CHAPTER IV

RESULTS

4.1 Screening, isolation and identification of biosurfactant-producing bacteria

4.1.1 Screening and isolation of biosurfactant-producing bacteria

Biosurfactants are synthesized by bacteria, yeasts and fungi during growth on various carbon sources, in particular during growth on hydrophobic substrates (Gerson and Zajic, 1979). The environmental sources of these strains were diesel oil and hydrocarbon contaminated bottom sediment and surface water (Broderick and Cooney, 1979), hydrocarbon contaminated subsurface soil (Macelwee *et al.*, 1990; Francy *et al.*, 1991) and unleaded gasoline contaminated soil (Allen *et al.*, 1992). Therefore, samples were collected from the various hydrocarbon contaminated with organic aqueous waste (pesticides) and oils (lubricating oils, automotive oils, hydraulic oils, and fuel oils) in Thailand (Table 4.1). One hundred and thirty isolates of bacteria were then separately grown in mineral salt medium (MSM) supplemented with 2% wv^{-1} glucose (As described in Method 3.2.1) while were also grown on mineral salt medium with distinguish bacteria as a control. The growth of bacteria at 37°C was observed by measuring the optical density at 600 nm using spectrophotometer during 3 days of incubation. Hence, bacteria were collected in culture broth after incubated in mineral salt medium (MSM) for 3 days. Subsequently, culture broth was centrifuged at 9000×g for 15 min to remove cells (As described in Method 3.3.2.1). Preliminary screening of bacteria were used 96 microwell plate with drop-collapse method (Bodour and Miller-Maier, 1998). Eighty-seven isolates tested gave positive results by this method in Table 4.1. While, five of

these isolates gave also positive for biosurfactant production by emulsification index (As described in Method 3.3.2.2). Thus, only five isolates of these bacterial isolates could be selected due to positive results of biosurfactant production detected by drop-collapse method and emulsification index (E_{24}). These bacterial isolates were designated as A102, A103, B202, P2 and P3.

Then, these five bacterial isolates were separately grown in mineral salt medium supplemented with 2% wv^{-1} glucose while they were also grown on mineral salt medium with no bacteria as a control. The growth of bacteria at 37°C was observed by measuring the optical density at 600 nm using spectrophotometer during 3 days of incubation. It was found that P2 and P3 grew in mineral salt medium containing 2% wv^{-1} glucose (Figure 4.1, Appendix A) better than the other these isolates. Moreover, P2 and P3 gave the higher value emulsification index (E_{24}) and also gave positive results by drop-collapse method (Table 4.1). Therefore, P2 and P3 were selected for further studies.

Table 4.1 Screening of biosurfactant-producing bacteria from various sources using the drop-collapse method and emulsification index (E_{24})

Source		Isolate number	Shown in Fig 4.2	Drop collapse [a]	Emulsification index (E_{24})[b]	Isolation
Contaminated area	1.Engine oil, Burirum province	1.1	C7	-	NE	
		1.2	C2	√	80.00	A102
		1.3	C10	-	NE	
		1.4	C3	√	80.00	A103
		1.5	NA	√	16.29	
		1.6	NA	-	NE	
		1.7	C4	√	75.00	B202
		1.8	C8	-	NE	
		1.9	NA	√	NE	
		1.10	C1	-	NE	
		1.11	C5	-	NE	
		1.12	NA	√	45.85	
		1.13	NA	√	48.00	
		1.14	C6	-	NE	
		1.15	C9	-	NE	
		1.16	NA	√	33.33	
	2. Petroleum oil, Konkaen province	2.1	D10	-	NE	
		2.2	E1	-	NE	
		2.3	E2	-	NE	
		2.4	E3	-	NE	
		2.5	E4	-	NE	
	3. Fuel oil, Thonburi location in Bangkok province	3.1	NA	√	NE	
		3.2	E6	√	93.33	P2
		3.3	E7	√	94.44	P3
		3.4	NA	√	NE	
		3.5	NA	√	46.67	
		3.6	NA	√	NE	
		3.7	NA	√	50.59	
	4. Machine oil, Chemical technique area Bangkok (Chulalongkorn University)	4.1	NA	-	NE	
		4.2	NA	-	NE	
		4.3	NA	√	22.22	
		4.4	NA	√	8.33	
		4.5	NA	-	NE	
		4.6	NA	√	NE	
		4.7	F8	-	NE	
		4.8	F9	-	NE	
		4.9	F10	-	NE	
	5. Contaminated soil with insecticide, Thonburi location in Bangkok province	5.1	J1	√	NE	
		5.2	J2	√	NE	
		5.3	J3	√	25.00	
		5.4	J4	√	NE	
		5.5	J5	√	NE	
	6. Contaminated soil, Department of Naval Dockyards, Bangkok province	6.1	K1	√	25.00	
		6.2	K2	√	38.46	
		6.3	K3	√	27.27	
		6.4	K4	√	66.67	
		6.5	K5	√	42.86	
		6.6	K6	√	16.67	
	7. Contaminated soil from food shop from Klongsan, Bangkok province	7.1	L1	√	46.15	
		7.2	L2	√	41.67	

(Continutes)

Source		Isolate number	Shown in Fig 4.2	Drop collapse [a]	Emulsification index (E_{24}) [b]	Isolation
Contaminated area	8. Contaminated soil from garage, Bangkok province	8.1	N1	√	54.55	
		8.2	N2	√	46.15	
		8.3	N3	√	41.67	
		8.4	N4	√	53.85	
		8.5	N5	√	30.77	
		8.6	N6	√	50.00	
Agricultural area	9. Cassava plantation, Rachaburi province	9.1	NA	-	NE	
		9.2	NA	√	NE	
		9.3	NA	√	NE	
		9.4	NA	-	NE	
	10. Orchard area I, Rayong province	10.1	NA	√	NE	
		10.2	NA	-	NE	
		10.3	NA	√	NE	
		10.4	NA	-	NE	
		10.5	NA	√	30.77	
		10.6	NA	-	NE	
		10.7	NA	√	48.72	
		10.8	NA	√	19.34	
		10.9	NA	√	33.33	
	11. Orchard area II, Rayong province	11.1	NA	√	NE	
		11.2	NA	√	NE	
		11.3	NA	√	NE	
		11.4	NA	√	NE	
		11.5	NA	√	34.94	
		11.6	NA	√	60.73	
		11.7	NA	√	NE	
		11.8	NA	-	NE	
	12. Mango plantation, Nakorn Patom province	12.1	E5	-	33.33	
		12.2	E8	-	22.22	
		12.3	E9	-	8.33	
		12.4	E10	-	NE	
		12.5	F1	-	46.67	
		12.6	F2	-	NE	
		12.7	F3	-	30.59	
		12.8	F4	-	35.29	
	13. Rambutan plantation, Chanthaburi province	13.1	NA	√	15.24	
		13.2	NA	√	18.82	
		13.3	NA	√	35.70	
		13.4	NA	√	28.00	
		13.5	NA	√	39.47	
		13.6	NA	√	34.87	
		13.7	NA	√	33.33	
		13.8	NA	√	33.33	
		13.9	NA	√	15.76	
	14. Lemon garden, Tak province	14.1	NA	√	NE	
		14.2	NA	√	NE	
		14.3	NA	√	NE	
		14.4	NA	√	NE	
		14.5	NA	√	NE	
	15. Orchard, Parchin Buri province	15.1	I1	√	46.15	
		15.2	I2	√	53.85	
		15.3	I3	√	38.46	
		15.4	I4	√	38.46	
		15.5	I5	√	41.67	
		15.6	I6	√	33.33	

(Continutes)

Source		Isolate number	Shown in Fig 4.2	Drop collapse [a]	Emulsification index (E_{24})[b]	Isolation
Agricultural area	16. Orchard at Suan Phueng I, Ratchaburi province	16.1	L3	√	41.67	
		16.2	L4	√	41.67	
	17. Orchard at Suan Phueng II, Ratchaburi province	17.1	M1	√	NE	
		17.2	M2	√	15.38	
		17.3	M3	√	38.46	
		17.4	M4	√	50.00	
		17.5	M5	√	25.00	
		17.6	M6	√	25.00	
		17.7	M7	√	25.00	
		17.8	M8	-	36.36	
Natural area	18. Slosh source, Chulalongkorn University	18.1	F5	-	NE	
		18.2	NA	√	NE	
		18.3	NA	√	NE	
		18.4	NA	√	NE	
		18.5	F6	-	NE	
		18.6	F7	-	NE	
		18.7	NA	√	NE	
		18.8	NA	√	NE	
		18.9	NA	√	NE	
	19. Pond full of water lilies, Chulalongkorn University	19.1	D1	-	NE	
		19.2	D2	-	NE	
		19.3	D3	-	NE	
		19.4	D4	-	NE	
		19.5	D5	-	NE	
		19.6	D6	-	NE	
		19.7	D7	-	NE	
		19.8	D8	-	NE	
		19.9	D9	-	NE	
Control	dH_2O	-		√	12.85	
	Culture broth (MSM)	-		√	33.33	
	Sodium laryl sulphate (SDS)	-		√	95.00	
	Tween80	-		√	95.00	

[a] √ = collapse, - = no collapse and NA = not available

[b] E_{24}: emulsification index shown as percentage of emulsion, whose E_{24} after 24 hours was >50% of the E_{24} after 2 hours (Willumsen and Karlson, 1997); NE = no detected.

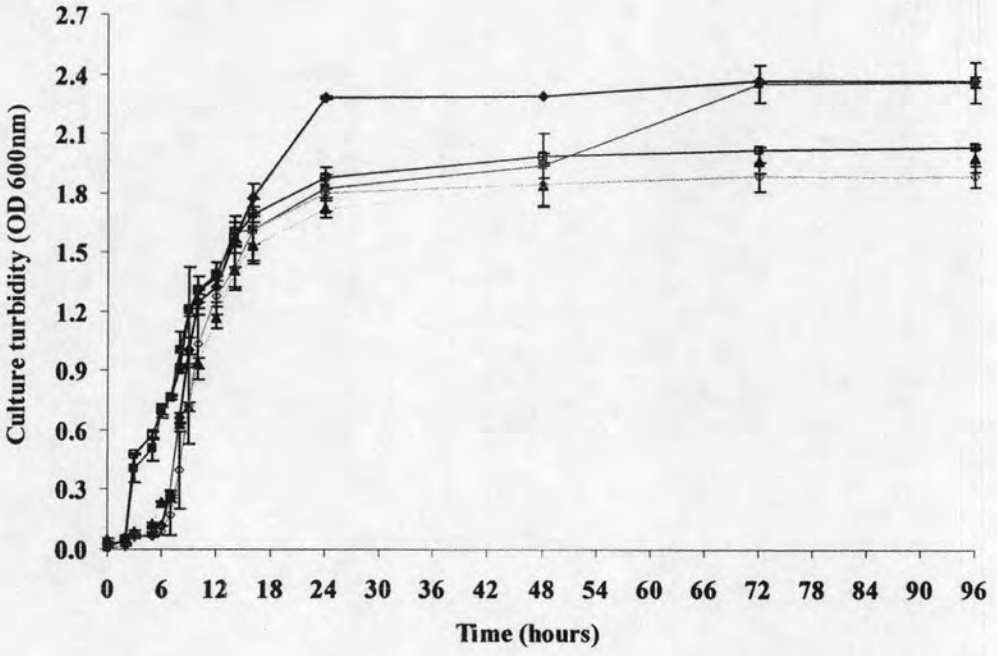


Figure 4.1 Growth of five bacterial isolates: P2 (-◆-), P3 (-■-), A102 (-▲-), A103 (-◇-) and B202 (-□-) in mineral salt medium in the presence of 2% wv^{-1} glucose as carbon source. The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

4.1.2 Preliminary screening of biosurfactant-producing bacteria

Five bacterial isolates were cultured in mineral salt medium (as described in Method 3.2.1) containing 2% wv^{-1} glucose as a carbon source and grown for 72 hours, with shaking at 250 rpm, at 37°C. After 72 hours growth, cells were harvested and culture supernatant was used to determine extracellular biosurfactant, while mineral salt medium as a control.

The biosurfactant-producing bacteria were screened by methods namely drop-collapse method and emulsification index (E_{24}) (as described in Method 3.3.2.2). The drop-collapse method depends on the principle that a drop of a liquid containing a biosurfactant will collapse and spread completely over the surface of oil

(Jain *et al.*, 1991; Bodour and Miller-Maier, 1998). For the emulsifying activity were determined using a modification of the method described by Bosch *et al.* (1988) and Willumsen and Karlson (1997). The emulsion stability was determined after 24 hours and the E_{24} was calculated as the ratio of the height of the emulsified layer to the height of the total oil phase and multiplying by 100. Biosurfactant-producing bacteria were designated as the one that should have an $E_{24} > 0.5$ (equals 50% emulsification of the diesel oil layer) 24 hours after shaking (Bosch *et al.*, 1988).

4.1.2.1 Drop-collapse method (Bodour and Miller-Maier, 1998)

Biosurfactant was prescreened by using the drop-collapse method as following. After 1 min of dropping the cultured broth on the diesel oil surface, the distribution of oil was observed. Biosurfactant-producing cultures gave flat drops with scoring system '√' corresponding to partial to complete spreading on the oil surface. Those cultures that gave rounded drops were scored as negative '_' indicative of the lack of biosurfactant production. For example, if the drop remained beaded, the results were scored as negative such as C1 and C5 until E5 (Figure 4.2). On the other hand, if the drop-collapse, the results were scored as positive such as C2, C3, C4, E6 and E7 of which their culture broth separated from the isolates designated as A102, A103, B202, P2 and P3, respectively. Tween 80 (A and G rows) and SDS (B and H rows) at the concentrations range of 0-90 $\mu g.\mu l^{-1}$ were used as positive controls.

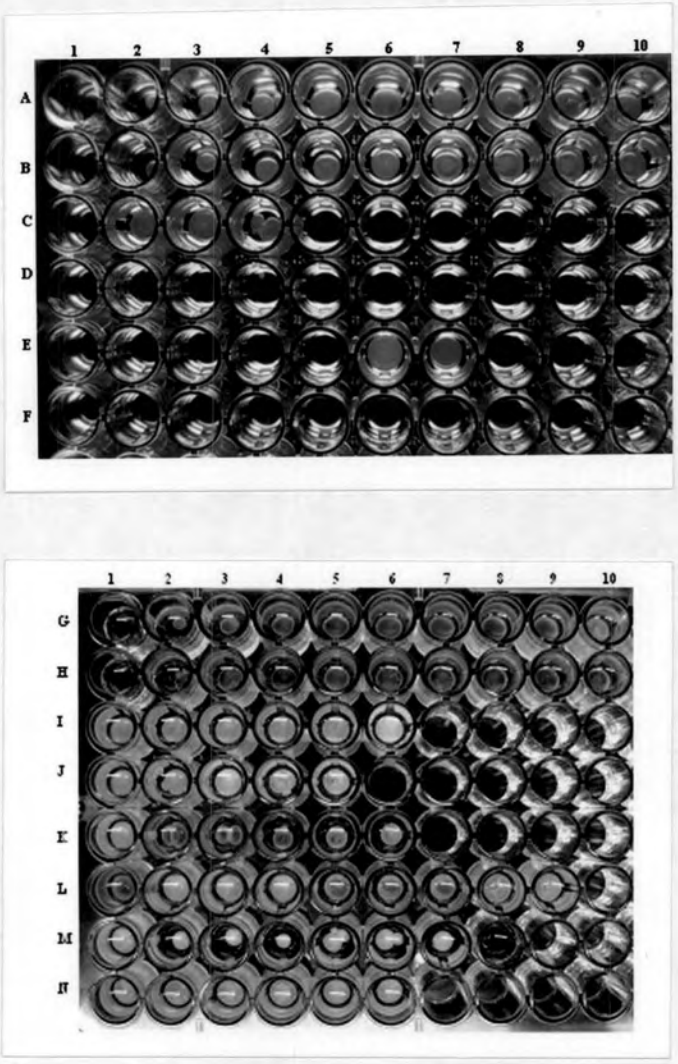


Figure 4.2 Characteristic of diesel oil after dropping culture broths from certain bacterial isolates. A and G rows contains various concentration of Tween 80 (0-90 $\mu g.\mu l^{-1}$), B and H rows contains various concentration of SDS (0-90 $\mu g.\mu l^{-1}$), C-F and I-N rows contains culture broths from certain bacterial isolates.

4.1.2.2 Emulsification index (E_{24})

The degree of emulsification and the stability of the emulsions formed by the isolates were presented in Table 4.2. Eighty-seven isolates were tested for emulsion activity by following the stability of emulsion. The higher degree of emulsification were detected in five isolates namely as A102, A103, B202, P2 and P3, after growing in mineral salt medium supplemented with 2% wv^{-1} glucose as carbon source (Figure 4.3). After calculation; the highest E_{24} was found in cultures broth of P3 following with the ones of P2, B202, A102 and A103, respectively as shown in Table 4.2 (The calculation is detailed in Appendix A). The experiments were carried out in triplicate which distilled water and distinguished bacteria on mineral salt medium supplemented with 2% wv^{-1} glucose as carbon source were used the control.

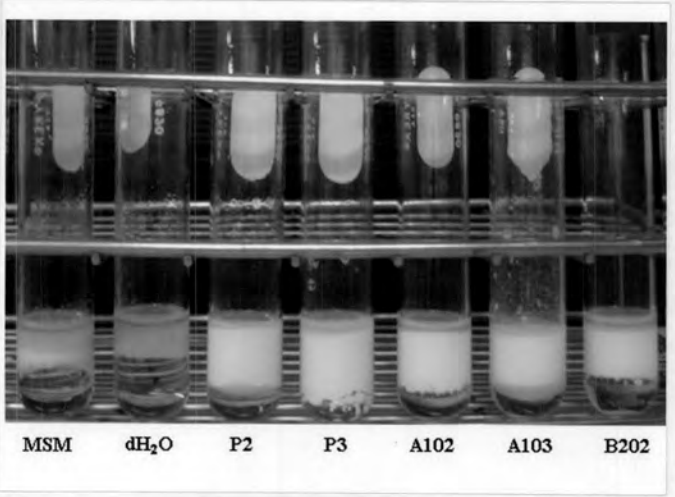


Figure 4.3 Emulsification activities after mixing of culture broths positively with diesel oil in water also standing for 24 hours.

Table 4.2 Configuration of biosurfactant production from five isolates by different methods (n = 4).

Isolates	Drop collapse [a]	Emulsification index [b] (E_{24})
A102	√	74.08 ± 0.101%
A103	√	67.86 ± 0.087%
B202	√	70.59 ± 0.074%
P2	√	85.88 ± 0.071%
P3	√	86.19 ± 0.060%

[a] Flat drops with scoring system '√' corresponding to partial to complete spreading on the oil surface. Rounded drops were scored as negative '_' indicative of the lack of biosurfactant production.

[b] An emulsion were secreted from biosurfactant-producing bacteria which was defined as stable if the E_{24} 50% or better (Bosch *et al.*, 1988).

4.1.3 Identification of biosurfactant-producing bacteria

4.1.3.1 The morphological characteristics of bacteria

Five bacteria isolates capable of biosurfactant-producing isolated from the contaminated soil with hydrocarbon compound (oil) were identified for their morphological characteristic by bacterial gram staining and chemical solution staining. After staining, the results showed that all five isolates were gram negative bacteria as shown in Figure 4.5 - 4.9. Furthermore, the characteristics of cells on Luria Bertani (As described in Method 3.2.2) agar have been shown in Table 4.3.

Table 4.3 Characteristic of cells of the bacterial isolates on Luria Bertani agar.

Cells characteristics	**Bacterial isolates**				
	A102	**A103**	**B202**	**P2**	**P3**
Color	White	White	White	White	White
Form	Circular	Circular	Circular	Circular	Circular
Diameter	1.0 mm	1.0 mm	1.0 mm	1.5-2.0 mm	1.0-1.5 mm
Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Edge	Entire	Entire	Entire	Entire	Entire

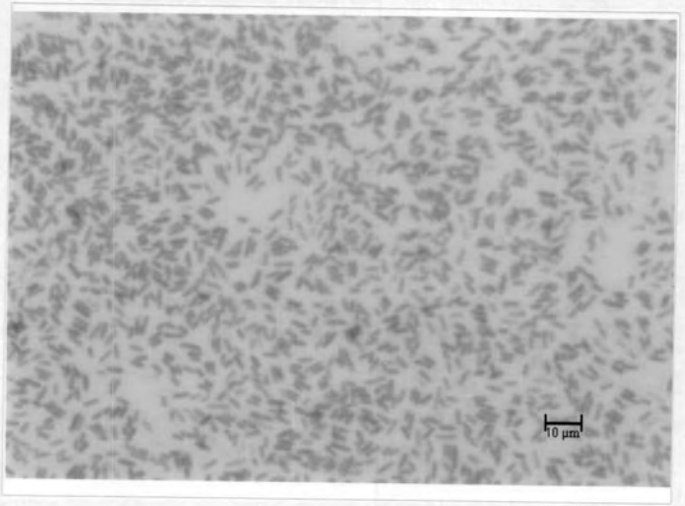


Figure 4.4 Gram's staining and morphology of isolate A102 (1000X)

1.0-1.5 µm × 1.5-2.5 µm

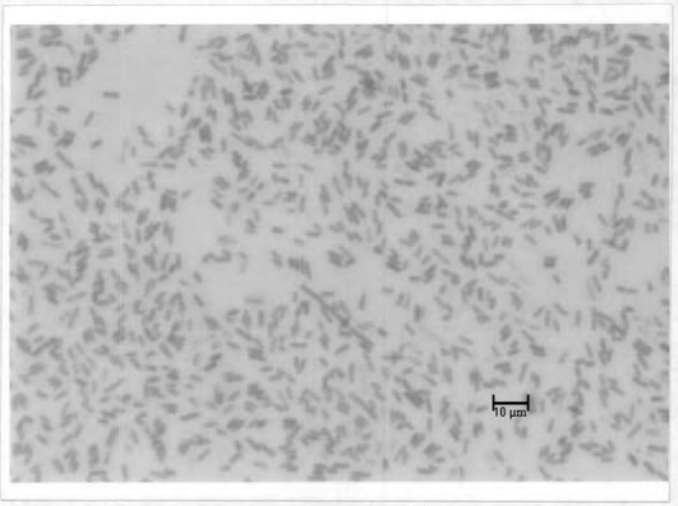


Figure 4.5 Gram's staining and morphology of isolate A103 (1000X)

1.0-1.5 µm × 1.5-2.5 µm

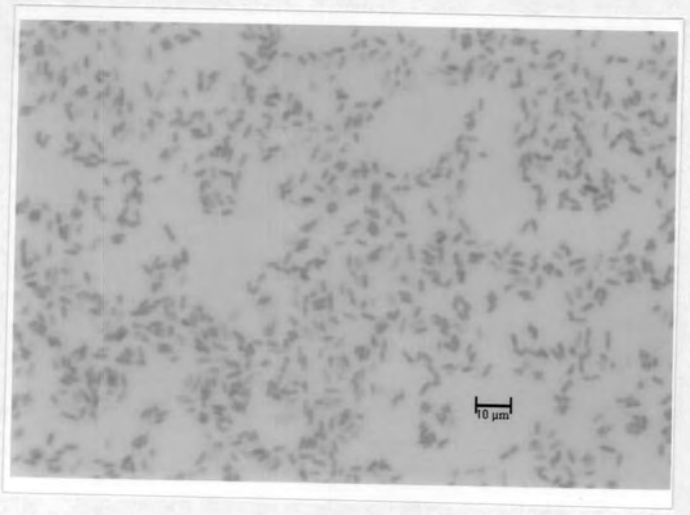


Figure 4.6 Gram's staining and morphology of isolate B202 (1000X)

1.0-1.5 μm × 1.5-2.5 μm

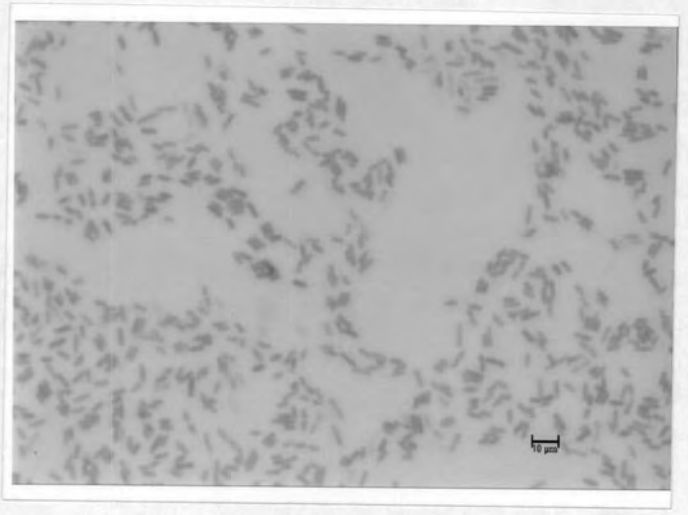


Figure 4.7 Gram's staining and morphology of isolate P2 (1500X)

1.0-1.5 μm × 1.5-2.5 μm

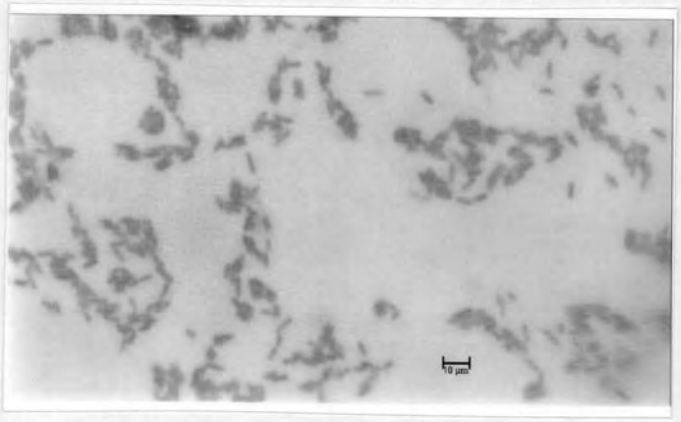


Figure 4.8 Gram's staining and morphology of isolate P3 (1500X)

1.0-1.5 μm × 1.5-2.5 μm

4.1.3.2 The biochemical test of the five bacterial isolates

From the results of morphological characteristic of five isolates of bacteria, these biosurfactant-producing bacteria were gram negative. Then, the biochemical tests were used to identify the species of bacteria. The biochemical characterization results (Table 4.4) were compared to the Manual of Clinical Microbiology supporting by the laboratory of Institution for Scientific Research, Department of Medical Sciences, Ministry of Public Health in Thailand. Five biosurfactant-producing bacteria; A102, A103, B202, P2 and P3 could be identified as *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Burkholderia cepacia*, respectively.

Table 4.4 Biochemical tests for biosurfactant-producing bacteria *

Biochemical Test	Bacterial isolates				
	A102	A103	B202	P2	P3
Oxidase test	+	+	+	-	+
Catalase test	-	-	-	-	+
TSI/ H_2S production	K/K / -	K/K / -	K/K / -	K/Agas / -	K/K /
SIM(H_2S/indole/motile)	-/-/+	-/-/+	-/-/+	-/ - /+	/ - / +
Citrate Utilization	+	+	+	+	+
Urease test	+	+	+	+	+
Nitrate reduction	+	+	+	-	-
N_2 gas production	+	+	+	-	-
Esculin hydrolysis	-	-	-	-	-
Acetate Utilization	+	+	+		+
Voges-Proskauer reaction				+	
Carbohydrate fermentation test					
Glucose/gas	+/	+/	+/	+ / +	+ /
Maltose	-	-		+	-
Lactose	-	-	-	-	-
Mannitol	+	+	+	+	-
D-Xylose	+	+	+	+	+
Rhamnose				+	
Inosital CTA				+	
Sorbital				+	
Raffinose				+	
Fructose	+	+	+		
Salicin				-	
Lysine Decarboxylase	-	-	-	-	-

(Continued)

Biochemical Test	Bacterial isolates				
	A102	**A103**	**B202**	**P2**	**P3**
Arginine Dihydrolase	+	+	+	+	+
Ornithine Decarboxylase	-	-	-	+	-
Bacteria identification	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Enterobacter cloacae*	*Burkholderia cepacia*

TSI: Triple Sugar Iron Agar Reaction, OF: Oxidation-Fermentation basal medium, NF; Non-fermentative gram-negative bacilli, SB: Sugar base, ASS: Ammonium salt sugar base, CTA: Cystine tryptic, +: positive result, -: negative result, K/K: alkaline butt and alkaline slant, blank: not tested, A/A: acid butt and acid slant

* From the laboratory of Institution for Scientific Research, Department of Medical Sciences, Ministry of Public Health in Thailand.

4.1.3.3 Determination of 16S ribosomal DNA gene for bacterial identification

The determination of 16S ribosomal DNA gene sequence was used to identify two biosurfactant-producing bacteria, P2 and P3. The specific primers (63f and 1387r) (Julian *et al.*, 1998) were used to amplify the target sequence as described in Materials and Methods 3.3.3.3. These primers were found to be more useful for 16S rDNA gene of bacterial species and environmental samples than PCR primers that are generally used. After PCR amplification, PCR products were analyzed by using 0.8% agarose gel electrophoresis. The length of the amplified 16S rDNA fragment product was approximately 1.3 kb. Subsequently, the blastN program was used to compare and analyze the 16S rDNA sequence against NCBI database (www.ncbi.nlm.nih.gov). The result of alignment of 16S rDNA sequences are shown in Appendix B and the results can be summarized in Table 4.5.

Table 4.5 Identification of biosurfactant-producing bacteria using 16S rDNA gene sequence comparison

Culture name	Sequence identities (%)	Sequence accession number	Bacteria	Source of bacteria	References	Note
P2	1) 99%	AJ853890	*Enterobacter*	Soil	Hammond. (2005)	-
	2) 99%	EF138627	*hormaechei*	Soil	Gao and Sheng	biodegradation of
			Enterobacter sp.		(Unpublished)	phthalate esters
	3) 99%	AM184248		Soil	Abraham *et al.*	-
Conclusion	-	-	*Enterobacter* sp.	-	(Unpublished)	
			***Enterobacter* sp.**			
P3	1) 99%	AB212239	*Burkholderia* sp.	Soil	Sakai *et al.*	bacteria isolated from Japan
	2) 99%	AF335494	*Burkholderia cepacia*	Soil	(Unpublished)	-
	3) 99%	AY677089	*Burkholderia cepacia*	Soil	Kim *et al.* (2004)	a common plant-associated bacterium
					Ka. (Unpublished)	
Conclusion	-	-	***Burkholderia cepacia***	-		

4.2 Characterization of biosurfactant for biosurfactant-producing bacteria

The analytical characterization of the glycolipids produced by *Enterobacter* and *Burkholderia* strains such as measurement of the surface tension, concentration of partial purified biosurfactant were determined. Then, the dependence of biosurfactant excretion on mineral salt medium supplemented with 2% wv^{-1} glucose and another medium such as Luria Bertani medium (LB) and nutrient broth (NB) were studied.

4.2.1 Partial purification of biosurfactant production

Extracellular biosurfactants produced by *P. aeruginosa* A102, *P. aeruginosa* A103, *Pseudomonas* sp. B202, *Enterobacter* sp. P2 and *B cepacia* P3 were partially purified biosurfactant (Described in Materials and Methods 3.4.1) and shown in Figure 4.9.

Figure 4.9 Partially purified of biosurfactant produced from *P. aeruginosa* A102, *P. aeruginosa* A103, *Pseudomonas* sp. B202, *Enterobacter* sp. P2 and *B. cepacia* P3, respectively.

4.2.2 Measurement of the surface tension of partial purified biosurfactant

The biosurfactant-producing bacteria was grown in mineral salt medium the presence of 2% wv^{-1} glucose. After 72 hours of incubation at 37°C, cell-free culture broths were collected and partially purified (Figure 3.1, Chapter 3). Biosurfactant in culture broth was analyzed by using Du Nouy ring method (Bodour and Miller-Maier, 1998). The lowest surface tension of biosurfactant from *B. cepacia* P3, *Enterobacter* sp. P2, *P. aeruginosa* A103, *Pseudomonas* sp. B202 and *P. aeruginosa* A102 were 25.0 ± 0.05 $mN.m^{-1}$, 26.0 ± 0.05 $mN.m^{-1}$, 30.0 ± 0.05 $mN.m^{-1}$, 32.0 ± 0.02 $mN.m^{-1}$ and 37.0 ± 0.02 $mN.m^{-1}$, respectively within 3 days of incubation (Figure 4.10).

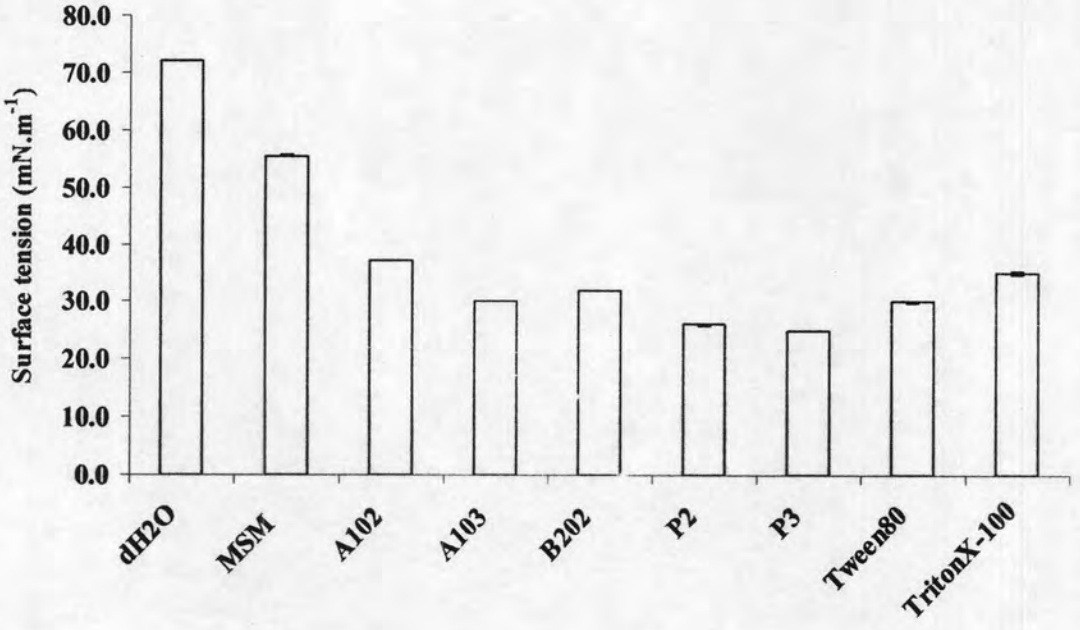


Figure 4.10 The surface tension reductions ($mN.m^{-1}$) of partial purified biosurfactant from A102, A103, B202, P2 and P3 after cultivated in medium. Each point represented the mean and standard deviation of triplicate samples.

4.2.3 Determination of biosurfactant concentration

Glycolipids produced by *Enterobacter* sp. P2 and *B. cepacia* P3 growing on 2% wv^{-1} glucose as carbon source were 17.49 ± 0.338 g glucose.l^{-1} and 37.02 ± 0.118 g glucose.l^{-1}, respectively.

4.2.4 Physiochemical properties and activity of biosurfactant

4.2.4.1 Critical micelle concentration (CMC)

The CMC of *Enterobacter* sp. P2 and *B. cepacia* P3 for this biosurfactant extract were estimated to be about 3.3 mg.l^{-1} and 1,995 mg.l^{-1} from the intercept of two straight lines from the concentration-dependent and concentration-independent sections of Figure 4.11 (The calculation is shown in Appendix F). Then, the surface tension decreased until value of 29 mN.m^{-1} and 30 mN.m^{-1} in *Enterobacter* sp. P2 and *B. cepacia* P3, respectively were reached (Figure 4.11). Maximum inverse CMC values of 29 mN.m^{-1} and 30 mN.m^{-1} in *Enterobacter* sp. P2 and *B. cepacia* P3 respectively were obtained.

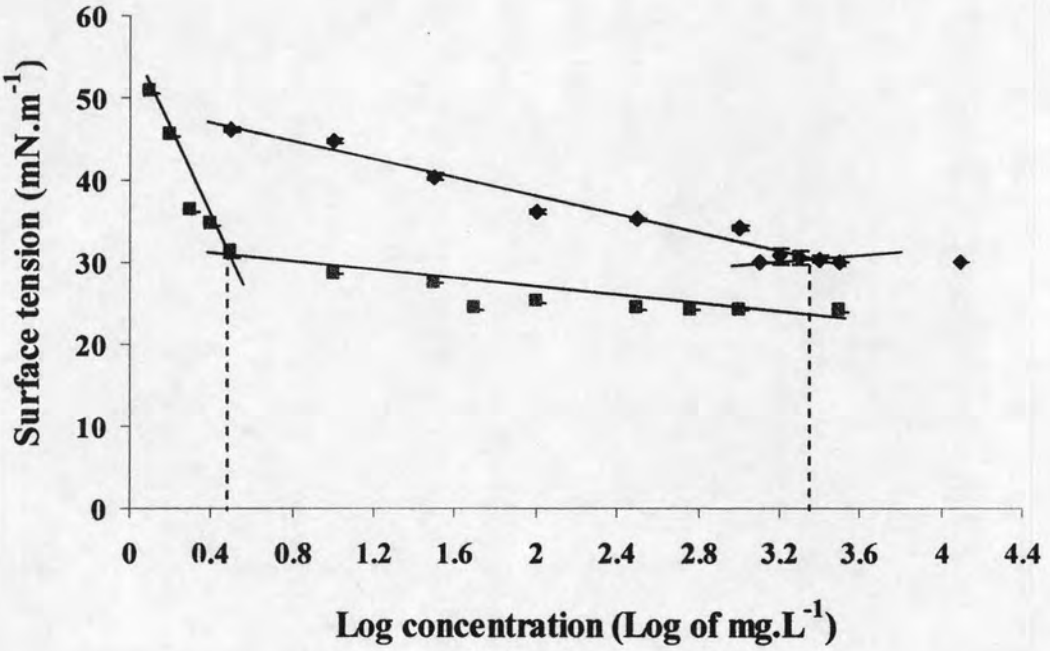


Figure 4.11 Critical micelle concentration of glycolipid produced by *Enterobacter* sp. P2 (◆) and *B. cepacia* P3 (■). Each point represented the mean and standard deviation of triplicate samples.

4.2.4.2 Stability of biosurfactant at various temperatures

The partially purify of biosurfactant was also maintained at a constant temperature of 30°C, 37°C and 45°C and used for the emulsification assay. Emulsification property and stability of biosurfactant produced by *Enterobacter* sp. P2 and *B. cepacia* P3 were determined using diesel oil (Castrol oil). In the present study, different temperatures e.g. 30°C, 37°C and 45°C were investigated on emulsification index (E_{24}). The cultures with control samples were placed on shaker at 250 rpm for 3 days in selected temperature (Yakimov *et al.*, 1995). The results showed in Table 4.6. Partial purification of biosurfactant of *Enterobacter* sp. P2 and *B. cepacia* P3 showed E_{24} at 30°C, 37°C and 45°C in Table 4.6 as compared with

Tween80 and SDS as controls. The results of the stability of biosurfactant at 30°C, 37°C and 45°C were shown in Figure 4.12-4.14 and Table F-3, Appendix F. Increase in temperature from 30 to 37°C had no effect on the activity of the biosurfactant, the emulsion indices obtained were the same (Figure 4.12-4.14). However a slight increase in the activity of the biosurfactant was detected at a temperature of 45°C (E_{24} = 79.29 ± 0.82% and 87.31 ± 3.11%). At temperatures of 60°C (E_{24} = 65.27 ± 0.20% and 70.28 ± 0.11%) and 75°C (E_{24} = 50.00 ± 0.22% and 55.81 ± 2.25%), the activities of the biosurfactant decreased. It was however noteworthy to mention that the biosurfactant retained approximately 56.26% and 62.91% of its original activity (E_{24} = 88.88 ± 0.77% and 88.71 ± 0.58%) at temperature of 75°C.

Table 4.6 Effect of change in temperature on activity of biosurfactant (n=3)

Temperature (°C)	**Emulsification index (E_{24})**			
	***Enterobacter* sp. P2**	***B. cepacia* P3**	**Tween 80**	**SDS**
30	83.77 ± 2.25	86.19 ± 0.55	94.74 ± 0.00	92.59 ± 0.00
37	88.88 ± 0.77	88.71 ± 0.58	99.95 ± 0.06	95.63 ± 0.06
45	79.29 ± 0.82	87.31 ± 3.11	92.59 ± 0.00	90.57 ± 1.20
60	65.27 ± 0.20	70.28 ± 0.11	76.83 ± 1.57	77.00 ± 0.89
75	50.00 ± 0.22	55.81 ± 2.25	63.00 ± 2.33	65.67 ± 4.03

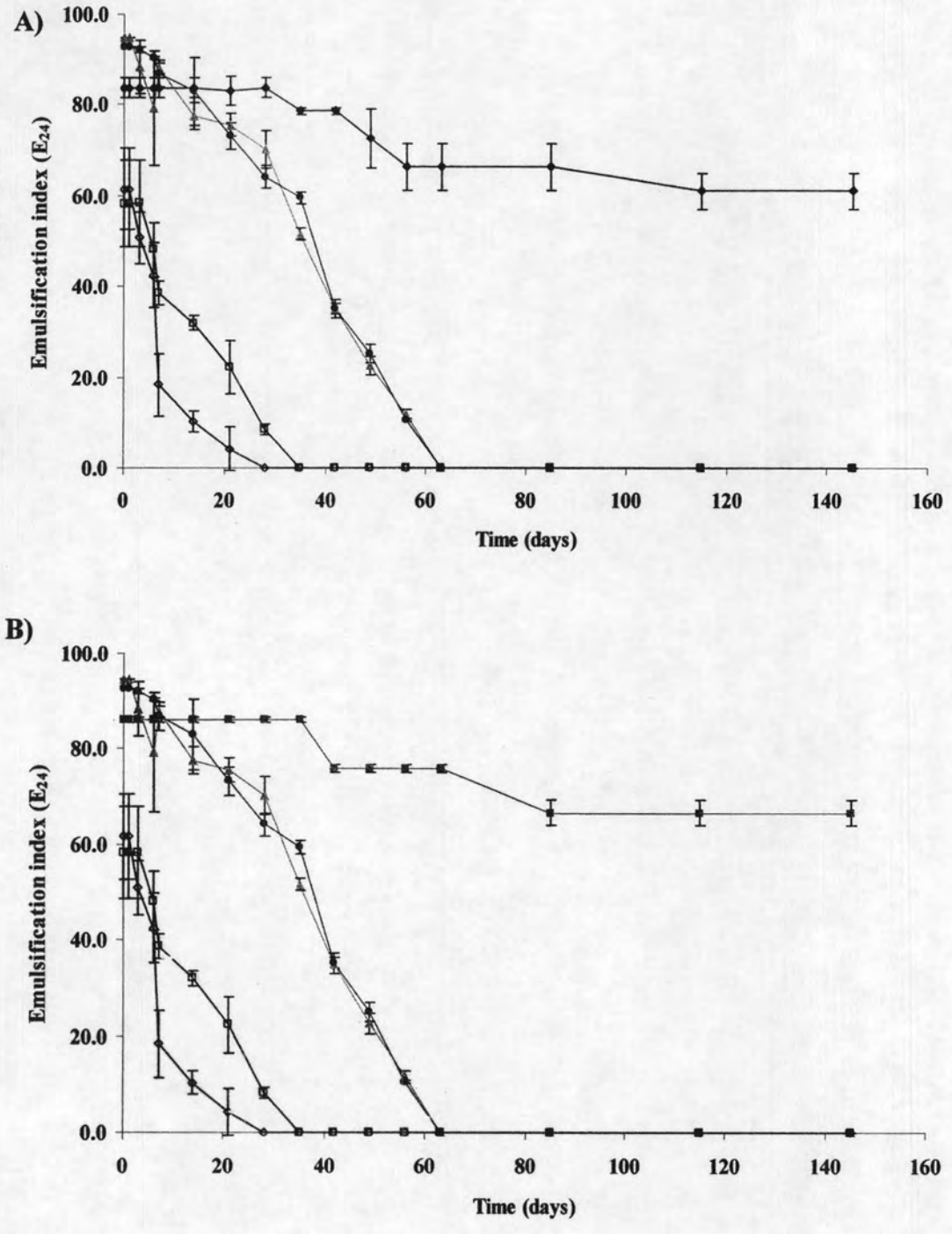


Figure 4.12 Stability of the emulsification index at 30°C of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 detected for the least one month which had the control as Tween80 (-▲-) and SDS (-●-); blank such as mineral salt medium supplemented with 2% wv^{-1} glucose (-◇-) and dH_2O (-□-).The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

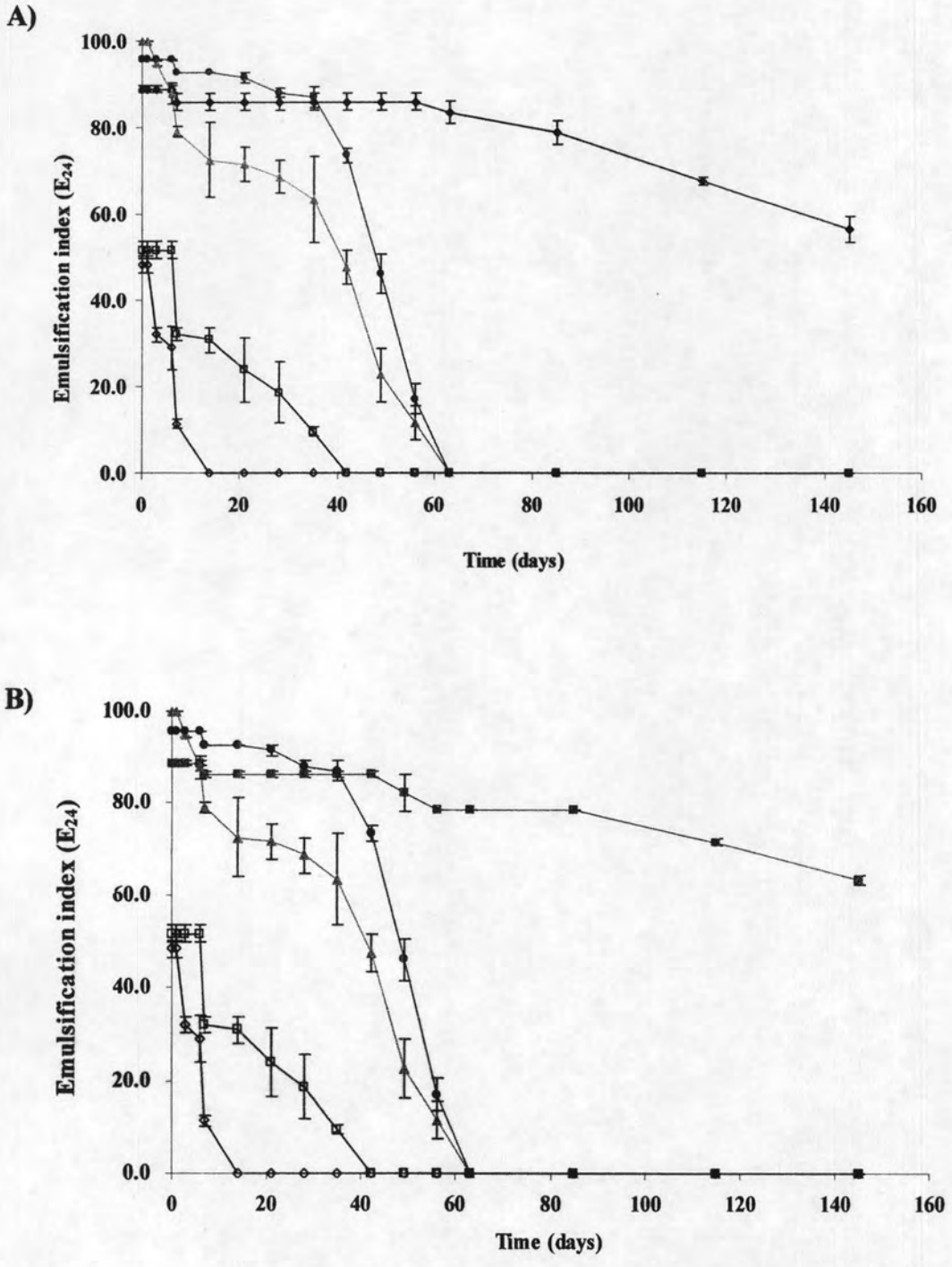


Figure 4.13 Stability of the emulsification index at 37°C of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 detected for the least one month which had the control as Tween80 (-▲-) and SDS (-●-); blank such as mineral salt medium supplemented with 2% wv^{-1} glucose (-◇-) and dH_2O (-□-).The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

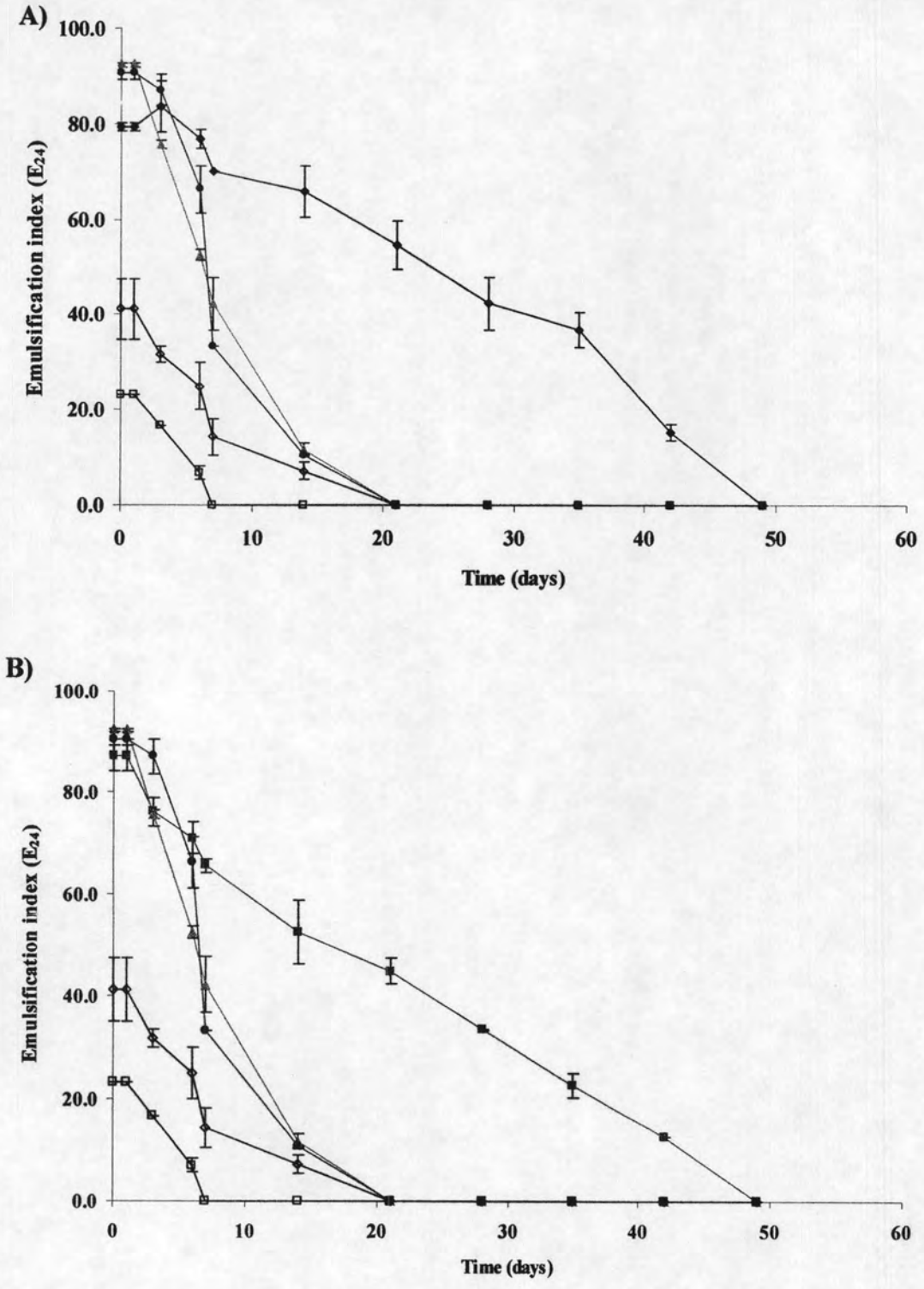


Figure 4.14 Stability of the emulsification index at 45°C of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 detected for the least one month which had the control as Tween80 (-▲-) and SDS (-●-); blank such as mineral salt medium supplemented with 2% wv^{-1} glucose (-◇-) and dH_2O (-□-).The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

4.2.4.3 Stability of biosurfactant at various of pH 2-12

Emulsification property and stability of biosurfactant at several of pH 2-12 produced by *Enterobacter* sp. P2 and *B. cepacia* P3 were determined using diesel oil. Partial purified of biosurfactant of *Enterobacter* sp. P2 and *B. cepacia* P3 showed emulsification index (E_{24}) of 94.87 ± 1.99% and 92.31 ± 3.44% at pH 7 which gave the best emulsion at 37°C. The activity of the biosurfactant was enhanced at pH of 6 and 7 nevertheless slight increase in the activity of the biosurfactants were detected at pH < 7 and pH > 7 (Figure 4.15-4.16). It was however noteworthy to mention that the biosurfactant retained approximately 50% of its original activity (E_{24} = 94.87 ± 1.99% and 92.31 ± 3.44%) at pH 4-9.

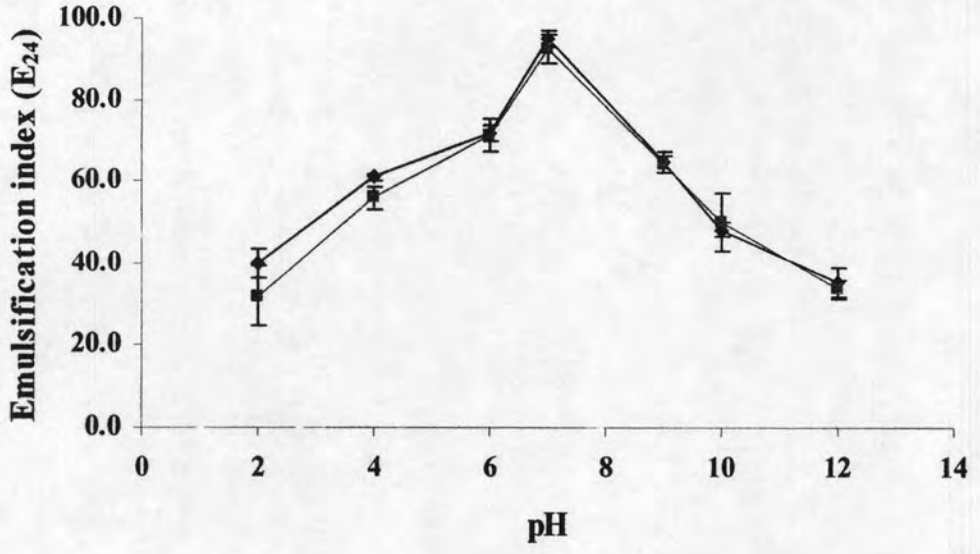


Figure 4.15 Effect of pH on activity of biosurfactant in *Enterobacter* sp. P2 (-•-) and *Burkholderia cepacia* P3 (-▪-). The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

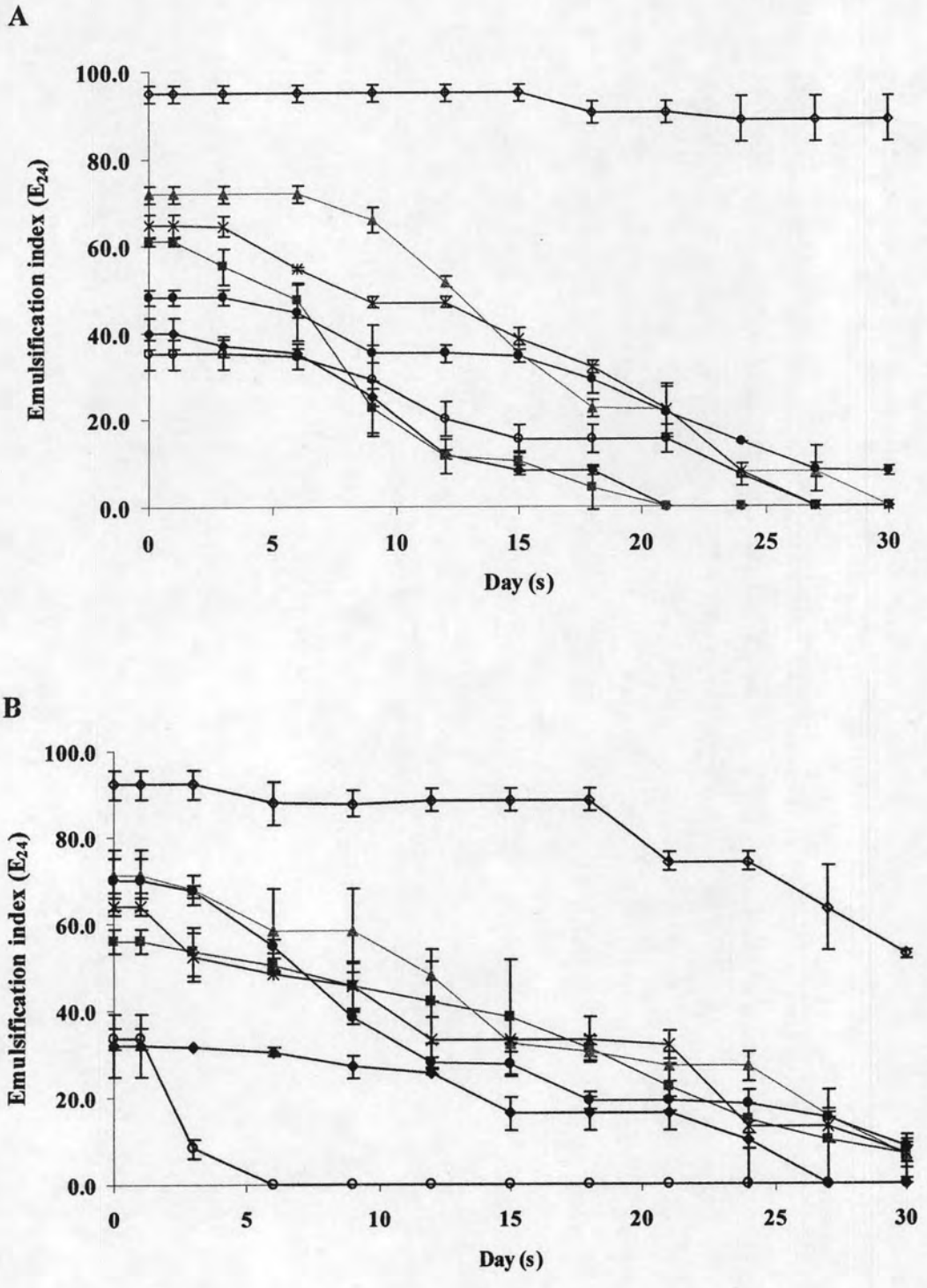
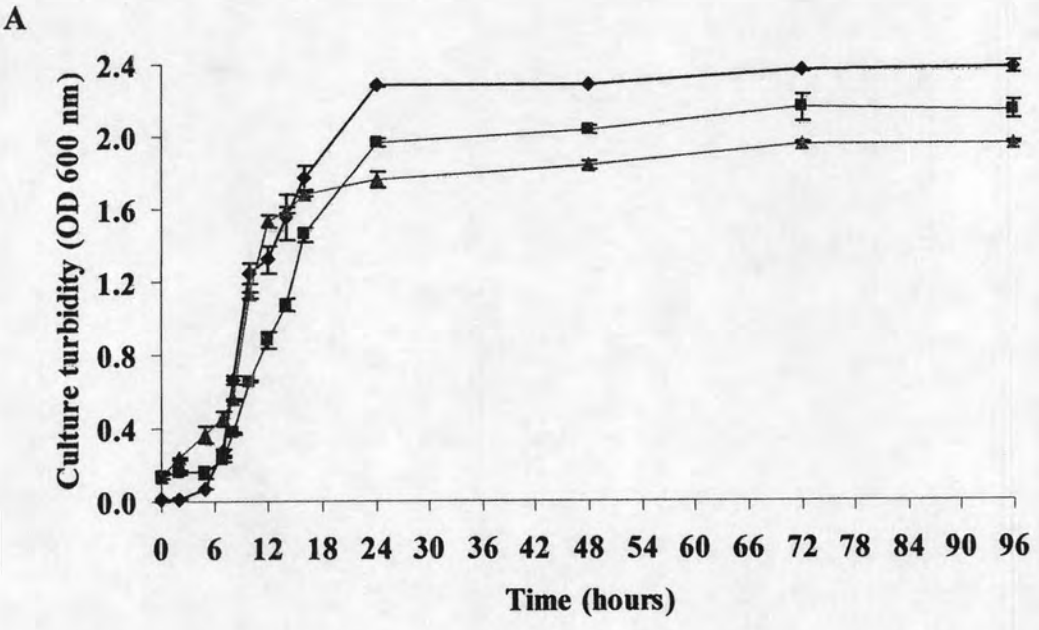


Figure 4.16 Stability of the emulsification index at various pH (A) of *Enterobacter* sp. P2 (B) and *B. cepacia* P3 detected for one month: pH 2 (—◆—), pH 4 (—■—), pH 6 (—▲—), pH 7 (—◇—), pH 9 (—✳—), pH 10 (—●—) and pH 12 (—○—). Each point represented the mean and standard deviation of triplicate samples.

4.2.5 Effect of biosurfactant-producing bacteria in Luria Bertani broth (LB) and nutrient broth (NB)

Furthermore, biosurfactant-producing bacteria were studied in LB and NB which replaced mineral salt medium (MSM) supplemented with 2% wv^{-1} glucose in order to compare the production of biosurfactant in bacteria. However, bacteria grown in LB and NB could produce a few biosurfactant as compared to the one grew in MSM (Figure 4.17). The results of the medium test revealed that the organism had good growth on LB and NB but had no effect on the production of biosurfactant (Figure 4.18). Detection of biosurfactant in the LB and NB occurred at the 72 hours. Then, the production slightly decreased were 12.44 ± 0.513 g glucose.l^{-1} and 13.95 ± 0.610 g glucose.l^{-1} in *Enterobacter* sp. P2; 26.85 ± 0.306 g glucose.l^{-1} and 27.14 ± 1.217 g glucose.l^{-1} in *B. cepacia* P3.



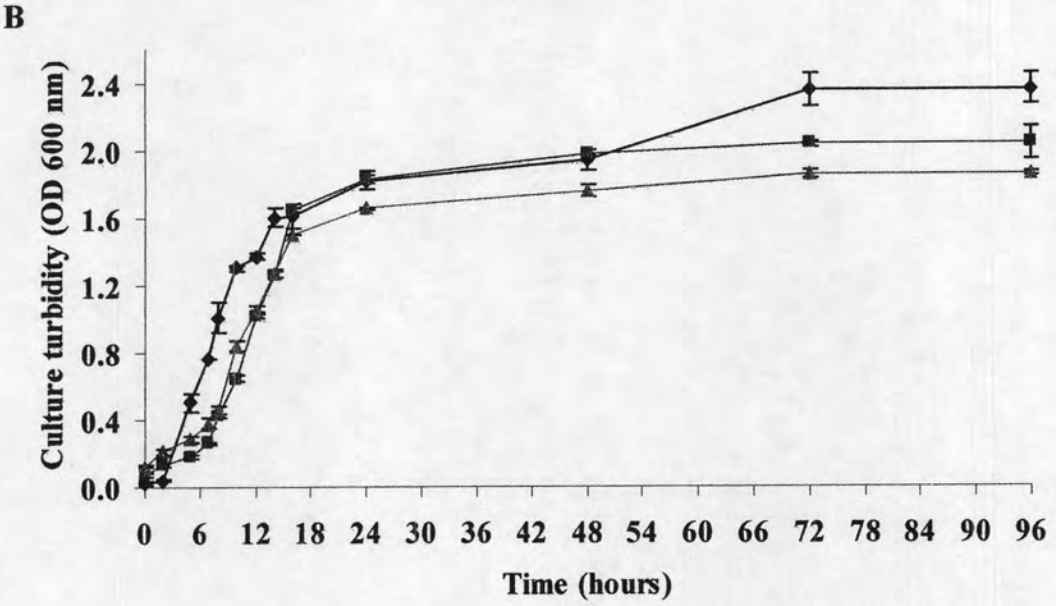


Figure 4.17 Growth (A) of *Enterobacter* sp. P2 (B) and *B. cepacia* P3 grown in mineral salt medium (MSM) containing 2% wv^{-1} glucose as carbon source: the control as MSM (—◆—), Luria Bertani broth (—■—) and nutrient broth (—▲—). Each point represented the mean and standard deviation of triplicate samples.

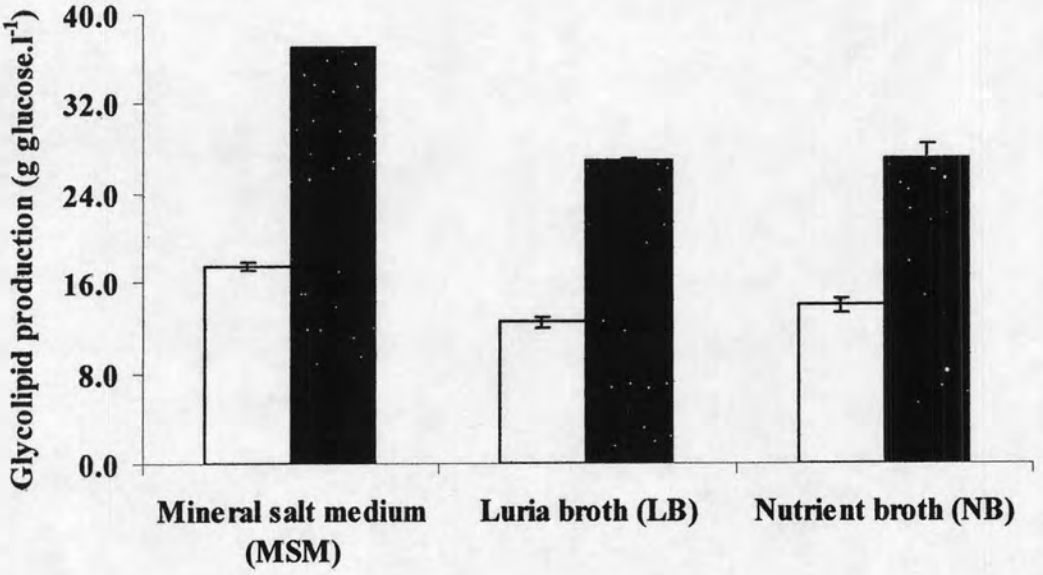


Figure 4.18 Glycolipid production of *Enterobacter* sp. P2 (□) and *B. cepacia* P3 (■) when cultivated in mineral salt medium supplemented with 2% wv^{-1} glucose, Luria Bertani broth (LB) and nutrient broth (NB). Each point represented the mean and standard deviation of triplicate samples.

4.3 Identification of the biosurfactant type

4.3.1 Thin-layer chromatography (TLC)

Samples were prepared for analysis of the sugar composition of biosurfactant (As described in Materials and Methods 3.4.3.1) before developed with TLC. In analysis of the sugar part on biosurfactant was one in the method in order to identify the type of biosurfactant. As well as, analysis of the sugar could verify the biosurfactant types.

The samples were developed with ethyl acetate/acetic acid/water (6:3:2). After air-drying, the chromatogram was sprayed with 0.2% wv^{-1} ninhydrin in acetone, followed by heating for detecting protein compounds. Carbohydrate components were located by the 5% vv^{-1} H_2SO_4 charring technique (As described in Materials and Methods 3.5.1). Glucose, mannose and rhamnose were used as standards which used 1 mM concentration and gave the R_f in Table 4.7. The partially purified biosurfactant of *P. aeruginosa* A102, *P. aeruginosa* A103, *Pseudomonas* sp. B202, *Enterobacter* sp. P2 and *B. cepacia* P3 gave sulfuric acid positive spots on silica TLC (Appendix D). Whereas, the samples spot from *Enterobacter* sp. P2 and *B. cepacia* P3 with the respective R_f values as 0.22 and 0.22 were detected and this value found on the same positive as 1 mM glucose did. The samples also showed positive reactions for sugars with α-napthol. But negative reaction for amino groups with 0.2% wv^{-1} ninhydrin in acetone and for ammonia or primary and secondary amines was found. These TLC analyses suggested and confirmed that the biosurfactant were a glycolipid composed of sugars as shown in Figure 4.19.

Table 4.7 Retardation factor (R_f) of sugar composition in biosurfactant detected by thin-layer chromatography (n=3).

Concentration of samples	Distance of solvent (cm.)	Distance of samples (cm.)	R_f (retardation factor)
1 mM glucose	5.0	1.1	0.23 ± 0.045
1 mM mannose	5.0	1.3	0.26 ± 0.018
1 mM rhamnose	5.0	2.3	0.45 ± 0.068
1.21 $\mu g.\mu l^{-1}$ *Enterobacter* sp. P2	5.0	1.1	0.23 ± 0.036
1.30 $\mu g.\mu l^{-1}$ *B. cepacia* P3	5.0	1.1	0.23 ± 0.045

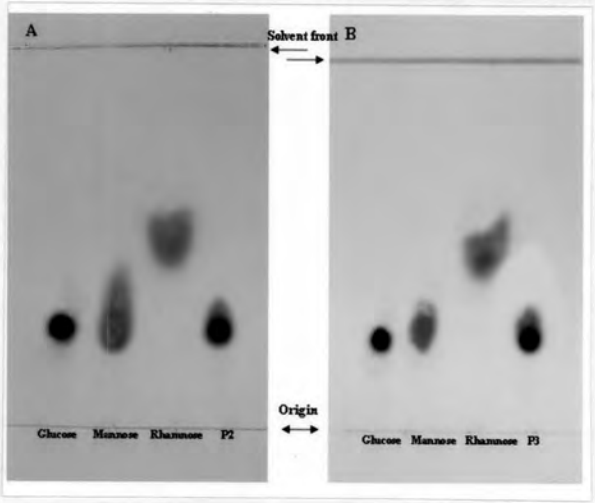


Figure 4.19 Thin layer chromatography analysis (A) of the partially purified biosurfactant of 1.21 $\mu g.\mu l^{-1}$ *Enterobacter* sp. P2 (B) of 1.30 $\mu g.\mu l^{-1}$ *B. cepacia* P3 which were 1 mM glucose, 1 mM mannose and 1 mM rhamnose as standard. Each sample (2 µl) was developed with solvent system (by volume): ethyl acetate/acetic acid/water (6:3:2). For detection of components, the plate was sprayed with 5% vv^{-1} H_2SO_4 in dH_2O and heated at 110°C for 20 minutes.

4.3.2 Nuclear magnetic resonance (NMR)

Enterobacter sp. P2 and *B. cepacia* P3 grew in a chemically defined mineral salt medium containing 2% wv^{-1} glucose as source of carbon and energy as well. Partial purified biosurfactant from *Enterobacter* sp. P2 and *B. cepacia* P3 were analyzed for chemical structure by NMR. Figures 4.20 and 4.21 showed the presence of methylene protons, not a methyne proton, with the relatively high δ values at 4.10 and 4.18, respectively, which indicated. That the glycerol moiety had a substituent at -OH group attached to C_1 as compared to the glycolipid components (Table 4.8). The singlet at 2.17 ppm from the *Enterobacter* sp. P2 and *B. cepacia* product pointed to the presence of an acetyl substituent, probably attached to a sugar moiety as previously reports (Shirley W. Hunter and Patrick J. Brennan, 1981). NMR clarified the component structure of biosurfactant. In addition, the constituents $-OCH_3$ groups in the range 3.68 to 4.0 ppm indicated from *Enterobacter* sp. P2 and *B. cepacia* P3 were detected which indicated that they were glycolipids.

Table 4.8 ^{1}H-NMR chemical shift data for glycolipid components (Sim *et al.*, 1997)

Aglycon	- CH_3	$-CH_2-$ CH_3	$-CH_2-CH_2-$ CH_3	$-CH_2-$ COO-	-COO- CH_2-	-O-C- H
More mobile component	0.88	1.21-1.33	1.60	2.63	5.38	4.50
Less mobile component	0.88	1.21-1.40	1.67	2.46	5.32	4.21
Sugar	1'-H	CH_5	2'-,3'-,5'-H	4'-H		
More mobile component	4.92	1.28	3.68-3.82	3.40		
Less mobile component	4.90	1.26	3.68-3.88	3.45		
	4.75			3.42		

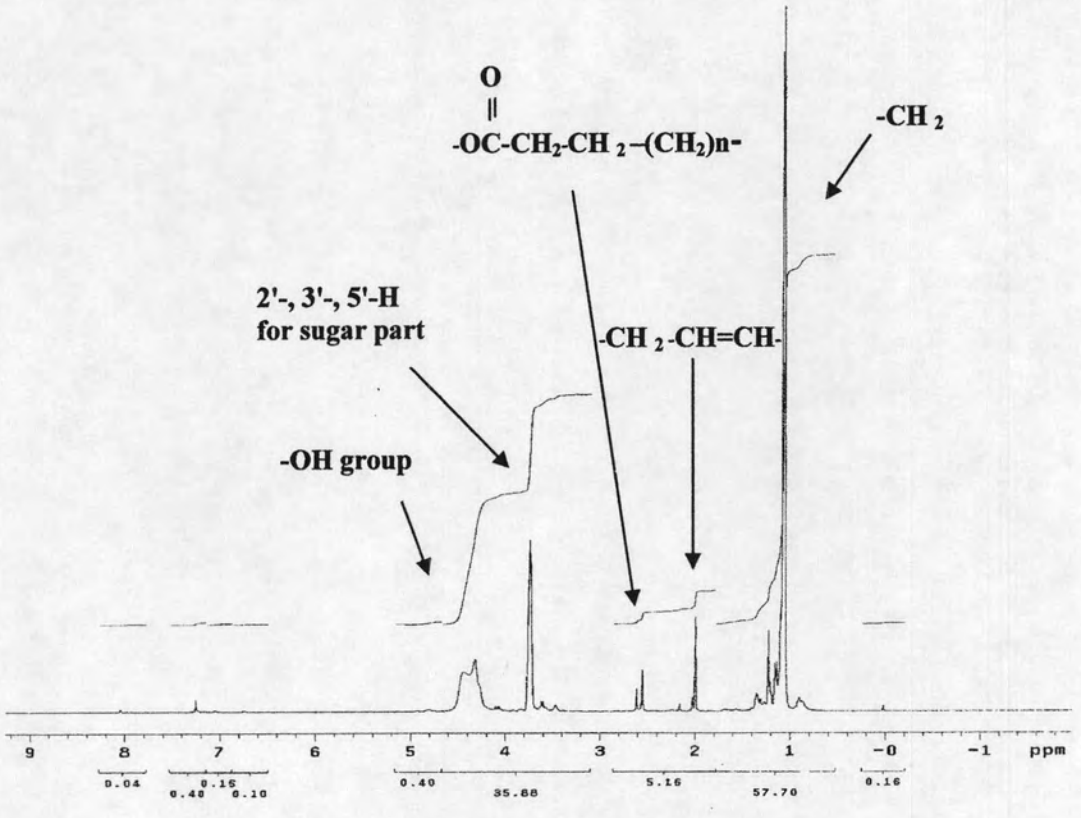


Figure 4.20 ^{1}H-NMR peak of *Enterobacter* sp. P2 in $CDCl_3$

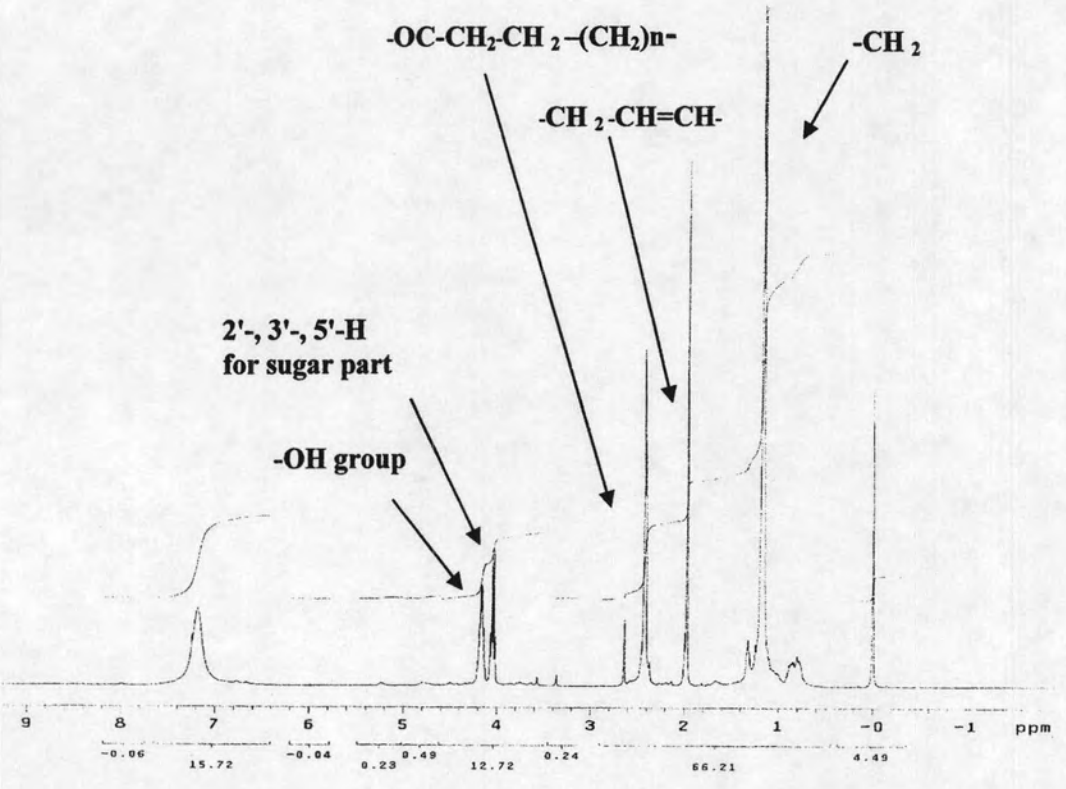


Figure 4.21 ^{1}H-NMR peak of *Burkholderia cepacia* P3 in $CDCl_3$

4.3.3 Fourier-transformed infrared spectroscopy (FTIR)

FTIR is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture (Rodrigues *et al.*, 2006). The FTIR spectra of both the partial purified biosurfactants of *Enterobacter* sp. P2 and *B. cepacia* P3 were shown in Figure 4.12-4.13. They had mostly similar absorption bands for example the point 1-4 in the Figure 4.22-4.23. The broad band at 3,433 cm^{-1} corresponded to the O-H stretch (Silverstaein and Webster, 1998). The asymmetrical stretching ($\nu_{as}CH_2$) and symmetrical stretching (ν_sCH_2) of methylene occurred at 2,926 and 2,857 cm^{-1}, respectively. The 1,624 cm^{-1} band was from stretching of the unsaturated C-C bonds. Lactones and esters had two strong absorption bands arising from C=O and C-O stretching. The C=O absorption band at 1,744 cm^{-1} may include contributions from that of lactones, esters, or acids. The stretch of C=O band of C(=O)-O-C in lactones appears at 1,157 cm^{-1}, while that from the acetyl esters at 1,247 cm^{-1}. Sugar C-O stretch of C-O-H groups was at 1,048 cm^{-1} (Mantsch and Chapman, 1996). The primary same between the spectra of the *Enterobacter* sp. P2 and *B. cepacia* P3 were the band at 1,448 and 1,423 cm^{-1} that corresponds to the C-O-H in-plane bending of carboxylic acid (–COOH) (Silverstaein and Webster, 1998). Next, at the spectra 3,390 and 3,366 cm^{-1} of the *Enterobacter* sp. P2 and *B. cepacia* P3, respectively showed O-H stretch of structure. The infrared spectra from both *Enterobacter* sp. P2 and *B. cepacia* P3 samples indicated that their products resemble the biosurfactant structures.

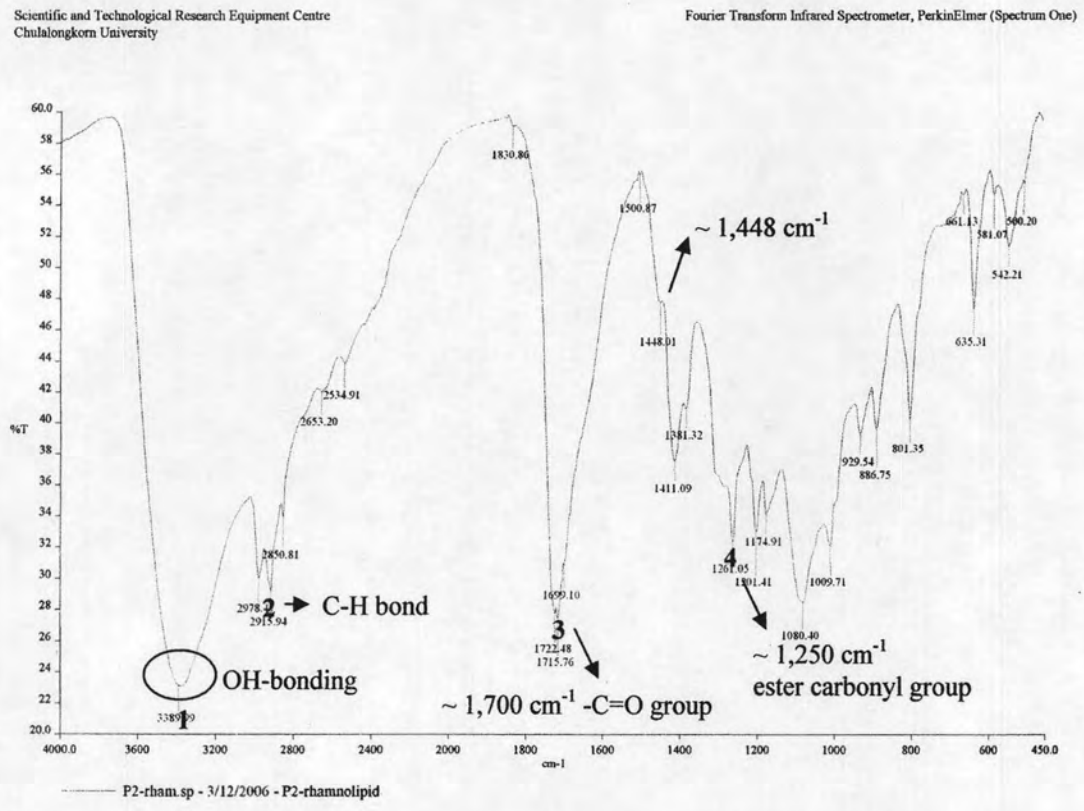


Figure 4.22 Fourier transform infrared absorption spectra of glycolipid biosurfactant obtained from *Enterobacter* sp. P2.

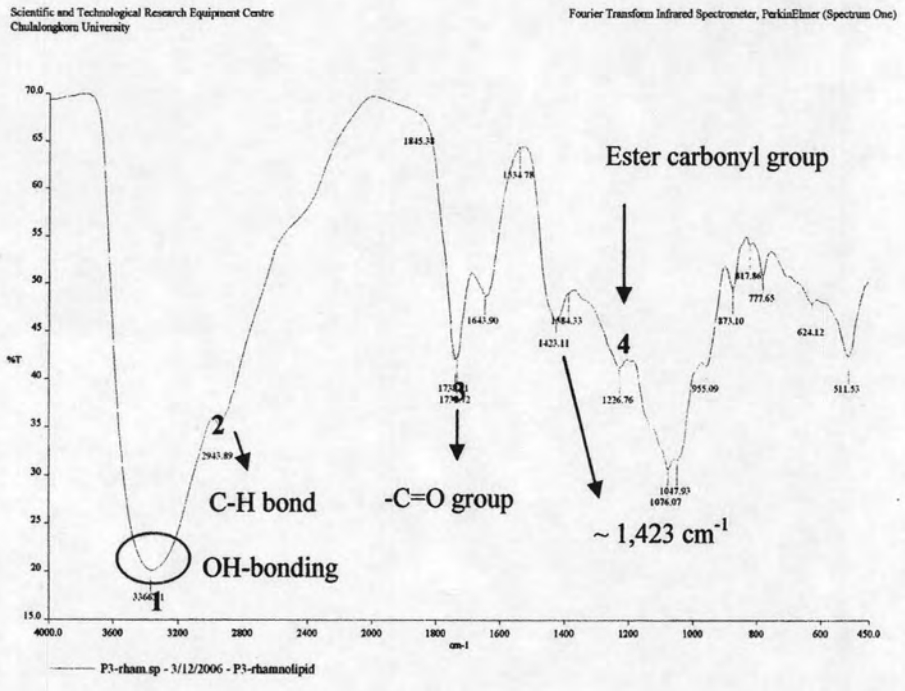


Figure 4.23 Fourier transform infrared absorption spectra of glycolipid biosurfactant obtained from *B. cepacia* P3.

4.3.4 Mass spectrometry (MS)

The partially purified biosurfactants of *Enterobacter* sp. P2 (Figure 4.24) and *B. cepacia* P3 (Figure 4.25) were reconfirmed for molecular weight determination by MS. The results of *Enterobacter* sp. P2 and *B. cepacia* P3 samples showed the negative-ion mass spectrum comprising one major band m/z 550.409 and m/z 550.363, respectively. The molecular mass of the main compound of the preparation was 551.409 Da. and 551.363 Da, consecutively. In agreement with the NMR spectroscopy data, molecular weight of *Enterobacter* sp. P2 and *B. cepacia* P3 products were found to approach the molecular weight of glycolipid which represented as the low molecular weight biosurfactant (Rosenberg and Ron, 1999). Our reports agreed with the previous reports in the literatures.

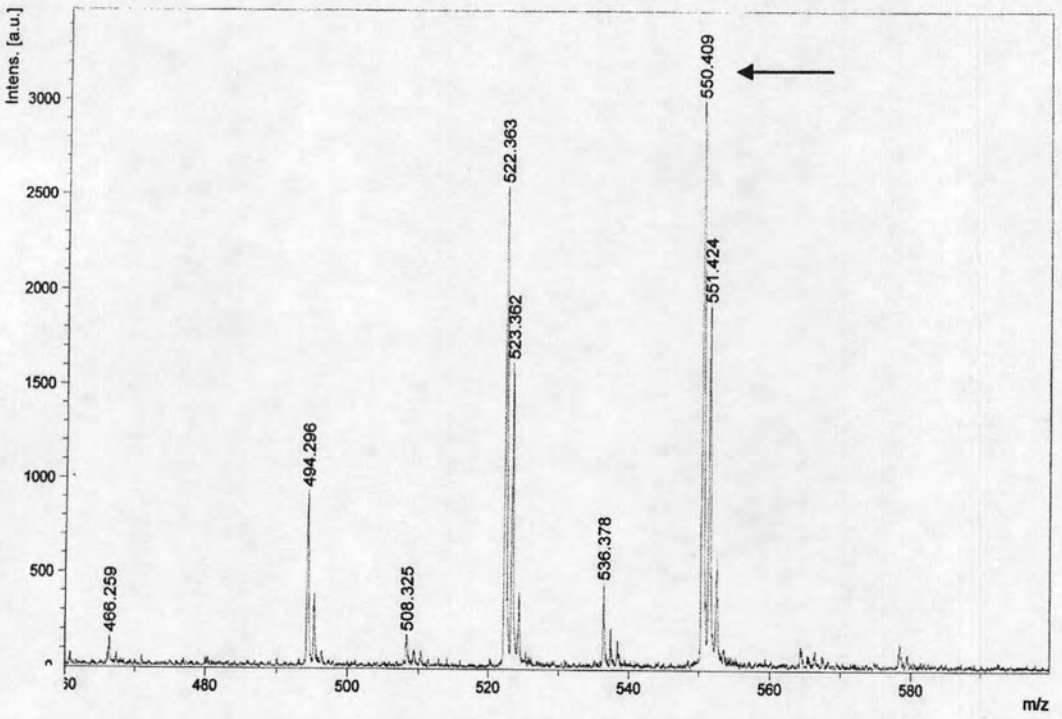


Figure 4.24 Mass spectrometry spectrograms of partially purified biosurfactant from *Enterobacter* sp. P2 cultured broth.

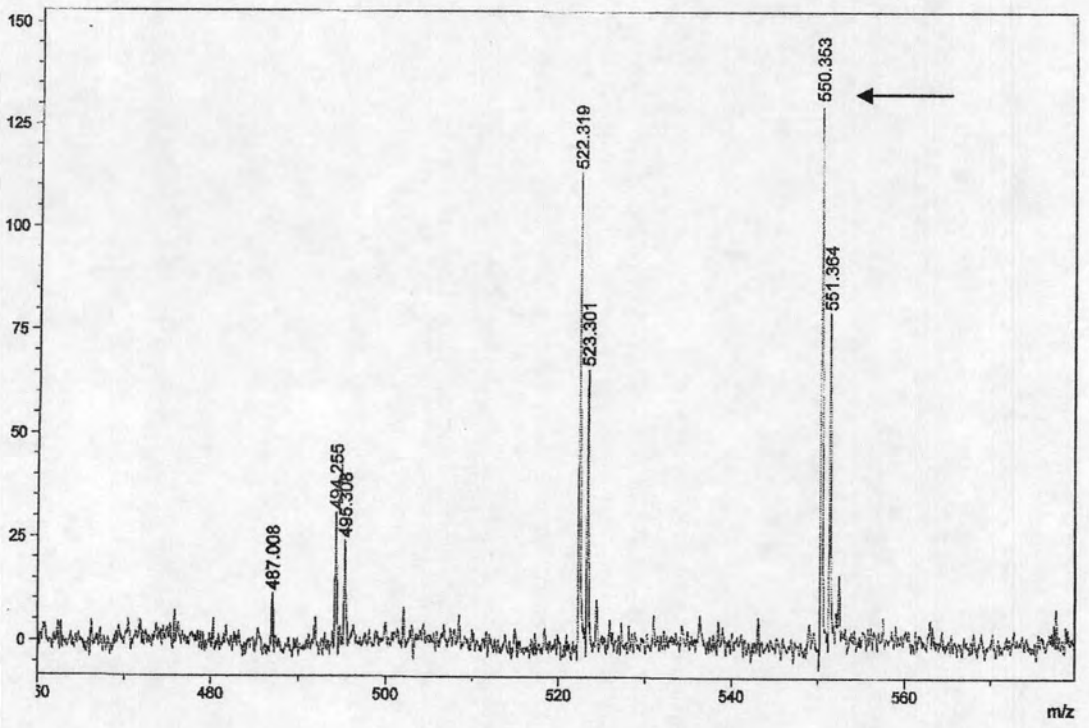


Figure 4.25 Mass spectrometry spectrograms of partially purified biosurfactant from *B. cepacia* P3 cultured broth.

4.4 Effect of carbon or/and nitrogen source on biosurfactant production

There were several nutritional and environmental factors for example carbon, nitrogen, pH, temperature and salinity (Guerra-Santos *et al.*, 1986) affected biosurfactant production and should be considered. Water-soluble carbon sources such as glucose, and vegetable oils were all used for glycolipid production by *Pseudomonas* sp. (Guerra-Santos *et al.*, 1984). Numerous reports revealed that the type of carbon substrates markedly affected the production yield of glycolipid (Lang and Wullbrandt, 1999). The major types of carbon sources used for glycolipid production were carbohydrate (Maier and Soberon-Chavez, 2000). In this work, three carbon sources, including glucose, maltose and sucrose were examined for their effectiveness on glycolipid production.

Previous report showed that nitrate is more effective in the production of glycolipids than ammonia and urea, which is in agreement with other studies reported in the literatures (Syldatk *et al.*, 1985; Ochsner and Reiser, 1995; Arino *et al.*, 1996). Thus, the nitrogen sources i.e. $NaNO_3$, $(NH_4)_2SO_4$ and CH_4N_2O were used in this study. However, biosurfactant production in certain *Pseudomonas* species can be enhanced when cells are grown under low nitrogen conditions (Guerra-Santos *et al.*, 1986).

Enterobacter sp. P2 and *B. cepacia* P3 grew well in mineral salt medium containing 11.1 mM (2% wv^{-1}) glucose as sole carbon source. The performance of glycolipid production with different carbon sources were depicted in Figure 4.26-4.28. It showed a general trend that glycolipid production initially decreased with increasing carbon substrate concentration. Moreover, the growth of *Enterobacter* sp. P2 and *B. cepacia* P3 completely terminated when increasing concentration of carbon source, resulting in negligible glycolipid production in the culture. The significant

inhibitory effect of increasing concentration of carbon source had not yet been revealed in the literature. The 2% wv^{-1} concentration of glucose was found to be optimum for biosurfactant formation. *Enterobacter* sp. P2 and *B. cepacia* P3 used in this study was able to utilize glucose, maltose, and sucrose for biosurfactant production.

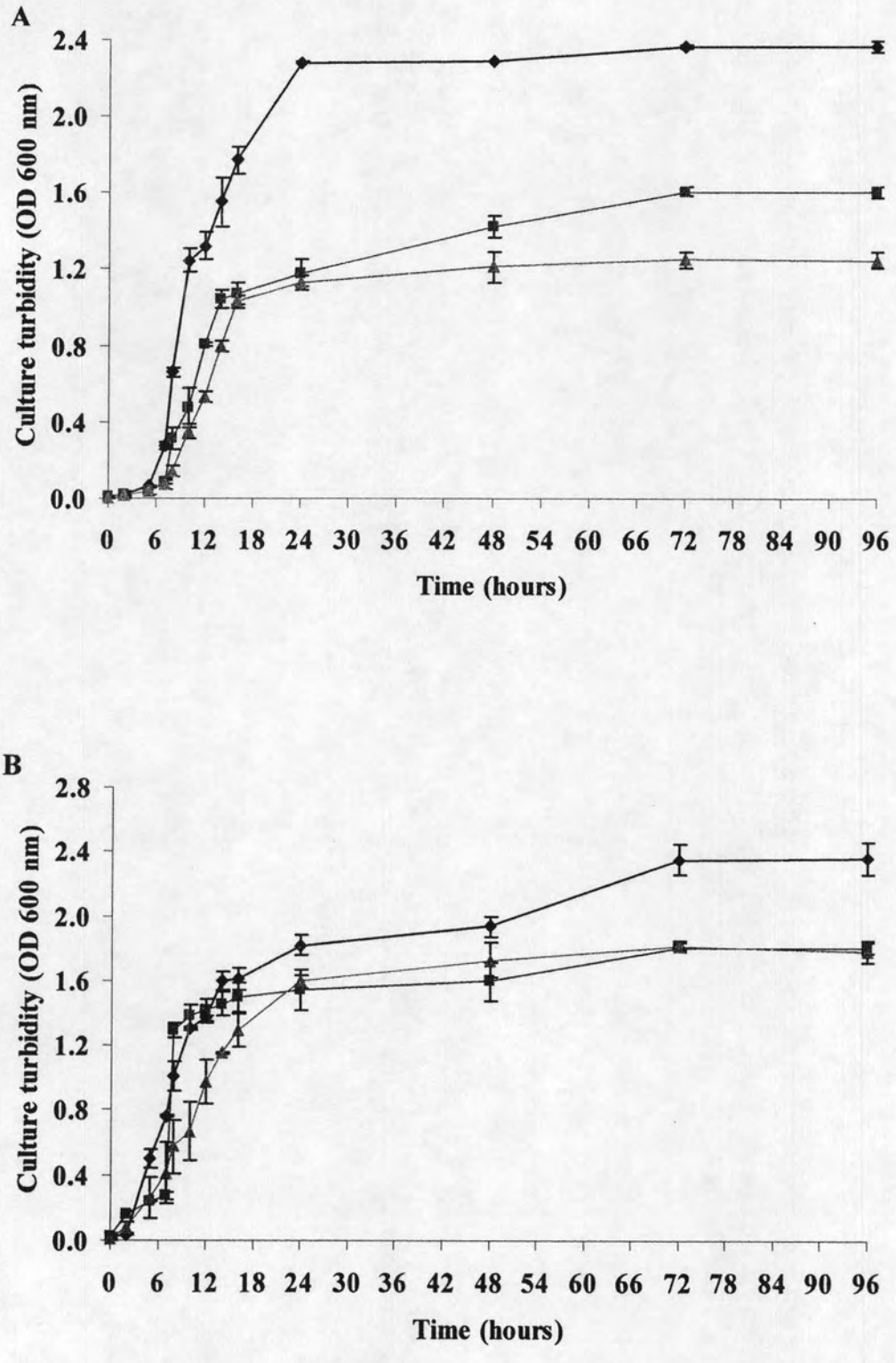


Figure 4.26 Growth (A) of *Enterobacter* sp. P2 (B) and *B. cepacia* P3 grown in mineral salt medium with 2% wv^{-1} carbon sources: glucose (—◆—), maltose (—■—) and sucrose (—▲—). Each point represented the mean and standard deviation of triplicate samples.

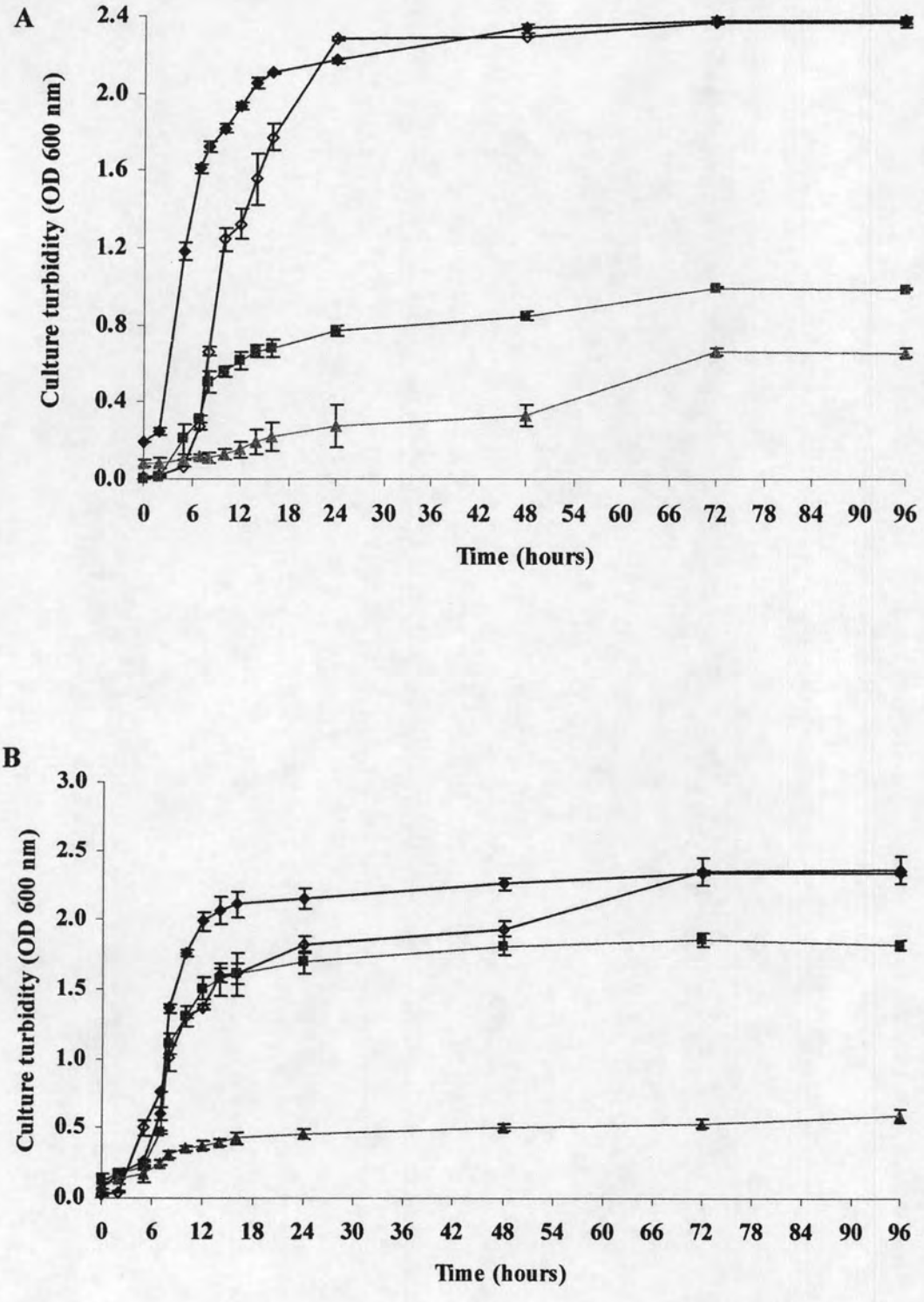


Figure 4.27 Growth of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 grown in mineral salt medium with 8% wv^{-1} (44.4 mM) carbon sources: supplemented with glucose (—◆—), maltose (—■—) and sucrose (—▲—) and control (—◇—). The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

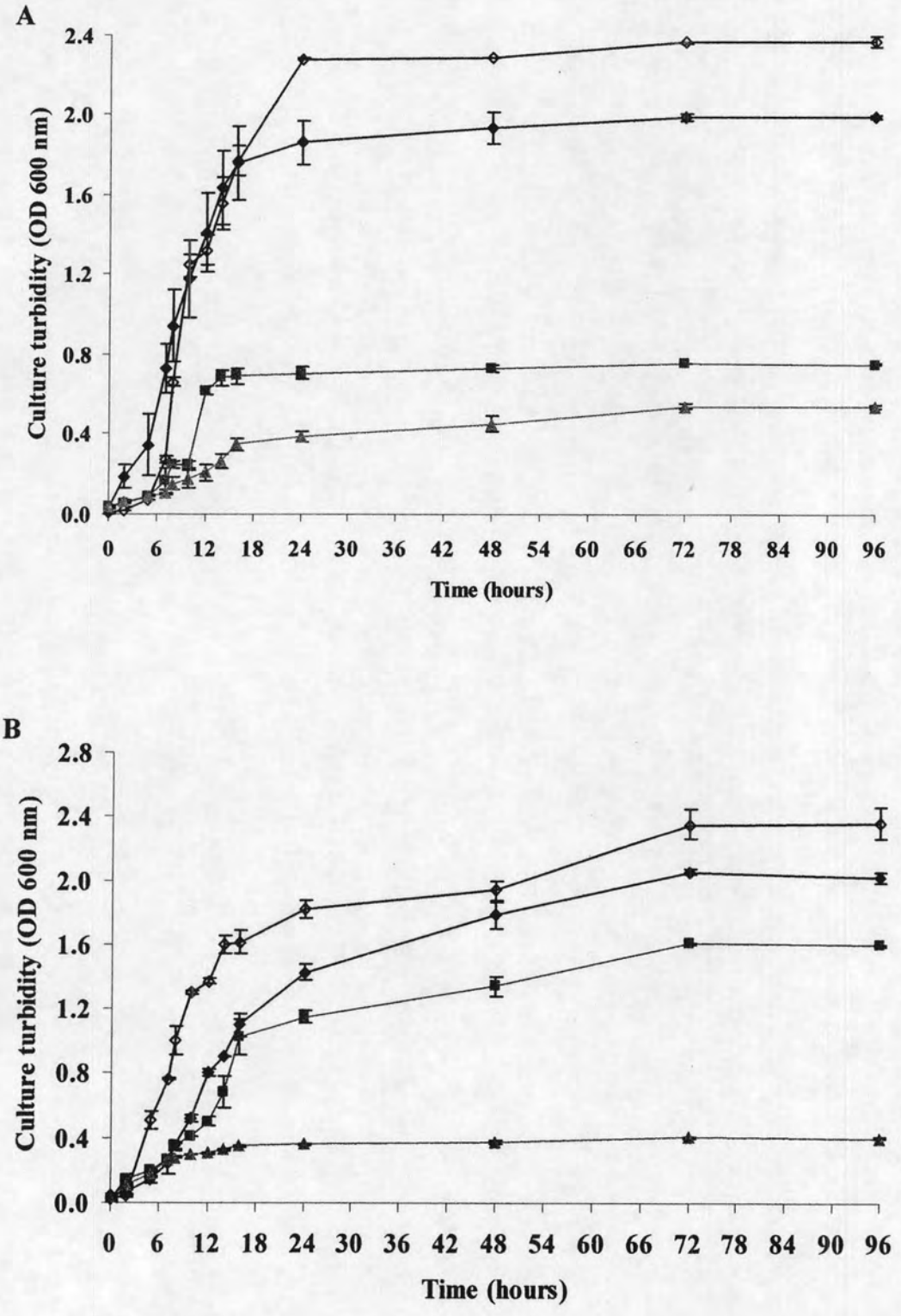
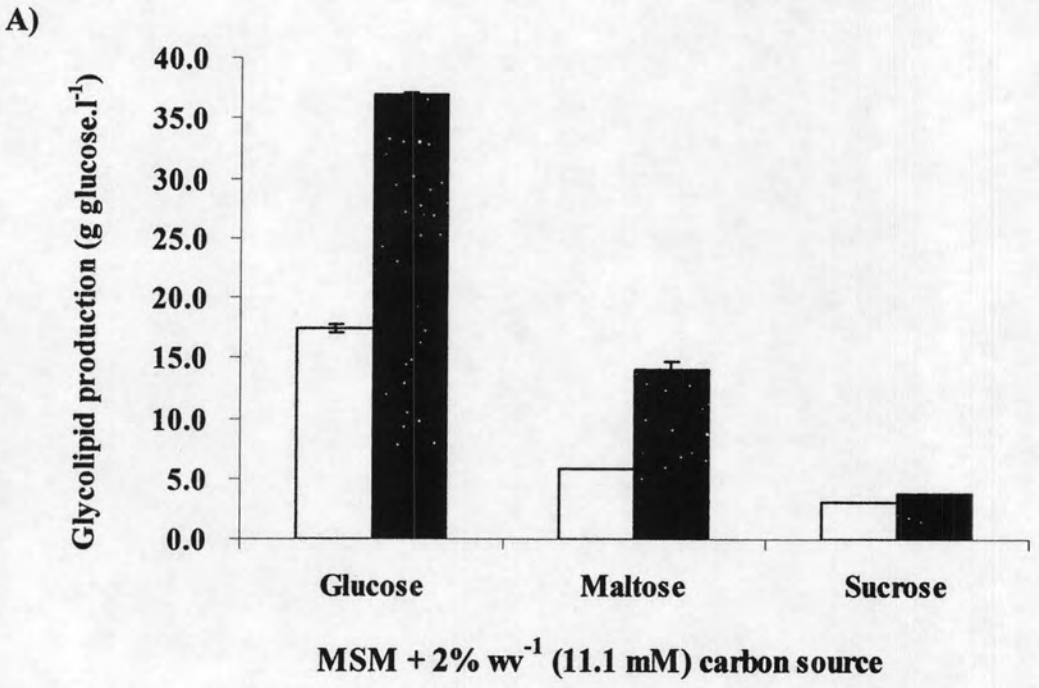


Figure 4.28 Growth of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 grown in mineral salt medium with 15% wv^{-1} (83.3 mM) carbon sources: supplemented with glucose (—◆—), maltose (—■—) and sucrose (—▲—) and control (—◇—). The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

Furthermore, the effect of different carbon sources on the glycolipid production was exhibited in Figure 4.29. *Enterobacter* sp. P2 and *B. cepacia* P3 were grown on each of three carbon sources with various concentrations e.g. 11.1 mM (2% wv^{-1}), 44.4 mM (8% wv^{-1}) and 83.3 mM (15% wv^{-1}). The results showed that *Enterobacter* sp. P2 and *B. cepacia* P3 were able to grow in medium containing any of the three substrates used. Biosurfactant was not synthesized at the high concentration condition of adding (15% wv^{-1}) 83.3 mM of either glucose or maltose, and sucrose as the carbon sources.



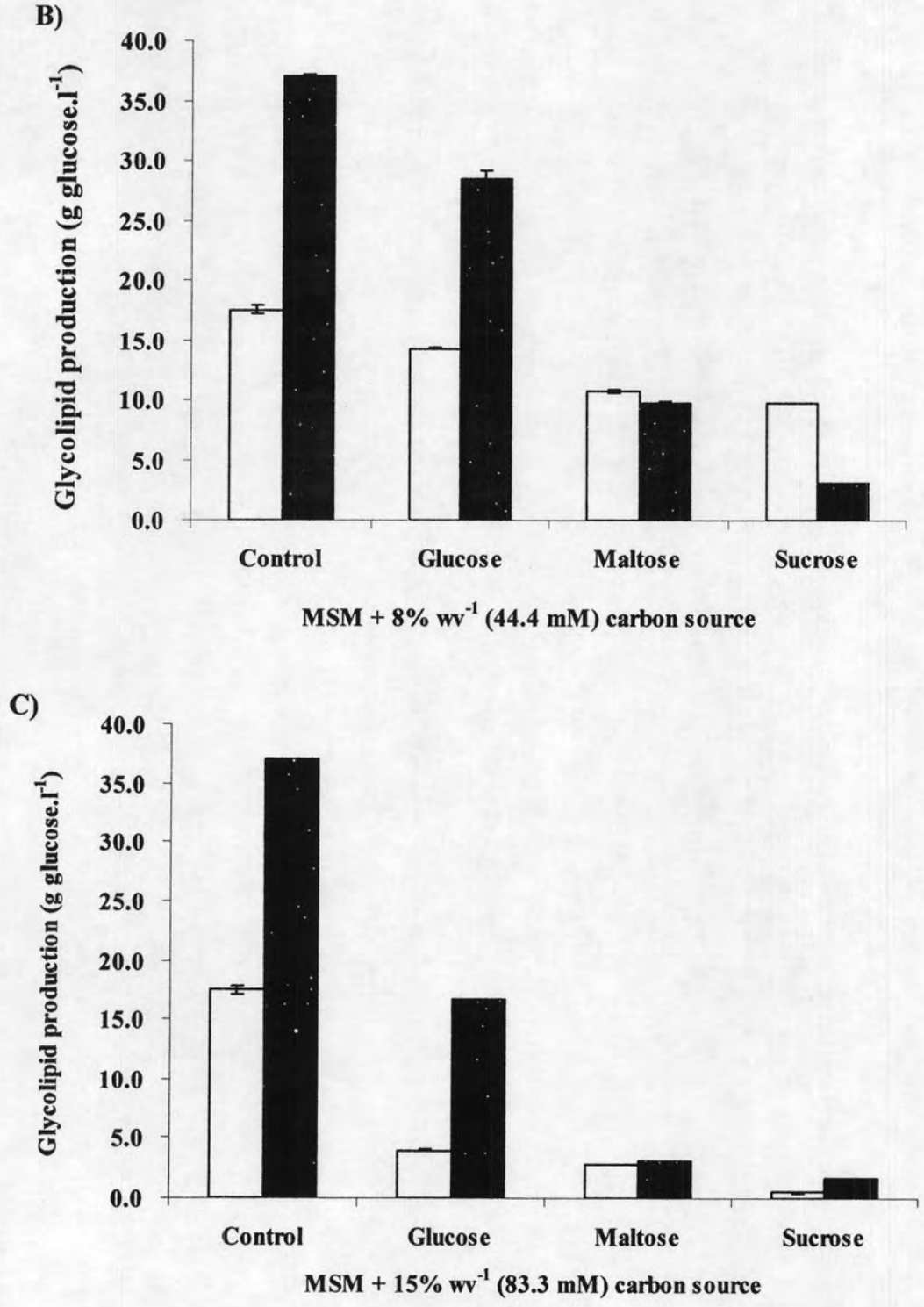


Figure 4.29 Effect of the type and the concentration of carbon source on glycolipid production (g glucose.l^{-1}) in batch culture of *Enterobacter* sp. P2 (□) and *B. cepacia* P3 (■) in MSM containing different carbon sources at 37°C and an agitation rate of 250 rpm; (A) 2% wv^{-1} carbon sources; (B) 8% wv^{-1} carbon sources and (C) 15% wv^{-1} carbon sources. The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

For evaluation of the appropriate nitrogen sources for the growth of *Enterobacter* sp. P2 and *B. cepacia* P3 were studied. Figure 4.30-4.32 showed that $NaNO_3$ affected the growth of broth isolates better than these from the $(NH_4)_2SO_4$ and CH_4N_2O. The best nitrogen concentration use was 0.5% wv^{-1}.

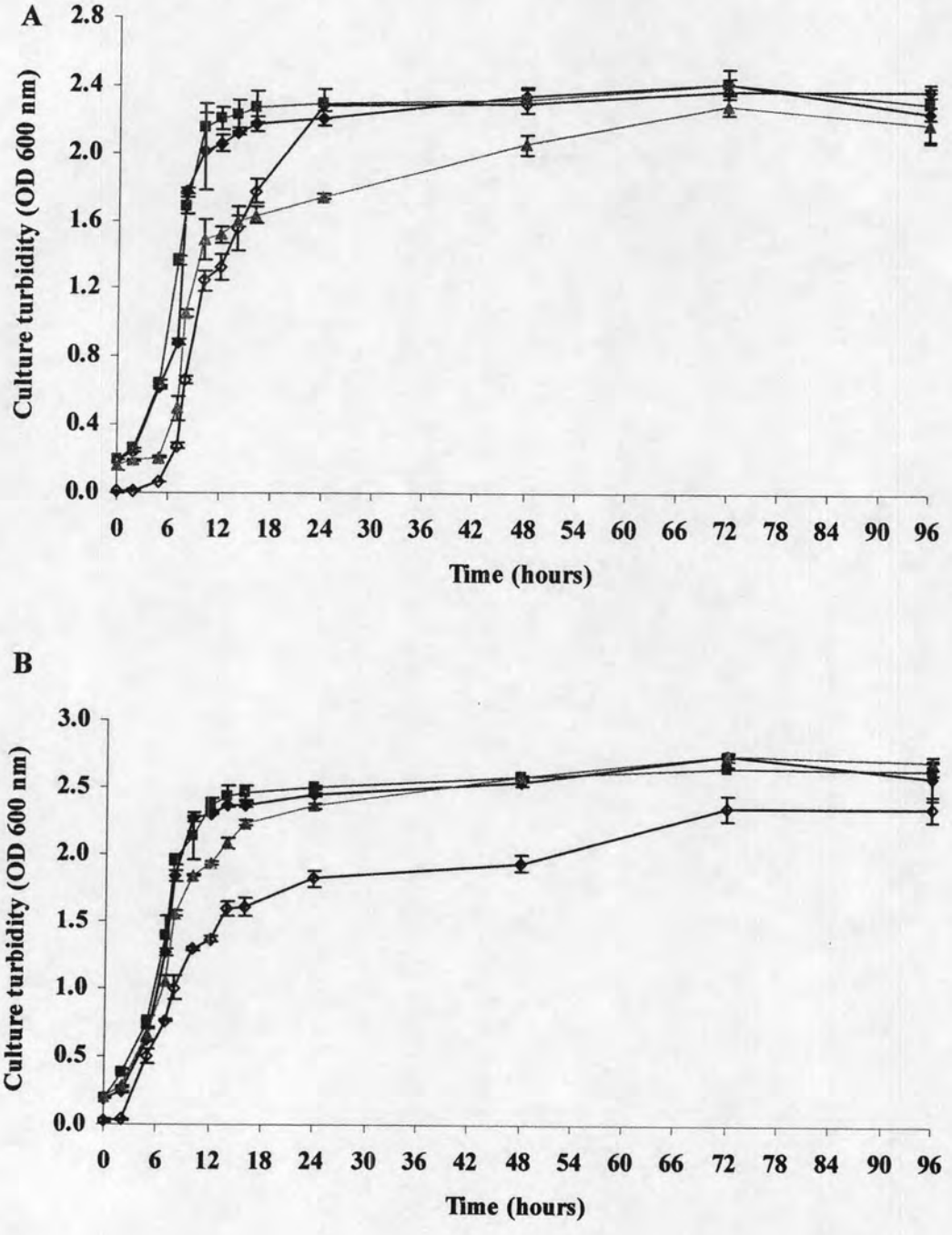


Figure 4.30 Growth of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 which was grown in mineral salt medium with 2% wv^{-1} glucose as carbon source supplemented with additional $NaNO_3$ as nitrogen source: 15 mM $NaNO_3$ (-◆-), 75 mM $NaNO_3$ (-■-),150 mM $NaNO_3$ (-▲-), 150 mM $NaNO_3$ and without adding the $NaNO_3$ as control (-◇-). Each point represented the mean and standard deviation of triplicate samples.

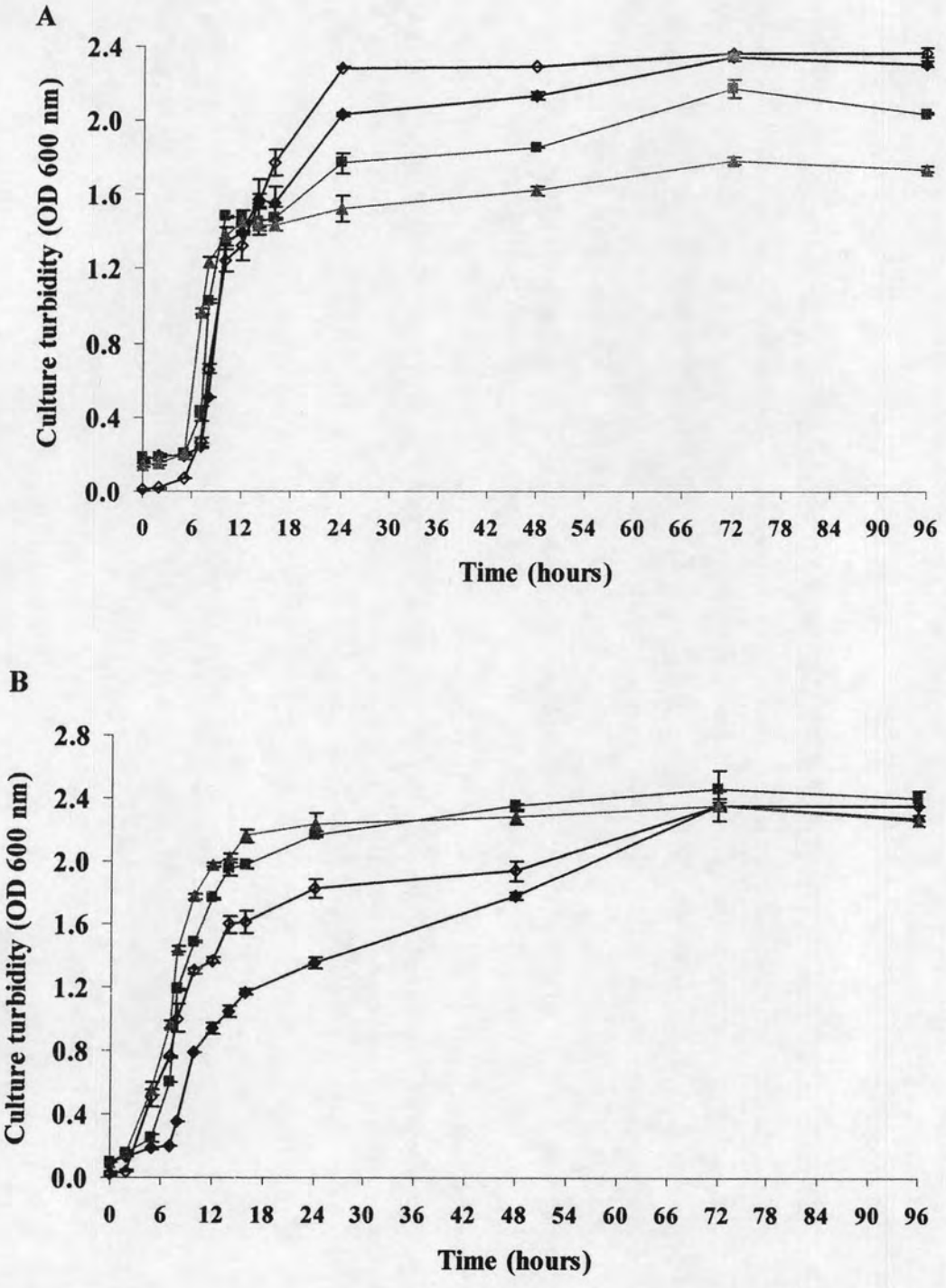


Figure 4.31 Growth (A) of *Enterobacter* sp. P2 (B) and *B. cepacia* P3 grown in mineral salt medium with 2% wv^{-1} glucose as carbon source supplemented with additional $(NH_4)_2SO_4$ as nitrogen source: 15 mM $(NH_4)_2SO_4$ (—◆—), 75 mM $(NH_4)_2SO_4$ (—■—), 150 mM $(NH_4)_2SO_4$ (—▲—), 150 mM $(NH_4)_2SO_4$ and without adding the $(NH_4)_2SO_4$ as control (—◇—). Each point represented the mean and standard deviation of triplicate samples.

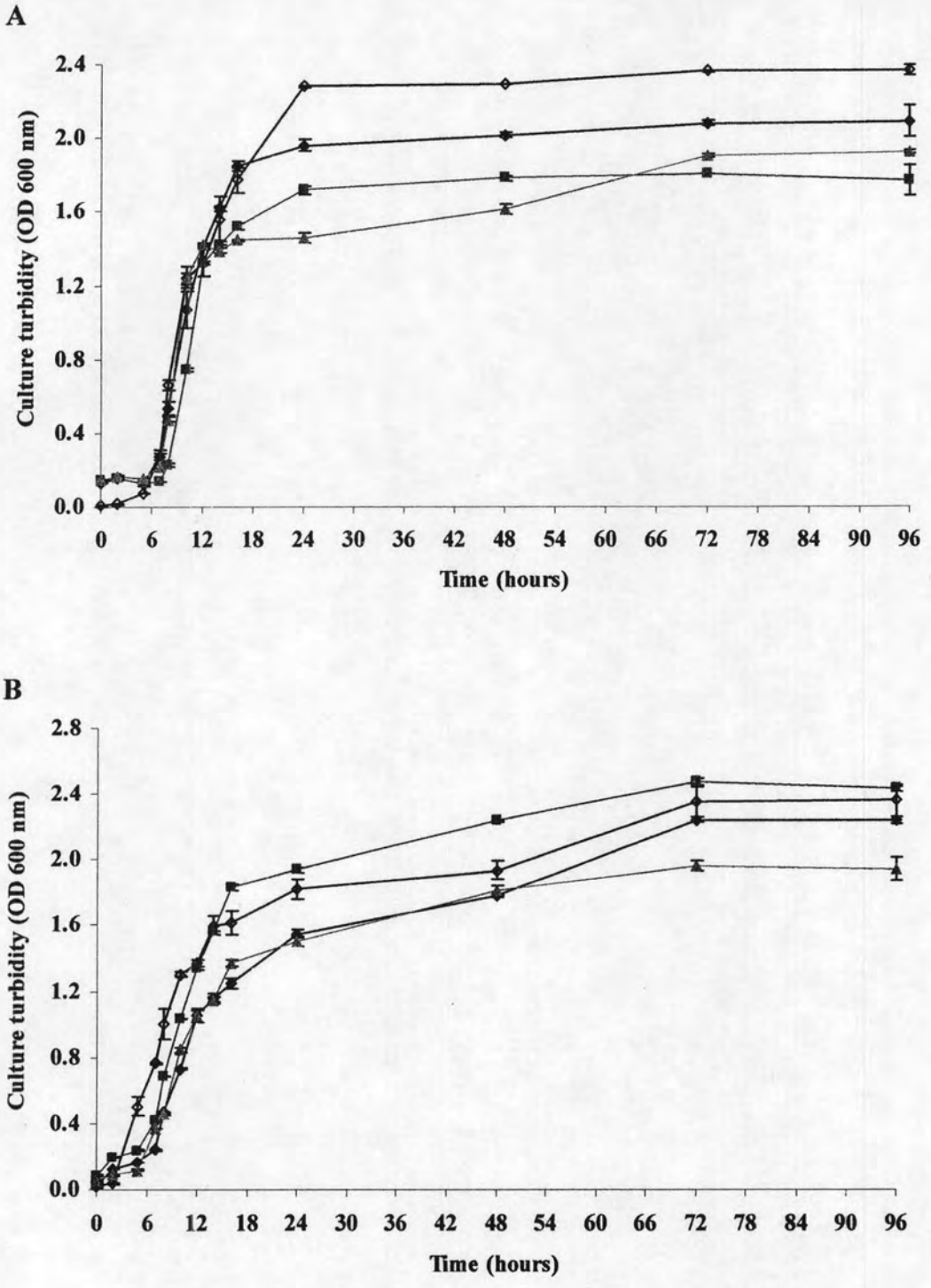
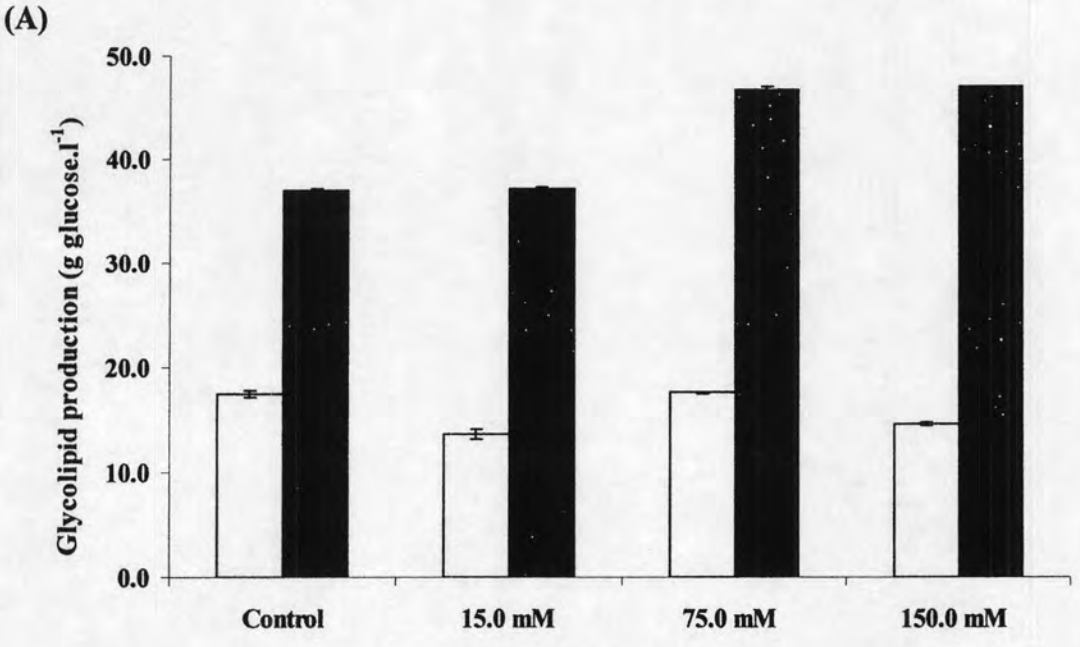


Figure 4.32 Growth of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 grown in mineral salt medium with 2% wv^{-1} glucose as carbon source supplemented with additional CH_4N_2O as nitrogen source: 15 mM CH_4N_2O (—◆—), 75 mM CH_4N_2O (—■—),150 mM CH_4N_2O (—▲—), 150 mM CH_4N_2O and without adding the CH_4N_2O as control (—◇—). Each point represented the mean and standard deviation of triplicate samples.

The nitrogen source affected biosurfactant production as depicted in Figure 4.33 at the end of 72 hours of cultivation. The use of 75.0 mM (0.5% wv^{-1}) $NaNO_3$ caused an increase in the production of glycolipid from 17.49 ± 0.338 g glucose.l^{-1} (in regular medium) to 17.58 ± 0.044 g glucose.l^{-1} (in regular medium + 0.5% wv^{-1} $NaNO_3$) in *Enterobacter* sp. P2; and from 37.01 ± 0.118 g glucose.l^{-1} (in regular medium) to 46.61 ± 0.414 g glucose.l^{-1} (in regular medium + 0.5% wv^{-1} $NaNO_3$) in *B. cepacia* P3. *Enterobacter* sp. P2 and *B. cepacia* P3 were able to use nitrogen sources such as ammonia or nitrate. To obtain high concentrations of glycolipids, it is necessary to have restrained conditions of this macronutrient. Our studies showed that nitrate was more effective in 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).



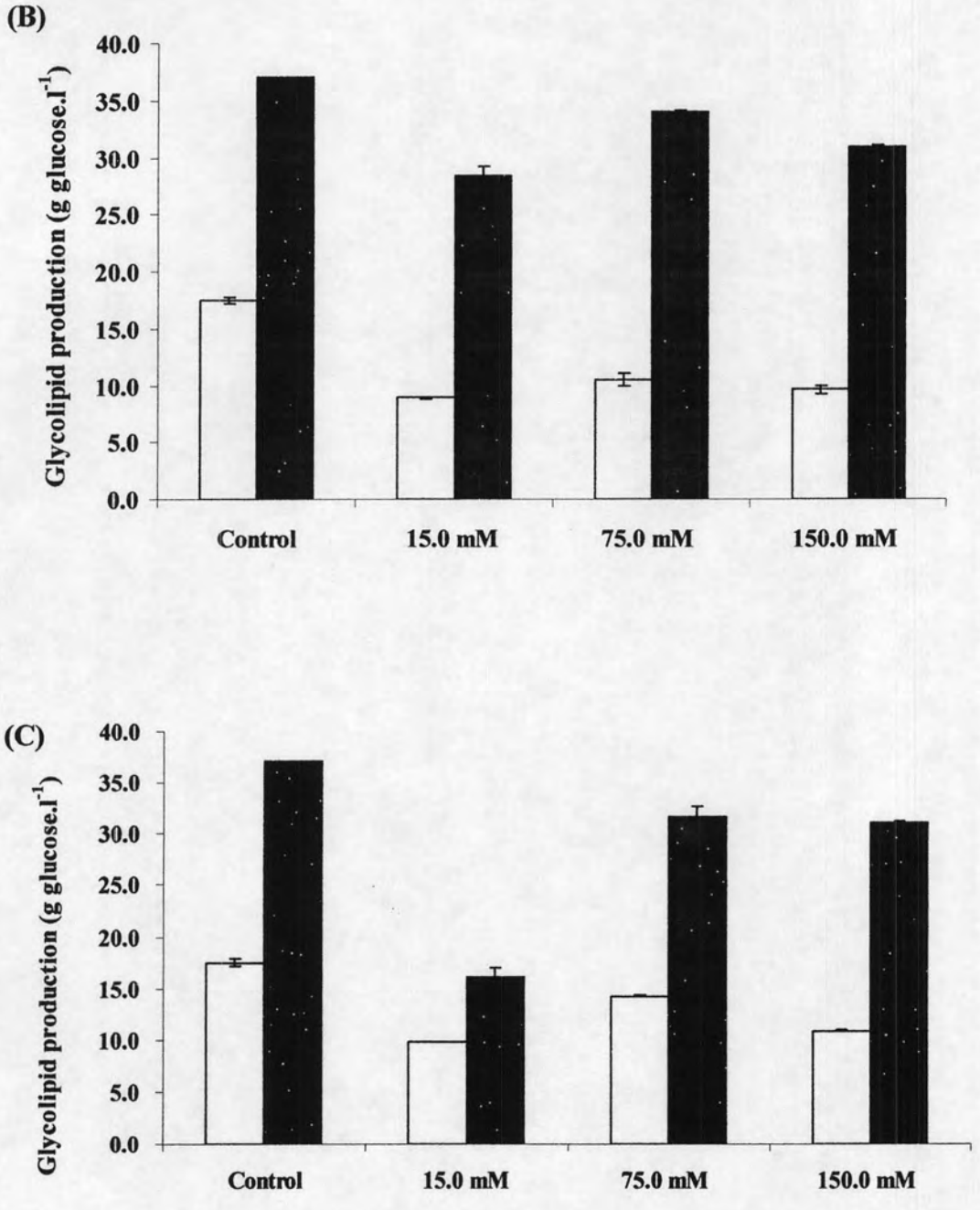


Figure 4.33 Effect of the type and the concentration of nitrogen source on glycolipid production (g glucose.l^{-1}) in batch culture of *Enterobacter* sp. P2 (□) and *B. cepacia* P3 (■) in mineral salt medium containing different carbon sources at a temperature of 37°C and an agitation rate of 250 rpm; (A) $NaNO_3$; (B) $(NH_4)_2SO_4$ and (C) CH_4N_2O. Each point represented the mean and standard deviation of triplicate samples.

The combination of carbon source and nitrogen source increased total biosurfactant production which was also investigated in each bacterial culture. The main criteria used to select so called, the optimum conditions were the increase of total biosurfactant production or growth of the isolates. Figure 4.29 and 4.33 showed the conditions that contained the high total biosurfactant production than the conditions without additional carbon and nitrogen source. After combination the optimum conditions of C- and N-sources, glycolipid production had been analyzed from partially purified biosurfactant of *Enterobacter* sp. P2, and *B. cepacia* P3. At the conditions of 44.4 mM glucose and 75 mM $NaNO_3$ showed was shown in Figure 4.34 the increase of glycolipid production (7.03% in *Enterobacter* sp. P2 and 9.89% in *B. cepacia* P3) after3 days incubation.

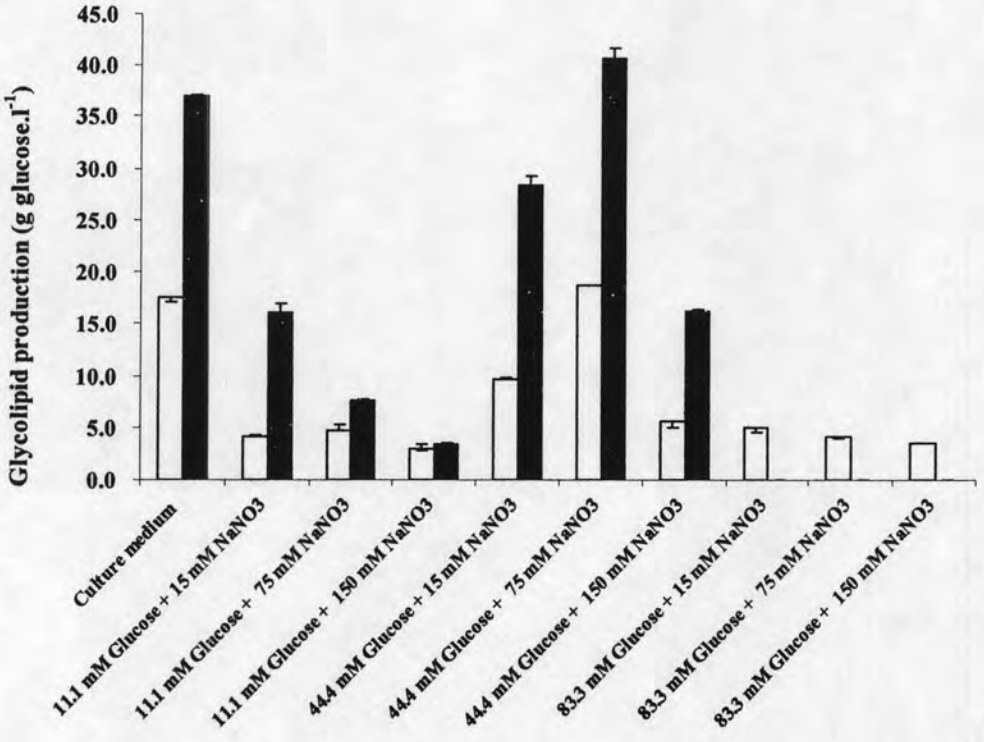


Figure 4.34 Biosurfactant production of *Enterobacter* sp. P2 (□) and *B. cepacia* P3 (■) in the combination of carbon or nitrogen source was cultured at 37°C, 250 rpm for 72 hours. The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).

For subculture of *Enterobacter* sp. P2 and *B. cepacia* P3, bacterial cultures were inoculated into the mineral salt medium with 2% wv^{-1} glucose as carbon source as the control. To investigate the affect of supplemented different carbon sources on glycolipid production 2% vv^{-1} olive oil, sunflower oil, soybean oil and diesel oil were used. The results (Figure 4.35) revealed that *B. cepacia* P3 grew better in the condition of supplementing with sunflower oil and olive oil more than the regular condition 48.86% and 20.78%, respectively. Whereas, poor growth was detected in *Enterobacter* sp. P2 in the condition of soybean oil and diesel oil supplementations.

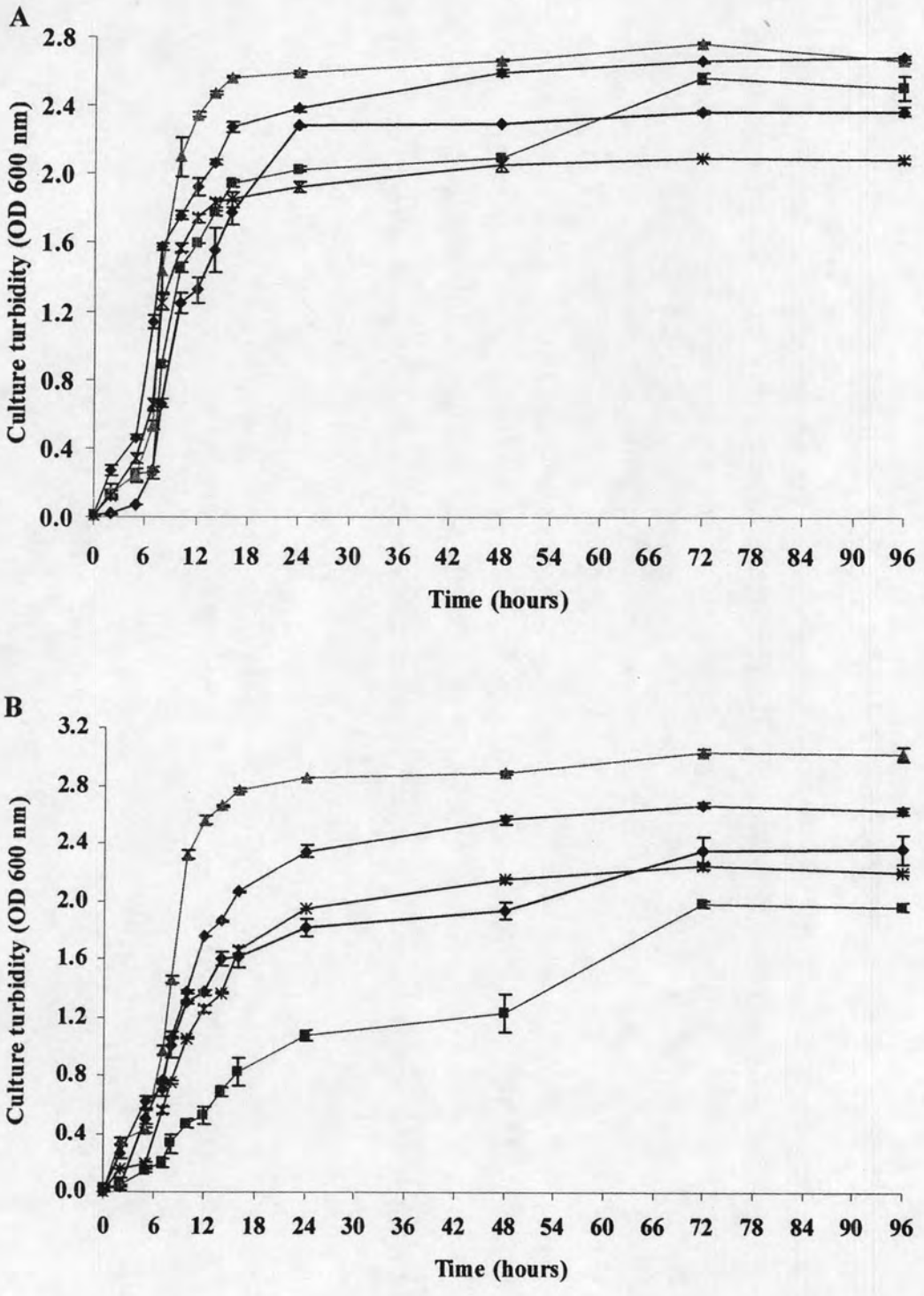


Figure 4.35 Growth of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 grown in mineral salt medium with 2% wv^{-1} glucose as carbon source supplemented with 2% vv^{-1} various type of oil as an additional carbon source: the control as mineral salt medium (-•-), diesel oil (-■-), sunflower oil (-▲-), olive oil (-◆-) and soybean oil (*). Each point represented the mean and standard deviation of triplicate samples.

Besides, biosurfactant production were also analyzed from the organism grew in MSM (2% wv^{-1} glucose) with oil as supplemented carbon source. The glycolipids production by the *Enterobacter* sp. P2 and *B. cepacia* P3, using supplemented substrates such as sunflower oil, olive oil, soybean oil and diesel oil, were displayed in Figure 4.36. It was found that *B. cepacia* P3 at the condition of supplementation of olive oil and sunflower oil, biosurfactant production increased 20.75% and 48.80%, respectively. But no increase of biosurfactant production in *Enterobacter* sp. P2 in any supplemented oil in medium was investigated.

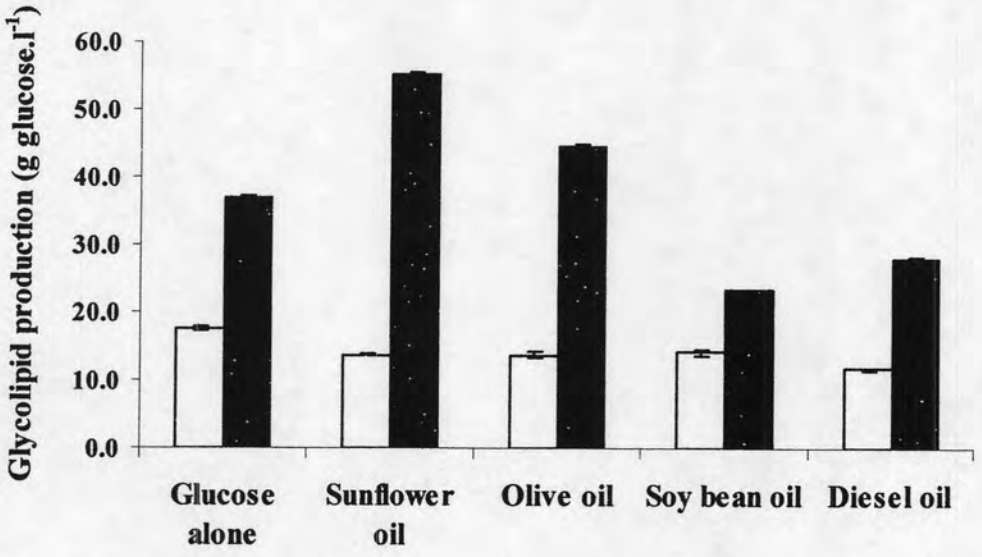


Figure 4.36 Effect of the type of supplement carbon sources on glycolipid produced (g glucose.l^{-1}) of *Enterobacter* sp. P2 (□) and *B. cepacia* P3 (■) in mineral salt medium supplemented with 2% wv^{-1} glucose with containing 2% vv^{-1} various types oil and control representing no additional oil. Each point represented the mean and standard deviation of triplicate samples.

4.5 Determination of the effect of temperature, NaCl and pH on the production of the biosurfactant

As the growth of *Enterobacter* sp. P2 and *B. cepacia* P3 at 30 and 37˚C was similar, it would be more economical to use 37˚C in practical applications. *Enterobacter* sp. P2 and *B. cepacia* P3 were unable to grow at 45˚C, leading to negligible glycolipid production at that temperature (Figure 4.37). 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.

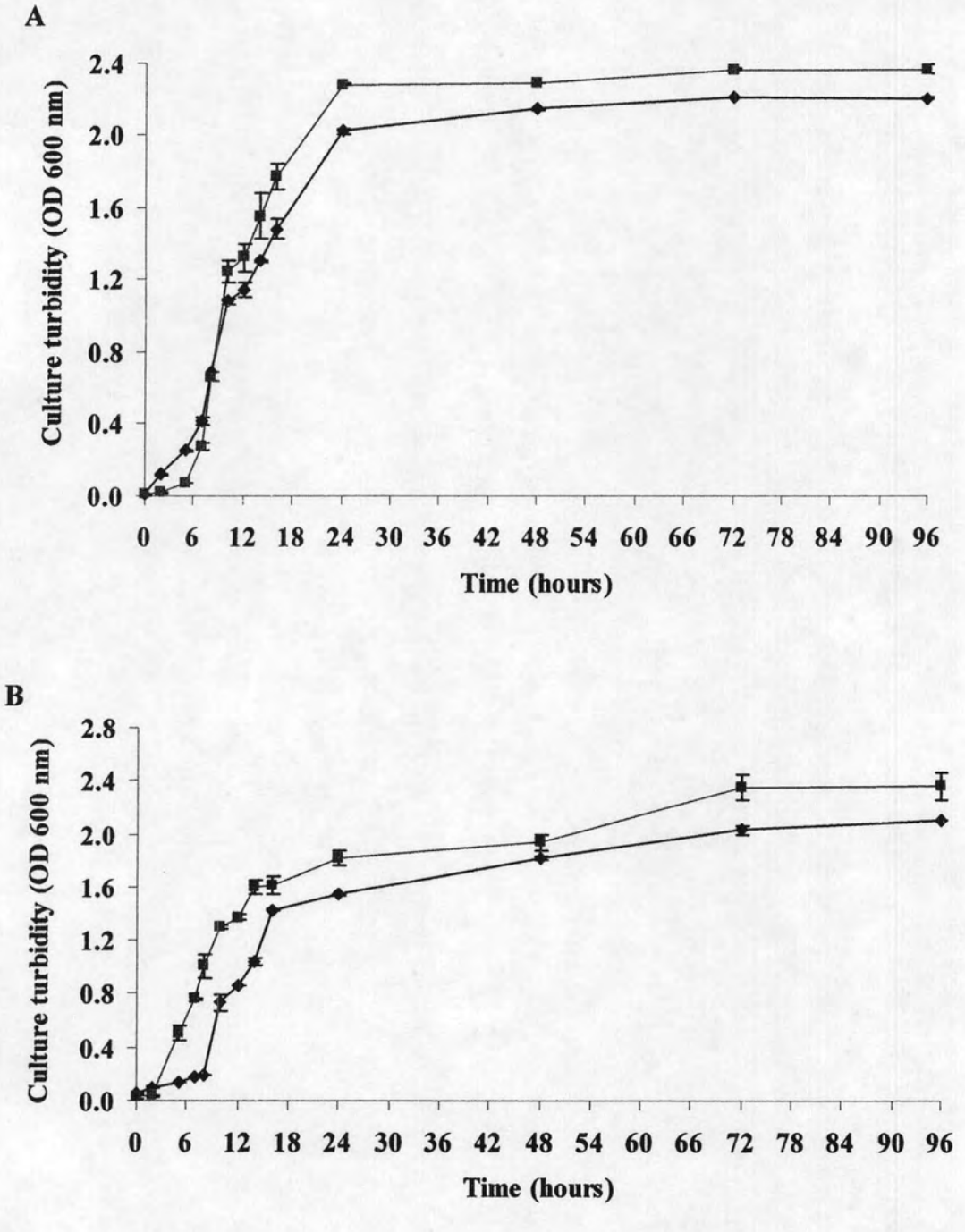


Figure 4.37 Growth of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 grown in mineral salt medium supplemented with 2% wv^{-1} glucose as carbon source at 30°C (—◆—), 37°C (—■—) and 45°C cannot detected. Each point represented the mean and standard deviation of triplicate samples.

Information regarding temperature to affect on glycolipid production with *Enterobacter* sp. P2 and *B. cepacia* P3 had been lacking. Hence, *Enterobacter* sp. P2 and *B. cepacia* P3 were grown in mineral salt medium supplemented with 2% wv^{-1} glucose at 30°C, 37°C and 45°C to explore the influence of culture temperature on glycolipid production. As indicated in Figure 4.38, glycolipid productions increased with temperature from 30 to 37°C, remained nearly constant for 30°C and 37°C (Appendix G), and no produced when temperature were further increased to 45°C.

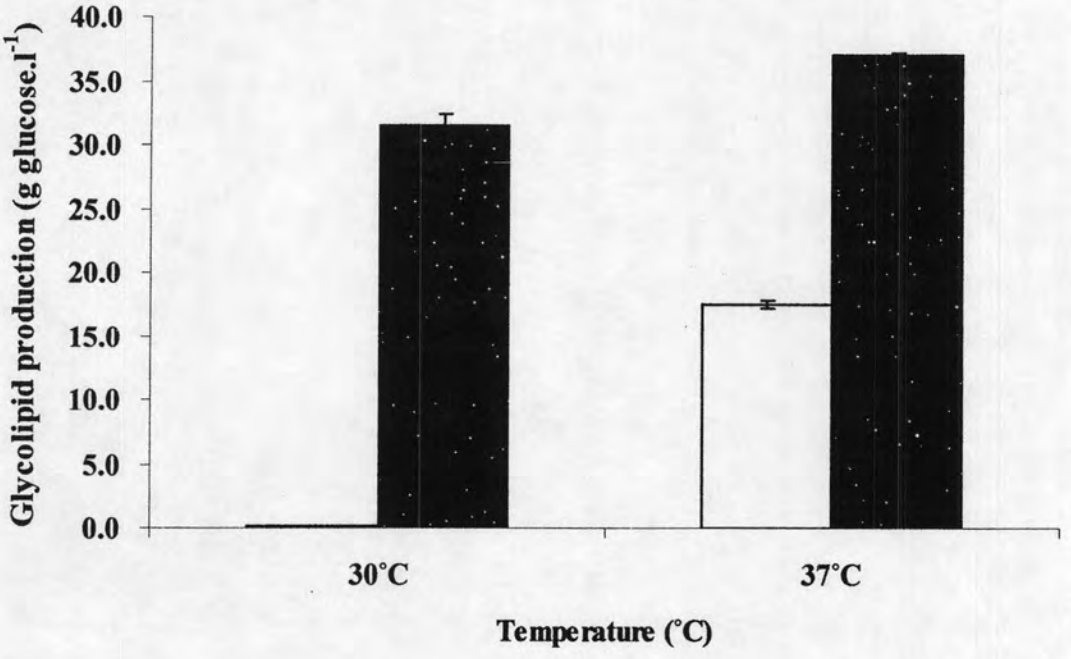


Figure 4.38 Glycolipid productions obtained from growth of *Enterobacter* sp. P2 (□) and *B. cepacia* P3 (■) on 2% wv^{-1} glucose as carbon source for 72 hours at 30°C and 37°C, respectively. Each point represented the mean and standard deviation of triplicate samples.

For growth studies and biosurfactant production at different NaCl concentrations and pH, the NaCl concentration and pH of the medium were adjusted accordingly. Growth studies were done using 2% wv^{-1} glucose as the carbon source. Experiments were done in triplicate and the results reported are averages of three independent experiments. As shown in Figure 4.39 the organism grew and produced biosurfactant at different NaCl concentrations (0.1, 0.5, 1.0 and 2.0% wv^{-1} or 17.1, 85.5, 171.0 and 342.0 mM) and pH values (4.5–10.5). Biosurfactant production of *Enterobacter* sp. P2 and *B. cepacia* P3 reduced in 0.1-2.0% wv^{-1} concentration, and no produced biosurfactant at 2.0% wv^{-1} NaCl concentration (Figure 4.40). This report described the biosurfactant production by *Enterobacter* sp. P2 and *B. cepacia* P3 were tested with various concentrations of NaCl in MSM (2% wv^{-1} glucose). Productions were repressed by NaCl at concentrations higher than 0.1% wv^{-1} addition cell growth was seriously affected by NaCl up to 0.1%. No biosurfactant were produced when the medium contained 2.0% wv^{-1} NaCl. These characteristics indicated that *Enterobacter* sp. P2 and *B. cepacia* P3 were non-halotolerant.

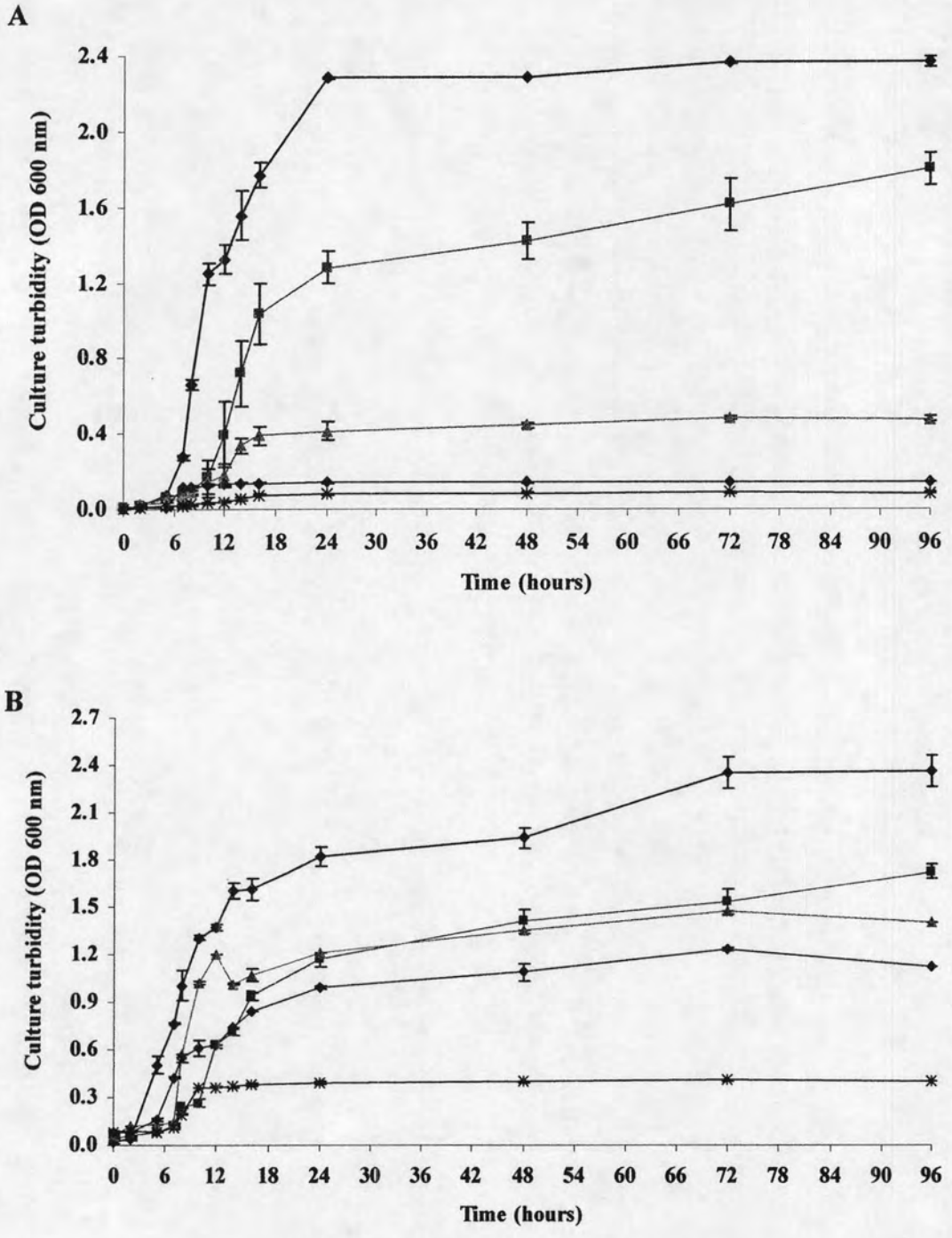


Figure 4.39 Growth of (A) *Enterobacter* sp. P2 and (B) *B. cepacia* P3 grown in mineral salt medium containing 11.1 mM glucose as carbon source which varied concentration of salt: without NaCl as the control (—◆—), 17.1 mM NaCl (—■—), 85.5 mM NaCl (—▲—), 171.0 mM NaCl (—◆—) and 342.0 mM NaCl (✳). Each point represented the mean and standard deviation of triplicate samples.

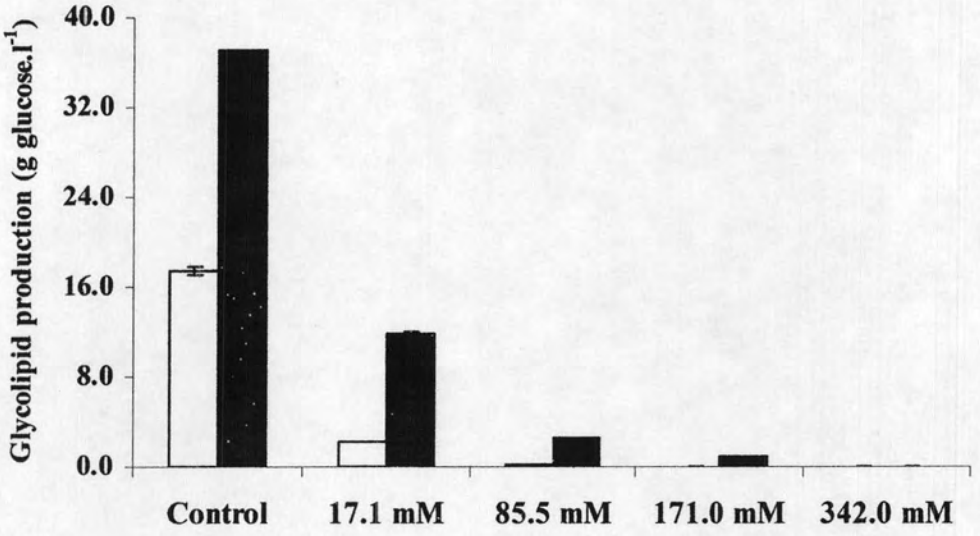


Figure 4.40 Effect of NaCl on production of *Enterobacter* sp. P2 (□) and *B. cepacia* P3 (■). The data were means from three independent experiments with vertical bars representing standard errors of the means (n=3).