CHAPTER V

DISCUSSIONS

5.1 Screening, isolation, and identification of biosurfactant-producing bacteria

One hundred and thirty isolates were screened from nineteen places. Five isolates, designated as biosurfactant-producing bacteria were all isolated from soil samples of engine oil with the history of hydrocarbon exposure. This statement agreed with Willumsen and Karlson (1997) who was also successful on isolating biosurfactant-producing bacteria from PAH-contaminated soils. Further Rashedi *et al.* (2005) could isolate biosurfactant-producing bacteria from oil contaminated. Thus, oil-contaminating soils should be the most suitable soil samples for biosurfactant-producing bacteria isolation. The environmental sources of biosurfactant-producing bacteria were hydrocarbon-contaminated soil (Macelwee *et al.*, 1990; Francy *et al.*, 1991) however hydrocarbon contaminated water (Broderick and Cooney, 1979) and marine water (Angelina Passeril *et al.*, 1992) were a few sources to find these bacteria (Table 5.1). Preliminary screening and isolation were used namely drop-collapse method. Similarly, Ahern *et al.* (2006), Bodour *et al.* (2003) and Jain *et al.* (1991) used this method for screening a range of diverse biosurfactant-producing microorganisms isolated from soils. Eighty-seven isolates gave positive results for drop-collapse method, which were detected in this work. Youssef *et al.* (2004) reported the drop-collapse method might not be sensitive in detecting low levels of biosurfactant production. For this reason, many authors suggested that this method should be supported by other techniques based on surface activity measurements (Mulligan *et al.*, 1984; Makkar and Cameotra, 1997). Another preliminary

drop-collapse method was used to screen and isolate, then followed with emulsification index (E_{24}) method in order to confirm the biosurfactant-producing bacteria. Also, Tabatabaee *et al.* (2005) isolated and confirmed the ability of isolates in biosurfactant production by emulsification test. In conclusion, the preliminary screening for use to screen biosurfactant-producing bacteria in large numbers should be firstly, examined the cultures by using the drop-collapse method. Then, the positive cultures from the drop-collapse method could be screened by the emulsification index (E_{24}).

In this study, five biosurfactant-producing bacteria isolated from oil-contaminated soils were designated as A102, A103, B202, P2 and P3. They were gram negative, rod bacteria and identified biochemically by the laboratory of Institution for Scientific Research, Department of Medical Sciences, Ministry of Public Health in Thailand, including 16S rDNA sequencing A102, A103, B202, P2 and P3, classified as *Pseudomonas aeruginosa, Pseudomonas aeruginosa, Pseudomonas* sp., *Enterobacter* sp. and *Burkholderia cepacia*, respectively, were studied.

Table 5.1 Microbial sources and properties of major classes of biosurfactants

Organisms	Source	Carbon source	Biosurfactant	Surface tension ($mN.m^{-1}$)	CMC ($mg.l^{-1}$)	Reference(s)
	Soil					
A. calcoaceticus	Petroleum-contaminated site	Crude oil	Lipopeptide	NA	NA	Nadarajah, 2002
Arthrobacter atrocyaneus	Petroleum-contaminated site	Petroleum hydrocarbons	glycolipid	NA	NA	Niepel, 1997
Arthrobacter sp. MIS38	PAH soil samples	PAH	Lipopeptide	24	NA	Morikawa, 1993
Bacillus licheniformis JF-2	Solid waste (crude oil)	Glucose, PAH (crude oil)	Lipoprotein	28	NA	Javaheri, 1985
Bacillus licheniformis	Soil	1% wv^{-1} glucose	Lipopeptide	27	15	Jenny, 1991
Bacillus subtilis MTCC 2423	Soil	2% wv^{-1} sucrose	Lipopeptide	24	NA	Makkar and Cameotra, 1997
Enterobacter sp. 214-6	solid waste crude oil	PAH	Rhamnolipid	53.4	NA	Toledo *et al.*, 2006
Listeria monocytogenes LO28	Contaminated soil	15% vv^{-1} glycerol	Surfactin	27.4	NA	Meylheuc *et al.* 2006
P. aeruginosa	Contaminated soil	Glucose	Rhamnolipid	29	NA	Guerra-Santos *et al.* 1984
Pseudomonas sp.	Contaminated soil	Glucose	Rhamnolipid	25-30	15	Guerra-Santos *et al.* 1986
P. aeruginosa	Contaminated soil	Hydrocarbon	Rhamnolipid	32-36	NA	Hisatsuka *et al.*, 1971
P. putida 21BN	Contaminated soil	2% vv^{-1} hexadecane or 2% wv^{-1} glucose	-	29	NA	Tuleva 2002
P. aeruginosa 47T2	Contaminated soil	2% vv^{-1} or 4% vv^{-1} frying oil	Rhamnolipid	34-36	NA	Haba *et al.*, 2000
P. fluorescens 378	Contaminated soil	50 $g.l^{-1}$ sucrose, n-pentane	Glycolipid	27	NA	Persson *et al.*, 1988
P. aeruginosa UW-1	Hydrocarbon-contaminated soil	Vegetable oil	Rhamnolipid	27.7-30.4	40	Sim *et al.*, 1997
Rhodococcus aurantiacus	Contaminated soil	n-alkanes	Glycolipid	26	NA	Ramsay *et al.*, 1988
Rhodococcus sp. H13A	Contaminated soil	Hexadecane	Glycolipid	NA	150	Finnerty and Singer 1984
Serratia sp. SVGG16	Tropical soil in Brazil	ethanol-blended gasoline	Rhamnolipid	34	NA	Cunha *et al.*, 2004
Torulobpsis apicola	Contaminated soil	Alkane/carbohydrate	Glycolipid	30	NA	Hommel *et al.*, 1987

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Organisms	Source	Carbon source	Biosurfactant	Surface tension ($mN.m^{-1}$)	CMC ($mg.l^{-1}$)	Reference(s)
	Water					
Aeromonas sp.	Tropical estuarine water (Lagos lagoon)	0.5% vv^{-1} crude oil	Glycolipid	NA	NA	Ilori *et al.*, 2005
Bacillus sp	Oil reservoirs	2% glucose	Glycolipid	36	310	Tabatabaee *et al.*, 2005
P. aeruginosa PA1	Oil production wastewater	2% vv^{-1} glycerol	Rhamnolipid	27.46	19	Anna *et al.*, 2002
P. aeruginosa	Contaminated-oil water	PAH	Rhamnolipid	NA	NA	Wang *et al.*, 2005
	Sea Water					
Alcaligenes sp.	Sea polluted by crude oil	Crude oil	Glycolipids	30	NA	Maneerat, 2005
Alcaligenes sp. MM1	Marine water, sea-water/sediment	n-hexadecane	Glycolipids	30	NA	Passeri *et al.*, 1992
Bacillus subtilis BBK-1	Sea water	Glucose	Lipopeptide (Surfactin)	28	NA	Niran *et al.*, 2002
Pseudomonas BOP 100 (marine bacterium)	Sea water	n-alkanes	Glycolipid	24.6	NA	Ishigami *et al.*, 1994
Myroides sp. SM1	Sea water	Crude oil	Surfactin	< 40	40	Maneerat *et al.*, 2005

NA = not available

Although in this study, five isolates were gram negative bacteria namely *Pseudomonas aeruginosa, P. aeruginosa, Pseudomonas* sp., *Enterobacter* sp. and *B. cepacia* however gram positive bacteria have been reported as biosurfactant-producing strains (Table 5.2). For example, *Bacillus licheniformis* produced lipopeptides (Jenny *et al.*, 1991), *Staphylococcus* sp. was able to produce extracellular biosurfactant (Nweke and Okpokwasili, 2003), *Rhodococcus erythropolis* produced glycolipids (Philp *et al.*, 2002), *Nocardia erythropolis* created the glycolipids (Macdonald *et al.*, 1981), *Gordonia amarae* excreted trehalose lipid biosurfactant (Ilhan Dogan *et al.*, 2006) were previously reported. Furthermore, *Flavobacterium* sp. MTN11 could produce a new class of biosurfactant which called flavolipids (Bodour *et al.*, 2004). While, yeasts and fungi were also able to produce biosurfactant. *Candida* (formerly *Torulopsis*) *bombicola*, one of a few yeast could produce biosurfactants with high yields of sophorolipids from vegetable oils and sugars (Mulligan, 2005) (Table 5.2). Moreover, mannosylerythritol lipids were produced from vegetable oils by the yeast strains of the genus *Pseudozyma*, namely, *Pseudozyma antarctica* (Kitamoto *et al.*, 2002), *P. aphidis* (Rau *et al.*, 2005), *P. rugulosa, P. fusiformata P. parantarctica* and *P. tsukubaensis* (Morita *et al.*, 2006). Furthermore, *Saccharomyces cerevisiae* produced an effective lipoproteins biosurfactant (Cameron *et al.*, 1988). In the part of biosurfactants, which were produced from fungi such as cellobiose lipids from coconut oil by *Ustilago maydis* ATCC 14826 (Frauiz *et al.*, 1986). Likewise, *Tsukamurella* sp. DSM 44370 isolated from an oil-contaminating soil produced a mixture of oligosaccharide lipids (lipopolysaccharides) from sunflower oil (Vollbrecht *et al.*, 1999).

Table 5.2 Major types of biosurfactants produced by microorganism (Banat *et al.*, 2000)

Microorganism	**Biosurfactant type**	**Reference**
Yeast		
Candida antarctica	Mannosylerythritol lipids	Kitamoto *et al.*, 1993
Candida bombicola	Sophorose lipid	Mulligan 2005
Candida apicola IMET 43747	Sophorose lipid	Hommel and Ratledge 1994
Candida sp. SY16	Mannosylerythritol lipid	Kim *et al.*, 1999
Lactobacillus sp.	Surfactin	Velraeds-Martine *et al.*, 1996b
Norcardia SFC-D	Trehalose lipid	Kosaric *et al.*, 1990
Saccharomyces cerevisae	Rhamnolipid	Vasileva-Tonkova *et al.*, 2001
Fungi		
Botrytis cinerea	Rhamnolipid	Abalos *et al.*, 2001
Rhizotecnia solani	Rhamnolipid	Abalos *et al.*, 2001
Ustilago maydis	Cellobiose lipids (glycolipid)	Hewald *et al.*, 2005
Bacteria		
Alcanivorax borkumensis	Glycolipid	Abraham *et al.*, 1998
Acinetobacter radioresistens	Alasan	Navon-Venezia *et al.*, 1995
Arthrobacter sp. EK1	Trehalose tetraester	Schulz *et al.*, 1991
Arthrobacter sp. MIS 38	Arthrofactin	Morikawa *et al.*, 1993
Bacillus pumilus A1	Surfactin	Thaniyavarn *et al.*, 2003
Bacillus subtilis	Surfactin	Makkar and Cameotra 1997
Bacillus subtilis C9	Surfactin	Kim *et al.*, 1997
Bacillus licheniformis	Lichenysin A	Yakimov *et al.*, 1995
Bacillus licheniformis JF-2	Lichenysin B	Lin *et al.*, 1994
Pseudomonas aeruginosa GL-1	Rhamnolipid	Arino *et al.*, 1996, Patel and Desai 1997
Pseudomonas aeruginosa UW-1	Rhamnolipid	Sim *et al.*, 1997
Pseudomonas fluorescens	Viscosin	Koch *et al.*, 1991
Pseudomonas marginalis PD 14B	Particulate-surfactant (PM factor)	Burd and Ward, 1996

(Continued)

Microorganism	**Biosurfactant type**	**Reference**
Bacteria		
P. maltophilla CSV 89	Biosur Pm	Poirier *et al.*, 1995
Rhodococcus sp. H13 A	Trehalose lipid	Singer *et al.*, 1990
Rhodococcus sp. ST 5	Trehalose lipid	Abu Ruwaida *et al.*, 1991
Serratia rubidea	Glycolipid	Matsuyama *et al.*, 1986
Serratia marcescens	Glycolipid	Pruthi and Cameotra 1997
Streptomyces tendae TU901/8c	Streptofactin	Richter *et al.*, 1998
Tsukamurella sp.	Glycolipid	Vollbrecht *et al.*, 1998

Pseudomonas aeruginosa (A102 and A103) and *Pseudomonas* sp. (B202) have been previously reported to involve in biosurfactant-producing bacteria. For example, *Pseudomonas aeruginosa* could produce glycolipid surface-active molecules (rhamnolipids) which had potential biotechnological applications (Gloria Soberón-Chávez *et al.*, 2005). Similarly, *P. aeruginosa* LBI was able to grow and to produce rhamnolipids when cultivated in the native oils tested (Siddhartha G.V.A.O. Costa *et al.*, 2005). Moreover, *Pseudomonas putida* 21BN grew on hexadecane as the sole carbon source and produced glycolipids when cultivated in the 2% wv^{-1} glucose (Tuleva *et al.*, 2002). Nevertheless, *Enterobacter* strains had a few studies to involve biosurfactant properties. For example, emulsification activity of a marine bacterial exopolysaccharide produced by *Enterobacter cloacae* (Anita Iyer *et al.*, 2006). In the case of *Burkholderia* strains their biosurfactant production such as rhamnolipid were originally described as being produced by the bacteria *Pseudomonas aeruginosa* and later by the bacteria *Burkholderia pseudomallei* (Jarvis and Johnson, 1949; Häussler *et al.*, 1998). In addition of the production of rhamnolipids was characteristic of *Pseudomonas aeruginosa*, some isolates of the nonpathogenic pseudomonads *P. putida* and *P. chlororaphis* as well as the pathogen *Burkholderia pseudomallei* were also recently shown to produce a variety of rhamnolipids (Häussler *et al.*, 1998; Tuleva *et al.*, 2002; Gunther *et al.*, 2005). As above-mentioned, there are a few reports on biosurfactant production by *Enterobacter* sp. and *B. cepacia*. Thus, it is interesting to investigate biosurfactant production from *Enterobacter* sp. and *B. cepacia*. In this study, *Enterobacter* sp. and *B. cepacia* grew in mineral salt medium (As described in Material and Method 3.2.1) supplemented 2% wv^{-1} glucose as carbon source and produced the biosurfactant.

5.2 Physiochemical properties and activity of biosurfactant

Effective physicochemical properties such as emulsification, foaming, dispersion (Desai and Banat, 1997), resistance to salts, anti-adhesive activity, antimicrobial activity, surface activity, critical micelle concentration (CMC), pH and temperature stability are characteristics of these compounds. This research was interested in studying the CMC, pH and temperature on biosurfactant stability because lack of knowledge of extreme pH and temperature on biosurfactant stability has been reported (Cameotra and Makkar, 1998).

5.2.1 Critical micelle concentration (CMC)

The CMC of *Enterobacter* sp. P2 and *B. cepacia* P3 were estimated to be about 3.3 $mg.l^{-1}$ and 1,995 $mg.l^{-1}$, respectively (Figure 4.11, Chapter 4 and The calculation is shown in Appendix F). Generally, the range of CMC values between 10-230 $mg.l^{-1}$ have been reported for rhamnolipids from other microbial sources (Marcia Nitschke *et al.*, 2005). Similarly, the CMC value of *Enterobacter* sp. P2 found to be nearly the value of glycolipids (10 $mg.l^{-1}$), which was investigated by Vollbrecht *et al.* (1999). In addition, the CMC of 3.3 $mg.l^{-1}$ was in agreement with other values reported previously in the literature (Table 5.3), and the tension-active properties of these molecules indicated good prospects for application in industry, when compared to the values of the CMC of chemical anionic surfactants. In the case of *B. cepacia* P3 the CMC values were estimated to be about 1,995 $mg.l^{-1}$ which was found to be high value as the same as biosurfactant produced by *Acinetobacter calcoaceticus* (Banat, 1995b). However, the CMC of this strain compared to chemical surfactants such as sodium dodecyl sulphate (SDS) that had the CMC values as 2,420 $mg.l^{-1}$ (Deshpande *et al.*, 1999) (Table 5.3) was still quite lower.

Table 5.3 Examples of critical micelle concentration of biosurfactants compared to chemical surfactants

Compound	Biosurfactant class	Surface tension ($mN.m^{-1}$)	CMC ($mg.l^{-1}$)	Reference
Synthetic surfactant				
Sodium dodecyl sulphate (SDS)		37	2,023-2,890	Mulligan and Gibbs 1993
Alkylate dodecylbenzene		47	590	Mulligan and Gibbs 1993
Cetyltrimethylammoniumbromide (CTAB)		42	NA	Neu and Poralla 1990
Dihydroamine fluoride		35	475	Busscher *et al.*, 1987
Oleylamine fluoride		30	270	Busscher *et al.*, 1987
Polyoxyethylene 20 sorbitan monooleat (Tween 80)		41	13	Kurt *et al.*, 1993
Alkylphenol ethoxylate ether (Triton X-100)		30	138	Laha and Luthy 1991
Biosurfactant from	**Low molecular weight**			
Bacillus subtilis	Surfactin	27	11	Mulligan and Gibbs 1993
Bacillus licheniformis JF-2	Lipopeptide	27	10	Lin *et al.*, 1994
Bacillus licheniformis	Lipopeptide	27	15	Jenny *et al.*,1991
Pseudomonas aeruginosa	Rhamnolipid	29	15	Mulligan and Gibbs 1993
Pseudomonas sp. DSM 2874	Rhamnolipid	200	30	Lang and Wullbrandt 1999
P. aeruginosa DSM 7107	Rhamnolipid	26	20	Lang and Wullbrandt 1999
P. aeruginosa UI 2979 1	Rhamnolipid	27	10	Lang and Wullbrandt 1999
P. aeruginosa IF0 3924	Rhamnolipid	25	200	Lang and Wullbrandt 1999
P. aeruginosa BOP 101	Rhamnolipid	30	200	Lang and Wullbrandt 1999
Pseudomonas fluorescens	Viscosin	27	150	Neu *et al.*,1990

(Continued)

Compound	Biosurfactant class	Surface tension ($mN.m^{-1}$)	CMC ($mg.l^{-1}$)	Reference
Biosurfactant from	**Low molecular weight**			
Pseudomonas fluorescens	Novel surface active (AP-6)	27	< 10	Persson *et al.*, 1988
Myroides sp. SM1	Surfactin	< 40	40	Maneerat *et al.*, 2005
Rhodococcus erythropolis	Glycolipid	37	15	Mulligan and Gibbs 1993
R. erythropolis SD-74	Succinoyl trehalose lipids	26	200	Ishigami *et al.*, 1987
Torulopsis bombicola	Sophorolipid	37	82	Mulligan and Gibbs 1993
Ustilago zeae	Corynomycolic acid	30	150	Lin 1999
Ustilago maydis	Cellobiose lipids	30	20	Lin 1999
	High molecular weight			
Acinetobacter calcoaceticus	Emulsan	35	1,900	Banat 1995a
Arthrobacter sp. SI 1	Trehalose tetraester and trehalose diester	30	1,500	Schulz *et al.*, 1991

NA = not available

5.2.2 Activity of biosurfactant at various temperatures of 30–75°C and pH

The biosurfactants activity was justified by measuring emulsification index (Method 3.3.2.2, Chapter 3) at the various temperature and pH in order to detect stability of the extracellular products from the two strains. The results showed in Figure 4.16, Chapter 4. The activity of the biosurfactant produced by the *Enterobacter* sp. P2 and *B. cepacia* P3 reduced as temperature increased. On the other hand, Evgenia Vasileva-Tonkova and Victoria Gesheva (2007) claimed that *Pantoea* sp. A13 (formerly *Enterobacter*) could show optimum emulsifying activity in kerosene at pH of 11.2 to 11.5 and a temperature of 30 to 32°C. According to the findings, NaCl had no effect on the activity. However, our results found that the optimum emulsifying activity of *Enterobacter* sp. P2 were at 37°C and pH 7. Although, the activity of the biosurfactant decreased when increasing temperature and extremes of pH nevertheless it was noteworthy to mention that the biosurfactant retained approximately 56.26% and 50% of its original activity (E_{24} = 88.88 ± 0.77% and 94.87 ± 1.99%) at temperature of 75°C and pH 4-9, respectively. Likewise, the activity of the biosurfactant of *B. cepacia* P3 was also expressed to maintain approximately 62.91% and 50% of its original activity (E_{24} = 88.71 ± 0.58% and 92.31 ± 3.44%) in the same condition. Moreover, the previous studies indicated that the bacterial de-emulsifiers were stable at high temperatures (Kosaric *et al.*, 1983). Also, the most known biosurfactants were less stable over such an extreme pH range (Kaplan and Rosenberg, 1982). Similarly, another of biosurfactant encouraged these results for instance liposan from *Candida lipolytica* found to be relatively stable between 30 and 90°C, but lost 60% of its activity after boiling for 1 hour (Cirigliano and Carman, 1984).

5.3 Identification of the biosurfactant type

The biosurfactant-producing bacteria were investigated using the protocol as described in Chapter 3.5 and exhibition of analysis as described in Result 4.3. The results of identification the biosurfactant types of both *Enterobacter* sp. P2 and *B. cepacia* P3 were glycolipids analyzed by chemistry techniques. Following in the Method 3.5.3.1 by thin-layer chromatography of biosurfactant was identified as glucose. The results showed since the spots of *Enterobacter* sp. P2 and *B. cepacia* P3 with R_f 0.22 and 0.22 (Figure 4.19, Chapter 4), were matched to 1 mM glucose spot. And they could react with α-napthol (Longas and Meyer, 1981). Similarly, Angelina Passeri *et al.* (1992) also identified the sugar moiety of the glycolipid by TLC measurement of the water-soluble product after acidic hydrolysis. Additionally, the determination structure of biosurfactant with nuclear magnetic resonance (NMR) (Sim *et al.*, 1997) and fourier-transform infrared spectroscopy (FTIR) (Silverstaein and Webster, 1998) specified the biosurfactant type as glycolipids. Correspondingly, Abraham *et al.* (1998) analyzed and identified the biosurfactant by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Moreover, the results of *Enterobacter* sp. P2 and *B. cepacia* P3, the negative-ion mass spectrum comprised one major band m/z 550.409 and m/z 550.363, respectively. Generally, molecular mass of biosurfactant was in the range from 500 to 1500 Da (Biermann *et al.*, 1987). According to Rosenberg and Ron (1999) explained the detail of the low molecular-mass biosurfactants such as glycolipids (504-650 Da). Thus, the biosurfactant of *Enterobacter* sp. P2 and *B. cepacia* P3 should be arranged glycolipid as the low molecular-mass biosurfactants since were in the range 504-650 Da. In addition, the type of biosurfactants secreted from *Enterobacter* sp. P2 and *B. cepacia* P3 could be classified as glycolipid biosurfactant. Previous report of Evgenia Vasileva-Tonkova

and Victoria Gesheva (2007) also showed that *Pantoea* sp. strain A-13 (formerly *Enterobacter*) (Gavini *et al.*, 1989) produced glycolipid biosurfactants. Moreover, it was identified as the corresponding glycolipid biosurfactants. Nevertheless *B. cepacia* reported somewhere that could produce the rhamnolipid biosurfactants (Davey *et al.*, 2003). A similar determination was reported by Jarvis and Johnson (1949) and Häussler *et al.* (1998) who found that rhamnolipids were originally described as being produced by *P. aeruginosa* and later by *B. pseudomallei* (Jarvis and Johnson, 1949; Häussler *et al.*, 1998). *B. cepacia* P3 in study could produce glycolipid biosurfactant that might indicate the difference in the sugar component on the biosurfactant.

5.4 Role of additional carbon or/and nitrogen source for biosurfactant production

Additional carbon and/or nitrogen source in the presence of 2% wv^{-1} glucose were affected in order to increase the production and/or improve cell growth as well as cell productivity. Moreover, the other media constituents could also affect biosurfactant production quite markedly. For example, the glycolipids were produced by *P. aeruginosa* (Robert *et al.*, 1989), *R. erythropolis* (Rapp *et al.*, 1979), *Mycobacterium* sp. (Cooper *et al.*, 1989) and *Torulopsis bombicola* (Gobbert *et al.*, 1984) which are some organisms reported to have produced biosurfactants that are glycolipid in nature. This study showed that carbon and nitrogen sources could affect the biosurfactants produced by *Enterobacter* sp. P2 and *B. cepacia* P3. *Enterobacter* sp. P2 and *B. cepacia* P3 were grown in various concentrations of carbon sources (glucose, maltose and sucrose). Biosurfactant productions achieved in both mineral salt medium with different carbon sources such as glucose, maltose and sucrose (Figure 4.26-4.28, Chapter 4). *Enterobacter* sp. P2 and *B. cepacia* P3 had ability to

use and produce the biosurfactant when cultivated in the three carbon sources when cultivated at 37°C, 250 rpm for 72 hours. The best biosurfactant production from *Enterobacter* sp. P2 and *B. cepacia* P3 in 2% wv^{-1} glucose as carbon source medium were 17.49 ± 0.338 $g.l^{-1}$ and 37.01 ± 0.118 $g.l^{-1}$, respectively. These results were similar to that of Matsufuji *et al.* (1997) who found that glucose gave the highest growth and biosurfactant production. The previous report, for example *P. aeruginosa* was known to produce the glycolipids on glucose (Guerra-Santos *et al.*, 1984) which were found to affect biosurfactant production by the organism. Recently Evgenia Vasileva-Tonkova and Gesheva (2007) reported that *Pantoea* sp. (formerly *Enterobacter*) (Gavini *et al.*, 1989) could produce 1.2 $g.l^{-1}$ of biosurfactant when incubated at 25°C, 130 rpm for 16 days on mineral salt medium with 2% vv^{-1} kerosene as a carbon source. This present study clearly exhibited the ability of *Enterobacter* sp. P2 that can produce the biosurfactant production more effectively than *Pantoea* sp. for instance, shorter time of incubation and obtained higher biosurfactant production. This statement agreed with Matsufuji *et al.* (1997) who were also successful on the use of glucose as a carbon source in order to improve growth of biosurfactant-producing bacteria. Furthermore, the glycolipid production has been examined with some carbon sources as *n*-paraffin, *n*-tetradecane or glucose (Syldatk *et al.*, 1985; Robert *et al.*, 1989) and plant oil was also good carbon source. The sugar specificity of other biosurfactant-producing microbes had also been studied. Both the sugar and the lipid moieties of the biosurfactant produced by the yeast *Torulopsis bombicola* were the same as depend on of carbon source (Gobbert *et al.*, 1984). This clearly indicated that the biosurfactant might be produced when the using non-hydrocarbon substrates. Biosurfactants have been reported to be produce on water-

soluble compounds such as glucose, sucrose, glycerol, or ethanol (Desai and Banat, 1997).

Numerous reports revealed that the type of carbon substrates markedly affected the production yield of glycolipids (Lang and Wullbrandt, 1999). Benincasa *et al.* (2002) studied by growing *P. aeruginosa* LBI in vegetable oils (sunflower oil). Jeong *et al.* (2004) reported 5.5 $g.l^{-1}$ yield of glycoolipid from *P. aeruginosa* BYK-2 grown at 2% vv^{-1} olive oil, which compared with the other sources. Subsequently, Wei (2005) demonstrated that olive oil was an excellent carbon source for rhamnolipid production with a maximum rhamnolipid concentration of nearly 3.6 $g.l^{-1}$ at an olive concentration of 10%. The most suitable substrates containing sunflower oil and olive oil for *B. cepacia* P3 to produce glycolipid as 18.34 ± 0.025 $g.l^{-1}$ and 14.88 ± 0.002 $g.l^{-1}$. On the other hand, the decrease biosurfactant production were found on all of oil (Figure 4.36, in Chapter 4) was found in *Enterobacter* sp. P2. Evgenia Vasileva-Tonkova and Gesheva (2007) found that when cultivate *Enterobacter* sp. P2 in 2% vv^{-1} kerosene as a carbon source its biosurfactant production is lower than these from *Enterobacter* sp. P2 after grown in the supplement of 2% vv^{-1} diesel oil. However, Haba *et al.* (2000) claimed sunflower oil was not as good a substrate as olive oil, neither for cell growth nor for biosurfactant production.

However, Syldatk *et al.* (1985) reported nitrogen limitation caused an overproduction of glycolipids. Then, this study determined the effect of different nitrogen sources ($NaNO_3$, $(NH_4)_2SO_4$ and CH_4N_2O) on biosurfactant production. Figure 4.30-4.32 in Chapter 4 showed that *Enterobacter* sp. P2 and *B. cepacia* P3 could grow in the presence of $NaNO_3$ more than the condition adding $(NH_4)_2SO_4$ or CH_4N_2O. The results showed that *Enterobacter* sp. P2 and *B. cepacia* P3 were able to

produce of glycolipid as 17.58 ± 0.044 g glucose.l^{-1} and 46.61 ± 0.414 g glucose.l^{-1}, respectively at the presence of nitrogen sources 75 mM $NaNO_3$ after cultivation for 72 hours (Figure 4.33). Nitrate showed more effective on the production of glycolipids than ammonia and urea, which were in agreement with other studies reported in the literatures (Syldatk *et al.*, 1985; Ochsner and Reiser, 1995; Arino *et al.*, 1996). For example, Anna *et al.* (2002) and Rashedi *et al.* (2005) studied the effect of nitrogen source using sodium nitrate the result of higher production of the glycolipid was detected. As well as, Kim *et al.* (1990) hypothesized the effectiveness of the nitrogen sources on overproduction of the trehalose-tetraester (glycolipids), subsequently the overproduction occurred after consumption of the nitrogen sources was found. For overproduction of glycolipids by *P. aeruginosa*, the following sources of carbon have been proven such as glycerol, glucose, n-alkanes and triglycerides. Syldatk and Wagner (1987) were claimed that suitable sources of nitrogen were both ammonium and nitrate ions. An essential precondition for overproduction of the above glycolipids was growth limitation, induced by appropriately limiting the concentration of nitrogen sources or multivalent ions, and an excess of the carbon source. As above-mentioned, both the sufficient carbon and nitrogen sources supported good growth and a substantial amount of glycolipids were produced.

5.5 Determination of the effect of NaCl, temperature and pH on the activity of the biosurfactant

NaCl activated biosurfactant activity of many strains which were isolated from seawater or petroleum reservoirs (Yakimov *et al.*, 1995). Some microorganisms could survive and grow over a wide range of salt concentrations. Also, salt tolerance was not surprising since the organism was able to grow in a medium containing up to 5% wv^{-1} NaCl (Abu-Ruwaida *et al.*, 1991). For example, *P. aeruginosa* BYK-2 was isolated from the southern sea of Korea that produced a glycolipid biosurfactant (Jeong *et al.*, 2004). However, there were very few reports on biosurfactant production in hypersaline environments. The most bacterial had the activity of biosurfactant when cultivated on higher salts was *Bacillus* strains. These statements agree with Jenneman *et al.* (1983) who reported the production of biosurfactant by a halotolerant *Bacillus* species and its potential in enhanced oil recovery. Then, *Bacillus licheniformis* BAS 50 was able to grow and produce a lipopeptide surfactant when cultured on variety of substrates at salinities up to 13% wv^{-1} NaCl (Yakimov *et al.*, 1995). *Enterobacter* sp. P2 and *B. cepacia* P3 at the concentration of NaCl from 0.1, 0.5, 1.0 and 2.0% wv^{-1} and pH of 10 showed the results of poor growth and little biosurfactant production. Since the increasing concentrate of NaCl resulted in higher pH values and consequent death occurred at 2.0% wv^{-1} NaCl. No biosurfactant production had been detected in *Enterobacter* sp. P2 and *B. cepacia* P3 when grew in the medium contained NaCl > 0.1 up to 2.0% wv^{-1}. These characteristics may indicate that *Enterobacter* sp. P2 and *B. cepacia* P3 were non-halotolerant. Opposite to previous studies, bacteria could grow in the absence of salt as well as in the presence of relative high salt concentrations (e.g., 8% in the case of *Staphylococcus aureus*) are designated as halotolerant (or extremely halotolerant if growth extends above 2.5 M).

A rare case of a bacterium that requires 2 M salt at least (optimal growth at 3.4 M), such as the actinomycete *Actinopolyspora halophila* (Mikesell and Boyd., 1986) was considered a borderline extreme halophile (Van der Tweel *et al.*, 1987). Moreover, previous report showed that the effect of pH and NaCl on the emulsifying activity tested by the optimum emulsifying activity to kerosene was observed at a pH of 11.2 to 11.5. According to their findings, NaCl had no effect on the activity (Evgenia Vasileva-Tonkova and Gesheva, 2007).

Temperatures are one of the most important parameters regulating the activities of microbes in natural environments which influenced the response of microbes directly by it were affected on growth rate, enzyme activity, cell composition and nutritional requirements. Considering the profound effected of temperature on the activities of microorganisms, it was somewhat surprising that many microbiologists neglect the fact that most natural environments were at low ambient temperatures. The optimal temperature of synthesis for glycolipids was 31-34°C. Below 30°C or above 37°C, the glycolipid yields were significantly reduced in continuous culture (Guema-Santos *et al.*, 1986). These results suggested that the optimal temperature for glycolipid production with the *Enterobacter* sp. P2 and *B. cepacia* P3 were in the range of 30–37°C. On the other hand, higher temperature (45°C) also affected in the decrease of the growth and no biosurfactant production of the bacteria detected. According to Visser *et al.* (1993) imposed temperature fluctuations may affect the bacteria type involved in the process, at temperatures exceeding the maximum value for growth; anyhow decay may exceed the growth rate of bacteria. This result was similar to that of Vasileva-Tonkova and Gesheva (2007) who found that the optimum emulsifying activity to kerosene was observed at a temperature of 30 to 32°C. Nonetheless, Zinder *et al.* (1984) mentioned that the

thermophilic *Methanothrix* enrichment was capable to grow at temperatures up to 70°C while *Methanosarcina* sp. grew faster at 55°C than a *Methanothrix* culture, while at 60°C, the doubling time of the two cultures were quite similar (~30 hours). They also mentioned that *Methanosarcina* sp., a predominant aceticlastic thermophilic methanogens, was sensitive to temperature elevations above 60°C. They postulated that the digester might withstand minor temperature fluctuations when operated closer to the optimum temperature of *Methanosarcina* sp. (55–58°C). Moreover, the other researchers had reported a slight increase temperature for the growth of bacteria.