = ax + b) and the equation:

$$\text{Enzyme activity(U/mL)} = \frac{(y-b)}{a} \text{x} \frac{\text{Vtotal}}{\text{Vanal}} \text{x} \frac{1}{\text{V.Enz}} \text{x} \frac{1}{\text{T.inc}} \quad (1)$$

Description:
V_{total}: Volume of enzyme + substrate + buffer + TCA + aquadest
V_{anal}: Total volume read on the spectrophotometer
V_{enz}: Volume of reacted and incubated enzyme
T_{inc}: Time of incubation

3. RESULT AND DISCUSSION

3.1 Plasmid DNA isolation of pET-28a(+) and pGEM T Easy:*ansB*

Figure 1A and 1B show a single DNA band exhibiting 5,396 bp and 4074 bp respectively which are corresponding with the size of the pET-28a+ and pGEM T Easy:*ansB* plasmid. The figures indicate the successful step of both plasmid DNA isolation. Electrophoresis data exhibited the presence of two bands. Those both two bands are assumed the supercoil-shaped DNA band (marked by the red box) and the open circular band (above the red box). The difference in the conformation of the DNA plasmid causes different migration rates. The measurement results using BioDrop showed that the concentration of plasmid DNA is 152 ng/µL and purity of 2.082 at adsorbent ratio of 260/280 (data not shown)

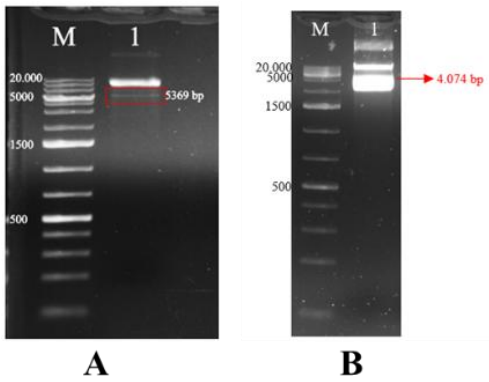


Figure 1. Electrophoresis of pET-28a(+) DNA plasmid. A= pET-28a+, B= and pGEM T Easy:*ansB* plasmid. M= Generic 1kb.

3.2 Digestion of pET-28a+ expression vector plasmid and *pSSPM3* synthetic promoter with *Bgl*II and *Nhe*I restriction enzymes

In order to fuse the *pSSPM3* Synthetic Promoter fragment to pET-28a+, restriction process was carried out so that the fragment ends between the pET-28a+ plasmid and the *pSSPM3* synthetic promoter were compatible for ligating. The restriction results of plasmid pET-28a+ and synthetic promoter *pSSPM3* were visualised using gel electrophoresis (Figure 2A and 2B). Following the restriction step, results of pET-28a+ plasmid and

pSSPM3 synthetic promoter were then purified from agarose gel using GeneJET Gel Extraction Kit (Thermo Scientific). The purified DNA was then measured for their concentration using BioDrop. The concentration of linear plasmid pET-28a+ on BioDrop showed 11 ng/µL with a purity of 1.833 using an absorbance ratio of 260/280, while the concentration of *pSSPM3* synthetic promoter is 5 ng/µL with a DNA purity of 1.677.

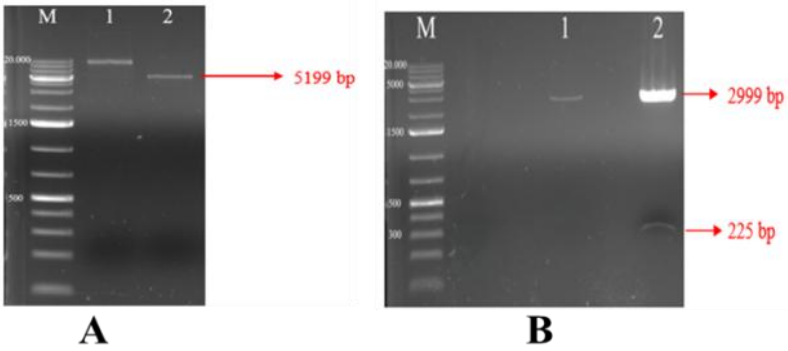


Figure 2. Restriction electrophoresis results of plasmid pET-28a+ (panel A) and pMG:*pSSPM3* using *Bgl*II and *Nhe*I (panel B). M = Generic 1kb.

3.3 Cloning of *pSSPM3* synthetic promoter into pET-28a+ plasmid

The purified linear pET-28a+ plasmid DNA and the synthetic promoter ***pSSPM3*** fragment were ligated to produce the pET-28a+:*pSSPM3* plasmid construct using T4 DNA Ligase enzyme. The ligation process was carried out at 4 ºC which is the optimum temperature of T4 DNA ligase enzyme. To confirm the ligation results of both pET-28a+ and *pSSPM3*, the PCR analysis was performed using specific primers T7PS and T7TR. The success of the ligation process is characterised by the presence of a band measuring 674 bp which can be seen in the figure 3.

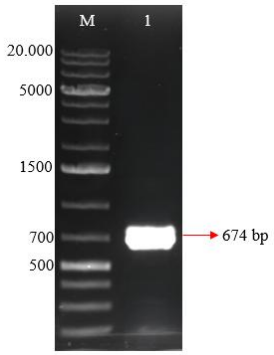


Figure 3. Confirmation of plasmid ligation results of pET-28a+:*pSSPM3*. M= Generic 1kb, 1= Amplicon sequence T7PS-T7TR.

Transformation of the recombinant plasmid pET-28a+:*pSSPM3* into competent cells of *E. coli* strain DH10B was performed using selective LB agar media treated with Kanamycin antibiotic. Single colonies of bacteria that grew were then observed and analyzed to confirm recombinant. The growing bacterial colonies were randomly selected for identification of transformant bacteria coded K1-K5.

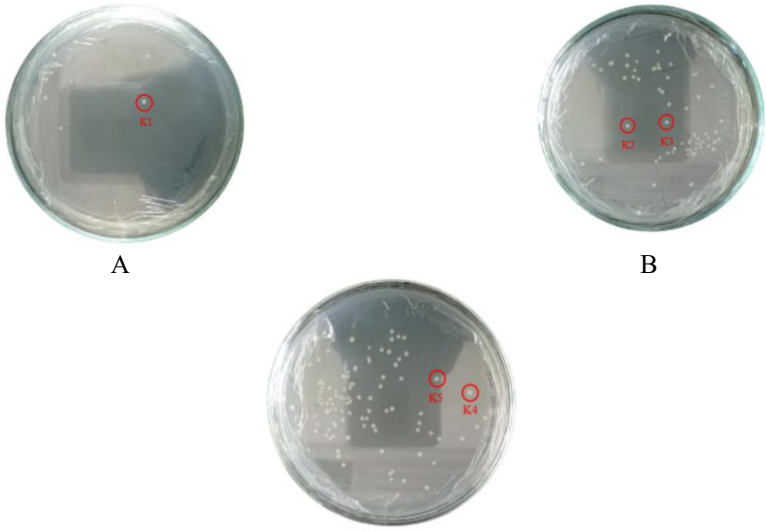


Figure 4. Single colonies of transformant bacteria grown on selective media. K1-K5 in panels A-C are single colonies used as samples in the analysis.

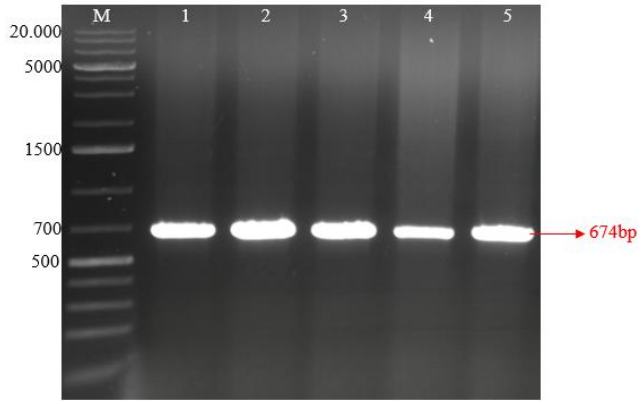


Figure 5. PCR amplicon of pET-28a+:*pSSPM3* transformant bacterial colonies with T7PS-T7TR primers. M=Generic 1kb, 1- 5= selected single colonies.

Identification of transformant *E. coli* Strain DH10 cells containing the pET-28a+: *pSSPM3* construct was performed by colony PCR using T7PS-T7TR specific primers. The amplification results with T7PS-T7TR primers can be seen in Figure 5. Based on the results of colony PCR using primer T7PS-T7TR on colony K1-K5, the amplicons product is in accordance with the target DNA of 674 bp so that the bacterial colony K1-K5 successfully transformed the recombinant plasmid pET-28a+:*pSSPM3* into *E.coli* strain DH10B bacteria.

3.4 Isolation of recombinant plasmid pET-28a+:*pSSPM3* from *E. coli* DH10B

K3 colonies which are transformant bacteria that have been confirmed using colony PCR previously (Figure 5) were used for further analysis. The recombinant plasmid DNA containing pET-28a+:*pSSPM3* are visualised using electrophoresis as shown in Figure 6. Concentration of plasmid DNA using Biodrop resulted 148 ng/µl and the purity of 2.027 at an adsorbent ratio of 260/280.

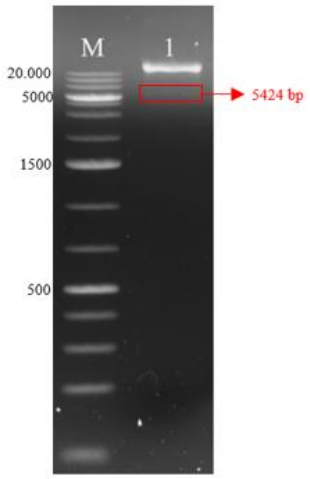
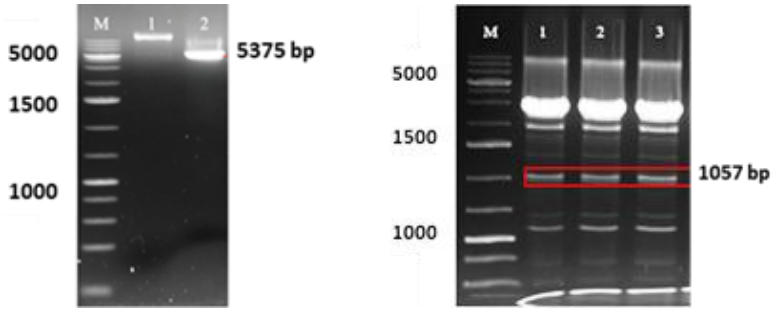


Figure 6. Isolated recombinant plasmid DNA of pET-28a+:*pSSPM3* from *E. coli* DH10B. M=Generic 1kb, 1= Isolated plasmid DNA of pET-28a+:*pSSPM3*).

3.5 Construction of pET-28a+:*pSSPM3*: *ansB* recombinant plasmid

The isolated recombinant plasmid DNA of pET-28a+:*pSSPM3* and pGEM T Easy:*ansB* were then digested using *Nhe*I and *Sac*I restriction enzymes. Restriction of plasmid DNA pET-28a+:*pSSPM3* and pGEM T Easy:*ansB* using *Nhe*I and *Sac*I enzymes was carried out so that both ends of the DNA fragments were compatible for ligating. The restriction results of plasmids pET-28a+:*pSSPM3* and pGEM T Easy:*ansB* were visualised using gel electrophoresis (Figure 7A and 7B). The restriction results of the recombinant plasmids pET-28a+:*pSSPM3* and pGEM T Easy:*ansB* were purified from agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific-USA). DNA purification results were measured for concentration using BioDrop. The concentration of recombinant plasmid pET-28a+:*pSSPM3* linear on BioDrop showed 6 ng/µL with a plasmid DNA purity value of 2,000 at an adsorbent ratio of 260/280. Concentration of *ansB* gene on BioDrop showed 7 ng/µL with a DNA purity of 1,400 at an adsorbent ratio of 260/280.



A **B**

Figure 7. Electrophoresis results of restricted recombinant plasmid pET-28a+:*pSSPM3* (A) and recombinant plasmid pGEM T Easy:*ansB* using *Nhe*I and *Sac*I enzymes. M = Generic 1kb, 1-3 are replications.

The purified pET-28a+:*pSSPM3* recombinant plasmid DNA and *ansB* gene were then ligated using T4 DNA ligase enzyme for the construction of pET-28a+:*pSSPM3:ansB* recombinant plasmid. The ligation process was carried out at 4 °C which is the optimum temperature for the performance of the T4 DNA ligase enzyme. To confirm the results of recombinant plasmid ligation pET-28a+:*pSSPM3:ansB*, PCR was performed using specific primers T7PS and T7TR. The success of the ligation process is characterised by the presence of a band measuring 1,682 bp which can be seen in Figure 8.

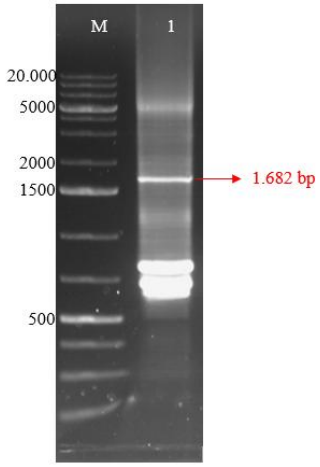


Figure 8. Confirmation of plasmid ligation results of pET-28a+:*pSSPM3*:*ansB*. M= Generic 1kb. 1= Amplicon sequence T7PS-T7TR.

The result of recombinant plasmid ligation pET-28a+:*pSSPM3:ansB* which has been confirmed using PCR, was then transformed using the heatshock method into competent cells of *E. coli* strain BL21. Selection of transformant bacteria was carried out using selective agar media that had been treated with kanamycin antibiotic. Single colonies of bacteria on selective agar media can grow because the recombinant plasmid pET-28a+:*pSSPM3:ansB* contains kanamycin resistant genes, so *E. coli* bacteria containing recombinant plasmid pET-28a+:*pSSPM3:ansB* can grow even in media that have been added with antibiotics. To confirm the success of transformation, colony PCR was then performed using T7PS-T7TR primers.

Figure 9. Single colonies of transformant bacteria grown on selective media. N1-N10 = single colonies used as samples.

Identification of transformant bacteria was performed by colony PCR using primers T7PS-T7TR. PCR amplification products with these primers will show a band measuring 1,682 bp if the recombinant plasmid pET-28a +:*pSSPM3:ansB* is present. The electrophoresis results of colony PCR products can be seen in Figure 10. Based on the results of colony PCR using T7PS-T7TR primers on colonies N1-N10, there is no visible band measuring 1,682 bp. This can occur if the DNA concentration is low enough and the DNA amplification was not occurred because the bacterial cell wall is not lysed during the denaturation process. Verification of the successful transformation of recombinant plasmid pET-28a+:*pSSPM3:ansB* was continued by performing PCR on recombinant plasmid DNA of pET-28a+:*pSSPM3:ansB*.

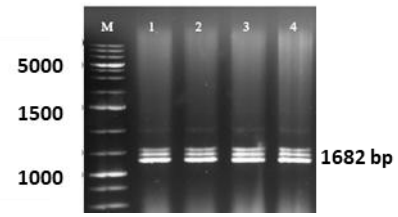


Figure 10. PCR colony of pET-28a+:*pSSPM3*:*ansB* transformant bacterial with T7PS-T7TR primers. M=Generic 1kb, 1-4 = selected single colony.

The PCR results of the previous colonies did not show any target bands as expected, so the success of recombinant plasmid transformation could not be confirmed. To confirm the transformant bacteria, it is necessary to isolate the recombinant plasmid DNA. Colonies of pET-28a+:*pSSPM3:ansB* transformant bacteria that grew on masterplate were then grown for the plasmid DNA isolation process. The results of recombinant plasmid isolation pET-28a+:*pSSPM3:ansB* visualised using electrophoresis can be seen in Figure 11. The isolated DNA was measured for concentration and purity using BioDrop. The concentration of isolated DNA was found to be 129 ng/µL with DNA purity of 2.048 at an adsorbent ratio of 260/280.

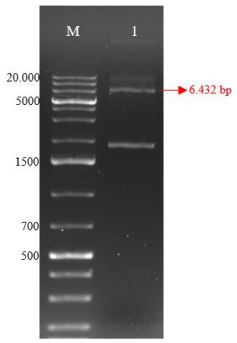


Figure 11. Isolated recombinant plasmid DNA of pET-28a+:*pSSPM3*:*ansB* from *E. coli* BL21 M=Generic 1kb, 1= Isolated plasmid DNA.

3.6 Verification of *ansB* gene in recombinant plasmid pET-28a+:*pSSPM3:ansB*

The isolated recombinant plasmid DNA pET-28a+:*pSSPM3:ansB* was then subjected to PCR using T7PS-T7TR primers to verify the presence of the *ansB* gene. The presence of the *ansB* gene can be verified with the T7PS-T7TR primer because this primer is designed using the pET-28a+ plasmid sequence, if there is an insertion gene in the MCS plasmid, the size of the amplification region increases to 1,682 bp. After PCR, PCR products were electrophoresed for visualisation and analysis of the results. The electrophoresis results can be seen in Figure 12. Based on the electrophoresis results, a band at the size of 1,682 bp was seen according to the target band. This indicates that the *ansB* gene is in the recombinant plasmid pET-28a+:*pSSPM3:ansB* and the cloning process in *E.coli* BL21 was successful.

Based on the results of plasmid DNA restriction in Figure 12B, there is a DNA band measuring 1057 bp with a relatively thin intensity. This condition can be caused by several factors, such as the less than optimal efficiency of the restriction process or the low concentration of the *ansB* gene that was successfully cloned. The presence of the DNA band still indicates that the *ansB* gene has been successfully inserted into the recombinant plasmid pET-28a+:*pSSPM3:ansB*. This result indicates that the cloning process of the *ansB* gene *in E. coli* BL21 has been successful.

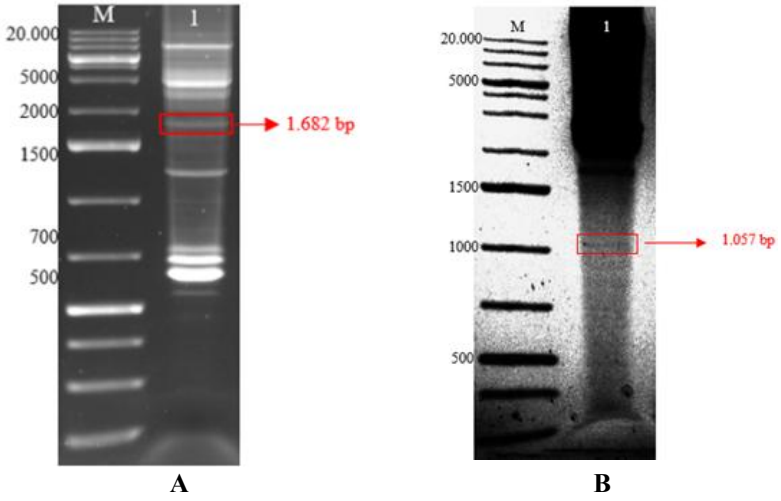


Figure 12. PCR amplicon of recombinant plasmid pET-28a+:*pSSPM3*:*ansB* with primers T7PS-T7TR (A). Restricted DNA plasmid pET-28a+:*pSSPM3*: *ansB* using *Nhe*I and *Sac*I enzymes. M = Generic 1kb, 1 = sample.

3.7 L-asparaginase II enzyme activity test using the Nessler method

The results of the enzyme activity test using supernatants from bacterial cultures showed that the group means were not significantly different. In *E. coli* Bl21 pET-28a+:*pSSPM3:ansB* transformant bacteria, there was a decrease in enzyme activity compared to *Serratia plymuthica* UBCF_13, while in *E. coli* DH10B pET-28a+:*pSSPM3:ansB* bacteria there was an increase in enzyme activity compared to *Serratia plymuthica* UBCF_13 (Table 1)

Table 1. Mean value of L-asparaginase II Enzyme Activity

Construct	Enzyme activity (U/mL)
S. plymuthica UBCF_13 native	0.598±0.082
E. coli BL21 *native*	0.621±0.013
E. coli BL21; pET-28a+*:ansB*	0.617±0.014
E. coli BL21; pET-28a+:*pSSPM3:ansB*	0.519±0.0142
E. coli DH10B ; pET-28a+:*pSSPM3:ansB*	0.636±0.0101

Based on table 1, the mean enzyme activity of all bacterial groups needs statistical analysis to see the differences between groups. The statistical analysis used was the One-Way ANOVA test. The test can show significant differences and compare the mean value of enzyme activity between bacterial groups.

Table 2. Results of One-Way ANOVA test of mean L-asparaginase II enzyme activity

Construct	**Enzyme activity (U/mL)**	***p Value***
S. plymuthica UBCF_13	0.598±0.082	
E. coli BL21 *native*	0.621±0.013	0.027
E.coli BL21 Transforman pET-28a+*:ansB*	0.617±0,014	
E.coli BL21; pET-28a+:*pSSPM3:ansB*	0.519±0.0142	
E.coli DH10B; pET-28a+:*pSSPM3:ansB*	0.636±0.0101	

Based on the table 2, there is a significant difference between groups of bacteria as evidenced by the significance value of 0.027 ($p<0.05$). The data shows a difference in means, so that further tests can be carried out with the Post-Hoc test to determine the significance difference between groups.

Table 3. Results of Post-Hoc test of L-asparaginase II enzyme activity

Construct	*S. plymuthica* UBCF_13	*E. coli* BL21 *native*	*E.coli* BL21 Transforman pET-28a+*:ansB*	*E.coli* BL21 Transforman pET-28a+:*pSSPM3:ansB*	*E.coli* DH10B Transforman pET-28a+:*pSSPM3:ansB*
S. plymuthica UBCF_13	1	0.981	0.991	0.591	0.910
E. coli BL21 *native*		1	0.991	0.004*	0.572
E.coli BL21 Transforman pET-28a+*:ansB*			1	0.005*	0.441
E.coli BL21 Transforman pET-28a+:*pSSPM3:ansB*				1	0.002*
E.coli DH10B Transforman pET-28a+:*pSSPM3:ansB*					1

*Significantly different ($p<0.05$)

Based on table 3, there was a significant difference between groups. Data showing a significance value of $p<0.05$ indicates a difference in results. The *E. coli* BL21 Transformant pET-28a+:*pSSPM3*:*ansB* group showed a significant difference from the native *E. coli*, *E. coli* BL21 Transformant pET-28a+:*ansB*, and *E. coli* DH10B Transformant pET-28a+:*pSSPM3*:*ansB* groups. This significant difference indicates that the *E. coli* BL21 Transformant pET-28a+:*pSSPM3*:*ansB* bacteria have different activity values from other groups of bacteria.

This study successfully achieved the molecular cloning of the *ansB* gene into the pET-28a+ vector featuring the synthetic promoter pSSPM3 within both *E. coli* DH10B and BL21, as confirmed by PCR and restriction digestion analyses. However, the central findings regarding L-asparaginase II activity revealed unexpected outcomes requiring deeper interpretation. Contrary to established knowledge, enzyme activity was higher in E. coli DH10B transformants compared to BL21, despite BL21's general superiority for recombinant protein expression. This counterintuitive result may stem from several factors: potentially lower plasmid stability or copy number in BL21 under these specific conditions (evidenced by initial colony PCR failures), strain-specific proteolytic degradation of the recombinant enzyme in BL21, or critically, the lack of optimized induction protocols essential for activating the T7 expression system inherent to the pET vector in BL21(DE3) strains. Our verification confirmed gene presence but did not assess induced expression levels, a significant limitation for cross-strain comparison.

Furthermore, the synthetic promoter pSSPM3, introduced specifically to enhance *ansB* expression, failed to deliver the anticipated effect; instead, activity data suggested it may have had a detrimental impact in the final BL21 construct. This lack of efficacy, or potential inhibition, could arise from pSSPM3's incompatibility with BL21's transcriptional machinery, suboptimal positioning within the vector disrupting regulatory elements, or unintended interference with gene expression. Crucially, while physical insertion of pSSPM3 was verified, the study lacked rigorous functional comparison (e.g., qRT-PCR for mRNA levels, Western blotting for protein) of constructs with versus without pSSPM3 under induced conditions in the same BL21 background, leaving its true performance unassessed.

Methodological aspects of the activity assay, specifically the use of crude supernatant without purification and unoptimized reaction parameters (pH, temperature, time), as highlighted by Arredondo-Nuñez et al. (2023), likely compounded the difficulty in accurately quantifying expression differences and preclude definitive conclusions about absolute promoter or strain efficiency. Future research must therefore prioritize direct functional validation of pSSPM3's strength relative to the native T7 promoter in BL21(DE3) under induced conditions, thorough investigation of the unexpected strain performance discrepancy (including

plasmid stability and protease activity assays), purification of the recombinant enzyme for specific activity measurements, and optimization of the activity assay itself to reliably evaluate the success of promoter engineering efforts.

4. CONCLUSION

This study successfully constructed the functional recombinant plasmid pET-28a+:*pSSPM3:ansB*, confirming the integration of the *Serratia plymuthica ansB* gene via molecular verification, thereby achieving its immediate objective of establishing a genetic platform for L-asparaginase II production. However, quantitative enzyme activity assays revealed that the synthetic promoter *pSSPM3* did not enhance expression in *E. coli* BL21 under the tested conditions. This outcome underscores unresolved challenges in promoter-host compatibility and necessitates further optimization of expression systems.

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