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A CONSTRUCTION AND AN ANALYSIS OF A GENE ENCODING SECRETORY LEUKOCYTE PROTEASE INHIBITOR AND PROTEIN DISULFIDE ISOMERASE USING A CO-EXPRESSION VECTOR IN *SACCHAROMYCES CEREVISIAE* BJ1824

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ABSTRACT

The secretory Leukocyte Protease Inhibitor (SLPI) is a protease inhibitor which can be found in saliva, bronchial mucus, seminal plasma and the amniotic membrane. It has been recognized for anti-inflammatory, antimicrobial and wound healing. Recombinant SLPI produced in Saccharomyces cerevisiae would be a useful biomaterial for wound healing. The yeast as a heterologous host holds an important role to express human SLPI. The yeast PDI is an enzyme which is required in the formation of disulfide bonds and folding of SLPI. A co-expression vector harboring SLPI gene and PDI1 gene is constructed aiming to strengthen the expression of SLPI in S. cerevisiae. The SLPI gene from the amniotic membrane is fused with HM1 signal peptide, namely hmSLPI. The HM1 signal peptide is used to mediate the translocation of SLPI across the membrane cells. The plasmids construction and characterization are done in Escherichia coli TOP10 prior to its transformation to S. cerevisiae BJ1824. The hmSLPI fusion gene and PDI1 gene are successfully amplified in sized 483 bp and 1,583 bp, respectively. The restriction analysis and nucleotide sequencing indicate that the recombinant plasmids, pAT_hmSLPI, pAT_PDI1 and pAT_PDI1_hmSLPI, are successfully constructed. All of the recombinant plasmids are also successfully introduced into S. cerevisiae BJ1824.

Keywords: *hmSLPI fusion gene, PDI1 gene, co-expression vector, S. cerevisiae BJ1824.*

INTRODUCTION

The amniotic membrane is a thin membrane that surrounds a developing fetus. This membrane contains numerous growth factors, cytokines, and protease inhibitors which are advantageous for wound healing and tissue regeneration [1]. In 1910, Davis has successfully applied, for the first time, an amniotic membrane as a surgical dressing on a skin graft [2]. Amniotic membranes are now commonly used as biological dressings for the management of burns, open and healing ulcers and mucosal defects in the oral cavity [1, 2]. They are also used for ocular surface reconstructions[3]. The advantages of their application as a wound dressing are abundant. They are less costly, and have low immunogenicity [5, 6]. The use of amniotic membrane products for clinical applications has increased exponentially.

Amniotic membranes accelerate wound healing by

promoting epithelialization and reducing inflammation, scarring, and vascularization [5, 6]. The active material that originates from them refers to the secretory leukocyte protease inhibitor (SLPI)[7]. The latter is a protein that mainly acts as a protease inhibitor - it inhibits elastase, cathepsin G, trypsin, chymotrypsin, chymase, and tryptase [8,9]. It consists of 107 amino acids organized into two similar domains, the C-terminal domain and the N-terminal domain, and contains twelve cysteine residues that form eight disulfide bridges stabilizing the protein [10]. The location of SLPI primary reaction with various serine proteases refers to the amino acid residue, Gly69-Gln70-Cys71-Leu72-Met73-Leu74, on the C-terminal domain of SLPI [10]. Although amniotic membranes are considered a promising, natural wound dressing, the donor variation and the potential risk while dealing with human tissue donations have restricted its use in clinical applications and its commercial availability [11]. Production of recombinant SLPI might overcome these limitations.

Saccharomyces cerevisiae has been widely used as a host for the expression of human heterologous proteins because it is an eukaryotic organism that is safe, easy to culture, grows quickly, and performs well following translational modifications, including disulfide bonds [12]. The target protein can also be expressed for secretion to extracellular media, simplifying its purification [13].

The promoter strength, the signal peptide sequence, the gene copy number, the folding and processing in the endoplasmic reticulum (ER), as well as disulfide bond formation [14] are the key factors in the secretion of heterologous proteins in *S. cerevisiae*. An unfolded or misfolded protein can either aggregate or degrade inside the ER [15]. The protein disulfide isomerase (PDI) catalyzes the disulfide bond formation in the ER and rearranges the incorrect disulfide pairings. PDI also acts as a chaperone by mediating the protein folding [16]. Therefore, the co-expression system of SLPI and PDI can strengthen the expression of SLPI.

The signal peptide sequence plays a role in the transportation of newly formed protein products across the membrane cells. It is also responsible for the co-translational translocation of proteins into the ER. A native signal peptide or a heterologous signal peptide can be used to secrete a protein into *S. cerevisiae*. In this study, the HM1 signal peptide from the killer toxin of *Hansenulla mrakii* IFO 0895 is fused to the *SLPI* gene. This signal peptide has successfully secreted α-L-arabinofuranosidase from *Geobacillus thermovorans* IT08 [17].

EXPERIMENTAL

Plasmid, Strain, and Media

E. coli TOP10 was used for plasmid propagation. *S. cerevisiae* BJ1824 *(MATa, Δura3, Δleu2, Δhis3, Δtrp1, Δpep4-3)*, used for the protein expression, were obtained from the Mie University Collection. A previously constructed plasmid, pET_ESLPI, and pYHM-1, were used as sources for the *SLPI* gene and the HM1 signal peptide sequence, respectively. The pAT425, obtained from YGRC (Japan), was used to construct the co-expression system [18]. Luria Bertani (LB) containing 100 µg/mL ampicillin and YPD (20 g/L D-glucose, 10 g/L yeast extract, 20 g/L peptone) were used to grow the yeast. A YNB medium without leucine (0.67 % yeast nitrogen base, supplemented with suitable amino acids and 2 % glucose) was used for the yeast selection.

Materials

All chemicals used were supplied by Sigma or Merck, except where specifically mentioned. Restriction enzymes (*Sac*1, *Eco*R1, *Pme*I, *Avr*II, *Not*I, M*lu*I), DNA ligase, the bacterial transformation kit, and Pfu DNA polymerase were obtained from Thermo Fisher Scientific. The primers used for PCR were synthesized by IDT.

An in-silico Analysis of *SLPI* Gene

A nucleotide sequence of the mature *SLPI* gene was retrieved from NCBI and added to the nucleotide sequence of HM1 signal peptide. The full sequence was then analyzed in silico using a rare codon analysis software (www.genscript.com). The signal peptide prediction was examined through a signal peptide analysis (http://www.cbs.dtu.dk/services/SignalP/).

Fusing the HM1 Signal Peptide and the SLPI Gene

The *SLPI* gene was first inserted upstream of the HM1 signal peptide in the pYHM1 plasmid. The recombinant plasmid of pET_ESLPI was used as a template to amplify the *SLPI* gene using a specific primer (pFSLPISac1 and pRSLPIEcoRI in Table 1). The process of PCR amplification followed the 3 min pre-denaturation at 95°C, followed by 30 cycles at 95°C for 30 s, at 59.3°C for 30 s, at 72°C for 2 min and a post-elongation at 72°C for 15

min. The amplified DNA fragment was digested with *Sac*I and *EcoR*I and purified using a gel extraction kit (Thermo Fisher Scientific). This fragment was then ligated into the I/*Eco*RI site of pYHM1 to generate pY_SLPI, which was transformed into *E.coli* TOP10-competent cells. The positive transformants were confirmed through colony PCR and the recombinant plasmid was evaluated through a restriction analysis and a nucleotide sequencing.

A Plasmids Construction

The construction of pAT_PDI1_hmSLPI was performed in two steps. They are outlined in Fig. 4. The *PDI1* gene was first introduced to multiple cloning sites-2 (MCS-2) of the pAT425 plasmid to yield pAT_PDI1. Next, the hmSLPI fusion gene was cloned into MCS-1 of the pAT_PDI1 plasmid in order to produce pAT_PDI1_hmSLPI. As an expression of a control, hmSLPI fusion gene was also cloned into MCS-1 of pAT425 yielding pAT_hmSLPI.

pAT_hmSLPI was constructed through an amplification of hmSLPI fusion gene from the pY_SLPI recombinant plasmid using the primers pFSLPIAvrII and pRSLPIPmeI (Table 1). The PCR product and pAT425 were then digested with the same restriction enzyme (*Avr*II/ *Pme*I) and were ligated into MCS-1 of pAT 425 producing pAT_hmSLPI.

The *PDI1* gene was amplified from BYP 7700 using the primers pFPDINotI and pRPDIMluI (Table 1) to obtain pAT_PDI1 recombinant plasmid.

The amplified product was digested by both *Not*I and *Mlu*I prior to its insertion into pAT425 at MCS-2 sites to yield pAT_PDI1.

The competent cells of *E. coli* TOP10 were used for a transformation. All transformed cells were grown in LB agar containing ampicillin, and verified through colony PCR, digestion by an appropriate restriction enzyme, and a nucleotide sequencing.

A Yeast Transformation

All recombinant plasmids (pAT_hmSLPI, and pAT_PDI1_hmSLPI were transformed into *S. cerevisiae* BJ1824. The yeast transformation was carried out through a modified lithium acetate procedure as described in ref. [19] without a single-stranded carrier DNA. A single colony of *S. cerevisiae* BJ1824 was grown overnight in YPD medium at 30°C until OD_{600} reached 1 - 1.2. The yeast cells were harvested by centrifugation and washed with sterile water. They were added to 254 µL of 50 % PEG 4000, 36 µL 1 M lithium acetate, 5 µL of recombinant plasmid, and 79 µL of sterile water and gently mixed. Then they were incubated at 30°C for 30 min and heat shocked at 42°C for 25 min. After that, the yeasts were centrifuged and resuspended in 100 µL sterile water. Subsequently they were plated in YNB medium without leucine and incubated at 30 C for 3 days. *S. cerevisiae* without plasmid were used as a negative control. The colony PCR was performed to ensure that *SLPI* gene has been introduced into the yeast recombinant.

The hmSLPI fusion gene was cloned into MCS-1 under the control of ADH promoter, while the *PDI1* gene was cloned into MCS-2 under the control of GAP promoter.

RESULTS AND DISCUSSION

A Bioinformatics Analysis

The protein expression level in a heterologous host corresponds to the values of the codon adaptation index (CAI), the frequency of the optimal codon (FOP) and the GC content. *The CAI score ranges from 0.0 to 1.0. Genes of a CAI score greater than 0.8 have a high probability of being expressed in a heterologous host. A FOP value lower than 30 can reduce the efficiency of the protein expression*. The ideal percentage range of GC content is between 30 % to 70 %. A high percentage range of GC

Table 1. Primers used in this study.

Primer Name	Sequence (5'to 3')
pFSLPI*Sac*I	GCGAGCTCATGTCTGGAAAGTCCTTCA
pRSLPI*Eco*RI	GCCGGAATTCTCAAGCTTTCACAGGG
pFSLPI*Avr*II	GCCGGCCTAGGATGAAATTTTCCTTCGT
pRSLPI*Pme*I	GCGCGTTTAAACTCAAGCTTTCACAGGG
pFPDI*Not*I	GGTATAACGCGTATGAAGTTTTCTGCTGGTG
pRPDI*Mlu*I	GTAATAGCGGCCGCTTA-CAATTCATCGTG

Underlining shows the sites restriction enzyme

content (> 70 %) may reduce and inhibit the translation, while a low percentage of GC content (< 30 %) may delay the transcription elongation [20].

When hmSLPI fusion gene is expressed in E.coli, the rare codon analysis results amount to 0.6 %, 33%, 49.18 % for CAI, FOP and GC content, respectively (Fig.1(A),1(B),1(C)). Meanwhile, the corresponding values referring to the expression in S. cerevisiae equal 0.71 %, 49.18% and 39%, respectively (Figs. 1(A), 1(B), 1(C)). The rare codon analysis results indicate that the probability of SLPI when expressed in S. cerevisiae is higher than that in E. coli.

Fusing the HM1 Signal Peptide and the *SLPI* Gene

The proteins, which are secreted, require a signal peptide located at their N-terminal. The signal peptide mediates the translocation of the nascent secretory proteins to the ER. Then the signal peptide is cleaved by signal peptidase and Kex2 protease, while the mature protein is delivered to the outside of cell membrane. In

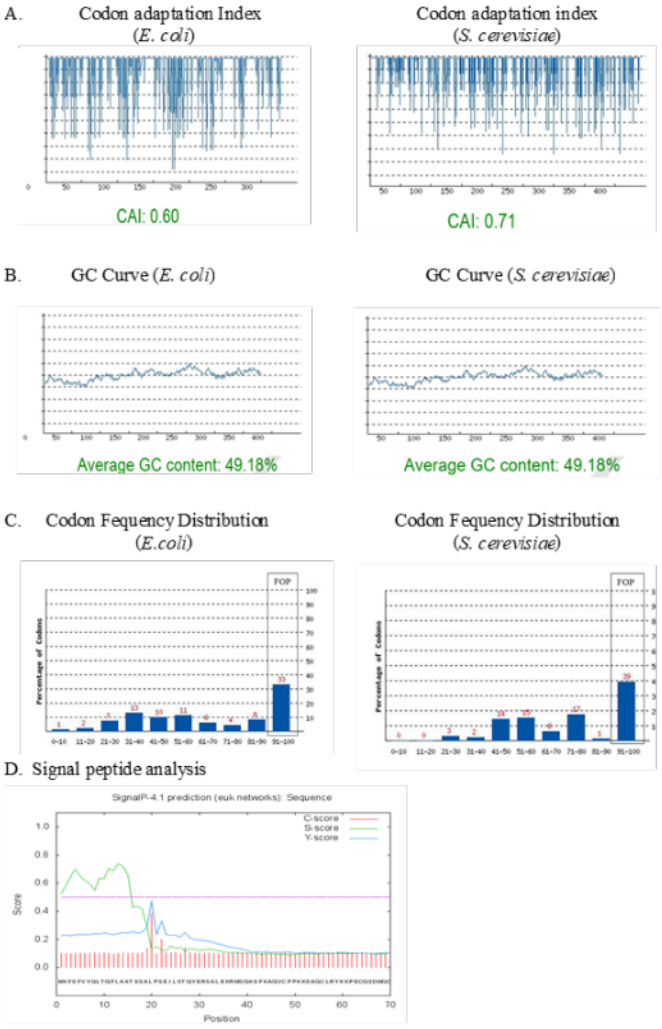


Fig. 1. Bioinformatic analysis of hmSLPI Fusion Gene:(A) Codon Adaptation Index of the SLPI gene, as expressed in *E. coli* and *S. cerevisiae*; (B) GC content of the SLPI gene, as expressed in *E. coli* and *S. cerevisiae*; (C) Codon Frequency Distribution of the SLPI gene, as expressed in *E. coli* and *S. cerevisiae*; (D) Signal peptide prediction of fusion protein of SLPI and HM1 signal peptide using the online signal peptide analysis software.

general, the signal peptides consist of basic amino acids at the N terminal, followed by hydrophobic residues [22].

The HM1- signal peptide used in this study originates from a yeast killer toxin. Several signal peptides derived from yeast killer toxins have successfully secreted heterologous proteins [24].

The signal peptide prediction using SignalIP demonstrates that the signal peptidase cleaves the HM1 signal peptide at A19-L20, while the Kex2-protease cleaves K36-R37 (Fig. 1(D)). The signal peptidase cleaves the pre-SLPI at the carboxyl side of A19, and the resulting pro-SLPI is then cleaved by the Kex2 protease. A schematic model of the fusion gene created by SLPI and the HM1 signal peptide is shown in Fig. 2(B).

Construction of the hmSLPI fusion gene is completed by inserting the *SLPI* gene into the pYHM1 plasmid. The restriction analysis and the nucleotide sequencing are performed to verify that the *SLPI* gene has successfully cloned into pYHM1 plasmid. The double digestion of pY_SLPI recombinant plasmid using *Sac*I and *Eco*RI yields two fragments: 5.9 kb, and 339 bp. One of the fragments (5.9 kb) refers to the linearized pYHM1, while the second one (339 bp) refers to the *SLPI* gene (Fig. 3A). This result indicates that the *SLPI* gene has successfully cloned into pYHM1 plasmid. The sequencing result also shows that pYHM1 carries the *SLPI* gene, which is in a frame with HM1 signal peptide (Fig. 3(B)).

A Construction of *hmSLPI* Fusion Gene and *PDI1* Gene Using a Co-Expression Vector

A co-expression of the PDI vector is initially created to enhance the yield of SLPI in *S. cerevisiae*. A schematic illustration of the construction of *SLPI* gene and

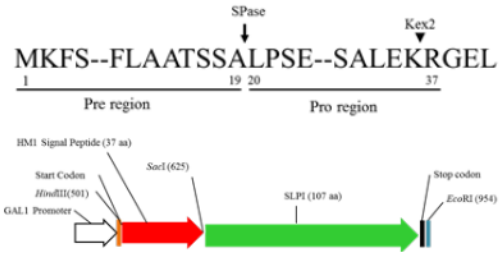
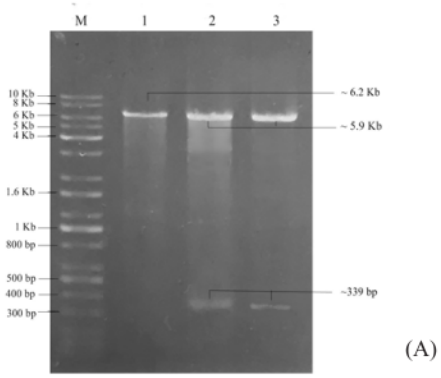


Fig. 2. Schematic model of fusion gene, *SLPI* gene and HM1 signal peptide. (A). HM1 signal peptide, where (↓) points to the cleavage site of the signal peptidase (SPase) and (▼) points to the cleavage site of Kex2. (B) Fusion gene of SLPI and HM1 signal peptide.

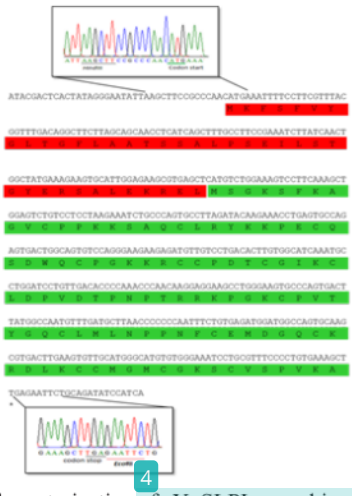
PDI1 gene in the pAT425 vector is presented in Fig. 4.

The *PDI1* gene is then cloned into MCS-2 of the pAT425 vector (pAT_PDI1). The pAT425 vector has two MCS cassettes, P_{ADH1}-MCS1-T_{DH1} and P_{TDH3}-MCS2-T_{DH3} (Fig. 4.). The positive transformants are verified through the PCR colony (Fig. 5(A)), the digestion by *Not*I/*Mlu*I (Fig. 5B) and the nucleotide sequencing.

The colony PCR and *Not*I/*Mlu*I digestion of the recombinant plasmids give the expected size of the *PDI1* gene, which of approximately 1.5 Kb. The NCBI BLAST of PDI, which of used in this study, is 100% identical to the *Saccharomyces cerevisiae PDI1* gene (D00842.1). The result of the NCBI BLAST indicated that the recombinant plasmid of pAT_PDI1 has been successfully constructed.



(A)



(B)

Fig. 3. Characterization of pY_SLPI recombinant plasmid. (A) Restriction analysis of pY_SLPI. Lane1: pY_SLPI digested by *Sac*I; lane2 and 3: pY_SLPI digested by *Sac*I and *Eco*RI. (B) Nucleotide sequencing of pY_SLPI.

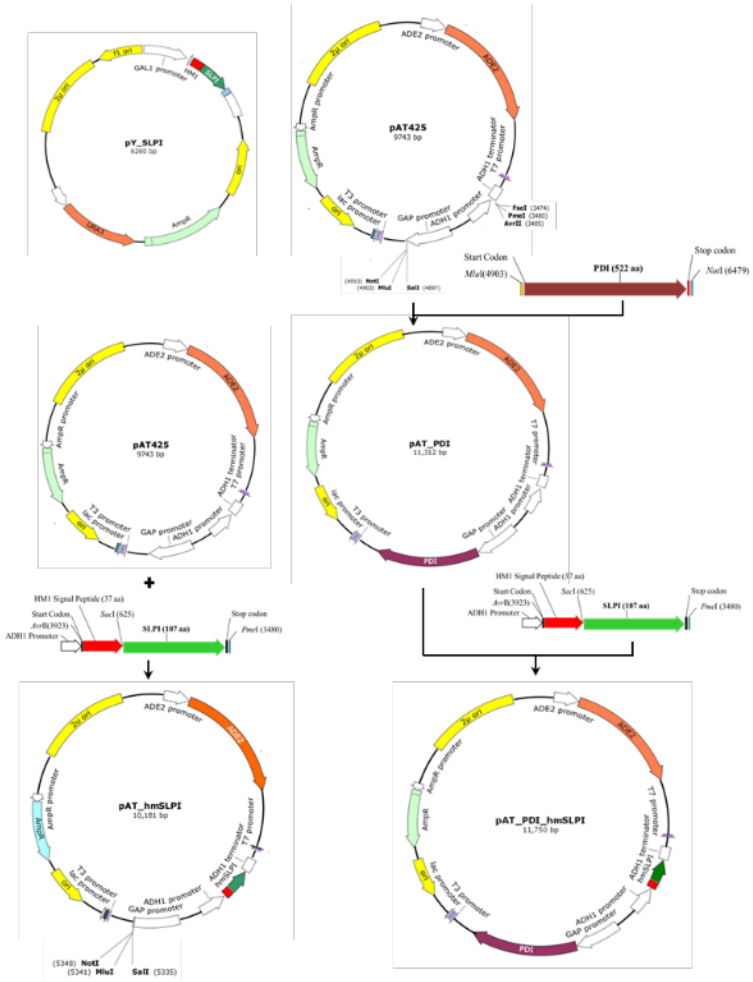


Fig. 4. Construction of co-expression vector of hmSLPI fusion gene and *PDI1* gene. pAT_PDI1: expression vector of PDI; pAT_hmSLPI: expression vector of SLPI; pAT_PDI1_hmSLPI: co-expression vector of SLPI and PDI. Ura3, Leu2, AmpR: selective marker gene.

PDI is a multifunctional protein that not only contributes to the disulfide bond formation, but also acts as a chaperone by promoting the correct folding of the protein [25]. Previous studies have reported that an overexpression of PDI increases the secretion of a recombinant protein, with or without disulfide bonds. The secretion of Apolipoprotein-A increases 2.5-5 times within the PDI overexpression system [26]. The increased expressions and the secretions are shown by AmyA_Opt, and GlaA_Opt proteins after they are co-expressed with PDI [27]. Hacker et al. [28] report that PDI enhances the secretion of an active transferrin receptor-targeting single-chain antibody (scFv). Moreover, the secretion levels of β-glucosidase, originating from *Pyrococcus furiosus,* increase 3-fold after overexpression of the PDI [29].

hmSLPI is then introduced to pAT_PDI1 recombinant plasmid on MCS-1. As a controlled expression, hmSLPI is also cloned into pAT425 on MCS-1, yielding pAT_hmSLPI. The colony PCR of transformants, harboring the recombinant plasmids, pAT_hmSLPI and pAT_PDI1_hmSLPI, can be seen in Fig. 6.

A single fragment of approximately 483 bp is obtained from the colony PCR of transformants that harbor the recombinant plasmid, pAT_hmSLPI (Fig. 6(A)). Fig. 6(B) shows a single fragment, approximately 1,583 bp and 458 bp, corresponding to the *PDI1* gene and *hmSLPI*

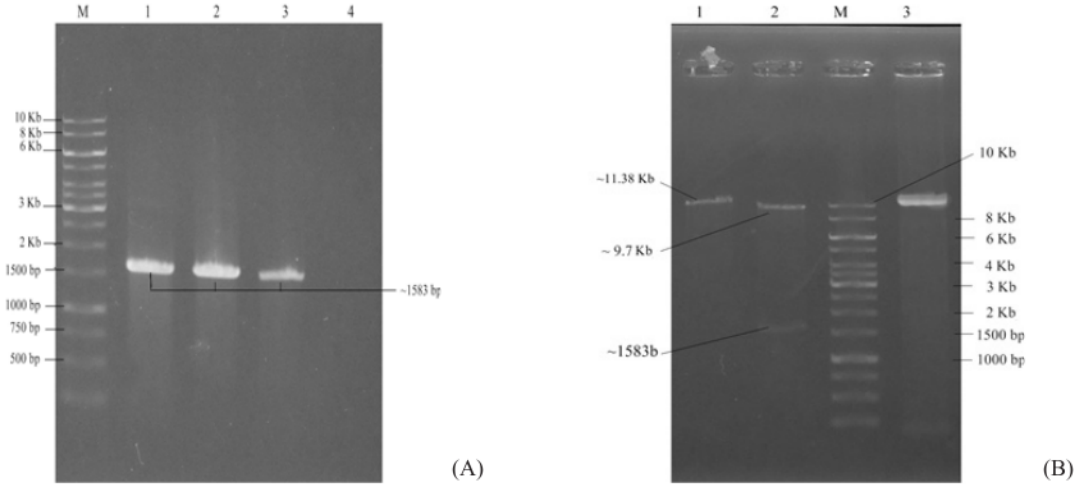


Fig. 5. Characterization of pAT_PDI1 recombinant plasmid. (A) Colony PCR of transformants. Lane 1 and 2: bacterial transformants; lane 3: positive control (*E.coli* TOP10_BY 7700). (B) Restriction analysis of pAT_PDI1. Lane 1: pAT_PDI1 digested by *Not*I, lane 2: pAT_PDI digested by *Not*I and *Mlu*I, lane 3: undigested pAT_PDI1.

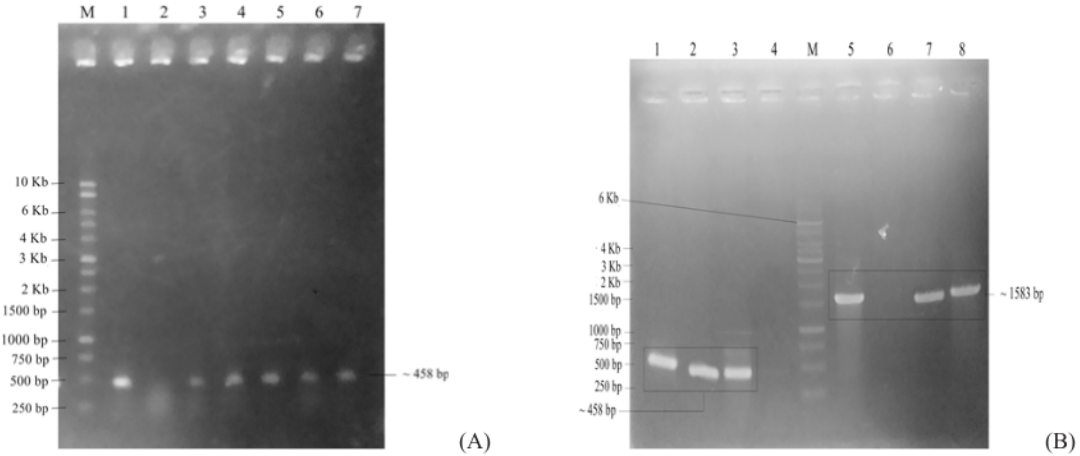


Fig. 6. Colony PCR of transformants. (A) *E.coli*_pAT_hmSLPI. Lane 1: positive control (*E. coli*_pET_ESLPI); lane 2 and 3: bacterial transformants (*E.coli*_pAT_hmSLPI); lane 4: negative control (*E. coli* TOP10 without plasmid). (B) *E.coli*_pAT_PDI1_hmSLPI. Lane 5: positive control (*E. coli*_BY 7700); lane 6: negative control (*E.coli* TOP10 without plasmid); lane 7 and 8: bacterial transformants (*E.coli*_pAT_PDI1_hmSLPI).

fusion gene, respectively. This result reveals that the PCR products are indeed hmSLPI fusion genes (Fig. 6).

The further characterization of the recombinant plasmids is carried out through a restriction analysis and a nucleotide sequencing. As shown in Fig. 7(A) and Fig. 7(B) the two predicted fragments are detected. They are composed of a vector and the hmSLPI fusion gene and of 458bp after *Avr*II/*Pme*I digestion. The BLAST analysis of the nucleotide sequence shows 100% identity with the human SLPI gene (EU116331). According to the results of the restriction analysis and the nucleotide sequencing, both pAT425 and pAT_PDI1 carry the hmSLPI fusion gene.

A Yeast Transformation

Three recombinant plasmids (pAT_hmSLPI, pAT_PDI1 and pAT_PDI1_hmSLPI) of a correct structure, as determined by the restriction analysis and the nucleotide

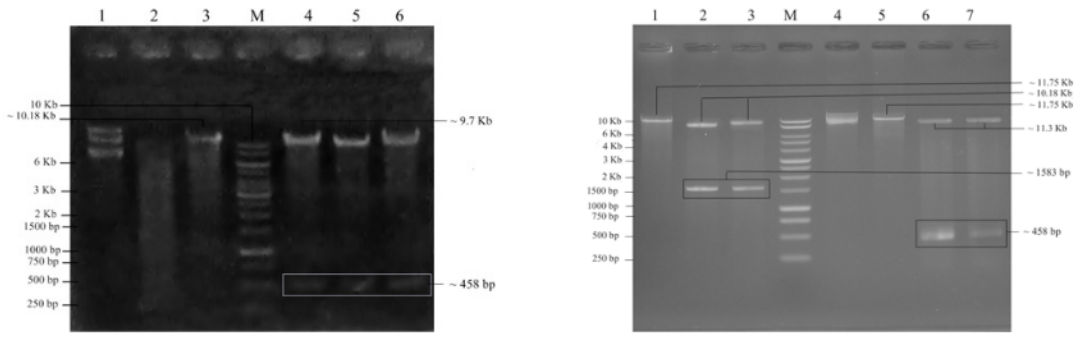


Fig. 7. Restriction analysis of pAT_hmSLPI and pAT_PDI1_hmSLPI. (A) pAT_ hmSLPI. Lane 1: undigested pAT-hmSLPI, lane 2 and 3: pAT_hmSLPI digested by *Pme*I, lane 4-6: pAT_hmSLPI digested by *Avr*II and *Pme*I. (B) pAT_PDI1_hmSLPI. Lane 1: pAT_PDI1_hmSLPI digested by *Not*I, lane 2 and 3: pAT_PDI1_hmSLPI digested by *Not*I and *Mlu*I, lane 4: undigested pAT_PDI1_hmSLPI, lane 5: pAT_PDI1_hmSLPI digested by *Pme*I, lane 6 and 7: pAT_PDI1_hmSLPI digested by*Avr*II and *Pme*I.

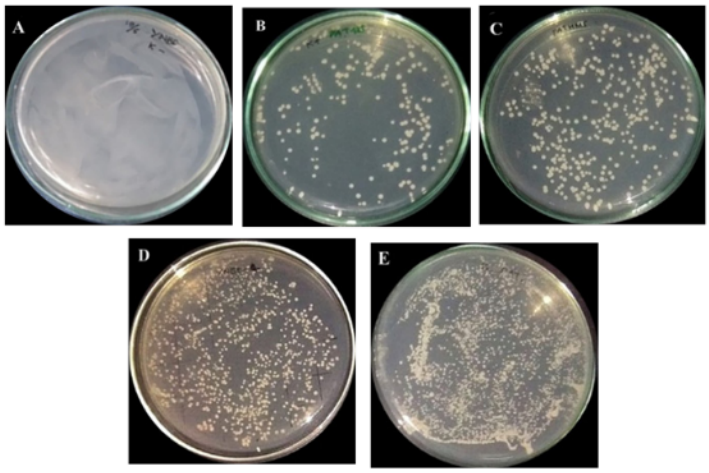


Fig. 8. Recombinant yeasts. (A) Negative control (*S. cerevisiae* without plasmid), (B) positive control (*S. cerevisiae*_pAT 425), (C) *S. cerevisiae*_pAT_hmSLPI, (D) *S. cerevisiae*_pAT_PDI1, (E) *S. cerevisiae*_pAT_PDI1_hmSLPI.

sequencing, are sub-cloned into *S. cerevisiae* BJ1824 using the lithium acetate method [19]. This method is chosen in this study for a yeast transformation because it is faster, simpler and easier to apply [30].

Fig. 8 shows that the positive control and the recombinant plasmids form colonies on YNB medium without leucine. The negative control (*S. cerevisisae* without plasmid) cannot not grow because it consists of leucine-auxotrophic strains. This result indicates that all recombinant plasmids have been successfully sub-cloned into *S. cerevisiae* BJ1824.

Colony PCR has also verified the presence of *SLPI* gene (Fig. 9). The PCR products of the putative transformants, pAT_hmSLPI and pAT_PDI1_hmSLPI are approximately 458 bp. This fragment is close to the theoretical size of the *hmSLPI* fusion gene.

CONCLUSIONS

The findings reported demonstrate that co-expression vectors which harbor *SLPI* gene and *PDI1* gene are successfully constructed using the pAT425 vector. This recombinant plasmid will be valuable for the production of SLPI in *S. cerevisiae*. Further studies are required to

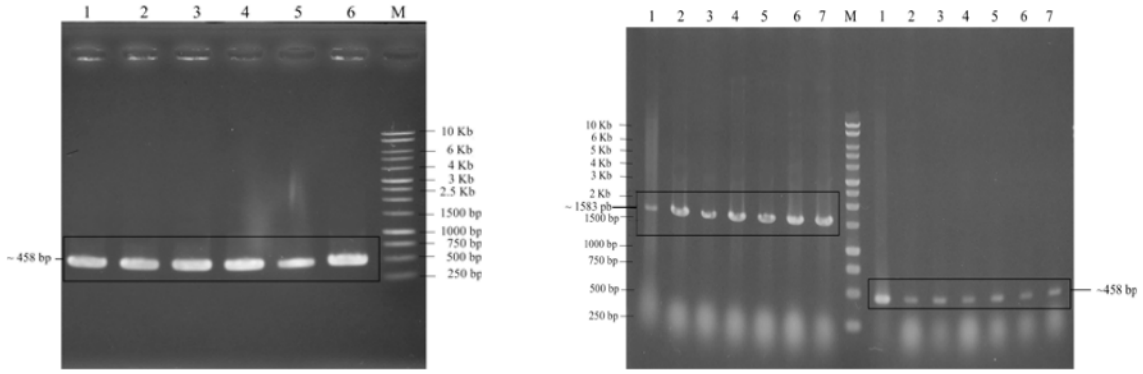


Fig. 9. Colony PCR of recombinant yeasts. (A) *S. cerevisiae* pAT _hmSLPI. (B) *S. cerevisiae* pAT_PDI1_hmSLPI.

determine the yield of SLPI in *S. cerevisiae*.

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