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ISOLATION AND ANALYSIS OF THE *rhlA* AND *rhlB* FROM RHAMNOLIPID PRODUCING BACTERIUM *Pseudomonas aeruginosa* strains S5

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PENGESAHAN

Dengan ini adalah disahkan bahawa projek yang bertajuk "**Isolation and Analysis of the *rhlA* and *rhlB* From Rhamnolipid Producing Bacterium *Pseudomonas aeruginosa* strains S5**" telah disiapkan serta dikemukakan kepada Jabatan Mikrobiologi oleh **Norhafizah Binti Azman** dengan matrik nombor **164294** sebagai syarat untuk kursus BMY4999 projek.

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ABSTRACT

Rhamnolipids are biotechnologically important biosurfactants, find applications in cosmetic and pharmaceutical industry, agriculture, environmental application and in bioremediation activities. *Pseudomonas aeruginosa* is one of the microbes that can produce rhamnolipids. The aim of this study is to test the rhamnolipids production of our *P. aeruginosa* strain and to analyze the genes required for the rhamnolipids biosynthesis. Based on the qualitative test on cetyltrimethyl ammonium bromide agar, *P. aeruginosa* strain S5 formed a blue halo around the culture which showed that the bacterium is a rhamnolipids producer. The emulsification index value of the rhamnolipids produced was 41%. Genes involved in rhamnolipids biosynthesis in *P. aeruginosa* strain S5; *rhlA* coding for rhamnosyltransferase 1 subunit A, and *rhlB*, coding for rhamnosyltransferase 1 subunit B were amplified, sequenced and analyzed. The sequencing results showed that the size of *rhlA* and *rhlB*, genes are 888 bp and 1281 bp, which corresponded to 295 and 426 amino acids protein, respectively. The genes sequence that were identified and analyzed could facilitate the understanding on the rhamnolipids biosynthesis in *P. aeruginosa* strain S5, hence the information can be further used in genetic modulation in order to produce natural biosurfactant in the economic yet safer manner which meets industrial and ecological prospect.

ABSTRAK

Rhamnolipid adalah biosurfaktan yang penting dalam bioteknologi termasuklah pengunaannya dalam kosmetik, industri farmaseutikal, pertanian, alam sekitar dan aktiviti bioremediasi. *Pseudomonas aeruginosa* adalah salah satu daripada mikrob yang boleh menghasilkan rhamnolipid. Tujuan kajian ini adalah untuk mengkaji pengeluaran rhamnolipid dari strain *P. aeruginosa* kami dan untuk menganalisis gen yang diperlukan untuk biosintesis rhamnolipid itu. Melalui ujian kualitatif pada agar cetyltrimethylammonium bromide, *P. aeruginosa* strain S5 telah menghasilkan halo biru di sekeliling kultur yang menunjukkan bakteria ini adalah pengeluar rhamnolipid. Keputusan indeks pengemulsian yang terhasil daripada rhamnolipid adalah 41 %. Gen-gen yang terlibat dalam biosintesis rhamnolipid oleh *P. aeruginosa* strain S5; *rhlA* yang mengekod untuk rhamnosyltransferase 1 subunit A, dan *rhlB* yang mengekod untuk rhamnosyltransferase 1 subunit B telah diperbanyakkan, dijujukkan dan dianalisis. Keputusan penjujukan menunjukkan bahawa saiz gen *rhlA* dan *rhlB* adalah masing-masing 888 bp dan 1281 bp yang mengekod untuk masing-masing 295 dan 426 asid amino protein. Jujukan gen-gen yang telah dikenalpasti dan dianalsis akan membantu memahami biosintesis rhamnolipid dalam *P. aeruginosa* strain S5, dan seterusnya maklumat tersebut boleh digunakan dalam modulasi genetik untuk menghasilkan biosurfaktan semulajadi dengan cara yang lebih selamat dan ekonomi yang akan memenuhi prospek industri dan ekologi.

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LIST OF ABBREVIATION

Abbreviation

PAS5	*Pseudomonas aeruginosa* strain S5
PA181	*Pseudomonas aeruginosa* strain 181
PAO1	*Pseudomonas aeruginosa* PAO1
EC	*Escherichia coli*
CTAB	Cetyltrimethylammonium bromide
MB	Methylene Blue
SDS	Sodium dodecyl sulfate
MS	Mineral salt
E_{24}	Emulsification Index
ml	mililiter
µl	microliter
ng/µl	nanogram per microliter
%	Percent
˚C	Degree Celsius
h	Hour
min	Minute
PCR	Polymerase Chain Reaction
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool

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CHAPTER 1

INTRODUCTION

Rhamnolipids are surfactants that act as surface-active compounds capable of reduce interfacial tension and surface at the interfaces between liquids, solids and gases, thereby allowing them to mix or disperse readily as emulsions in water or other liquids (Banat et al., 2000). Rhamnolipids are important industrially because they are normally used as a detergent, emulsifier, foaming agent, wetting agents and also dispersant (Makkar et al., 2002).

The study about *Pseudomonas aeruginosa* strain S5 was initially performed by in the Enzyme Microbial Technology Laboratory (EMTech lab) for its ability to produce lipase (Baharum et al, 2003). It is an aerobic, gram-negative rod and motile bacterium. It was locally isolated from soil samples and known to be BTEX degrader (Baharum et al, 2003). Since rhamnolipids are normally produced by certain species of *Pseudomonas* (Pornsunthorntawee et al., 2010), the study of the potentiality of *P. aeruginosa* strain S5 for as a rhamnolipids producer is important. *Pseudomonas aeruginosa* produces primarily two forms of rhamnolipids: mono-rhamnolipids and di-rhamnolipids. Rhamnosyltransferase 1 catalyses the synthesis of mono-rhamnolipids from dTDP-L-rhamnose is encoded by the *rhlAB* operon and β-hydroxydecanoyl-β-hydroxydecanoate, whereas the second rhamnosyltransferase, encoded by *rhlC* catalyses the di-rhamnolipid is produced from mono-rhamnolipid and dTDP-L-rhamnose (Deziel et al., 2003).

This study will elucidate the rhamnolipids biosynthesis pathway in *Pseudomonas aeruginosa* strain S5 and determine the availability of the *rhlA* and *rhlB* genes in this strain. The objectives of this study were (1) to determine the rhamnolipids production of *Pseudomonas aeruginosa* strain S5, (2) to isolate *rhlA* and *rhlB* from *Pseudomonas aeruginosa* strain S5 and (3) to analyze the *rhlA* and *rhlB* genes required for rhamnolipids biosynthesis.

REFERENCES

Abdel-mawgoud, A. M., Hausmann, R., Le, F., De, E., & Markus, M. M. (2011). Rhamnolipids: Detection, Analysis, Biosynthesis, Genetic Regulation, and Bioengineering of Production. *Biosurfactants*. (G. Soberón-Chávez, Ed.) (Vol. 20, pp. 13–56). Berlin, Heidelberg: Springer Berlin Heidelberg.

Al-araji, L. I. Y., Noor, R., Raja, Z., Rahman, A., Basri, M., & Salleh, A. B. (2007). Optimisation of Rhamnolipids Produced by *Pseudomonas aeruginosa* 181 using Response Surface Modeling. *Annals of Microbiology*, *57*(4), 571–575.

Baharum, S. N., Salleh, A. B., & Basri, M. (2003). Organic Solvent Tolerant Lipase by *Pseudomonas* sp. strain S5 : Stability of Enzyme in Organic Solvent and Physical Factors Affecting Its Production. *Annals of Microbiology*, *53*, 75–83.

Banat, I. M., Makkar, R. S., & Cameotra, S. S. (2000). Potential Commercial Applications of Microbial Surfactants. *Applied Microbiology and Biotechnology*, *53*(5), 495–508.

Banat, I. M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M. G., Fracchia, L., Marchant, R. (2010). Microbial Biosurfactants Production, Applications and Future Potential. *Applied Microbiology and Biotechnology*, *87*(2), 427–44.

Bunster, L., Fokkema, N. J., & Schippers, B. (1989). Effect of Surface-Active *Pseudomonas* spp. on Leaf Wettability. *Applied Environment Microbiology.*, *55*(6), 1340–1345.

Burger, M. M., Glaser, L., & Burton, R. M. (1963). The Enzymatic Synthesis Of A Rhamnose-Containing Glycolipid by Extratcs of *Pseudomonas aeruginosa*. *The Journal of Biological Chemistry*, *238*(8), 2595-2602.

Cappuccino, J. G., & Sherman, N. (2005). *Microbiology: A laboratory manual*. San Francisco, CA: Pearson Benjamin Cummings.

Das, P., Mukherjee, S., & Sen, R. (2008). Genetic Regulations of the Biosynthesis of Microbial Surfactants: An Overview. *Biotechnology and Genetic Engineering Reviews*, *25*, 165-186.

Deziel, E. (2003). rhlA is Required for The Production of A Novel Biosurfactant Promoting Swarming Motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), The Precursors of Rhamnolipids. *Microbiology*, *149*(8), 2005–2013.

Edwards, J. R., & Hayashi, J. A. (1965). Structure of a rhamnolipid from *Pseudomonas aeruginosa*. *Archives of Biochemistry and Biophysics*, *111*(2), 415-421.

El-Sersy, N.A., (2012). Plackett-Burman Design to Optimize Biosurfactant Production by Marine *Bacillus subtilis* N10. *Romanian Biotechnological Letter, 17*(2), 7049–7064.

Hazra, C., Kundu, D., & Chaudhari, A. (2012). Biosurfactant-assisted Bioaugmentation in Bioremediation. *Microorganisms in Environmental Management*. (T. Satyanarayana & B. N. Johri, Eds.) (pp. 631–664). Dordrecht: Springer Netherlands.

Hirayama, T., & Kato, I. (1982). Novel methyl rhamnolipids from *Pseudomonas aeruginosa*. *FEBS Letters*, *139*(1), 81-85.

Kosaric, N. (1992). Biosurfactants in Industry. *Pure and Applied Chemistry*, *64*(11), 1731-1737.

Lang, S., & Wullbrandt, D. (1999). Rhamnose lipids - biosynthesis, Microbial Production and Application Potential. *Applied Microbiology and Biotechnology*, *51*(1), 22–32.

Maier, R. M., & Sobero, G. (2000). *Pseudomonas aeruginosa* Rhamnolipids : Biosynthesis and Potential Applications. *Applied Microbiology and Biotechnology*, 625–633.

Makkar, R. S., Cameotra, S. S., & Banat, I. M. (2011). Advances in Utilization of Renewable Substrates for Biosurfactant Production. *AMB Express*, *1*(1), 5.

Mani, A., Hameed, S. S., Ramalingam, S., & Kaliamoorthi, R. (2011). Effect of Rhamnolipid Potential on Biodegradation of Endosulfan by *Pseudomonas aeruginosa* in Batch Studies. *Journal of Bioscience and Technology*, *2*(3), 268–278.

Mathiyazhagan, N. (2011). Amplification of Biosurfactant Producing Gene (rhlB) from *Pseudomonas aeruginosa* Isolated from Oil Contaminated Soil, *International Journal of Pharma and Bio Sciences*, *2(1,)* 497–504.

Medina, G. (2003). Transcriptional Regulation of *Pseudomonas aeruginosa* rhlR, Encoding A Quorum-sensing Regulatory Protein. *Microbiology*, *149*(11), 3073–3081.

Mulligan, C. N. (2005). Environmental Applications For Biosurfactants. *Environmental Pollution*, *133*(2), 183-98.

Nitschke, M., Costa, S. G. V. a. O., & Contiero, J. (2011). Rhamnolipids and PHAs: Recent Reports on Pseudomonas-derived Molecules of Increasing Industrial Interest. *Process Biochemistry*, *46*(3), 621–630.

Ochsner, U. R. S. A., Reiser, J., Fiechter, A., & Witholt, B. (1995). Production of *Pseudomonas aeruginosa* Rhamnolipid Biosurfactants in Heterologous Hosts, *Applied and Environmental Microbiology*, *61*(9), 3503–3506.

Ochsner, U. A., Fiechter, A., & Reisers, J. (1994). Isolation, Characterization, and Expression in *Escherichia coli* of The *Pseudomonas aeruginosa rhlAB* Genes Encoding A Rhamnosyltransferase Involved in Rhamnolipid Biosurfactant Synthesis. *The Journal of Biological Chemistry*, (31), 19787–19795.

Ortiz, A., Aranda, F. J., & Teruel, J. A. (2010). Interaction of Dirhamnolipid Biosurfactants with Phospholipid Membranes: A Molecular Level Study. *Biosurfactant*, (R. Sen, Ed.) (Vol. 672, pp. 42–53). New York, NY: Springer New York.

Pinzon, N. M., & Ju, L. (2009). Analysis of Rhamnolipid Biosurfactants by Methylene Blue Complexation, *Applied Microbiology and Biotechnology*, 975–981.

Pornsunthorntawee, O., Wongpanit, P., & Rujiravanit, R. (2010). Rhamnolipid Biosurfactants: Production and their Potential in Environmental Biotechnology. *Biosurfactant*, 211-221.

Rahim, R., Ochsner, U. A., Olvera, C., Graninger, M., Messner, P., Lam, J. S., & Sobero, G. (2001). Cloning and Functional Characterization of The *Pseudomonas aeruginosa* rhlC Gene That Encodes Rhamnosyltransferase 2 , An Enzyme Responsible for Di-rhamnolipid Biosynthesis. *Molecular Microbiology*, *40*(3), 708–718.

Reis, R. S., Pacheco, G. J., Pereira, A. G., & Freire, D. M. G. (2013). Biosurfactants : Production and Applications, Biodegradation- Life of Science, Dr Rolando Chanmy (Ed.), InTech, 2013 (Vol.01, pp. 1-370).

Satpute, S. K., Bhuyan, S. S., Pardesi, K. R., Mujumdar, S. S., Dhakephalkar, P. K., Shete, A. M., & Chopade, B. A. (2010).Molecular Genetics of Biosurfactant Synthesis in Microorganisms. *Biosurfactants*. (R. Sen, Ed.) (Vol. 672, pp. 14–41). New York, NY: Springer New York.

Siegmund, I., & Wagner, F. (1991). New Method for Detecting Rhamnolipids Excreted by *Pseudomonas* Species During Growth on Mineral Agar. *Biotechnology Techniques*,*5*(4), 265-268.

Soberón-Chávez, G., Lépine, F., & Déziel, E. (2005). Production of Rhamnolipids by *Pseudomonas aeruginosa*. *Applied Microbiology and Biotechnology*, *68*(6), 718–25.

Solaiman, D. K. Y., Ashby, R. D., Gunther, N. W., & Zerkowski, J. a. (2015). Dirhamnose-lipid Production by Recombinant Nonpathogenic Bacterium *Pseudomonas chlororaphis*. *Applied Microbiology and Biotechnology*, *99*(10), 4333–4342.

Sotirova, A., Spasova, D., Vasileva-Tonkova, E., & Galabova, D. (2009). Effects of Rhamnolipid-Biosurfactant on Cell Surface of *Pseudomonas aeruginosa*. *Microbiological Research*, *164*(3), 297–303.

Stanghellini, M. E., & Miller, R. M. (1997). BIOSURFACTANTS: Their Identity and Potential Efficacy in the Biological Control of Zoosporic Plant Pathogens. *Plant Disease*, *81*(1), 4-12.

Toribio, J., Escalante, A. E., & Soberón-Chávez, G. (2010). Rhamnolipids: Production in Bacteria Other Than *Pseudomonas aeruginosa*. *European Journal of Lipid Science and Technology*, *112*(10), 1082–1087.

Vatsa, P., Sanchez, L., Clement, C., Baillieul, F., & Dorey, S. (2010). Rhamnolipid Biosurfactants as New Players in Animal and Plant Defense Against Microbes. *International Journal of Molecular Sciences*, *11*(12), 5095–108.

Walter, V., Syldatk, C., & Hausmann, R. (2010). Screening Concepts for the Isolation of Biosurfactant Producing Microorganisms. *Biosurfactant*. (G. Soberón-Chávez, Ed.) (Vol. 20, pp. 1-13). Berlin, Heidelberg: Springer Berlin Heidelberg.

Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R. M., & McInerney, M. J. (2004). Comparison of Methods to Detect Biosurfactant Production by Diverse Microorganisms. *Journal of Microbiological Methods*, *56*(3), 339–47.

Zhang, L., Veres-Schalnat, T. A., Somogyi, A., Pemberton, J. E., & Maier, R. M. (2012). Fatty acid Cosubstrates Provide β-oxidation Precursors for Rhamnolipid Biosynthesis in *Pseudomonas aeruginosa*, as Evidenced by Isotope Tracing and Gene Expression Assays. *Applied and Environmental Microbiology*, *78*(24), 8611–22.

Zhu, K., & Rock, C. O. (2008). RhlA Converts Beta-hydroxyacyl-acyl Carrier Protein Intermediates in Fatty Acid Synthesis to the Beta-hydroxydecanoyl-beta-hydroxydecanoate Component of Rhamnolipids in *Pseudomonas aeruginosa*. *Journal of Bacteriology*, *190*(9), 3147–54.