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TROUBLESHOOTING THE HETEROLOGOUS EXPRESSION OF RIBOFLAVIN SYNTHASE FROM *PHOTOBACTERIUM* SP. J15

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ABSTRACT
Riboflavin synthase is an enzyme involved in riboflavin biosynthesis pathway that catalyzes the formation of riboflavin (vitamin B_2). Riboflavin synthase is a homotrimeric structure that consists of three subunits. The enzyme catalyzes the riboflavin production from two substrate of 6,7-dimethyl-8-ribityllumazine. Riboflavin synthase of many microorganisms such as *B. subtilis* and *E. coli* has been studied previously and the structure and mechanism of this enzyme was reported. However, till date there has been no report on riboflavin synthase from marine bacteria. In this study, a 612 bp gene of riboflavin synthase was isolated from *Photobacterium* sp. J15. The gene was amplified by PCR and cloned into pTrcHis and pET-32b(+) vectors, respectively. The expression of riboflavin synthase gene in *E. coli* Top10 using pTrcHis system was very low even at insoluble fractions. Analysis of the DNA sequence of this riboflavin synthase shows 6 rare codons, in which common *E. coli* strains are not able to supply the corresponding tRNAs; thus the *E. coli* strain Rosetta-gami B (DE3) pLysS was selected as the expression host, which is able to supply rare tRNAs by carrying a chloramphenicol resistant plasmid (pRARE2) and also enhance disulfide bond formation in cytoplasm. The riboflavin synthase was expressed in the cytoplasm of recombinant clone at high level in inclusion body fraction. The identified bands of riboflavin synthase in SDS-PAGE shows the size of ~40 kDa, which represent the riboflavin synthase (~23 kDa) tagged with Trx-Tag (~12 kDa), S-Tag (~4 kDa) and His-Tag (~1 kDa). The overexpression of proteins provides the sufficient amount of enzyme for further analysis such as charachterization, structure studies through protein crystallography and finally inhibition studies.

KEYWORDS: Riboflavin synthase, heterologous expression, rare codons.

1. INTRODUCTION
Riboflavin, also known as vitamin B2, is the precursor of the two widely used coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) which both play key roles in basic functions of cell metabolism (Bereswill et al. 1999; Gerhardt et al., 2002; Fischer et al. 2003). Riboflavin is biosynthesized by plants and many microorganisms. It is required by all flavoproteins and is essential in maintaining health in humans and animals. In humans, the deficiency of riboflavin is called ariboflavinosis, which causes cracked and red lips, inflammation of the lining of mouth and tongue, mouth ulcers, cracks at the corners of the mouth (angular cheilitis), and sore throat (Boubakri et al., 2013; Powers, 2003).

In biosynthesis pathway of riboflavin, riboflavin synthase catalyzes the final reaction of riboflavin biosynthesis from two molecules of 6,7-dimethyl-8-ribityllumazine (Bacher et al. 1980; Fischer et al. 2005; Fischer and Bacher 2006; Fischer and Bacher 2008; Cohen 2010). One unit of riboflavin synthase catalyzes the formation of 1 nmol riboflavin per hour under assay conditions (Bacher et al., 1980). There are no cofactor requirements for riboflavin synthase to catalyse the reaction. The enzyme is nonexistent in humans and therefore, is a potential target for antimicrobial agents of organisms whose pathogenicity and survival is dependent on their ability to biosynthesis riboflavin (Liao et al., 2001).

The successful application of enzymes in industrial production of riboflavin is a remarkable example of biotechnical process replacing chemical process. The

first microorganism used for industrial production of riboflavin was *Ashbya gossypii*, which is a plant pathogen and a natural producer of riboflavin. This microorganism can use vegetable oil as carbon source to produce riboflavin. Later microbial production of riboflavin was also done using special strains of *B. subtilis* and *Candida famata* (Tatsuya and Enoch, 2012; Abbas et al., 2011; Sugimoto et al., 2009; Schlosser et al., 2007). In an attempt to establish a higher efficient production of the riboflavin synthase enzyme through heterologous expression, riboflavin synthase gene from *Photobacterium* sp. strain J15 was overexpressed in *E. coli* Rosetta-gami B (DE3) pLysS host.

2. MATERIAL AND METHODS

E. coli Top10 and *E. coli* Rosetta-gami B (DE3) pLysS were purchased from Novagen, USA. Both *E. coli* strains were cultured in Luria Bertani (LB) medium whereas *E. coli* Rosetta-gami B (DE3) pLysS in presence of three antibiotics (0.24 µg/ml tetracycline, 0.34 µg/ml chloramphenicol and 0.24 µg/mlkanamycin) but *E. coli* Top10 was cultured without antibiotic at 37°C with agitation at 220 rpm.

2.1 Extraction of genomic DNA of *Photobacteium* sp. strain J15

Photobacterium sp. strain J15 was cultured in marine agar and incubated for overnight at 30˚C. Single colony was inoculated into marine broth for overnight at 30 ˚C under agitation rate of 200 rpm. The culture was harvested by centrifugation at 12,000 ×*g* and the genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's protocol.

2.2 Cloning of riboflavin synthase gene into pTrcHis vector

Amplification of riboflavin synthase gene

Genome map of previously sequenced genomic DNA of *Photobacterium* sp. strain J15 revealed a gene encoding for riboflavin synthase. A pair of primers was designed according to riboflavin synthase gene sequence as follows:

Forward-RS: 5'-ATG TTT ACC GGT ATT GT-3'
Reverse-RS: 5'-TTA TAA TGC TTT TTC TGC C-3'

The amplified riboflavin synthase gene that contained deoxyadenosine ('A') overhang in the 3´ terminus of the gene, was cloned into linear pTrcHis vector provided by the TOPO TA Expression Kit which contained overhanging 3´ deoxythymidine (T) residues. All the procedures were continued according to TOPO TA Expression Kits (Invitrogen, USA) protocol.

Transformation of Recombinant pTrcHis into *E. coli* Top10

The ligation mixture of riboflavin synthase gene and pTrcHis vector was used to transform *E. coli* Top10 competent cell according to the manufacturer's instruction. The transformed cells were plated on LB agar plate containing 50 µg/ml ampicillin and incubated overnight at 37°C.

Direct PCR analysis of positive clones

Colony PCR is a technique to quickly determine size and orientation of insert in transformed grown colonies. The orientation of insert was checked by PCR using a combination of forward primer from riboflavin synthase gene (For-RS) and reverse from vector (pTrcHis reverse priming site; bases 574-591). A single colony of four randomly chosen transformants were picked and added to a mixture containing 12 µl of 2x PCR master mix (Fermentas, Germany), 5 pmol of each forward and reverse primers and 11 µl of nuclease-free deionized water. The PCR mixture was subjected to amplification at pre-denaturation of 94°C for 4 min, 30 cycles of denaturation (94°C; 1 min), annealing (42°C; 1 min) and extension (72°C; 1 min) and final extension at 72°C for 7 min. The product of amplification was electrophoresed on 1% (w/v) agarose gel.

2.3 Expression of riboflavin synthase gene in *E. coli* Top10

A loopful of glycerol stock of recombinant *E. coli* Top10 containing recombinant plasmid pTrcHis/RiS, was cultured on LB agar plate containing 50 µg/ml ampicillin and incubated overnight at 37°C. A single colony was transferred into 10 ml LB broth containing 100 µg/ml ampicillin and incubated in 37°C at 200 rpm for overnight. One ml of recombinant *E. coli* Top10 (pTrcHis/HiS) culture was transferred into 100 ml LB broth containing 100 µg/ml ampicillin in 500 ml blue cap bottle incubated in 37°C, 200 rpm until an OD_{600} ~ 0.5. As the culture reached OD_{600} ~ 0.5, one ml culture was collected by centrifuging for 10 min at 12,000 ×*g* at 4°C and remaining culture was induced with 1 mM IPTG. To check the level of expression, 1 ml of sample was collected at 4, 8, 12, 20, 24 and 48. The harvested cells pellets were resuspended in 0.4 ml phosphate buffer pH 7 prior to ultra-sonification to break the cells wall and release of the intercellular proteins. The cell lysates were centrifuged at 12,000 ×g and the supernatants were collected as the soluble fractions and the pellet as the insoluble fractions. All the fractions were analysed by using SDS-PAGE.

2.4 Cloning of riboflavin synthase gene into pET-32b(+) Vector

To improve the heterologous expression of riboflavin synthase, it is recommended to test different combination of vector (pET-32b(+) and expression host (*E. coli* Rosetta-gami B (DE3) pLysS). Both the vector and host were purchased from Novagene (Germany).

Amplification of riboflavin synthase gene

Riboflavin synthase gene was analyzed for restriction endonuclase sites using Bio Lab NEB Cutter V02 Software (http://tools.neb.com/NEBcutter2/). A set of primers was designed based on the riboflavin synthase gene in the genome of *Photobacterium* sp. strain J15.

The restriction endonuclase sites, *Nco* I and Bpu *1102I* (underlined in primers sequences), were incorporated at forward and reverse primers, respectively. The set of primers used is as follows:
Forward-RiS: 5'-CAG AGC CAT GGT TAT GTT TAC CGG TAT TGT C-3'
Reverse-RiS: 5'-ACA GGC TCA GCC ATT ATA ATG CTT TTT CTG CCA A-3'

2.5 Transformation of recombinant plasmid pET/RiS into *E. coli* Rosetta-gami B (DE3) pLysS

The amplified riboflavin synthase gene and pET-32b(+) vector both were double digested with *Nco* I and *Bpu* 1102I restriction endonucleases. Both double digested riboflavin synthase gene and plasmid were electrophoresed on 1% (w/v) agarose gel and the desired bands were excised with a sterile and sharp scalpel and kept in sterile eppendorf tube. The DNA fragments were extracted using QIAquick gel extraction kit (Qiagen, Germany) according to manufacturer's instructions. The purified double digested pET-32b(+) plasmid and riboflavin synthase gene were ligated using T4 DNA ligase. Recombinant plasmid pET/RiS (4 µl) was mixed with 100 µl of *E. coli* Rosetta-gami B (DE3) pLysS competent cell and incubated on ice for 30 min. Then, the mixture was heat shocked at 42°C for 40 s and immediately incubated on ice. LB broth (0.25 ml) was added to mixture and incubated at 37°C with shaking for 1 h. The transformants were spread on pre-warmed LB agar containing 50 µg/ml ampicillin, 0.34 µg/ml chloramphenicol, 0.24 µg/ml tetracycline and 0.24 µg/ml kanamycin and incubated at 37°C for 24 h.

2.6 Expression of riboflavin synthase in *E. coli* Rosetta-gami B (DE3) pLysS

Expression of riboflavin synthase was conducted by culturing *E. coli* Rosetta-gami B (DE3) pLysS in LB broth containing 50 µg/ml ampicillin, 0.34 µg/ml chloramphenicol, 0.24 µg/ml tetracycline and 0.24 µg/ml kanamycin at 37°C for 24 h and the culture was harvested by centrifugation at 10,000 ×*g*. The harvested pellet was resuspended in 0.4 ml of 100 mM phosphate buffer pH 7 and subjected to sonification to break the cells wall. The cell lysate was centrifuged at 12,000 ×*g* and the supernatant was collected as the soluble intracellular protein and the pellet as the inclusion body. All the fractions was subjected to SDS-PAGE to analyze the level of riboflavin synthase expression in *E. coli* Rosetta-gami B (DE3) pLysS.

3. RESULTS AND DISCUSSION

3.1 Isolation of genomic DNA and riboflavin synthase gene

The ratio of A_{260}/A_{280} was 1.895 indicated that the protein contamination was successfully removed during extraction process and the purity was suitable for further usage. The highly concentrated genomic DNA was indicated by intense bands on agarose gel (Figure 1A). The PCR amplified riboflavin synthase gene is illustrated in figure 1B.

The recombinant plasmids pTrcHis/RiS extracted from 3 positive clones were sent to 1st Base Company (Selangor, Malaysia) for sequencing using pTrcHis forward and riboflavin synthase gene reverse primers. The sequence alignment confirmed the successful cloning of riboflavin synthase gene into pTrcHis vector (Figure 2).

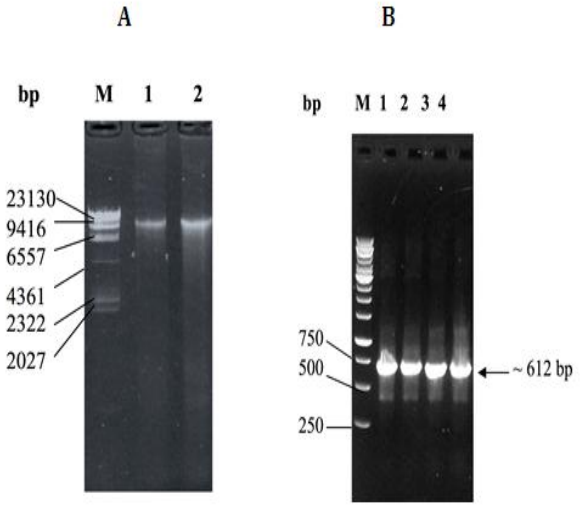

Figure 1: A. Genomic DNA extraction of *Photobacterium* sp. J15. Lane M: Lambda DNA/*Hin*dIII ladder. Lanes 1 and 2: Genomic DNA of *Photobacterium* sp. strain J15. B. Amplification of riboflavin synthase gene from *Photobacterium* sp. strain J15 using riboflavin synthase gene forward and reverse primers. Lane M: GeneRuler 1kb DNA Ladder (Fermentas, Canada). Lanes 1 to 4: Gene encoding riboflavin synthase. Arrow locates the PCR product with the size of ~ 612 bp.

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CLUSTAL W (1.81) multiple sequence alignment

1st_Base_Colony_A1    AAGGATCCAACCCTTATGTTTACCGGTATTGTCCAGGGTACAGCTGAAGTGGTGGATATT
RiS_Gene_             ----------ATGTTTACCGGTATTGTCCAGGGTACAGCTGAAGTGGTGGATATT
                                 **************************************************

1st_Base_Colony_A1    AAGAAAAAAGACCGCTTTCAAACGCATGTGATTCGTATGCCATCTGAATTATTATCTGGC
RiS_Gene_             AAGAAAAAAGACCGCTTTCAAACGCATGTGATTCGTATGCCATCTGAATTATTATCTGGC
                      ************************************************************

1st_Base_Colony_A1    CTTGAAACCGGGGCATCTGTGGCACATAACGGTTGTTGTTTAACAGTTACAGACATTCTA
RiS_Gene_             CTTGAAACCGGGGCATCTGTGGCACATAACGGTTGTTGTTTAACAGTTACAGACATTCTA
                      ************************************************************

1st_Base_Colony_A1    GATGACCTGGTTGCATTCGACCTTATGCAAGAGACATTAAGAGTAACTAACCTCGGGCTC
RiS_Gene_             GATGACCTGGTTGCATTCGACCTTATGCAAGAGACATTAAGAGTAACTAACCTCGGGCTC
                      ************************************************************

1st_Base_Colony_A1    TTAGCGATTGGCGATCATGTCAACGTAGAACGTGCCGCTAGGTTTGGTGATGAAATTGGC
RiS_Gene_             TTAGCGATTGGCGATCATGTCAACGTAGAACGTGCCGCTAGGTTTGGTGATGAAATTGGC
                      ************************************************************
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1st_Base_Colony_A1   GGCCATTCAATGTCTGGACATATTAATGTGGCGACGGAAATCTGCGACATTATTACGACA
RiS_Gene_            GGCCATTCAATGTCTGGACATATTAATGTGGCGACGGAAATCTGCGACATTATTACGACA
                     ************************************************************

1st_Base_Colony_A1   CCAAATAACTGTACAGTATGGTTCAAGTTACCAGCCGATTTTTCCAAATATGTTCTTCGT
RiS_Gene_            CCAAATAACTGTACAGTATGGTTCAAGTTACCAGCCGATTTTTCCAAATATGTTCTTCGT
                     ************************************************************

1st_Base_Colony_A1   AAAGGCTATATCGGTATTGACGGATGTTCTTTGACTATTGGTGATGTTAAAGAGGATTGC
RiS_Gene_            AAAGGCTATATCGGTATTGACGGATGTTCTTTGACTATTGGTGATGTTAAAGAGGATTGC
                     ************************************************************

1st_Base_Colony_A1   TTTTGTGTTCACTTGATCCCTGAAACCTTAAATCGAACCTTGTTTGGTCAACGTAAGATT
RiS_Gene_            TTTTGTGTTCACTTGATCCCTGAAACCTTAAATCGAACCTTGTTTGGTCAACGTAAGATT
                     ************************************************************

1st_Base_Colony_A1   GGGGATGTTGTAAATATCGAGATTGATCCTCAAACACAAGCGATAGTTGATACAGTCGAA
RiS_Gene_            GGGGATGTTGTAAATATCGAGATTGATCCTCAAACACAAGCGATAGTTGATACAGTCGAA
                     ************************************************************

1st_Base_Colony_A1   AGGGTGTTGGCAGAAAAAGCATTATAAAGGGCGAATTCAATTCGAAGCTTGGCTGTTTT
RiS_Gene_            AGGGTGTTGGCAGAAAAAGCATTATAA----------------------------------
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Figure 2. Sequence alignment of recombinant plasmid pTrcHis/RiS carrying riboflavin synthase gene with the sequence of the riboflavin synthase gene from the genome sequence of *Photobacterium* sp. strain J15. The '*' below the aligned sequences indicating identical nucleotides and the '–' showing lack of matching neucleotide. The alignment confirmed a successful cloning of riboflavin synthase gene into pTrcHis vector.

3.2 Expression of riboflavin synthase in *E. coli* Top10

Both the supernatant and pellet of collected sample from recombinant *E. coli* Top10 were run on 10% SDS-PAGE for detection of recombinant riboflavin synthase. The N-terminal tag on pTrcHis plasmid increase the size of fusion protein by 3~4 kDa. As expected, the size of fusion riboflavin synthase was about 27 kDa as observed on SDS-PAGE. The total protein expressed in recombinant *E. coli* Top10 (pTrcHis/RiS) did not contain high amount of expressed riboflavin synthase (Figure 3). The expression of riboflavin synthase gene in *E. coli* Top10 using pTrcHis system was very low even at insoluble fractions. In order to troubleshoot the expression, riboflavin synthase gene codons were analyzed. In molecular biology the level of heterologous protein expression is related to the host system and expression vector; an appropriate combination of expression vector and host is neccessary to obtain high level of protein expression.

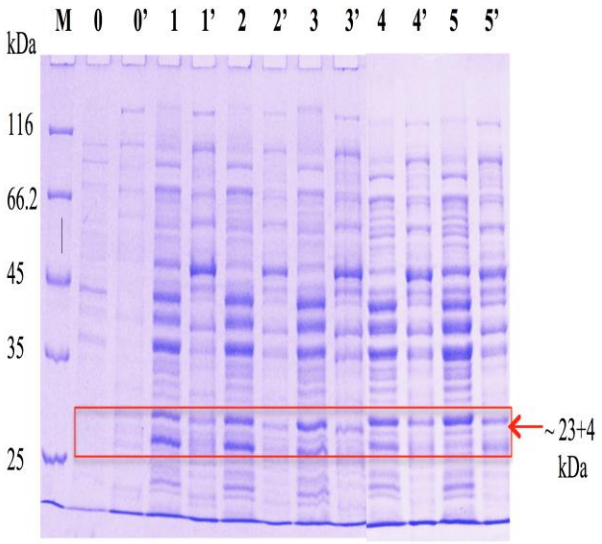

Figure 3. SDS-PAGE (10%) analysis of riboflavin synthase expression in *E. coli* Top10 at different incubation time. Lane M: Unstained Protein Molecular Weight Marker (Fermentas, Canada). Lane 0: Soluble fraction of 0 h incubation. Lane 0': Inclusion body fraction of at 0 h incubation. Lane 1: Soluble fraction at 4 h incubation. Lane 1': Inclusion body fraction at 4 h incubation. Lane 2: Soluble fraction at 8 h incubation. Lane 2': Inclusion body fraction at 8 h incubation. Lane 3: Soluble fraction at 12 h incubation. Lane 3': Inclusion body fraction at 12 h incubation. Lane 4: Soluble fraction at 16 h incubation. Lane 4': Inclusion body fraction at 16 h incubation. Lane 5: Soluble fraction at 20 h incubation. Lane 5': inclusion body fraction at 20 h incubation. The arrow indicates the expected size of fusion riboflavin synthase (~ 23+4 kDa)(4 kDa is the size of N-terminal tag that introduced to riboflavin synthase by pTrcHis vector).

3.3 Examining the codon usage of riboflavin synthase gene

Not all 64 mRNA codons are used equally in organisms as different organisms selectivity utilize different set of codons. Major codons are mostly participate in protein expression and supplied by many host cells, whereas the minor or rare codons are involving in low level at protein expression thus rarely being used and supplied by organisms. The nucleotide sequence and codon analysis of riboflavin synthase gene is illustrated in Table 1. Six rare codons (AGG, AGA, CGA, GGA, ATA, CTA) could rarely be supplied by *E. coli* cells. Alternatively, *E. coli* Rosetta-gami B (DE3) pLysS, which is able to supply rare tRNAs by carrying a chloramphenicol resistant plasmid (pRARE2).

Table 1: Codon analysis of riboflavin synthase gene sequence.

Amino Acids	Codons (number of each codon in riboflavin Synthase gene)	Rare Codons
Alanine	GCT (2), GCA (5), GCG (3), GCC (2)	
Arginine	CGC (1), CGT (4), GCA (1), AGA (1), AGG (2), CGA (1)	AGG (2), AGA (1), CGA (1)

Asparagine	AAT (4), AAC (4)	
Aspartic acid	GAT (10), GAC (6)	
Cystein	TGT (5), TGC (2)	
Glutamic acid	GAA (9), GAG (3)	
Glutamine	CAA (5), CAG (1)	
Glycine	GGT (7), GGC (5), GGG (3), GGA (2)	GGA (2)
Histidine	CAT (5), CAC (1)	
Isoleucine	ATT (13), ATC (4), ATA (1)	ATA (1)
Leucine	TTA (8), CTT (3), CTA (1), CTG (1), CTC (2), TTG (4)	CTA (1)
Lysine	AAA (6), AAG (3)	
Methionine	ATG (4)	
Phenylalanine	TTT (6), TTC (2)	
Proline	CCA (3), CCT (2)	
Serine	TCT (5), TCA (1), TCC (1)	
Tyrosine	TAT (2)	
Threonine	ACC (4), ACA (8), ACG (3), ACT (2)	
Tryptophan	TGG(1)	
Valine	GTG (6), GTT (7), GTC (3), GTA (4)	

3.4 Restriction endonuclease site analysis of riboflavin synthase gene

The restriction endonuclease sites are specific site of DNA sequences which can be recognized and specifically cut by restriction enzymes. To facilitate the restriction digestion and ligation of gene into cloning vector, it is necessary to introduce restriction sites in each 5' terminus end of the gene by using primers. Therefore, it is recommended to analyze the gene sequence for the absence of the selected enzyme endonuclease sites. The possible existing of restriction sites in riboflavin synthase gene sequence were recognized and shown in Figure 4.

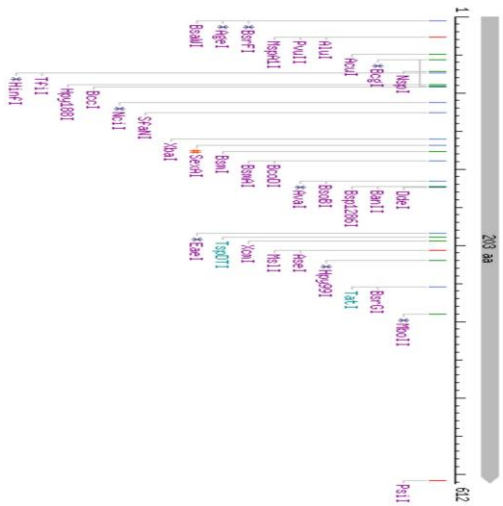

Figure 4. Restriction site analysis of riboflavin synthase gene. Restriction sites (35 sites) were recognized in the nucleotide sequence of riboflavin synthase gene using BioLab NEB Cutter V02 Software (http://tools.neb.com/NEBcutter2/). These restriction sites, which are existing in riboflavin synthase gene sequence could not be used to ligate the riboflavin synthase gene into vector.

3.5 Cloning of riboflavin synthase gene in pET-32b(+) system

The riboflavin synthase gene was successfully amplified by PCR from *Photobacterium* sp. J15 genome using RiS-forward and RiS-reverse primers containing *Nco* I and *Bpu* 1102I restriction sites, respectively (Figure 5A). Introducing restriction endonuclease site in primer is an useful method in molecular cloning to facilitate the ligation of a target DNA into a particular plasmid containing the same pair of restriction sites. Both of riboflavin synthase gene and pET-32b(+) were digested by *Nco* I and *Bpu* 1102 I to produce complementary sites. The complementary and cohesive terminus of digested gene and plasmid with two different restriction endonuclease provided an appropriate orientation for the insertion of riboflavin synthase gene into the pET-32b(+) plasmid, which then was ligated using T4 DNA ligase (Figure 5B). A combination of forward primer targeting at T7 promoter region and a reverse primer targeting at 3' end of riboflavin synthase gene was employed to confirm the appropriate orientation of riboflavin synthase gene inserted in pET-32b(+) plasmid. Among 20 analyzed colonies, 3 of them gave the PCR product, which confirm the presence of insert at desired orientation in pET-32b(+) vector. The clones were sent to 1st Base Company (Selangor, Malaysia) for sequencing, which confirmed the presence of riboflavin synthase gene in pET-32b(+) (Figure 6).

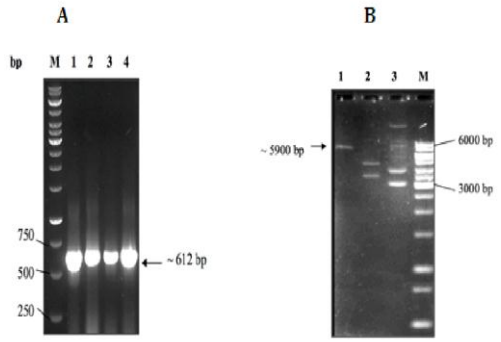

Figure 5. A: Amplification of riboflavin synthase gene from *Photobacterium* sp. J15. Lane M: Gene Ruler™ 1kb DNA Ladder (Fermentas, Canada). Lane 1 to 4: Gene encoding riboflavin synthase gene flanked with *Nco* I and *Bpu* 1102 I restriction sites at the 5'- and 3' –terminus, respectively. Arrow locates the PCR product with the size of ~ 612 bp. B: Digested, recombinant and empty pET-32b(+) plasmid. Lane 1: *Nco* I and *Bpu* 1102 I digested empty pET-32b(+) plasmid. Lane 2: Uncut recombinant pET-32b(+) plasmid carrying riboflavin synthase gene after ligation by T4 DNA ligase. Lane 3: Uncut empty pET-32b(+) plasmid. Lane M: GeneRuler™ 1kb DNA Ladder (Fermentas, Canada). Lanes 2 and 3 are circular pET-32b(+) plasmids that showing the smaller size than the linear pET-32b(+).

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B_r1_rev    CCCAGATCTGGGTACCGACGACGACGACAAGGCCATGGTTATGTTTACCGGTATTGTCCA
Gene_       ----------------------------------------ATGTTTACCGGTATTGTCCA
                                                    ********************

B_r1_rev    GGGTACAGCTGAAGTGGTGGATATTAAGAAAAAAGACCGCTTTCAAACGCATGTGATTCG
Gene_       GGGTACAGCTGAAGTGGTGGATATTAAGAAAAAAGACCGCTTTCAAACGCATGTGATTCG
            ************************************************************

B_r1_rev    TATGCCATCTGAATTATTATCTGGCCTTGAAACCGGGGCATCTGTGGCACATAACGGTTG
Gene_       TATGCCATCTGAATTATTATCTGGCCTTGAAACCGGGGCATCTGTGGCACATAACGGTTG
            ************************************************************

B_r1_rev    TTGTTTAACAGTTACAGACATTCTAGATGACCTGGTTGCATTCGACCTTATGCAAGAGAC
Gene_       TTGTTTAACAGTTACAGACATTCTAGATGACCTGGTTGCATTCGACCTTATGCAAGAGAC
            ************************************************************

B_r1_rev    ATTAAGAGTAACTAACCTCGGGCTCTTAGCGATTGGCGATCATGTCAACGTAGAACGTGC
Gene_       ATTAAGAGTAACTAACCTCGGGCTCTTAGCGATTGGCGATCATGTCAACGTAGAACGTGC
            ************************************************************

B_r1_rev    CGCTAGGTTTGGTGATGAAATTGGCGGCCATTCAATGTCTGGACATATTAATGTGGCGAC
Gene_       CGCTAGGTTTGGTGATGAAATTGGCGGCCATTCAATGTCTGGACATATTAATGTGGCGAC
            ************************************************************

B_r1_rev    GGAAATCTGCGACATTATTACGACACCAAATAACTGTACAGTATGGTTCAAGTTACCAGC
Gene_       GGAAATCTGCGACATTATTACGACACCAAATAACTGTACAGTATGGTTCAAGTTACCAGC
            ************************************************************

B_r1_rev    CGATTTTTCCAAATATGTTCTTCGTAAAGGCTATATCGGTATTGACGGATGTTCTTTGAC
Gene_       CGATTTTTCCAAATATGTTCTTCGTAAAGGCTATATCGGTATTGACGGATGTTCTTTGAC
            ************************************************************

B_r1_rev    TATTGGTGATGTTAAAGAGGATTGCTTTTGTGTTCACTTGATCCCTGAAACCTTAAATCG
Gene_       TATTGGTGATGTTAAAGAGGATTGCTTTTGTGTTCACTTGATCCCTGAAACCTTAAATCG
            ************************************************************

B_r1_rev    AACCTTGTTTGGTCAACGTAAGATTGGGGATGTTGTAAATATCGAGATTGATCCTCAAAC
Gene_       AACCTTGTTTGGTCAACGTAAGATTGGGGATGTTGTAAATATCGAGATTGATCCTCAAAC
            ************************************************************

B_r1_rev    ACAAGCGATAGTTGATACAGTCGAAAGGGTGTTGGCAGAAAAAGCATTATAAATGCTGAG
Gene_       ACAAGCGATAGTTGATACAGTCGAAAGGGTGTTGGCAGAAAAAGCATTATAA--------
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Figure 6. Sequence alignment of recombinant plasmid pET/RiS carrying riboflavin synthase gene with the sequence of the riboflavin synthase gene from the genome sequence of *Photobacterium* sp. J15. The alignment of sequencing result confirmed a successful insertion of riboflavin synthase gene into pET-32b(+) vector. The identical nucleotides are shown by '*' and lack of identical nucleotide is shown by '–' at the third line of aligned sequences.

3.6 Expression of riboflavin synthase in *E. coli* Rosetta-gami B (DE3) pLysS

The pET System (Novagen, USA) is developed for the cloning and high-level expression of recombinant proteins in *E.coli*. The strong bacteriophage T7 promoter in pET vector provides an independent transcription which can express the recombinant gene out of the *E. coli* transcription control, by IPTG induction and providing T7 RNA polymerase in the *E. coli* cell (Studier *et al*., 1990). The pET-32b(+) vector sequence includes various restriction sites for the insertion of the target gene and also carrying ampicillin resistance gene to provide a selective expression of recombinant clone.

E.coli Rosetta-gami B (DE3) pLysS host strains combined the advantages of Rosetta 2 and Origami 2 strains to improve codon bias and enhance disulfide bond formation in the cytoplasm when heterologous proteins are expressed in *E. coli* cells (Novagen, USA). These *trxB*/*gor* mutants are compatible with kanamycin-resistant vectors, which enhance disulfide bond formation and carry the chloramphenicol-resistant pRARE2 plasmid, which supplies seven rare tRNAs. DE3 indicates that the host carries a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter. The strains are suitable for the production of protein from target genes cloned into pET vector upon induction with IPTG.

The expression level was significantly higher for *E. coli* Rosetta-gami B (DE3) pLysS (pET/RiS) as compared to *E. coli* Top10 (pTrcHis/RiS) because the *E. coli* Rosetta-gami B (DE3) pLysS is able to supply rare tRNAs including 8 rare codons found in riboflavin synthase gene sequence. Also the T7 promoter in pET/RiS system is stronger than the *trc* promoter in pTrcHis/RiS. The fractions of total proteins, inclusion bodies, extracellular and soluble proteins were analyzed for the presence of riboflavin synthase. The riboflavin synthase was expressed in the cytoplasm of recombinant clone at high level (Figure 7). The identified bands of riboflavin synthase in SDS-PAGE shows the size of ~40 kDa, which represent the RiS (~23 kDa) tagged with Trx-Tag (~12 kDa), S-Tag (~4 kDa) and His-Tag (~1 kDa).

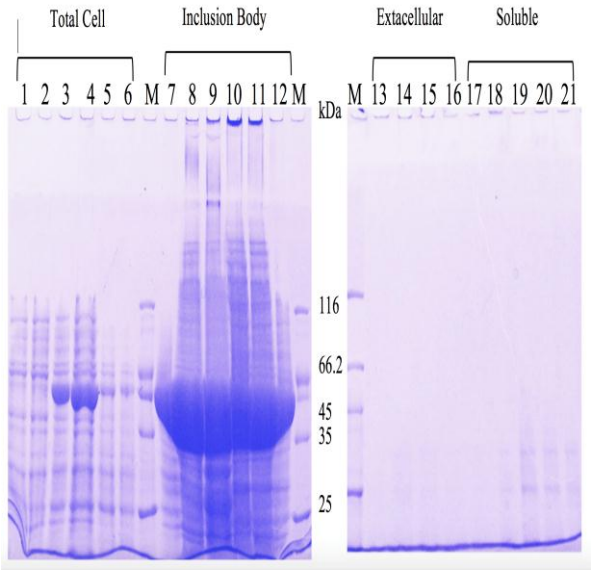

Figure 7. SDS-PAGE (12 %) analysis of riboflavin synthase expression in *E. coli* Rosetta-gami B (DE3) pLysS. The recombinant *E. coli* Rosetta-gami B (DE3) pLysS (pET/RiS) was grown at 37°C overnight with 0.4 mM of IPTG as inducer. Lanes M: Unstained Protein Molecular Weight Marker (Fermentas, Canada). Lanes 1 to 6: Total protein for recombinant culture at 0, 4, 8, 12, 16 and 20 h incubation. Lanes 7 to 12: Inclusion body (Insoluble) fraction collected respectively at 0, 4, 8, 12, 16 and 20 h incubation. Lanes 13 to 16: Extracellular fractions collected from the medium respectively at 8, 12, 16 and 20 h incubation. Lanes 17 to 21: Soluble fraction collected respectively at 8, 12, 16 and 20 h incubation.

4. CONCLUSION

In vitro studies of riboflavin synthase has been investigated over past decades. The chemical production of riboflavin and many vitamins has successfully been replaced by microbial technologies through overexpression of certain enzymes. A novel riboflavin synthase from *Photobacterium* sp. J15 was selected in this study to expresse the protein in heterologous system. The riboflavin synthase gene from *Photobacterium* sp. J15 includes 6 rare codons and 7 Cysteine residues that might form the disulfide bond in enzyme complex. Therefore the *E. coli* Top10 host cell was replaced by *E. coli* Rosetta-gami B (DE3) pLysS, which could supply rare tRNAs and promote the disulfide bond formation in cytoplasm. Finally heterologous expression of riboflavin synthase of *Photobacterium* was successfully carried out using *E. coli* Rosetta-gami B (DE3) pLysS as the expression host.

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