Chapter 4

Conclusion

Bacillus sp. MUV4 was grown on Mckeen medium containing various carbon sources (2.0%) with the initial pH of 7.0 at 30 °C for 72 h. Glucose was the best carbon source for growth and biosurfactant production and gave higher ODA and EC (9.76 cm^2 and 0.89%, respectively) compared to sucrose, glutamate and mollases. *Bacillus* MUV4 lowly grew and produced biosurfactant when using weathered oil, palm oil and n-hexadecane as carbon sources. The optimal concentration of glucose was found to be 2.5 % giving and highest ODA and EC (10.74 cm^2 and 0.95%, respectively). Monosodium glutamate with concentration of 1.0% was selected as nitrogen source for further study. Addition of 0.3% yeast extract in the medium was evelated growth and biosurfactant production (ODA 78.5 cm^2, 81.82% EA and 4.72% EC). The optimal medium containing of 2.5% glucose as carbon source, 1.0% monosodium glutamate and 0.3% yeast extract as nitrogen source was improved the growth and biosurfactant production that the growth was increased 1.9 folds of growth and the ODA and EC were increased 8.0 and 5.8 folds, respectively from those of basal Mckeen medium.

The environmental conditions for biosurfactant production in fermentor were as following : initial pH of 7.0, temperature of 30 °C, agitation rate of 200 rpm. Control of pH at 7.0 during cultivation was unnecessary for broth growth and biosurfactant production. The optimum aeration rate was 1.0 vvm.

The partial purification of biosurfactant was performed by precipitation of 60 h culture supernate with 6 N HCl, neutralized with 2.0 N NaOH to pH 7.0 and freeze-drying. The acid precipitated biosurfactant yield was 0.8 g/l. This acid precipitate biosurfactant was soluble in water, alkaline water, chloroform, methanol and ethanol but was insoluble in acetonitrile, acetone and hexane. pH

had much effect on ODA and EC. Relative ODA and EC of culture broth was stable at the pH range 6.0-10.0 while relative EA was stable at the pH range 4.0-14.0. Relative ODA, EA and EC of acid precipitate biosurfactant were stable at the pH range 6.0-12.0. The maximum ODA, EA and EC values retained more than 80% at pH 8.0. NaCl concentration had much effect on ODA and EA. At 15-20% NaCl the relative ODA was less than 10% and EA was not detectable while the relative EC was higher than 60% in culture broth and 25% in acid precipitate biosurfactant. Temperature had much effect on ODA and EC than EA. The relative EA value had little effect by temperature even at 100 °C for 12 h, the biosurfactant in culture broth still retained more than 80% EA activity.

The biosurfactant from *Bacillus* MUV4 were presumptively characterized by TLC analysis and chemical tests. They were lipid containing amino acid compounds. The biosurfactant showed activity against the growth of *Bacillus anthracis, Shigella* sp. and *Streptococcus feacalis* ATCC29212. The acid precipitate biosurfactant (0.1%) effect in recovery of kerosene oil from sandpack column was 50.04%.

Suggestions

The results of this work lead to the following suggestions :

1. Characterization on the composition and structure of the biosurfactant produced by *Bacillus* MUV4 by HPLC and GC/MS method
2. Determination the concentration of biosurfactant and age of innoculum in the antibiotic activity tested.
3. Applications of biosurfactant such as biodegradation of polycyclic aromatic hydrocarbon (PAH)
4. Strain improvement for higher biosurfactant yield by mutation and genetic engineering techniques.

References

Abu-Ruwaida, A.S., Banat, I.M., Haditirto, S., Salem, S. and Kadri, M. 1991a. Isolation of biosurfactant producing bacteria-product characterization and evaluation. Acta. Biotechnol. 11: 315-324.

Abu-Ruwaida, A.S., Banat, I.M., Haditirto, S. and Khamis, A. 1991b. Nutrition requirements and growth characteristics of biosurfactant producing *Rhodococcus* bacterium. World. J. Microbiol. Biotechnol. 7: 53-61.

Banat, I.M. 1993. The isolation of a thermophilic biosurfactant producing *Bacillus* sp. Biotechnol. Lett. 15: 591-594.

Banat, I.M. 1995. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation : a review. Biores. Technol. 53: 1-12.

Banat, I.M., Makkar, R.S. and Cameotra, S.S. 2000. Potential commercial applications of microbial surfactants. Appl. Microbiol. Biotechnol. 53: 459-508.

Banat, I.M., Samarah, N., Murad, M., Horne, R. and Banerjee, S. 1991. Biosurfactant production and used in oil tank clean-up. World. J. Microbiol. Biotechnol. 7: 80-88.

Barathi, S. and Vasudevan, N. 2001. Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from a petroleum-contaminated soil. Environ. International. 26: 413-416.

Barry, A.L. and Thronsberry, C. 1980. Susceptibility : Diffusion Test Procedures. ***In*** Manual of Clinical Microbiology (ed. E.H. Lenette). pp. 463-473. Washington, DC : American Society for Microbiology, Press.

Banincasa, M., Contiero, J., Manresa, M.A. and Moraes. 2001. Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source. J. Food. Eng. 6-12.

Besson, F. and Michel, G. 1992. Biosynthesis of iturin and surfactin by *Bacillus subtilis* : evidence for amino acid activating enzymes. Biotechnol. Lett. 14: 1013-1018.

Bryant, F.O. 1990. Improved method for the isolation of biosurfactant glycolipids from *Rhodococcus* sp. strain H13A. Appl. Environ. Microbiol. 56: 1497-1496.

Budsabun, T. 1996. Surface tension reduction of *Bacillus subtilis* 3/38 culture broth obtained from different conditions. Master of Science thesis in Microbiology, Chulalongkorn University, Bangkok, Thailand 100p.

Busscher, H.J., Van der Mei, H.C. and Van der Mei, H.C. 1996. Biosurfactants from thermophilic dairy streptococci and their potential role in the fouling control of heat exchanger plates. Appl. Microbiol. Biotechnol. 16: 15-21.

Carmeotra, S. S., and Singh, H. D. 1990. Purification and characterization of alkane solubilizing factor produced by *Pseudomonas* PG-1. J. Ferment Bioeng. 69: 341-344.

Cirigliano, M.C. and Carman, G.M. 1984. Isolation of bioemulsifier from *Candida lipolytica*. Appl. Environ. Microbiol. 48: 474-750.

Cirigliano, M.C. and Carman, G.M. 1985. Purification and characterization of liposan, and bioemulsifier from *Candida lipolytica*. Appl. Environ. Microbiol. 51: 846-850.

Clint, J. H. 1992. Nature of surfactant. ***In*** Surfactant Aggregation. pp. 1-11. New York: Chapman and Hall.

Cooper, D.G. and Goldenberg, B.G. 1987. Surface active agents from to *Bacillus* species. Appl. Environ. Microbiol. 53: 224-229.

Cooper, D.G, MacDonald, C.R., Duff, S. J.B. and Kosaric, N. 1981. Enhanced production of surfactin from *B. subtilis* by continuous product removal and metal cation additions. Appl. Environ. Microbiol. 42: 408-412.

Cooper, D.G. and Paddock, D.A. 1983. *Torulopsis petrophillum* and surface activity. Appl. Environ. Microbiol. 46: 1426-1429.

Cooper, D.G. and Paddock, D.A. 1984. Production of biosurfactant from *Torulopsis bombicola*. Appl. Eviron. Microbiol. 47: 173-176.

Denger, K. and Schink, B. 1995. New halo- and thermotolerant fermenting bacteria producing surface-active compounds. Appl. Microbiol. Biotechnol. 44: 161-166.

Davila, A., Marohal, F. and Vandecasteele, J. 1992. Kenetics and balance of a fermentation free from product inhibition : sophorose lipid production by *Candida bombicola*. Appl. Microbiol. Biotechnol. 38: 6-11.

Dawson, R.M.C., Elliott, D.C., Elliot, W.F. and Johns, K.M. 1986. Method for detection of biochemical compounds on paper and thin-layer chromatograms with some notes on separation. ***In*** Data for Biochemical Research. 3rd ed. pp. 453-502. The Bath Press, Avon.

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

Deziel, E., Paquette, G., Villemur, R., Lepine, F. and Bisaillon, J. 1996. Biosurfactant production by soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 62: 1908-1912.

Fiechter, A. 1992. Biosurfactant : moving towards industrial application. Trends. Biotechnol. 10: 208-217.

Finnerty, W.R. 1994. Biosurfactants in environmental biotechnology. Curr. Opin. Biotechnol. 5: 291-295.

Fox, S.L. and Bala, G.A. 2000. Production of surfactant from *Bacillus subtilis* ATCC 21332 using potato substrates. Biores. Technol. 75: 235-400.

Ghurye, G.L. and Vipulanandan, D. 1994. A practical approach to biosurfactant production using non-aseptic fermentation of mixed cultures. Biotechnol. Bioeng. 44: 661-666.

Gobbert, U., Lang, S. and Wanger, F. 1984. Sophorose lipid formation by resting cells of *Torulopsis bombicola.* Biotechnol. Lett. 6: 225-230.

Goswami, P. and Singh, H.D. 1991. Different modes of hydrocarbon uptake by two different *Pseudomonas* spp. Biotechnol. Bioeng. 37: 1-11.

Guerra-Santos, L.H., Kappelli, O. and Fiechter, A. 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol. 48: 301-305.

Guerra-Santos, L.H., Kappelli, O. and Fiechter, A. 1986. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl. Microbiol. Biotechnol. 24: 443-448.

Gurjar, M., Khire, J.M. and Khan, M.I. 1995. Bioemulsifier production by *Bacillus stearothermophilus* VR-8 isolate. Lett. Appl. Microbiol. 21: 83-86.

H-Kittikun, A., Abe, N., Kamio, Y. and Izaki,K. 1993. Isolation, purification and identification of antibiotics produced by *Bacillus* MUV4. Annual Report. ICBiotech. 6: 379-390.

Hommel, R.K., Weber, L., Weiss, A., Himelreich, U., Rilke. O. and Kleber, H.P. 1994. Production of sophorose lipid by *Candida* (*Torulopsis*) *apicola* grown on glucose. J. Biotechnol. 26: 199-205.

Horowitz, S., Gilbert, J.N. and Griffin, W.M. 1990. Isolation and characterization of a surfactant produced by *Bacillus licheniformis* 86. J. Ind. Microbiol. 6: 243-248.

Horowitz, S. and Griffin, W.M. 1991. Structural analysis of *Bacillus licheniformis* 86 surfactant. J. Ind. Microbiol. 7: 45-52.

Islam, J. 2001. Screening of biosurfactant producing bacteria and optimization of production process. Master of science thesis in Biotechnology. Prince of Songkla University.

Javaheri, M., Jenneman, G.E., McInerney, M.J and Knapp, R.M. 1985. Anaerobic production of biosurfactant by *Bacillus licheniformis* JF-2. Appl. Environ. Microbiol. 44: 698-700.

Jenny, K., Kappeli, O. and Fiechter, A. 1991. Biosurfactants from *Bacillus licheniformis*: structure analysis and characterization. Appl. Microbiol. Biotechnol. 36: 5-13.

Johnson, V., Singh, M., Saini, V.S., Dilip, Adhikari, K., Sista, V. and Yadov, N.K. 1992. Bioemulsifier production by an Oleaginous Yeast *Rhodotorula glutinis* IIP-30. Biotechnol. Lett. 14: 487-490.

Jung, H.K., Lee, J.B., Yim, G.B. and Kim, E.K. 1995. Properties of microbial surfactants, S-acid. Korean. Biotechnol. Bioeng. 10: 71-77.

Kappeli, O. and Finnerty, W.R. 1979. Partition of alkane by and extracellular vesicle derived from hexadecane-grown *Acinetobacter.* J. Bacteriol. 140: 707-712.

Khire, J.M. and Khan, M.I. 1994. MEOR : Microbes and the subsurface environment. Enzyme. Microb. Technol. 16: 258-259.

Kim, S.H., Lim, E.J., Lee, J.D. and Lee, T.H. 2000. Purification and characterization of biosurfactant from *Nocardia* sp. L-417. Biotechnol. Appl. Biochem. 31: 249-253.

Kim, H.S., Yoon, B.D., Choung, D.H. , Oh, H.M., Katsuragi, T. and Tani, Y. 1999. Characterization of a biosurfactant, mannosylerythritol lipid produced from *Candida* sp. SY16. Appl. Microbiol. Biotechnol. 52: 713-721.

Kim, H.S., Yoon, B.D., Lee, C.H., Suh, H.H., Oh, H.M., Katsuragi, T. and Tani, Y. 1997. Production and properties of a lipopeptide biosurfactant from *Bacillus subtilis* C9. J. Ferment. Bioeng. 84: 41-46.

Kitamoto, D., Yanagishita, H., Shinbo, T. Nakane, T. Kamosawa, C. and Nakahara, T. 1993. Surface active properties and antimicrobial activities

of mannosylerythritol : lipids as biosurfactants produced by *Candida antarctica*. J. Biotechnol. 29: 91-96.

Kretschmer, A., Bock, H. and Wagner, F. 1982. Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erythropolis* grow on n-alkane. Appl. Environ. Microbiol. 44: 864-870.

Lee, L.H. and Kim, J.H. 1993. Distribution of substrate carbon is sophorose lipid production by *Torulopsis bombicola*. Biotechnol. Lett. 15: 263-266.

Li, Z.Y., Lang, S., Wangner, F., Witte, L. and Wray, V. 1984. Formation and identification of interfacial-active glycolipids from resting microbial cells of *Arthrobacter* sp. and potential use in tertiary oil recovery. Appl. Environ. Microbiol. 48: 610-617.

Lin, C., Carswell, K.S., Sharma, M.M. and Georgiou. 1994. Continuous production of the lipopeptide biosurfactant of *Bacillus licheniformis* JF-2. Appl. Microbiol. Biotechnol. 41: 281-285.

MacDonald, C.R., Cooper, D.G. and Zajic, J.E. 1981. Surface-active lipids from *Nocardia erythropolis* grown on hydrocarbons. Appl. Environ. Microbiol. 41: 117-123.

Makkar, R.S. and Cameotra, S.S. 1997a. Biosurfactant production by thermophilic *Bacillus subtilis* strain. J. Ind. Microbiol. Biotechnol. 18: 37-42.

Makkar, R.S. and Cameotra, S.S. 1997b. Utilization of molasses for biosurfactant production by two *Bacillus* strains on thermophilic conditions. J. Am. Oil. Chem. Soc. 74: 887-889.

Makkar, R.S. and Cameotra, S.S. 1998. Synthesis of biosurfactant in extreme conditions. Appl. Microbiol. Biotechnol. 50: 520-529.

McInerney, M.J., Javaheri, M. and Nagle, D.P. 1990. Properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2. J. Ind. Microbiol. 5: 95-102.

MeNeill, G.P. and Yamane, T. 1991. Further improvements in the yield of monoglycerides during enzymatic glycerolysis of fats and oils. J. Am. Oil. Chem. Soc. 68: 6-10.

Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y. and Imanaka, T. 1993. A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS 38. J. Bacteriol. 175: 6459-6466.

Mulligan, C.N. and Gibbs, B.F. 1989. Correlation of nitrogen with biosurfactant production by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 55: 3016-3019.

Navon-Venezia, S. Zosim, Z., Gottlieb, A., Legmann, R. Carmeli,, S.,Ron, E.Z. and Rosinberg, E. 1995. Alasan, a new bioemulsifier from *Acinetobacter radioresistens*. Appl. Environ. Microbiol. 61: 290-297.

Neu, T.R., Hartner, T. and Poralla, K. 1990. Surface active properties of viscosin-a peptidolipid antibiotic. Appl. Microbiol. Biotechnol. 32: 518-520.

Neu, T.R. and Poralla, K. 1990. Emulsifying agent from bacteria isolated during screening for cells with hydrophobic surfaces. Appl. Microbiol. Biotechnol. 32: 521-525.

Ohno, A., Ano, T. and Shoda, M. 1995. Effect of temperature on production of lipopeptide antibiotics, iturin A and surfactin by a dual producer, *Bacillus subtilis* RB17, in solid-state fermentation. J. Ferment. 80: 517-519.

Peypoux, F. and Michel, G. 1992. Controlled biosynthesis of Val-7 and Leu-7 surfactins. Appl. Microbiol. Biotechnol. 36: 515-517.

Pruthi, V. and Cameotra, S.S. 1997a. Production of a biosurfactant exhibiting excellent emulsification and surface active properties by *Serratia marcescens* : short communication. World. J. Microbiol. Biotechnol. 13: 133-135.

Pruthi, V and Cameotra, S.S. 1997b. Production and properties of biosurfactant synthesized by *Arthrobacter protophormiae*–an antarctic strain : short communication. World. J. Microbiol. Biotechnol. 13: 137-139.

Passeri, A. 1992. Marine biosurfactants IV. Production, characterization and biosynthesis of anionic glucose lipid from marine bacterial strain MM1. Appl. Microbiol. Biotechnol. 37: 281-286.

Robert, M., Mercade, M.E., Bosch, M.P., Parra, T.L., Espuny, M.J., Manresa, M.A. and Guinea, J. 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T. Biotechnol. Lett. 11: 871-874.

Roongsawang, N., Haniyavarn, J. and Thaniyavarn, S. 1999. Properties and biosurfactant produced by *Bacillus* sp. strain KP-2. Thai J. Biotechol. 1: 54-60.

Rosenberg, E. and Ron, E.Z. 1999. High- and low-molecular mass microbial surfactants. Appl. Microbiol. Biotechnol. 52: 154-162.

Rosenberg, E., Zukerberg, A., Rubinovitz, V. and Gutnick, D.L. 1979. Emulsifier Arthrobacter RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. 37: 402-408.

Sandrin, C., Peypoux, F. and Michel, G. 1990. Coproduction of surfactin and Iturin A, lipopeptides with surfactant and antifungal properties, by *Bacillus subtilis*. Biotechnol. Appl. Biochem. 12: 370-375.

Sheppard, J. D. and Cooper, D. G. 1990. The effect of biosurfactant on oxygen transfer in a cyclone column reactor. J. Chem. Technol. Biotechnol. 48: 325-336.

Singh, M., Saini, V., Adhikari, D.K., Desai, J.D. and Sista, V.R. 1990. Production of bioemulsifier by SCP producing strain of *Candida tropicalis* during hydrocarbon fermentation. Biotechnol. Lett. 12: 743-746.

Suttivanitchakul, B., Thaniyavarn, J. and Thaniyavarn, S. 1999. Biosurfactant production by *Bacillus licheniformis* F2.2. Thai. J. Biotechnol. 46-53.

Thimon, L, Peypoux, F.. and Michel, G. 1995. Interactions of surfactant from *Bacillus subtilis*, with inorganic cations. Biotechnol. Lett. 14: 713-718.

Van Dyke, M.I., Couture, P., Brauer, M., Lee, H. and Trevors, J.T. 1993. *Pseudomonas aeruginosa* UG2 rhamnolipid biosurfactant : structural characterization and their use in removing hydrophobic compounds from soil. Can. J. Microbiol. 39: 1071-1078.

Vollbrecth, E., Heckmann, R., Wray, V., Nimtz, M. and Lang, S. 1998. Production and structure education of di- and oligosaccharide lipids (biosurfactants) from *Tsukamurella* sp. nov. Appl. Microbiol. Biotechnol. 50: 530-537.

Wang, S.D. and Wang, D.I.C. 1990. Machanisms for biopolymer accumulation in immobilized *Acinetobacter calcoaceticus* system. Biotechnol. Bioeng. 36: 402-410.

Yakimov, M.M., Timmis, K.N., Wray, V. and Fredrickson, H.L. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant substrate *Bacillus licheniformis* BAS50. Appl. Microbiol. Biotechnol. 61: 1706-1713.

Yakimov, M.M., Fredrickson, H.L. and Timmis, K.N. 1996. Effect of heterogeneity of hydrophobic moieties on surface activity of lichenycin A, a lipopeptide biosurfactant from *Bacillus licheniformis* BAS50. Biotechnol. Appl. Biochem. 23: 13-18.

Zhang, Y. and Miller, R.M. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl. Environ. Microbiol. 56: 3276-3282.

Zhang, Y., Maier,W.J. and Miller, R.M. 1997. Effect of rhamnolipids on the dissolution, bioavailability, and biodegradation of phenanthrene. Environ. Sci. Technol. 31: 2211-2217.

Appendices

Appendix A

Medium and chemicals

1. Mckeen medium

Glucose	20.0	g
DL-glutamic acid	5.0	g
$MgSO_4.7H_2O$	1.02	g
K_2HPO_4	1.0	g
KCl	0.5	g
Distilled water	1,000	ml
Trace elements	1.0	ml
pH	7.0	

Method: dissolved all ingredients in 1000 ml distilled water and autoclaved at 15 pond/inch^2 at 121 ^{o}C for 15 minutes.

Note: Trace elements containing (g/100 ml distilled water)

$MgSO_4.4H_2O$	0.5	g
Cu $SO_4.5H_2O$	0.16	g
Fe $SO_4.7H_2O$	0.015	g

2. Nutrient broth

Beef extract	3.0	g
Peptone	5.0	g
pH	7.0	

Method: dissolved all ingredients in 1000 ml distilled water and autoclaved at 15 pond/inch^2 at 121^{o}C for 15 minutes.

3. Nutrient agar

Beef extract	3.0	g
Peptone	5.0	g
Agar	15.0	g
pH	7.0	

Method: dissolved all ingredients in 1000 ml distilled water, then mixed well and boiled. Autoclaved at 15 pond/inch^2 at 121 ^{o}C for 15 minutes.

4. Mueller Hinton agar

Beef extract powder	2.0	g
Acid digest of casein	17.5	g
Soluble starch	1.5	g
Agar	15.0	g
pH	7.3	

Method: dissolved all ingredients in 1000 ml distilled water, then mixed well and boiled. Autoclaved at 15 pond/inch^2 at 121 ^{o}C for 15 minutes.

5. Method and reagents for the detection of biochemical compounds on thin layer chromatograms (Dawson *et al.*, 1968)

4.1 Amino acids

4.1.1 Ninhydrin

For general use as a locating agent use 0.25% w/v ninhydrin in acetone. The spots may be developed by heating at 100^{o}C for 5 min or for a lighter background, by leaving at room temperature for few hours. The amino acid give purple spots except for histidine and glycine (red-gray); phenylalanine, tyrosine and aspartic acid (blue); tryptophane (brown); asparagine (dirty yellow); proline (yellow).

4.1.2 Ultraviolet light

The amino group can react with free aldehyde groups in the paper. The resulting Schiff's bases fluoresces blue in ultraviolet light. Heat the paper at 100 ^{o}C for 30 min N-substituted amino acids give dark spots.

4.2 Carbohydrates

4.2.1 Alkaline potassium permanganate

Spray with 1.0% aq. $KMnO_4$ containing 2.0% Na_2CO_3. Dry at room temperature or rapidly at 100 ^{o}C. Yellow spots on a purple ground, then gray spots on brown ground are given by sugar alcohols, glycosides, reducing and non-reducing sugars. Not given by methyl or acetyl sugars.

4.2.2 Iodine vapour

Expose for 15 min to iodine vapour. Brown spots are given by sugar mercaptals and alcohols, glycosides, N.acylamino sugars, neutral and acidic polysaccharides.

4.2.3 Anisaldehyde

Anisaldehyde 0.5 ml in 20 ml of methanol and 1 ml of sulfuric acid were used as anisaldehyde reagent for detecting sugar. Gray spot are given by the sugar.

4.3 Lipids

4.3.1 Rhodamine 6G

0.001% aq. rhodamine 6G in 0.25 M K_2HPO_4. View wet under UV light. Purple, blue and yellow spots against rose background.

4.3.2 Iodine vapour

Detect all lipids, nitrogenous compounds, non-reducing carbohydrates. Expose for 15 min to iodine vapour. Brown spots are given by all lipids.

6. **Tris-HCl buffer was prepared by the method of Bates and Bower** (1956, cited by Stoll and Blanchard, 1990)

   A: 0.02 M Tris (hydroxymethyl) aminomethane

   B: 0.02 M HCl

   50 ml of A + X ml of B

B solution (ml)	pH
46.6	7.0
45.7	7.1
44.7	7.2
43.4	7.3
42.0	7.4
40.3	7.5
38.5	7.6
36.6	7.7
34.5	7.8
32.0	7.9
29.2	8.0
26.2	8.1
22.9	8.2
19.9	8.3
17.2	8.4
14.7	8.5
12.4	8.6
10.3	8.7
8.5	8.8
7.0	8.9

Appendix B

Table 1 Time course of growth and biosurfactant production of *Bacillus* MUV4 in Mckeen medium under shake-flask culture

Time (h)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)
0	7.00^{bc}	0^{i}	0^{g}	0^{d}
6	6.88^{bc}	0.29^{h}	0^{g}	0^{d}
12	6.66^{c}	2.05^{g}	0^{g}	0^{d}
18	6.71^{bc}	4.97^{d}	0.12^{f}	0.66^{c}
24	6.81^{bc}	5.57^{c}	4.63^{e}	0.76^{b}
36	7.26^{a}	6.82^{a}	6.15^{d}	0.74^{b}
48	7.13^{bc}	5.73^{b}	9.76^{a}	0.89^{a}
60	6.98^{bc}	4.98^{e}	9.45^{b}	0.80^{b}
72	6.65^{c}	4.74^{h}	9.07^{c}	0.65^{c}

Table 2 Growth, final pH and biosurfactant production of *Bacillus* MUV4 at 48 h cultivation in the Mckeen medium with various carbon sources (2.0%) on shaker (200 rpm) at 30°C

Types of carbon source	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)
Glucose	7.13^{b}	5.73^{b}	9.76^{a}	0.89^{a}
Sucrose	7.19^{b}	7.52^{a}	7.88^{b}	0.64^{b}
Mollases	8.98^{a}	5.86^{b}	1.01^{c}	0.21^{c}
Glutamate	8.79^{a}	3.69^{c}	0.87^{d}	0.11^{d}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for shake-flask cultures

Table 3 Growth, final pH and biosurfactant production of *Bacillus* MUV4 at 48 h cultivation in the Mckeen medium with various hydrophobic carbon sources (0.1%) on shaker (200 rpm) at 30^{o}C

Types of hydrophobic carbon source	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)
Weathered oil	8.26^{b}	3.13^{b}	0.50^{a}	0.15^{a}
Palm oil	8.52^{a}	3.59^{a}	0.38^{b}	0.05^{b}
n-hexadecane	8.53^{a}	2.77^{c}	0.50^{a}	0.14^{a}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for shake-flask cultures

Table 4 Growth, final pH and biosurfactant production of *Bacillus* MUV4 at 48 h cultivation in the Mckeen medium with various weathered oil concentration on shaker (200 rpm) at 30^{o}C

Conc. of weathered oil (%)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)
0.1	8.15^{a}	2.98^{a}	0.50^{b}	0.14^{a}
0.3	8.18^{a}	2.41^{a}	1.33^{a}	0.19^{a}
0.5	8.26^{a}	2.47^{a}	0.79^{b}	0.13^{a}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for shake-flask cultures

Table 5 Growth, final pH and biosurfactant production of *Bacillus* MUV4 at 48 h cultivation in the Mckeen medium with various glucose concentration on shaker (200 rpm) at 30^{o}C

Glucose concentration (%)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)
1.0	7.44^{ab}	2.09^{e}	8.16^{c}	0.61^{c}
2.0	7.78^{a}	5.45^{c}	10.36^{b}	0.67^{c}
2.5	7.12^{b}	5.89^{a}	10.74^{a}	0.93^{a}
3.0	6.61^{c}	5.71^{b}	6.75^{d}	0.78^{b}
3.5	6.65^{c}	5.25^{cd}	6.16^{e}	0.83^{b}
4.0	6.70^{c}	5.12^{d}	5.56^{f}	0.64^{c}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for shake-flask cultures

Table 6 Growth, final pH and biosurfactant production of *Bacillus* MUV4 at 48 h cultivation in the Mckeen medium contained 2.5% as carbon source with various nitrogen sources on shaker (200 rpm) at 30°C

Type of nitrogen source	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)
KNO_3	6.49^{b}	0.76^{c}	0^{f}	0^{g}
NH_4NO_3	4.30^{e}	2.53^{e}	0^{f}	0^{g}
$(NH_4)_2\ SO_4$	4.49^{e}	1.45^{f}	0.10^{f}	0.12^{f}
$(NH_4)_2HPO_4$	5.20^{d}	7.32^{a}	4.91^{c}	1.24^{c}
$(NH_4)H_2PO_4$	5.77^{c}	5.24^{b}	3.71^{d}	0.89^{d}
NH_4HCO_3	5.75^{c}	3.81^{d}	2.41^{e}	0.45^{e}
$NaNO_3$	6.37^{b}	0.54^{g}	0.10^{f}	0.16^{f}
DL-glutamic acid	7.67^{a}	4.99^{bc}	10.75^{b}	2.30^{b}
L-glutamic acid	7.59^{a}	5.09^{b}	11.35^{a}	2.60^{a}
MSG	7.57^{a}	5.28^{b}	11.25^{a}	2.58^{a}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for shake-flask cultures

Table 7 Growth, final pH and biosurfactant production of *Bacillus* MUV4 at 48 h cultivation in the Mckeen medium contained 2.5% as carbon source with various monosodium glutamate (MSG) concentration (%) on shaker (200 rpm) at 30 ^{o}C

MSG concentration (%)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)	EA (%)
0.1	6.64^{d}	1.66^{d}	1.54^{e}	0.77^{d}	67.82^{d}
0.3	6.65^{d}	4.11^{cd}	5.89^{de}	1.67^{c}	73.33^{c}
0.5	7.30^{c}	6.73^{abc}	10.75^{c}	2.61^{b}	74.70^{b}
1.0	7.70^{a}	8.23^{a}	44.28^{a}	2.94^{a}	80.00^{a}
1.5	7.63^{b}	7.31^{ab}	34.94^{b}	2.53^{b}	74.60^{b}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for shake-flask cultures

Table 8 pH change and cell growth and biosurfactant production from *Bacillis* MUV4 when cultivated in the medium containing yeast extract and bacto peptone at various concentrations for 48 hrs (M+Y1=Medium+ 0.1% yst extract, M+Y3=Medium+0.3% yst extract, M+Y5=Medium+ 0.5 % yst extract, A+P1= Medium+0.1% bacto peptone, M+P3=Medium+ 0.3% bacto peptone and M+P5=Medium+0.5% bacto peptone, M=Medium contained 2.5% as carbon source and 1.0% monosodium glutamate)

Type and concentration of yeast extract and bacto peptone	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)	EA (%)
M	7.21^{a}	8.18^{f}	44.28^{c}	2.74^{f}	80.00^{a}
M+Y1	6.52^{bc}	9.18^{e}	60.79^{bc}	2.93^{e}	81.00^{a}
M+Y3	6.65^{b}	9.49^{d}	78.50^{a}	4.72^{a}	80.13^{a}
M+Y5	7.10^{a}	9.84^{c}	66.44^{ab}	3.35^{d}	81.00^{a}
M+P1	6.40^{c}	10.28^{a}	42.70^{bc}	2.49^{g}	78.18^{a}
M+P3	6.63^{b}	10.19^{ab}	47.73^{c}	3.69^{b}	81.00^{a}
M+P5	7.44^{a}	9.93^{bc}	45.22^{c}	3.47^{c}	80.00^{a}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for shake-flask cultures

Table 9 Time course of growth and biosurfactant production of *Bacillus* MUV4 in optimal Mckeen medium under shake-flask culture (200 rpm) at 30°C

Time (h)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)	EA (%)
0	7.00^{d}	0^{e}	0^{g}	0.09^{g}	0^{e}
6	5.89^{h}	1.38^{d}	0^{g}	0.11^{g}	0^{e}
12	6.17^{g}	9.34^{c}	3.40^{f}	0.61^{f}	57.64^{d}
18	6.87^{e}	9.51^{c}	7.10^{e}	1.41^{e}	63.64^{c}
24	7.17^{c}	10.43^{b}	40.69^{d}	1.89^{d}	71.93^{b}
36	7.69^{a}	12.96^{a}	66.44^{c}	4.38^{b}	79.42^{a}
48	7.62^{b}	12.62^{b}	78.50^{a}	4.29^{b}	81.13^{a}
60	6.65^{ab}	10.10^{bc}	78.50^{a}	5.18^{a}	81.82^{a}
72	6.63^{f}	9.96^{bc}	72.35^{b}	4.06^{c}	78.18^{a}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for shake-flask cultures

Table 10 Time course of growth and biosurfactant production of *Bacillus* MUV4 in optimal medium in 2.0-l fermentor culture under uncontrolled pH (pH 7.0) at 30^{o}C

Time (h)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)	EA (%)
0	7.10^{c}	0^{f}	0^{e}	0^{e}	0^{e}
6	6.26^{g}	1.41^{e}	0^{e}	0^{e}	0^{e}
12	6.49^{f}	2.66^{d}	0.79^{e}	0.23^{e}	12.96^{d}
18	6.71^{e}	3.76^{c}	3.46^{e}	1.14^{d}	56.60^{c}
24	6.79^{de}	4.24^{c}	5.31^{e}	1.54^{c}	62.62^{bc}
36	6.83^{de}	5.44^{b}	11.30^{d}	1.77^{c}	63.64^{b}
48	6.91^{d}	7.41^{a}	32.10^{c}	3.50^{b}	72.72^{a}
60	7.95^{a}	5.36^{b}	60.79^{a}	4.21^{a}	74.54^{a}
72	7.41^{b}	5.89^{b}	50.24^{b}	4.10^{a}	72.72^{a}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for fermentor cultures

Table 11 Time course of growth and biosurfactant production of *Bacillus* MUV4 in optimal medium under 2.0-l fermentor culture under controlled pH (pH 7.0) at 30°C for 72 h

Time (h)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)	EA (%)
0	7.05^{a}	0^{I}	0^{g}	0^{f}	0^{e}
6	6.94^{b}	1.37^{h}	0^{g}	0^{f}	0^{e}
12	7.04^{b}	2.00^{g}	0.79^{g}	0.10^{f}	36.36^{d}
18	9.96^{b}	2.64^{f}	3.14^{f}	0.92^{e}	36.36^{d}
24	9.94^{b}	4.19^{e}	4.90^{e}	1.13^{de}	47.27^{c}
36	7.02^{a}	5.14^{d}	7.07^{d}	1.25^{d}	60.00^{b}
48	7.05^{a}	6.69^{a}	9.07^{c}	1.72^{c}	72.72^{a}
60	7.03^{a}	6.46^{b}	32.10^{a}	2.80^{a}	72.72^{a}
72	7.04^{a}	6.21^{c}	28.26^{b}	2.50^{b}	63.63^{b}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for fermentor cultures

Table 12 pH change, cell growth and biosurfactant production from *Bacillus* MUV4 during cultivation in fermentor at various aeration rates for 60 h

Aeration rates (vvm)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)	EA (%)
0	4.89^{d}	1.19^{d}	0.57^{d}	0.53^{c}	9.09^{d}
0.5	7.95^{a}	5.36^{c}	60.79^{bc}	4.00^{b}	73.23^{b}
1.0	7.74^{b}	7.16^{b}	72.34^{a}	4.49^{a}	81.48^{a}
1.5	7.38^{c}	7.98^{a}	55.39^{c}	3.70^{b}	60.00^{c}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for fermentor culture

Table 13 Time course of growth and biosurfactant production of *Bacillus* MUV4 under optimal condition (uncontrolled pH, aeration rate 1.0 vvm, agitation rate 200 rpm, pH 7.0) at 30^{o}C

Time (h)	pH	Cell growth (OD660nm)	ODA (cm^2)	EC (%)	EA (%)
0	7.01^{c}	0^{h}	0^{c}	0^{g}	0^{f}
6	6.26^{f}	1.52^{g}	0^{c}	0^{g}	0^{f}
12	6.62^{e}	2.10^{f}	1.77^{c}	0.54^{f}	12.70^{e}
18	6.85^{d}	3.76^{e}	4.91^{c}	0.62^{f}	50.91^{d}
24	7.03^{c}	3.91^{e}	6.15^{c}	0.85^{e}	55.55^{c}
36	7.01^{c}	5.00^{d}	45.34^{b}	2.28^{d}	74.07^{b}
48	7.85^{a}	6.62^{b}	47.75^{b}	3.57^{c}	81.48^{a}
60	7.74^{b}	7.13^{a}	72.34^{a}	4.49^{a}	81.48^{a}
72	7.88^{a}	6.26^{c}	50.25^{b}	4.09^{b}	8036^{a}

Note : The same supscript-letter means not significantly different ($p<0.05$) These values are the average of triplicate for fermentor cultures

Table 14 Effect of pH on stability of culture broth biosurfactant from *Bacillus* MUV4 (controlled pH =7.74)

pH	Parameters		
	ODA relative (%)	EA relative (%)	EC relative (%)
2	6.25	57.77	17.45
4	7.29	74.69	24.50
6	77.44	98.72	97.31
7.74 (control)	100	100	100
8	84.64	99.58	95.97
10	82.1	98.18	94.70
12	64.00	86.67	25.50
14	51.83	82.22	29.36

Table 15 Effect of pH on stability of acid precipitated biosurfactant from *Bacillus* MUV4 (controlled pH =7.44)

pH	Parameters		
	ODA relative (%)	EA relative (%)	EC relative (%)
2	7.29	0	23.53
4	19.55	15.79	52.29
6	82.08	80.28	82.35
7.44 (control)	100	100	100
8	89.61	91.13	83.88
10	84.52	90.13	82.16
12	75.15	85.71	71.91
14	57.84	67.89	62.75

Table 16 Effect of NaCl concentration on stability of culture broth biosurfactant from *Bacillus* MUV4

NaCl concentration (%)	Parameters		
	ODA relative (%)	EA relative (%)	EC relative (%)
0	100	100	100
5	95.95	74.27	95.94
10	69.33	0	67.57
15	69.33	0	56.42
20	9.22	0	63.51
25	6	0	58.11
30	6	0	55.74
35	6	0	55.74

Table 17 Effect of NaCl concentrations on stability of acid precipitated biosurfactant from *Bacillus* MUV4

NaCl concentration (%)	Parameters		
	ODA relative (%)	EA relative (%)	EC relative (%)
0	100	100	100
5	93.02	73.23	90.20
10	33.22	0	39.21
15	9.80	0	26.67
20	5.15	0	20.39
25	1.32	0	18.48
30	0	0	16.86
35	0	0	10.58

Table 18 Effect of temperature on stability (%ODA relative)of culture broth biosurfactant from *Bacillus* MUV4

Incubation times (h)	Temperature (^{o}C)				
	4	30	55	80	100
0	100	100	100	100	100
6	97.64	97.64	86.87	89.97	86.97
12	95.31	92.25	79.57	77.44	64.00
18	95.31	92.50	77.44	64.00	64.00
24	91.49	82.64	64.00	64.00	59.59
36	84.81	77.69	67.90	67.90	50.12
48	84.81	77.66	64.00	64.00	47.87

Table 19 Effect of temperature on stability (%EA relative)of culture broth biosurfactant from *Bacillus* MUV4

Incubation times (h)	Temperature (^{o}C)				
	4	30	55	80	100
0	100	100	100	100	100
6	99.34	99.58	97.90	97.89	97.15
12	98.51	98.96	96.41	97.15	94.12
18	98.51	98.51	94.51	90.37	88.54
24	97.92	97.58	94.73	86.04	86.04
36	96.59	95.67	78.87	78.04	77.28
48	96.43	90.78	78.43	77.28	77.28

Table 20 Effect of temperature on stability (%EC relative)of culture broth biosurfactant from *Bacillus* MUV4

Incubation times (h)	Temperature (^{o}C)				
	4	30	55	80	100
0	100	100	100	100	100
6	95.75	96.25	92.52	90.16	91.80
12	81.30	80.98	84.42	80.32	80.00
18	69.12	66.57	71.96	69.84	69.72
24	67.76	60.52	57.32	49.84	38.80
36	65.44	56.14	41.43	39.68	38.80
48	58.73	41.21	35.82	30.79	25.87

Table 21 Effect of temperature on stability (%ODA relative) of acid precipitated biosurfactant from *Bacillus* MUV4

Incubation times (h)	Temperature (^{o}C)				
	4	30	55	80	100
0	100	100	100	100	100
6	97.55	94.25	91.80	84.02	91.50
12	89.58	76.56	75.62	75.62	70.41
18	74.68	75.62	75.62	70.41	63.31
24	63.07	62.67	63.31	54.39	61.62
36	61.13	56.24	59.23	50.17	49.61
48	61.13	56.24	50.17	44.44	42.53

Table 22 Effect of temperature on stability (%EA) of acid precipitated biosurfactant from *Bacillus* MUV4

Incubation times (h)	Temperature (^{o}C)				
	4	30	55	80	100
0	100	100	100	100	100
6	98.18	99.82	98.82	98.85	98.85
12	98.18	98.18	98.18	98.85	98.20
18	99.79	98.18	95.86	88.22	82.35
24	95.40	87.66	76.46	73.52	73.52
36	80.00	83.31	73.52	73.52	73.52
48	74.28	77.35	73.72	58.81	58.81

Table 23 Effect of temperature on stability (%EC relative) of acid precipitated biosurfactant from *Bacillus* MUV4

Incubation times (h)	Temperature (^{o}C)				
	4	30	55	80	100
0	100	100	100	100	100
6	95.24	94.61	88.80	87.90	91.06
12	93.65	95.38	64.80	67.74	69.92
18	58.73	56.14	47.20	48.38	47.15
24	57.94	55.38	32.60	39.52	34.15
36	57.14	55.38	35.20	24.19	20.32
48	57.14	55.38	32.80	23.39	20.32

Publication

Prommachan, O., H-kittikun and Kawai, F. 2001. Production of biosurfactant from *Bacillus* MUV4. The Annual Meeting of the Thai society for Biotechnology, BioThailand 2001: From Research to Market. November, 7-10, 2001, Bangkok, Thailand.

Vitae

Name Miss Onanong Prommachan

Birth date 28 November 1977

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biotechnology)	Prince of Songkla University	1999

Scholarship Award during Enrolment

Graduate Study Scholarship for Outstanding Scholastic Achievement, Prince of Songkla University