MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.11, No.1, March 2017, p 28-34

Available online at
http://jurnal.permi.or.id/index.php/mioline
DOI: 10.5454/mi.11.1.5

Construction and Expression of Single Recombinant Peptide Surfactant for Enhanced Oil Recovery (EOR) Application

CUT NANDA SARI[1], USMAN[1], RIESA KW ROHMAT[2], LENI HERLINA[1], KEN SAWITRI SULIANDARI[1], ONIE KRISTIAWAN[1], DWIYANTARI[2], TATI KRISTIANTI[2], AND SONY SUHANDONO[2*]

[1] *PPPTMGB LEMIGAS, Cipulir, Kebayoran Lama, Jakarta 12330, Indonesia;*
[2]*School of Life Sciences and Technology, Institut Teknologi Bandung, Jalan Ganesha 10, Bandung 40132, Indonesia.*

Surfactant is generally synthetic chemical, which is effective and reliable. However, the chemicals usually cannot be degraded easily in the environment and could cause damage to the environment. The other possible alternative to produce surfactant is using genetic engineering in order to produce peptide based surfactant. In this research, peptide surfactant was produced using a gene construct which was created using overlapped polymerase chain reaction method (OE-PCR). SDS-PAGE analysis showed that single surfactant peptide construction can be expressed by induction of IPTG 1 mM and after at least twice sonication. This research proved that both two constructions have been successfully expressed by producing peptide in expected size (approximately 15 kDa).

Key words: enhanced oil recovery, overlapped PCR method, surfactant peptide

Surfaktan digunakan untuk meningkatkan perolehan minyak bumi (*enhanced oil recovery*). Surfaktan hasil sintesis ini bersifat cepat dan efektif, tetapi banyak menimbulkan masalah lingkungan. Alternatif lain yang bisa digunakan untuk menghasilkan surfaktan dengan rekayasa genetika untuk produksi rekombinan peptida bersifat surfaktan dalam bakteri. Pada penelitian ini dilakukan konstruksi genetik untuk menghasilkan surfaktan peptida dengan menggunakan metode *overlapped-PCR* (OE-PCR). Hasil analisis SDS-PAGE menunjukkan konstruksi surfaktan peptida dapat diekspresikan dengan cara diinduksi IPTG 1 mM dan setelah dua kali pemecahan sel. Penelitian ini membuktikan bahwa konstruksi genetik berhasil mengekspresikan peptida pada ukuran yang sesuai (sekitar 15 kDa).

Kata kunci: *enhanced oil recovery*, metode *overlapped-PCR*, peptida surfaktan

Oil recovery from production wells still cannot fulfill the world oil demands (Bachmann *et al.* 2014). Oil is non-renewable resource, as the world demand of crude oil increased, the oil industry find difficulties to discover new oil resources. The industry should more focus on mature or even abandoned wells (Brown and Vadie 2000). Primary oil recovery requires high cost with the result 20% - 50% from original oil in place (OOIP) (Sandrea and Sandrea 2007). Therefore, the better oil recovery method with less production cost is needed.

One of the existing technologies is using surfactant in order to mixed oil and water. The emulsion between oil and water results oil recovery and may increase crude oil recovery up to 30% - 200%. However, the existing surfactant is not eco-friendly, because it is difficult to degrade in nature. One of the non eco-friendly surfactant is Oleyl alcohol ($CH_3(CH2)_7$-CH=CH-$(CH_2)_8OH$) which is produced synthetically (Salter *et al.* 2011). This compound causes damage in hydrodynamics open water and cause CO_2 exchange effectivity that will harm the life at sea. Beside that, Oleyl alcohol is also irritant to human especially skin and eyes. One of eco-friendly surfactant may be produced using genetic engineering, a biosurfactant. Biosurfactant production using natural microbial is usually called microbial enhanced oil recovery (MEOR). MEOR is the technology using microbial and the metabolic way for oil recovery (Bachmann *et al*. 2014). Oil recovery using microbial that is developed into biosurfactant has become advanced as seen from the numerous researches that studied and generated about biosurfactant (Desai and Banat 1997; Van Hamme *et al*. 2003; Sandrea and Sandrea 2007; Maneerat 2005; Dwyer *et al.* 2014; Pacheco *et al.* 2010). Meanwhile, biosurfactant has also weakness, the quality is hard to be standardized. Biosurfactant need to be developed in recombinant in order to generate in large scale with assured quality. Peptide based biosurfactant in this research has been designed by LEMIGAS. This peptide sequence is then called as

*Corresponding author: Phone: +62-22-2511575, Fax:+62-22-2534107; Email: sony@sith.itb.ac.id

SUPEL. Earlier the peptide was synthesized chemically, which was very expensive. In order to make SUPEL production cheaper, genetic engineering will be used in this research. Peptide sequence was translated into DNA sequence with consideration of optimum codon usage for *Escherichia coli*, then the DNA is made synthetically and constructed into plasmid pET 32-b+, which is then called as SS (Single SUPEL) construct. Peptide will be produced by *E. coli* cell naturally and the result will be analyzed using Poly Acrilamid Gel Electrophoresis (PAGE).

MATERIALS AND METHODS

Optimization of SUPEL Codon Usage . This research will use *E. coli* strain BL21 (DE3), therefore codon optimisation for translation in *E. coli* is needed for efficient production of the peptide. This codon usage optimation was made by Codon Adaptation Indexed (CAI) with reference of highly expressed gene (Maloy *et al.* 1996). The optimise codon usage of the SUPEL presented in Table 1.

Single SUPEL Construction Design. The DNA sequenced was ordered to be synthesised chemically at Genescript. The cloned gene was inserted with Tobacco Etch Virus (TEV) protease peptide recognition side. This has been made by inserting DNA sequence into a primer that was designed to amplified SUPEL. A pair of primer is designed to have (30 bp) sequence that complements each other at tip point 3' (TEV-F primer and TEV-R primer). As a result the SUPEL fragment contain DNA sequence encode TEV protease peptide recognition side. The cloned DNA inculding the protease recognition site was construted into pET32b+ using overlapped PCR. Specific primer pair which is used for amplification [TEV + surfactant's composer nucleotide] is: TEV-F primer and TEV-R primer.

PCR profile of TEV amplification and surfactant's coding nucleotide is started with pre-denaturation 95 °C for 3 min, cycle 1 is 15 times (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min), cycle 2 is 10 times (95 °C for 30 s, annealing temperature touchdown 50 - 55 °C for 30 s, 72 °C for 1 min), cycle 3 is 15 times (95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min), additional elongation temperature 72 °C for 7 min and 4 °C to deactivate the enzyme. The further PCR product becomes template for a pair of primer that overlapped PCR. Figure 1 shows PCR design of TEV construction and nucleotide. Primer was designed having Tm = 60-65 °C at part that overlapped with pET-32b vector. The overlapped primer with pET-32b vector has 25-35 bp in length. Primer for PCR overlap extension has 15-20 bp in length at part that overlapped with template [TEV + nucleotide surfactant].

The PCR product was digested with *Dpn*I enzyme which will destroy the template (pET-32b+ vector). This enzyme will degrade wild type of pET-32b+ vector because this vector endogenously methylated. The addition result of *Dpn*I enzyme will only leave the PCR overlap product and can be transformed into *E.coli* strain BL21 (DE3).

SUPEL insert confirmation with PCR (Polymerase Chain Reaction), restriction enzyme and sequencing. Putative recombinant plasmid is amplified using **TEV-PEP_F** Forward Primer sequence and **TEV-PEP_R**

Table 1 Codon usage for single peptide SUPEL at E.coli

Amino Acid	*Codon Usage*	% Emergence	Amino Acid	*Codon Usage*	% Emergence
M	ATG	100	**L**	CTG	55
D	GAC	41	**E**	GAA	70
F	TTC	49	**N**	AAC	61
S	TCT	19	**I**	ATC	46
S	TCT	19	**L**	CTG	55
M	ATG	100	**D**	GAC	41
A	GCT	19	**K**	AAA	76
K	AAA	76	**A**	GCT	19
L	CTG	55	**R**	CGT	42
C	TGC	57	**N**	AAC	61
H	CAC	48	**S**	TCT	19
T	ACC	43	**stop**	TAA	62

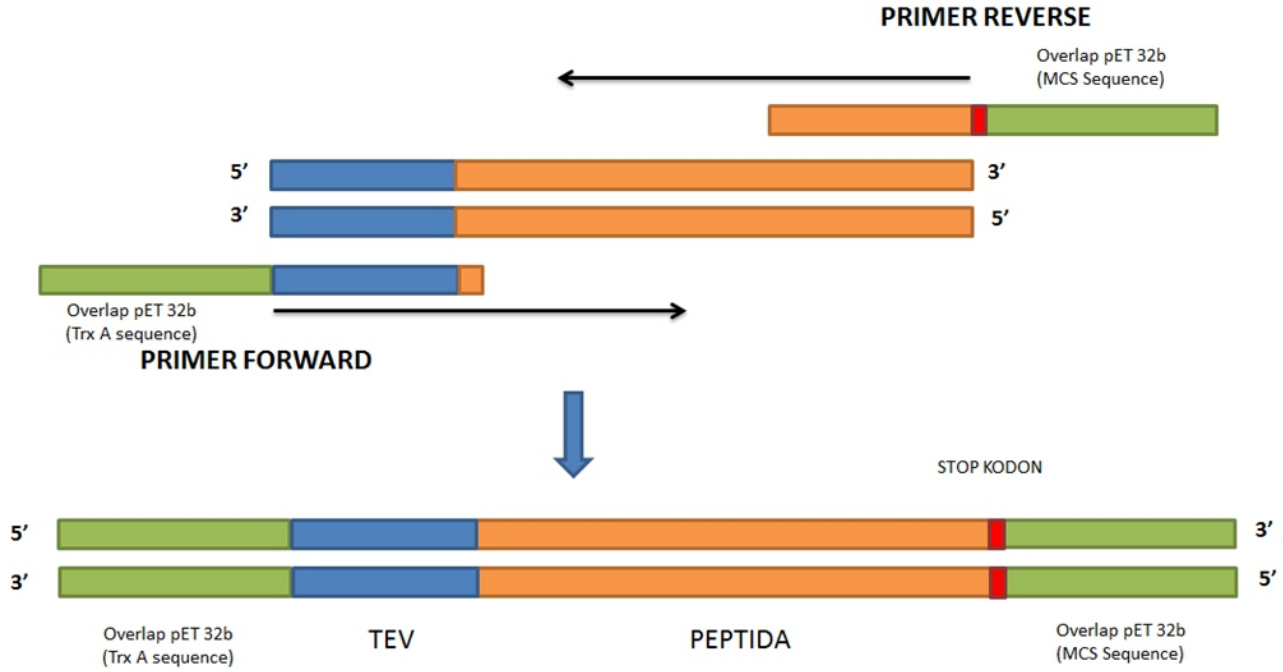

Fig 1 Primer design for PCR overlap extension. Forward primer has total length of 56 bp that consists of 30 bp that overlaps with Trx tag from pET-32b (green) and 26 bp that overlaps with TEV sequence and nucleotide the composer of surfactant (blue and orange). Reverse primer has total length of 51 bp that consists of 28 bp and overlaps with MCS part from pET-32b (green), 3 bp codon stop (red), and 20 bp with nucleotide the composer surfactant (orange).

Reserve Primer with using Green Taq Ready Mix. Sequencing is done in Macrogen Inc., South Korea with two ways, using universal primer T7 promoter and T7 terminator. Sequencing result is then analyzed and aligned using some softwares such as Bioedit and SnapGene™v.1.1.3.

Expression Test for Single SUPEL Construction. Single SUPEL construction result using PCR overlap is then transformed and made glycerol stock. From that glycerol stock, solid LB (Luria Bertani) is streaked into bowl and made into liquid culture. Overnight culture result 1 mL is put into erlenmeyer 100 mL of LB. The culture is incubated into incubator 37 °C with shaker 180 rpm for 2-4 h to obtain OD_{600} 0.5-0.7. After OD_{600} reach that number, 5 mL from culture is separated as non-inducted culture and then the other 95 mL is inducted using 1 mM IPTG (final concentration). Culture is then incubated for 4 hours measured by OD_{600} using pattern: V x OD_{600} = 1 to uniform SDS-PAGE sample amount. The further culture is pelleted at the speed of 14000 rpm for 10 min. The pelet was sonicated once and twice. The further sonicated cell result was then analysed by SDS-PAGE is done using 15% or 20% Tris-Glisin gel.

RESULTS

Single SUPEL Construction. PCR amplicon to synthetize self SUPEL peptide template using a pair of primer that has 30bp overlapped PCR, was obtained with the size around 120 bp (Fig 2). This PCR product is then become template to make overlapped PCR using mega primer.

PCR product with the further mega primer is used to overlap PCR using expression vector pET-32b as a template. Through this overlap PCR of TEV + SUPEL + codon stop will insert between Trx*tag and His*tag sequence of pET32b+, producing construction map as shown in Fig 3.

Based on the map above, SUPEL peptide is above signal strength control of transcription and translation T7 bacteriophage, where the expression is induced by the availability of T7 RNA polymerase in cell and induced with IPTG at final concentration 1 mM (Novagen 2003). The TEV sequnce presence is useful for target protein purification. The sequence existence confirmation result of TEV + SUPEL + codon stop is validated using PCR with specific primer to amplify TEV and SS construction coding sequence, that is

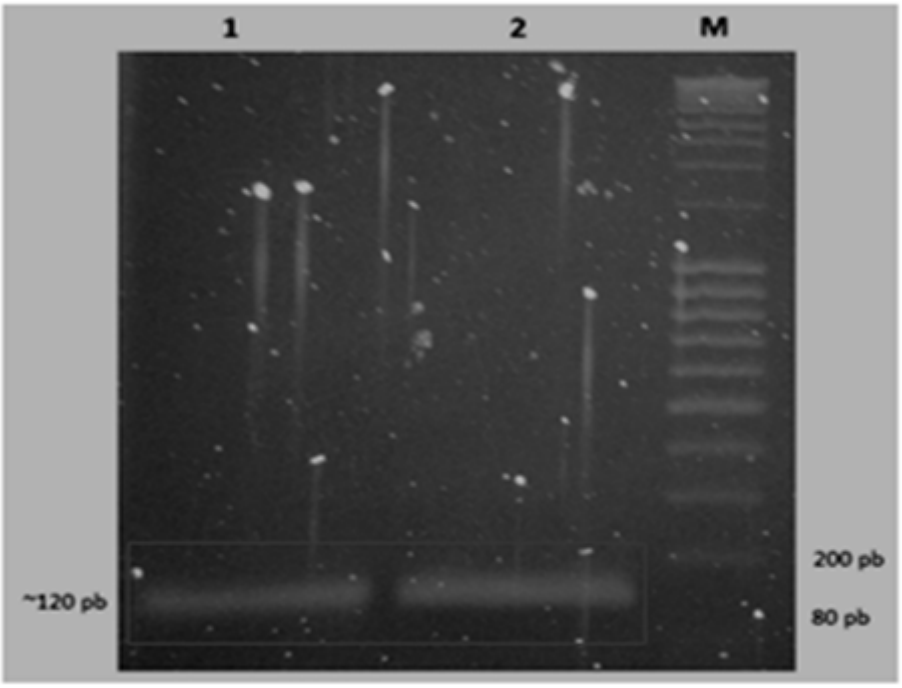

Fig 2 PCR Electrophoregram of SUPEL; 1 and 2 of PCR samples, M. DNA 1 Kb Ladder.

Fig 3 Single SUPEL construction Map.

amp_F and amp_R. PCR result shows the size under ladder tape 250 bp, has been successfully amplified because the size is supposed to be 81 bp (Fig 4).

Expression Analysis of Recombinant Single SUPEL Peptide (SS). Expression from SUPEL peptide was tested with SDS-PAGE analysis. Supernatant of culture was used for SDS-PAGE analysis (Fig 5). SS construction expressed SUPEL peptide protein at the size under ladder 15 kDa. This result is according to online calculation result of the combination of TEV protein molecule weight, 1x SUPEL, and codon stop which is obtained ~ 14.9 kilo Dalton. This result indicates that SUPEL protein has been expressed correctly. Protein expression occured after 2 to 3 times sonication. This result can be assumed that 1x SUPEL protein is expressed intracellular.

DISCUSSION

Amino acid series at this SUPEL causes peptide has two polar of different side chain, that is hydrophobic and hydrophylic. Modelling using online software innovagen http://pepcalc.com/, around 67% of amino acid is hydrophobic and the other 33% is hydrophylic (Fig 6). This indicates that SUPEL peptide can bind with non-polar compound like oil. The condition of both characteristic in this SUPEL peptide causes SUPEL peptide is amphipathic when in liquid, which is the hydrophylic is inside the water while the hydrophobic will be on the water surface. This SUPEL peptide is designed to form helix structure. The helix structure is designed in order that peptide has surfactant characteristic, depleted in water, and stable

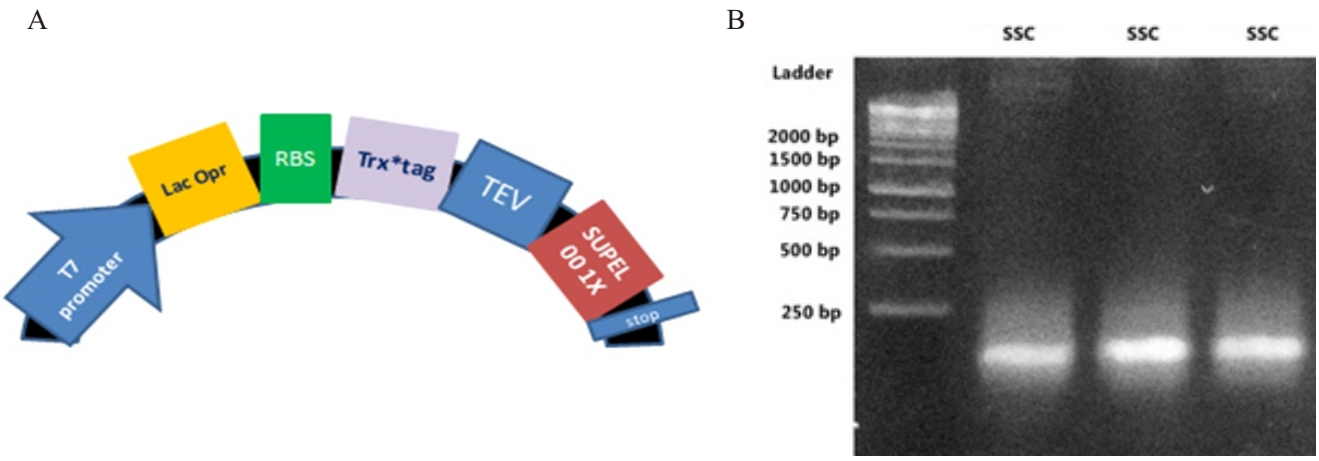

Fig 4 (A) Side attachment of prime ramp_F and amp_R in sequence; (B) Electrophoregram of existence confirmation result of TEV sequence and 1x SUPEL peptide sequence.

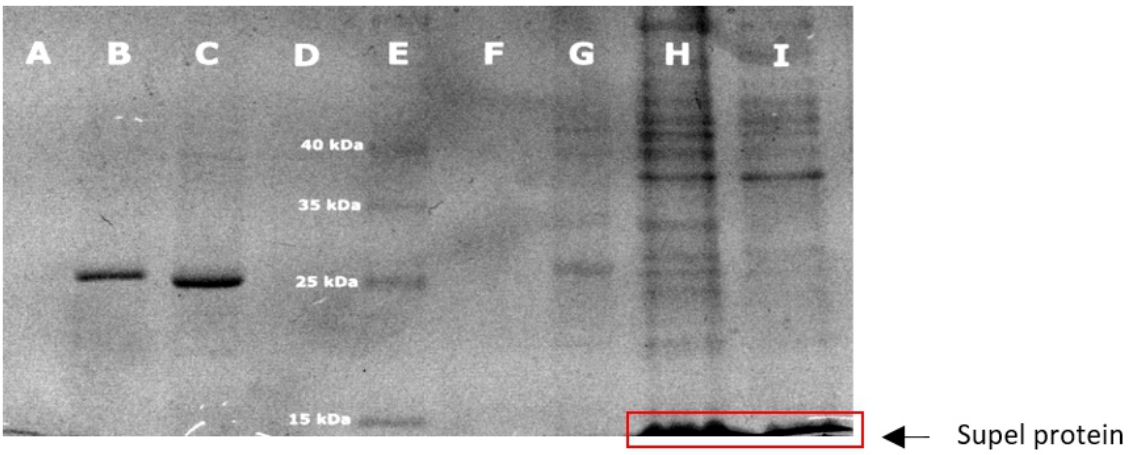

Fig 5 SDS-PAGE of SS construction supernatant sample; A. Lysed cells of BL21 DE3 with no plasmid, B. pET non-induction, C. pET induction, D. Recombinant non-induction, E. Ladder, F. Recombinant 1 induction non sonication, G. Recombinant induction and sonication, H. Recombinant induction and two times sonication, I. Recombinant induction and three times.

Fig 6 Simulation of hydropathy in silico using innovagen (http://pepcal.com/).

at high temperature and high salinity (Dwyer *et al.* 2014).

Peptide surfactant must have the characteristic that similar to surfactant which can be used in crystalization, that is peptide surfactant must be designed to have hydrophylic residual and 3 – 6 hydrophobic residual at the tail (Yeh *et al.* 2005; Kiley *et al.* 2005; Matsumoto *et al.* 2009). The tail of SUPEL

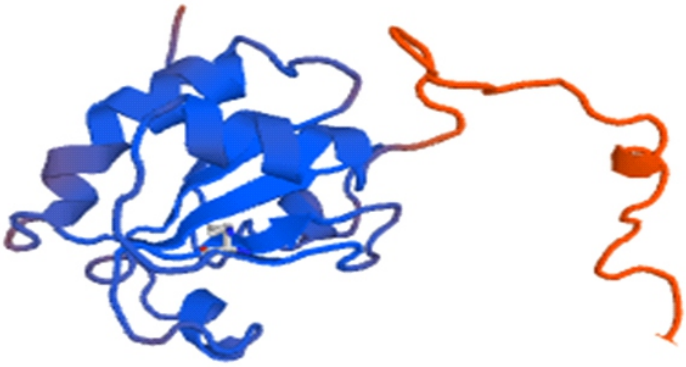

Fig 7 1x peptide that still fused with TRX and TEV. Blue protein shows Thioredoxine while the orange shows TEV and peptide surfactant.

peptide is designed to have 3 amino acid residual of hydrophobic, that is arginine (R), lysine (K) and aspargarine (N).

Prediction of protein structure of 1x SUPEL peptide that fused with Thioredoxine and TEV is analyzed in silico with bioinformatics program. This structure shows the presence of Trx protein that forms alphahelix and TEV and also 1x SUPEL peptide forms linear structure (Fig 7).

Simulation of fusion protein structure between Thioredoxine, TEV and single peptide SUPEL shows that in fused condition, single peptide SUPEL forms linear structure so it is assumed that peptide hasn't had surfactant characteristic yet. That's why, the separation of single peptide SUPEL from thioredoxine using TEV protease needs to be made before surfactant characteristic test.

In conclusion, construct of surfactant SUPEL sequences (SS) has been successfully conducted into pET-32b expression vector using overlapping PCR methods. Constructs of SS can express SUPEL peptide at sizes below 15 kDa ladder by SDS-PAGE analysis. This is consistent with the result of online program calculation which show molecular weight of combination of TEV protein, 1x SUPEL, and stop codon at the size of ~ 14.9 kilo Dalton

REFERENCES

Bachmann RT, Johnson AC, Edyvean RG. 2014. Biotechnology in petroleum industry: an overview. Int Biodeterior Biodegradation. 86:225-237. doi: 10.1016/j.ibiod.2013.09.011.

Brown LR and Vadie AA. 2002. Slowing production decline and extending the economy life of an oil field: new MEOR technology. SPE Reservoir Eval Eng. 5(1): 33-41. doi: 10.2118/75355-PA.

Desai, J. D., Banat, I. M. 1997. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev. 61(1): 47-64.

Dwyer MD, Brech M, Yu L, Middelberg, Anton PJ. 2014. Intensified expression and purification of a recombinant biosurfactant protein. Chem Eng Sci. 105: 12-21. doi: 10.1016/j.ces.2013.10.024.

Kiley P, Zhao X, Bruce BD, Baldo M, Zhang S. 2005. Self-assembling peptide detergents stabilize isolated photosy I on a dry surface for an extended time. PLoS Biol. 3:1181–1186. doi: 10.1371/journal.pbio.0030230.

Maloy S, Stewart V, Taylor R. 1996. *Genetic analysis of pathogenic bacteria*. New York: Cold Spring Harbor Laboratory Press.

Maneerat, Suppasil. 2005. Production of biosurfactants using substrates from renewable-resources. Slongklanakarin J Sci Technol. 27 (3): 675-683.

Matsumoto K, Koutsopoulos S, Vaughn M, Bruce BD, Zhang S. 2009. Designer lipid-like peptide surfactants stabilize functional photosy I membrane complex in solution. J Phys Chem. 115:75–83. doi: 10.1021/jp8021425.

Novagen, 2003. *pET Sy Manual*. US: Novagen.

Pacheco GJ, Ciapina, Elisa MP, Gomes, Edelvio de B, Pereira Junior, Nei. 2010. Biosurfactant production by *Rhodococcus erythropolis* and its application to oil removal. Braz J Microbiol. 41: 685-693. doi: 10.1590/S1517-83822010000300019

Salter ME, Upstoll-Goddard RC, Nihtingale PD, Archer SD, Blomquist B, Ho DT, Huebert B, Schlosser P, Yang M. 2011. Impact of an artificial release on air-sea gas fluxes during deep ocean gas exchange experiment. J Geophys Res. 116: 1-6. doi: 10.1029/2011JC007023.

Sandrea I and Sandrea R. 2007. Global oil reserves-recovery factors leave vast target for EOR technologies. Oil Gas J. 1-8.

Van Hamme JD, Singh A, Ward OP. 2003. Recent advances in petroleum microbiology. Microbiol Mol Biol Rev. 67

(4): 503-549. doi: 10.1128/MMBR.67.4.503-549.2003.

Yeh JI, Du S, Tordajada A, Paulo J, Zhang S. 2005. Peptergent: Peptide detergents that improve stability and functionality of a membrane protein glycerol-3-phosphate dehydrogenase. Biochemistry. 44:16912-16919. doi: 10.1021/bi051357o.