CHAPTER IV

RESULTS

4.1 Screening, isolation, and identification of 4-chloroaniline-degrading bacteria

4.1.1 Screening and isolation of 4-chloroaniline-degrading bacteria

4-Chloroaniline (4CA) is one of the intermediates of microbial degradation of phenylurea, phenylcarbamate herbicides. As the contamination and accumulation of 4-chloroaniline has been reported in soil and water (Boehncke et al., 2003). 4-Chloroaniline degrading bacteria can be isolated from soil. Therefore, soil samples were collected from the various agricultural fields contaminated with herbicide as well as natural areas in Thailand (Table 4.1). Enrichment technique was applied to increase quantity of bacteria capable of 4-chloroaniline degradation. Screening on mineral medium agar plate containing of 25 ppm (0.2 mM) 4-chloroaniline, twenty-one colonies of 4-chloroaniline degrading bacteria. Twenty-one strains of bacteria were then separately grown in mineral liquid medium supplemented with 25 ppm 4-chloroaniline (as described in Method 3.2.1), while they were also grown on mineral medium with methanol as a control. The growth of bacteria at room temperature was observed by measuring the optical density at 590 nm using spectrophotometer during 15 days of incubation. The results showed that only five strains could be selected because growth of these bacterial strains in 4-chloroaniline-containing medium was apparently greater than those of the control (Fig.4.1). These bacterial strains were designated as 4CA-2, 4CA-16, 4CA-17, 4CA-19 and 4CA-20.

Then, these five bacterial strains were cultured in mineral media containing 0.1% yeast extract and 25 ppm (0.2 mM) 4-chloroaniline dissolved in methanol. Cell

were grown for 18 days. During the incubation, cell suspension was collected to analyze 4-chloroaniline utilization using High Performance Liquid Chromatography (HPLC). The results show that all five bacteria, (4CA-2, 4CA-16, 4CA-17, 4CA-19 and 4CA-20) could degrade 4-chloroaniline by 58.00%, 75.64%, 83.19%, 30.00% and 25.00% within 18 days of incubation (Fig.4.2 and Table 4.2). Within the first 12 days of incubation, the percentage of degradation was dramatically decreased for 4CA-2, 4CA-16, and 4CA-17 strains with the specific degradation rate of 2.6, 6.7 and 8.1 nmole 4-chloroaniline/(min.mg protein), respectively, whereas 4CA-19 and 4CA-20 strains showed slower the specific degradation rate. The three strains, 4CA-2, 4CA-16, and 4CA-17, were, therefore selected for further studys.

Table 4.1 Source of screening of 4-chloroaniline-degrading bacteria in Thailand

Source		Screening		Isolation
		Pre-enrichment	Enrichment	
Agricultural area	1. Tangerine groove I	2	1	4CA-2, 4CA-16
	2. Tangerine groove II	1	1	4CA-17
	3. Tangerine groove I, Chiangmai province	0	0	-
	4. Tangerine groove II, Chiangmai province	0	0	-
	5. Pineapple plantation I	2	1	-
	6. Pineapple plantation II	0	0	-
	7. Rice field I	0	0	-
	8. Rice field II	0	0	-
	9. Rice field III	0	0	-
	10.Chinese vegetable plantation I	0	0	-
	11.Chinese vegetable plantation II	0	0	-
	12.Chilli plantation I	0	0	-
	13.Chilli plantation II	0	0	-
	14.Cassava plantation I	1	1	-
	15.Cassava plantation II	0	0	-
	16.Agricultural area I, Rayong province	2	2	-
	17.Agricultural area II, Rayong province	2	1	-
	18. Agricultural area I, Tak province	0	0	-
	19. Agricultural area II, Tak province	0	0	-
Natural area	20. Hot spring I	2	2	4CA-19, 4CA-20
	21. Hot sprong II	0	0	

Table 4.2 biodegradability and growth of five bacteria for screening 4-chloroaniline-degraing bacteria

Bacterial name	Growth		Degradation	
	Growth rate (h^{-1})	Final OD	Specific degradation rate [nmole 4CA (min.mg protein)$^{-1}$]	Total degradation (18 days)
2	0.096 ±0.0009	0.689 ± 0.055	2.6 ± 1.22	58.00% ± 3.44
16	0.080 ± 0.0009	0.504 ± 0.052	6.7 ± 1.57	75.64% ± 3.46
17	0.086 ± 0.0009	0.758 ± 0.0043	8.1 ± 2.30	83.19% ± 3.56
19	ND	0.859 ± 0.034	ND	30.00% ± 3.51
20	ND	0.958 ± 0.021	ND	25.00% ± 3.87

ND = Not determined

4.1.2 Identification of 4-chloroaniline-degrading bacteria

4.1.2.1 The morphological characteristic of bacteria

Three bacterial strains cabaple of 4-chloroaniline degradation isolated from the soil sample of tangerine groove were identified for their morphological characteristic by bacterial gram staining and chemical solution staining (APPENDIX A). After staining, the results showed that all three strains are gram-negative bacteria as shown in Figure 4.3 - Figure 4.5. Furthermore, the characteristic of colonies on mineral medium agar plate containing 25 ppm 4-chloroaniline has been shown in Table 4.3.

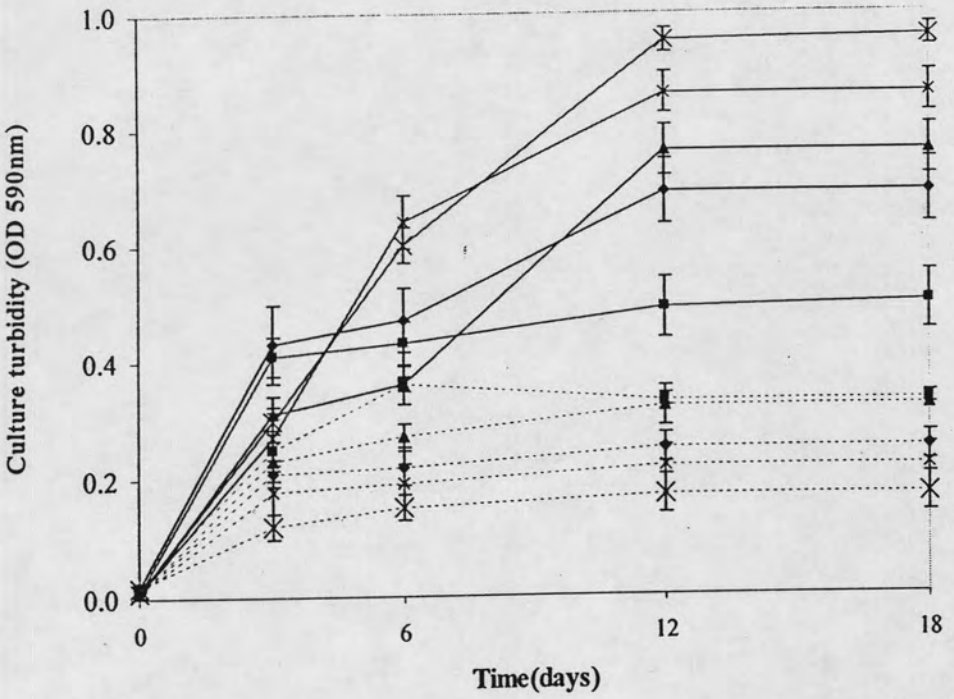

Figure 4.1 Growth of five bacterial strains: 4CA-2 (—◆—, --◆--), 4CA-16(—■—, --■--), 4CA-17(—▲—, --▲--), 4CA-19(—◇—, --◇--), and 4CA-20(—⊟—, --⊟--) in mineral medium in the presence of 25 ppm (0.2 mM) of 4-chloroaniline dissolved in metanol (solid line) and in methanol in the absence of 4-chloroaniline with only methanol as a control. (dash line). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=4)

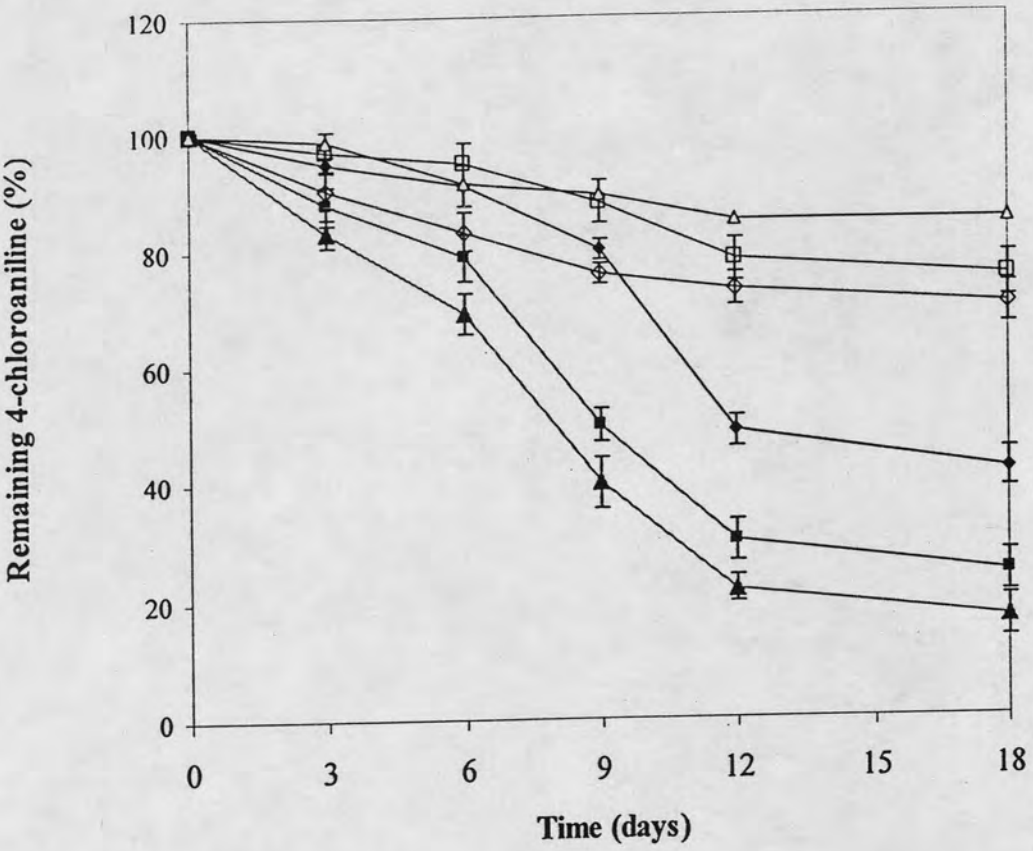

Figure 4.2 4-Chloroaniline degradation of five bacterial strains: 4CA-2 (—◆—), 4CA-16(—■—), 4CA-17(—▲—), 4CA-19(—◇—),4CA-20(—□—) and abiotic control(—△—) grown in mineral medium containing of 25 ppm of 4-chloroaniline. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=4)

Table 4.3 Characteristic of colonies of the bacterial isolates on mineral medium agar plate containing 25 ppm 4-chloroaniline.

Colonies charactistics	Bacterial isolates		
	4CA-2	4CA-16	4CA-17
Color	White	White	White
Form	Punctiform	Circular	Circular
Diameter	0.5-1mm	1mm	1mm
Surface	Smooth	Smooth	Smooth
Edge	Entire	Entire	Entire

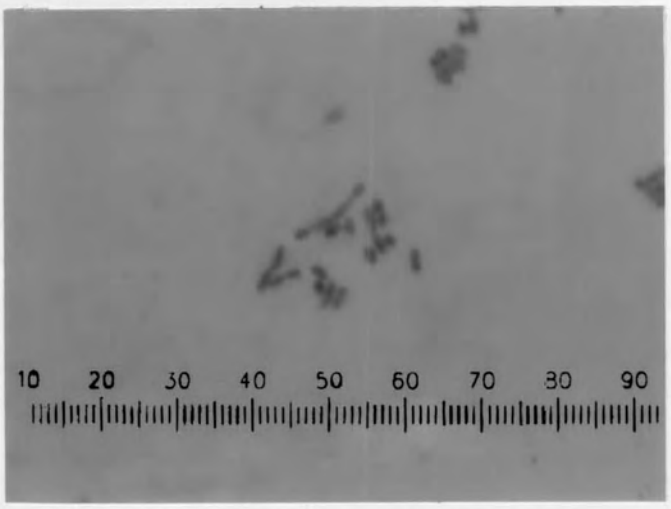

Figure 4.3 Gram's staining and morphology of strain 4CA-2 (1500X)
1.0-1.5 μm × 1.5-2.5 μm

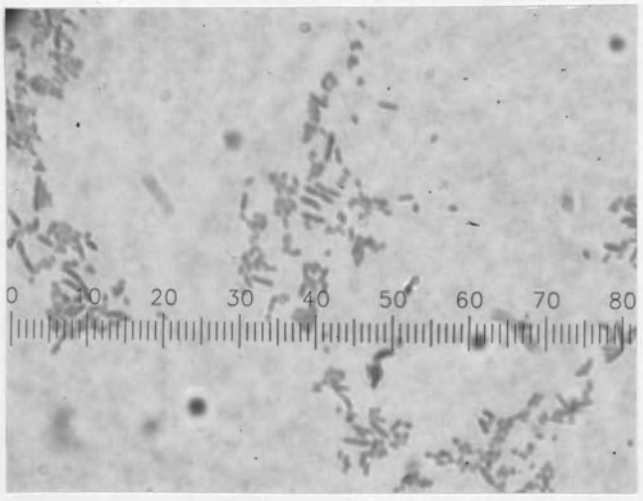

Figure 4.4 Gram's staining and morphology of stain 4CA-16 (1500X)
0.5-1.0 μm × 1.5-4.0 μm

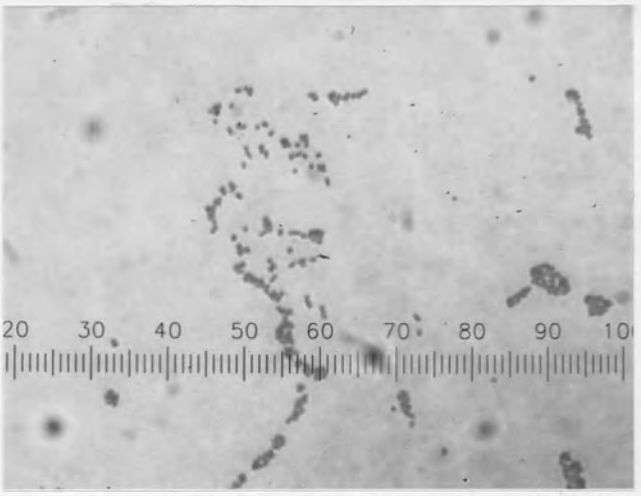

Figure 4.5 Gram's staining and morphology of stain 4CA-17 (1500X)
0.3-1.5 μm × 0.6-2.5 μm

4.1.2.2 The biochemical test of the three bacterial isolates

From the results of morphological characteristic of three strains of bacteria, these 4CA-degrading bacteria are gram-negative. Then, the biochemical test was used to identify the species of bacteria.

When the biochemical characterization results (shown in Table 4-2) were compared to the Manual of Clinical Microbiology (Murray, 1999), three 4-chloroaniline-degrading bacteria; 4CA-2, 4CA-16 and 4CA-17, could be identified as *Acinetobacter baumannii, Pseudomonas putida* and *Klebsiella pneumoniae*, respectively

Table 4.4 Biochemical test for 4-chloroaniline–degrading bacteria *

Biochemical Test	Bacterial isolates		
	4CA2	4CA16	4CA17
Oxidase test	-	+	-
Catalase test	+	+	+
TSI/ H_2S production	K/K / -	K/K /	A/A / -
SIM(H_2S/indole/motile)	/ - / -	/ - / +	- / - / -
Citrate Utilization	+	+	+
Urease test	-	+	+
Nitrate reduction	-	-	
N_2 gas production	-	-	
Esculin hydrolysis	-	-	+
Acetate Utilization	+	+	
Voges-Proskauer reaction			+
Malonate Utilization	+		+
Carbohydrate fermentation test			
Glucose/gas	+ /	+ /	+ / +
Maltose	-	-	+
Lactose	+	-	+
Mannitol	-	-	+
D-Xylose	+	+	+
Rhamnose			+
Inosital CTA			+
Sorbital			+
Raffinose			+

Table 4.4 (continued)

Biochemical Test	4CA2	4CA16	4CA17
Carbohydrate fermentation test			
Inosital CTA			+
Sorbital			+
Raffinose			+
Fructose	-	+	
Salicin			+
Lysine Decarboxylase	-	-	+
Arginine Dihydrolase	-	+	-
Ornithine Decarboxylase	-	-	-
Bacterial identification	*Acinetobacter baumannii*	*Pseudomonas putida*	*Klebsiella pneumoniae*

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.

4.1.2.3 Determination of *16S* ribosomal DNA gene for bacterial identification

16S ribosomal DNA gene sequence was used to identify three 4-chloroaniline-degrading bacteria: 4CA-2, 4CA-16 and 4CA-17. The specific primers (63f and 1387r) (Marchesi et al, 1998) were used to amplify the target sequence as described in Materials and Methods 3.3.2.3. 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 kbps. 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 D and the results can be summarized in Table 4.5.

Table 4.5 Identification of 4-chloroaniline degrading bacteria using 16S rDNA gene sequence comparison

Culture name	Sequence identities (%)	Sequence accession number	Source of bacteria	References	Bacteria	Note
4CA-2	1) 99%	AY847284.1	Soil	Tan (unpublished)	*Acinetobacter baumannii*	Biocontrol in citrus canker
	2) 99%	AJ888983.1	Soil	Dijkshoorn et al. (unpublished)	*Acinetobacter calcoaceticus*	-
	3) 99%	DQ226213	Soil	Shukor and Dahalan (unpublished)	*Acinetobacter sp.*	-
Conclusion	-	-	-	-	***Acinetobacter baumannii***	-
4CA-16	1) 99%	AY741157.1	Soil	Muthukumarasamy and Kang (unpublished)	*Pseudomonas putida*	Diazotrophic bacteria
	2) 99%	AM403529.1	Soil	Wang (unpublished)	*Pseudomonas* sp.	Deep-sea sediments bacteria
	3) 99%	AM184288.1	Water	Abraham et al., (unpublished)	*Pseudomonas putida*	Pathogenic bacteria along River downstream
Conclusion	-	-	-	-	***Pseudomonas putida***	-
4CA-17	1) 96%	AB114634.1	Soil	Meunchang et al.(unpublished)	*Klebsiella* sp.	nitrogen fixing bacteria
	2) 95%	AF511429.1	Water	Ovesen et al. (unpublished)	*Klebsiella pneumoniae*	-
	3) 96%	AJ783916.1	Soil	Kaempfer and Ruppel	*Klebsiella variicola*	-
Conclusion	-	-	-	-	***Klebsiella pneumoniae***	-

4.2 Degradation of 4-chloroaniline by 4-chloroaniline-degrading bacteria

4.2.1 4-Chloroaniline degradation kinetic

4-Chloroaniline-degrading bacteria were cultured in mineral medium containing 25 ppm (0.2 mM) 4-chloroaniline for 12 days (Fig 4.6A). During the incubation, cell suspension was interval taken, centrifuged and the supernatant was analyzed for the degradation of 4-chloroaniline using High Performance Liquid Chromatography (HPLC). The total degradation of 4-chloroaniline of *Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16), *Klebsiella pneumoniae* (4CA-17), and control were 61.00% ± 1.68, 59.82 ± 0.71, 62.82% ± 3.87, while the decrease of 4-chloroaniline in abiotic control was 12.75% ± 0.08 (Fig 4.6B). *Klebsiella pneumoniae* (4CA-17) exhibited the highest percentage of total degradation compared to those of *Acinetobacter baumannii* (4CA-2) and *Pseudomonas putida* (4CA-16). Loss of 4-chloroaniline in abiotic control might be caused by the effect of photo-oxidation. 4-Chloroaniline biotransformation kinetics was determined as the specific 4-chloroaniline degradation rate expressing the disappearance rate of 4-chloroaniline in the first four days on cell protein basis. Abiotic control (without cell inoculum) was also performed as a control. The specific 4-chloroaniline degradation rate was calculated from the rate of 4-chloroaniline degradationh within the indicated period (e.g. within the first four days in Fig 4.7) divided by cell protein concentration (the calculation is shown in Appendix C). The initial specific degradation rate calculated within the first four days of incubation and growth rate of *Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16) and *Klebsiella pneumoniae* (4CA-17) are 8.70 ± 1.60, 13.60 ± 2.50 19.00 ± 2.30 nmol (min.mg protein)$^{-1}$and 0.0075, 0.0060 and 0.0059 (h^{-1}), respectively. While *Acinetobacter baumannii* (4CA-2) showed good growth in the presence of 25 ppm 4-chloroaniline, *Klebsiella pneumoniae* (4CA-17)

showed the highest of percentage of total degradation 62.82% ± 3.87 and the specific degradation rate 19.00 ± 2.30 nmol (min.mg protein)$^{-1}$

Table 4.6 The specific degradation rate of 4-chloroaniline by three bacterial isolates

Bacterial culture *	**Cell protein * concentration** (g.protein/liter)	**4-Chloroaniline degradation rate**** nmol(liter.4 days)$^{-1}$	**Specific 4-chloroaniline degradation rate**** nmol(min.mg protein)$^{-1}$
Acinetobacter baumannii (4CA-2)	0.3868	-19.4 ± 1.2	8.70 ± 1.60
Pseudomonas putida (4CA-16)	0.1981	-15.5 ±2.50	13.60 ± 2.50
Klebsiella pneumoniae (4CA-17)	0.2830	-31.0 ±.2.87	19.00 ± 2.30

* Bacterial culture was grown in total volume of 100 ml of 25 ppm 4-chloroaniline (0.2 *m*M) containing medium.

According to previous studies of biodegradation of chorinated compounds, free chloride released during the degradation could be used to confirmed the degradation process (Radianingtyas et al, 2003). Therefore, in our experiment, free chloride concentration was monitored using both ion selective electrode (ISE) and colometric method (as described in method 3.7.2). During 12 days of 4-chloroaniline degradation, dechlorination of 4-chloroaniline was observed in all three bacteria (Fig 4.7). Free chloride concentration was increased from 0.05 mM to 0.14 mM (70%) for *Acinetobacter baumannii*; 0.05 mM to 0.13 mM (65%) for *Pseudomonas putida*; 0.05 mM to 0.14 mM (70%) for *Klebsiella pneumoniae*.

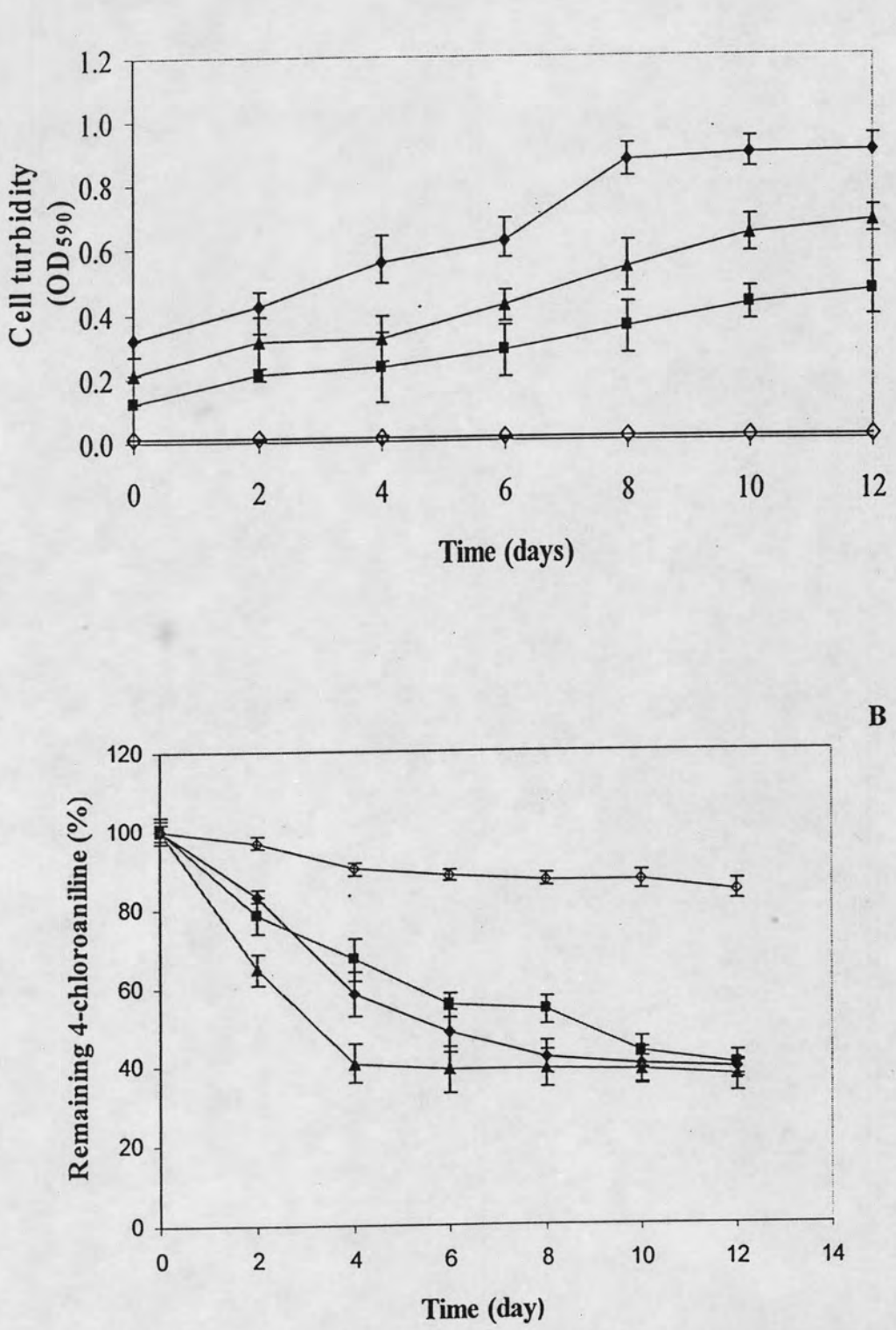

Figure 4.6 Growth (A) and 4-chloroaniline-degradation (B) *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—) and control (—◇—) (B) in mineral medium containing 25ppm of 4-chloroaniline as a sole source of carbon and nitrogen. The data are means from three independent experiment with vertical bars representing standard errors of the means (n=3).

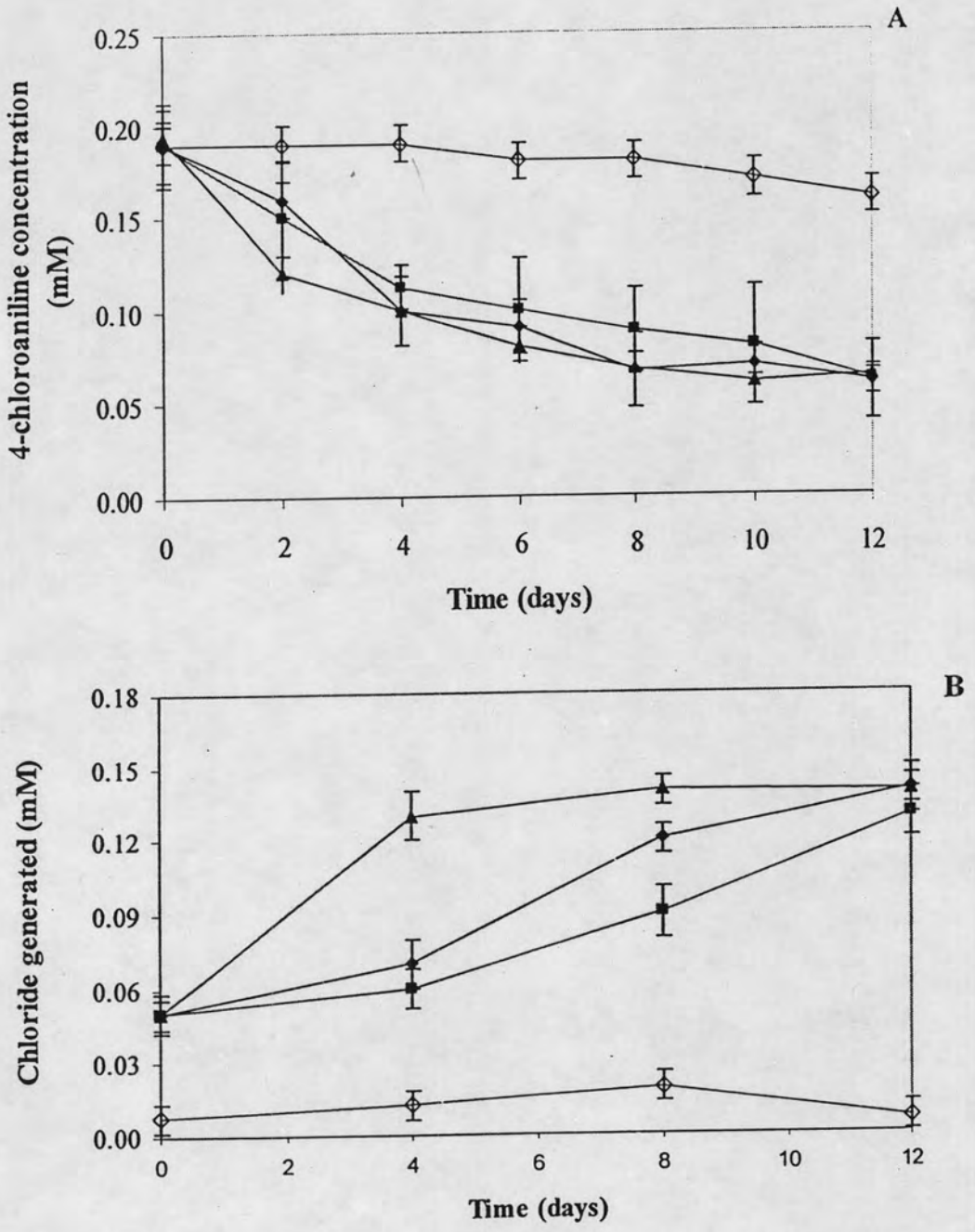

Figure 4.7 The degradation of 4-chloroanine (A) and the apparent free chloride ion from the degradation (B) in mineral medium containing 25 ppm of 4-chloroaniline as a source of carbon and nitrogen of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—) , and abiotic control (—◇—), respectively. The data are means from three independent experiment with vertical bars representing standard errors of the means (n=3).

4.2.2 Determination of enzymes involving 4-chloroaniline degradation

Ortho-cleavage and a modified *ortho*-cleavage pathway are convergent pathways for aromatic compound degradation (Harwood and Parales, 1996). Nevertheless, the modified *ortho*-cleavage pathway reveals the enzymes that have been evolved to handle chlorinated substrates which are different from *ortho*-cleavage pathway (Schlomann, 1994). For instance, chlorocatechol 1,2-dioxygenase is an important enzyme that can be detected in modified *ortho*-cleavage pathway while catechol 2,3-dioxygenase can be detected in *meta*-cleavage pathway (Harwood and Parales, 1996). Catechol 2,3-dioxygenase catalyzes the degradation of the methylated aromatic hydrocarbons. (Harayama and Kok, 1992). In the present study, we investigated these enzyme activities including catechol 1,2-dioxygenase (*ortho*-cleavage pathway), catechol 2,3-dioxygenase of which catechol was used as a substrate, chlorocatechol 2,3-dioxygenase (*meta*-cleavage pathway) and chlorocatechol 1,2-dioxygenase (modified *ortho*-cleavage pathways) of which 4-chlorocatechol was used as a substrate from cell grown in four different growth conditions. The specific activity of these enzymes were determined by measuring the apparent product using a spectrophotometer, i.e *cis,cis*-muconate as a product of catechol 1,2-dioxygenase, 3-chlorocis,cis-muconate as a product of chlorocatechol 1,2-dioxygenase, 2-hydroxumuconic acid as a product of catechol 2,3-dioxygenase, 5-chloro-2-hydroxymuconoc acid as a product of chlorocatechol 2,3-dioxygenase in Chapter 3 (3.4.2.4) and the results are shown in Table 4.7-4.9. Chlorocatechol 1,2-dioxygenase was apparently detected in all three bacteria in 2 days in 4-chloroaniline-inducing condition (4-chloroaniline-containing medium), i.e. 191.8 nmole(min.mg protein)$^{-1}$ for *Acinetobacter baumannii*, 205.8 nmole(min.mg protein)$^{-1}$ for *Pseudomonas putida* and 223.4 nmole(min.mg protein)$^{-1}$ for *Klebsiella pneumoniae*. In 8 days,

chlorocatechol 1,2-dioxygenase was detectable in lower extent than that of the second day of incubation. Catechol 1,2-dioxygenase, representing inducible enzyme in ortho-cleavage pathway, was also detected in all three bacterial isolates, although 4-6 times lower than that of chloro-1,2 dioxygenase, suggesting not only the presence of the modified *ortho*-cleavage pathways but also the *ortho*-cleavage pathway.

Table 4.7 Specific activities of enzymes* (nmole/min.mg protein) involving 4CA degradation in *Acinetobacter baumannii* (4CA-2) in different growth conditions

Pregrown	*Ortho*-cleavage pathway and modified *ortho*-cleavage pathway				*Meta*-cleavage pathway			
	2 Days		8 Days		2 Days		8 Days	
	Catechol 1,2 dioxygenase	Chlorocatechol 1,2 dioxygenase	Catechol 1,2 dioxygenase	Chlorocatechol 1,2 dioxygenase	Catechol 2,3 dioxygenase	Chloroatechol 2,3 dioxygenase	Catechol 2,3 dioxygenase	Chloroatechol 2,3 dioxygenase
mmye + 4CA[a]	50.6 ± 17.5	**191.8** ± 47.5	55.9 ± 24.2	47.3 ± 16.4	4.6 ± 1.5	0 ± 0	3.9 ± 0.6	0 ± 0
mmye[b]	24.7 ± 1.5	9.2 ± 4.0	2.6 ± 1.1	6.2 ± 1.5	1.0 ± 0.3	0 ± 0	1.3 ± 0.6	0 ± 0
LB + 4CA[c]	0.7 ± 0.2	8.6 ± 2.5	1.7 ± 0.8	4.1 ± 1.0	0.3 ± 0.1	0 ± 0	0.4 ± 0.3	0 ± 0
LB[d]	0.6 ± 0.3	3.8 ± 1.9	1.4 ± 0.2	2.9 ± 1.3	0. ± 0	0 ± 0	0.1 ± 0.1	0 ± 0

*determined in cell-free extract

[a]mm + ye + 4CA: mineral medium containing 0.1% yeast extract and 25ppm 4-chloroaniline

[b]mm + ye: mineral medium containing 0.1%yeast extract

[c]LB + 4CA : Luria bertani medium containing 25ppm 4-chloroaniline

[d]LB : Luria bertani medium

Table 4.8 Specific activities of enzymes* (nmole/min.mg protein) involving 4CA degradation in *Pseudomonas putida* (4CA-16) in different growth conditions

Pregrown	*Ortho*-cleavage pathway and modified *ortho*-cleavage pathway				*Meta*-cleavage pathway			
	2 Days		8 Days		2 Days		8 Days	
	Catechol 1,2 dioxygenase	Chlorocatechol 1,2 dioxygenase	Catechol 1,2 dioxygenase	Chlorocatechol 1,2 dioxygenase	Catechol 2,3 dioxygenase	Chloroatechol 2,3 dioxygenase	Catechol 2,3 dioxygenase	Chloroatechol 2,3 dioxygenase
mmye + 4CA[a]	33.8 ± 14.6	**205.8** ± 68.6	36.9 ± 16.0	125.1 ± 21.7	3.0 ± 2.0	0 ± 0	3.8 ± 0.8	0 ± 0
mmye[b]	4.0 ± 1.8	6.8 ± 2.4	3.5 ± 1.5	9.4 ± 2.0	1.2 ± 0.3	0 ± 0	1.2 ± 0.2	0 ± 0
LB + 4CA[c]	2.4 ± 0.6	6.0 ± 1.0	1.3 ± 0.1	6.6 ± 3.1	0.3 ± 0.1	0 ± 0	0.4 ± 0.2	0 ± 0
LB[d]	2.2 ± 1.5	5.2 ± 1.6	0.2 ± 0.1	3.7 ± 2.6	0.2 ± 0.1	0 ± 0	0.2 ± 0.1	0 ± 0

*determined in cell-free extract

[a]mm + ye + 4CA: mineral medium containing 0.1% yeast extract and 25ppm 4-chloroaniline

[b]mm + ye: mineral medium containing 0.1%yeast extract

[c]LB + 4CA : Luria bertani medium containing 25ppm 4-chloroaniline

[d]LB : Luria bertani medium

Table 4.9 Specific activities of enzymes* (nmole/min.mg protein) involving 4CA degradation in *Klebsiella pneumoniae* (4CA-17) in different growth conditions

Pregrown	*Ortho*-cleavage pathway and modified *ortho*-cleavage pathway				*Meta*-cleavage pathway			
	2 Days		8 Days		2 Days		8 Days	
	Catechol 1,2 dioxygenase	Chlorocatechol 1,2 dioxygenase	Catechol 1,2 dioxygenase	Chlorocatechol 1,2 dioxygenase	Catechol 2,3 dioxygenase	Chloroatechol 2,3 dioxygenase	Catechol 2,3 dioxygenase	Chloroatechol 2,3 dioxygenase
mmye + 4CA[a]	41.2 ± 14.3	**223.4** ± 35.7	62.9 ± 27.2	74.6 ± 18.5	7.1 ± 3.2	0 ± 0	6.0 ± 1.2	0 ± 0
mmye[b]	8.5 ± 2.8	9.0 ± 2.2	4.3 ± 1.1	9.1 ± 1.4	2.9 ± 1.4	0 ± 0	3.1 ± 1.1	0 ± 0
LB + 4CA[c]	6.5 ± 1.9	3.9 ± 1.0	3.8 ± 1.9	4.3 ± 1.5	0.1 ± 0	0 ± 0	0 ± 0	0 ± 0
LB[d]	1.8 ± 0.9	2.5 ± 1.1	0.5 ± 0.2	3.1 ± 1.4	0. ± 0	0 ± 0	0 ± 0	0 ± 0

*determined in cell-free extract

[a]mm + ye + 4CA: mineral medium containing 0.1% yeast extract and 25ppm 4-chloroaniline

[b]mm + ye: mineral medium containing 0.1%yeast extract

[c]LB + 4CA : Luria bertani medium containing 25ppm 4-chloroaniline

[d]LB : Luria bertani medium

4.3 Optimum conditions of 4-chloroaniline degradation

4-Chloroaniline can serve as a sole carbon and nitrogen source for *Acinetobacter baumannii* (4CA-2), *Pseudomonas putida* (4CA-16), *Klebsiella pneumoniae* (4CA-17) exhibiting the degradation of 61.00% ± 1.68, 59.82% ± 0.71, 62.82% ± 3.87, respectively. The optimization of growth conditions were attempted and investigated in order to increase the total degradation as well as the degradation rate, i.e, to improve cell growth and/or cell degradability. Type and concentration of additional carbon and/or nitrogen source was varied to promote cell growth and cell degradability. Previous report of *Moraxella* sp. strain G showed that the strain could degrade 2.5 mM 4-chloroaniline and grew well on succinate and citrate (Zeyer and Kearney, 1982). Nitrogen repression was not affected when culture medium contained 10 mM sodium nitrate or 10 mM ammonium chloride (Zeyer et al., 1985). Besides 4-chloroaniline, aniline was used as an inducer for 4-chloroaniline degradation (Surovtseva et al., 1980). Previous report and preliminary test of this study exhibited that the additional carbon or nitrogen source at 4 mM showed the increasing of total 4-chloroaniline degradation. Therefore, additional carbon and/or nitrogen source were supplemented as followed: 4 mM citrate or 4 mM succinate for carbon source; 4 mM NH_4Cl or 4 mM $NaNO_3$ for nitrogen source; 1 mM aniline for carbon and nitrogen source. The sole or combination of carbon source and nitrogen source which increased total 4-chloroaniline degradation or 4-chloroaniline rate was also investigated in each bacterial culture. The main criteria used to select so called, the optimum conditions were the increase of total 4-chloroaniline degradation or 4-chloroaniline rate or growth rate of the isolates. From Table 4.8, the conditions that contained the high % total degradation than the conditions without additional carbon and nitrogen source were selected to do the next optimum condition step.

The mineral medium containing 25 ppm (0.2 mM) 4-chloroaniline supplemented with 4 *mM* citrate and 4 mM NH_4Cl was selected in the first optimum condition of *Acinetobacter baumannii (*4CA-2). The concentration was ranging from 4 mM, 8 mM, and 18 mM in citrate and NH_4Cl (Fig.4.8 and Table 4.9). In this case, carbon source (citrate) was combined with nitrogen source (NH_4Cl) at a concentration ranging from 4 mM, 8 mM, and 18 mM. From the Figure 4.9 A, concentration of 4*m*M citrate showed growth rate which was higher than bacterial cell without additional carbon source. Then, the concentration of 8 mM citrate and 18 mM citrate were toxic with bacterial cells (4CA-2). 4 mM Citrate promoted cell growth and total degradation than those when 8 mM or 18 mM citrate was provides From Figure 4.10, the conditions that contained NH_4Cl at all concentrations of this experiment exhibited degradation curve and growth curve were similar in culture *Acinetobacter baumannii (*4CA-2) without additional carbon source. Therefore, the conditions that supplemented with NH_4Cl were not affected to the growth and degradation of *Acinetobacter baumannii (*4CA-2). From figure 4.11, the combination of 4 mM citrate and 4 mM NH_4Cl showed the maximum total degradation (70.16%) and specific degradation rate (9.25 $(nmole/min.mg.protein)^{-1}$ within 4 days. On the contrary, the combination of 8 mM citrate and 8 mM NH_4Cl, 18 mM citrate and 18 mM NH_4Cl inhibited cell growth to 0.035 (h^{-1}), 0.040 (h^{-1}) and reduced the total degradation to 27.62% , 31.65%. A sole carbon source or nitrogen source used in these experiment in the absence of 25 ppm (0.2 mM) 4-chloroaniline was not enough to support growth (Figure 4.12-4.13).

The mineral medium containing 25 ppm (0.2 mM) 4-chloroaniline supplemented with 4 mM citrate and 4 mM succinate was selected in the first optimum condition of *Pseudomonas putida* (4CA-16) The concentration was ranging

from 4 mM, 8 mM, and 18 mM in citrate and succinate (Fig.4.15 and Table 4.13). The suitable conditions for *Pseudomonas putida* (4CA-16) were 4 mM citrate and 4 mM succinate (Fig.4.15-4.19). Total degradation of 4 mM citrate and 4 mM succinate was higher than *Pseudomonas putida* (4CA-16) without additional carbon and nitrogen source as 8.42% and 8.40%. Growth rate of *Pseudomonas putida* (4CA-16) with 4 mM citrate and 4 mM succinate was higher than the growth rate without additional carbon and nitrogen source.

The mineral medium containing 25 ppm (0.2 mM) 4-chloroaniline supplemented with 1 mM aniline was selected in the first optimum condition of *Klebsiella pneumoniae* (4CA-17). Various concentrations of aniline was further studied, ranging from 2 mM, 4 mM, 8 mM and 16 mM of aniline. From Figure 4.20-4.23 total degradation of 1 mM aniline and 2 mM was higher than *Klebsiella pneumoniae* (4CA-17) without additional carbon and nitrogen source as 5.56% and 4.37%. Growth rate of 1 mM aniline was higher than *Klebsiella pneumoniae* (4CA-17) without additional carbon and nitrogen source to. The concentration of aniline at 1 mM and 2 mM exhibited total degradation and growth was greater than 4 mM, 8 mM, 16 mM aniline and the culture medium of *Klebsiella pneumoniae* (4CA-17) without supplemented with carbon and/or nitrogen source. The concentration of aniline at 8 mM and 16 mM, cell growth and degradation were not different from control. The result of suitable condition in each bacterium exhibited in Table 4.15.

Table 4.10 Biodegradation ability of the three bacterial isolates in various conditions

Bacterial culture	Degradation condition (in the presence of 25ppm (0.2 mM) 4-chloroaniline	Growth rate (h^{-1})	% Total degradation (12 days)	Specific degradation nmole (min. mg.protein)$^{-1}$ within 4 days
4CA-2	Culture medium	0.086 ±0.011	61.00 ± 1.68	**8.70 ± 1.60**
	4 mM succinate	0.033 ± 0.008	26.75 ± 2.95	**7.93 ± 2.50**
	4 mM citrate	**0.088 ± 0.011**	**64.06 ± 2.95**	9.51 ± 3.15
	4 mM NH_4Cl	**0.088 ± 0.009**	**68.21 ± 2.95**	7.67 ± 2.50
	4 mM $NaNO_3$	0.035 ± 0.006	29.54 ± 3.31	**9.98 ± 2.50**
	1 mM Aniline	0.041 ± 0.006	44.74 ±3.31	**8.29 ± 1.87**
4CA-16	Culture medium	0.057 ± 0.0012	59.82 ± 0.71	**13.6 ± 2.50**
	4 mM succinate	**0.065 ± 0.0009**	**68.31 ± 2.87**	16.14 ± 2.25
	4 mM citrate	**0.065 ± 0.0010**	**68.24 ± 2.55**	14.20 ± 1.85
	4 mM NH_4Cl	0.036 ± 0.0009	39.25 ± 4.22	**5.26 ± 1.85**
	4 mM $NaNO_3$	0.053 ± 0.0009	22.97 ± 3.88	**5.26 ± 1.85**
	1 mM Aniline	0.044 ± 0.0009	43.63 ± 2.87	**11.51 ± 2.50**
4CA-17	Culture medium	0.053 ±0.0010	62.82 ± 3.87	**19.0 ± 2.30**
	4 mM succinate	0.019 ± 0.0005	30.88 ± 2.87	**6.25 ± 2.30**
	4 mM citrate	0.032 ± 0.006	30.43 ± 3.31	**8.88 ± 1.87**
	4 mM NH_4Cl	0.027 ±0.006	24.09 ± 3.31	**3.57 ± 2.50**
	4 mM $NaNO_3$	0.023 ± 0.005	23.97 ± 3.87	**1.57 ± 2.50**
	1 mM Aniline	0.064 ± 0.0011	68.38 ± 3.31	21.11 ± 2.50

Culture medium = mineral medium containing 0.1% yeast extract and 25 ppm 4-chloroaniline

4CA-2, 4CA-16 and 4CA-17 were designated *Acinetobacter baumannii, Pseudomonas putida and Klebsiella pneumoniae, respectively.*

Table 4.11 Biodegradation ability of *Acinetobacter baumannii* (4CA-2) in additional carbon or nitrogen source

Degradation condition	Growth rate (h^{-1})	% Total degradation (12 days)	Specific degradation nmole (min. mg.protein)$^{-1}$ within 4 days
Culture medium + 4CA	0.086 ± 0.011	61.00 ± 1.68	**8.70 ± 1.60**
4 mM citrate + 4CA	**0.088 ± 0.011**	**64.06 ± 2.95**	9.51 ± 3.15
8 mM citrate + 4CA	0.029 ± 0.008	24.00 ± 1.77	**1.75 ± 0.55**
18 mM citrate + 4CA	0.016 ± 0.008	23.48 ± 1.25	**3.19 ± 1.22**
4 mM citrate	0.024 ± 0.003	-	-
8 mM citrate	0.026 ± 0.005	-	-
18 mM citrate	0.012 ± 0.002	-	-
Culture medium + 4CA	0.086 ± 0.011	61.00 ± 1.68	**8.70 ± 1.60**
4 mM NH_4Cl + 4CA	**0.088 ± 0.009**	**68.21 ± 2.95**	**7.67 ± 2.50**
8 mM NH_4Cl + 4CA	0.083 ± 0.006	58.33 ± 1.69	**4.49 ± 1.60**
18 mM NH_4Cl + 4CA	0.082 ± 0.006	59.33 ± 2.87	**7.63 ± 1.22**
4 mM NH_4Cl	0.012 ± 0.002	-	-
8 mM NH_4Cl	0.019 ± 0.002	-	-
18 mM NH_4Cl	**0.013 ± 0.003**	-	-

Culture medium = mineral medium containing 0.1% yeast extract and 25 ppm 4-chloroaniline

Table 4.12 Biodegradation ability of *Acinetobacter baumannii* (4CA-2) in the combination of carbon or nitrogen source

Degradation condition	**Growth rate (h^{-1})**	**% Total degradation (12 days)**	**Specific degradation nmole (min. mg.protein)$^{-1}$ within 4 days**
Culture medium + 4CA	0.086 ± 0.011	61.00 ± 1.68	**8.70 ± 1.60**
4 mM citrate + 4 mM NH_4Cl + 4CA	**0.091 ± 0.009**	**70.16 ± 1.56**	9.25 ± 2.87
8 mM citrate +8 mM NH_4Cl + 4CA	0.035 ± 0.009	33.38 ± 2.87	**3.25 ± 1.60**
18 mM citrate +18 mM NH_4Cl + 4CA	0.040 ± 0.009	29.35 ± 1.68	**4.48 ± 1.60**
4 mM citrate + 4 mM NH_4Cl	0.078 ± 0.011	-	-
8 mM citrate + 8 mM NH_4Cl	0.040 ± 0.011	-	-
18 mM citrate +18 mM NH_4Cl	0.042 ± 0.009	-	-

Culture medium = mineral medium containing 0.1% yeast extract and 25 ppm 4-chloroaniline

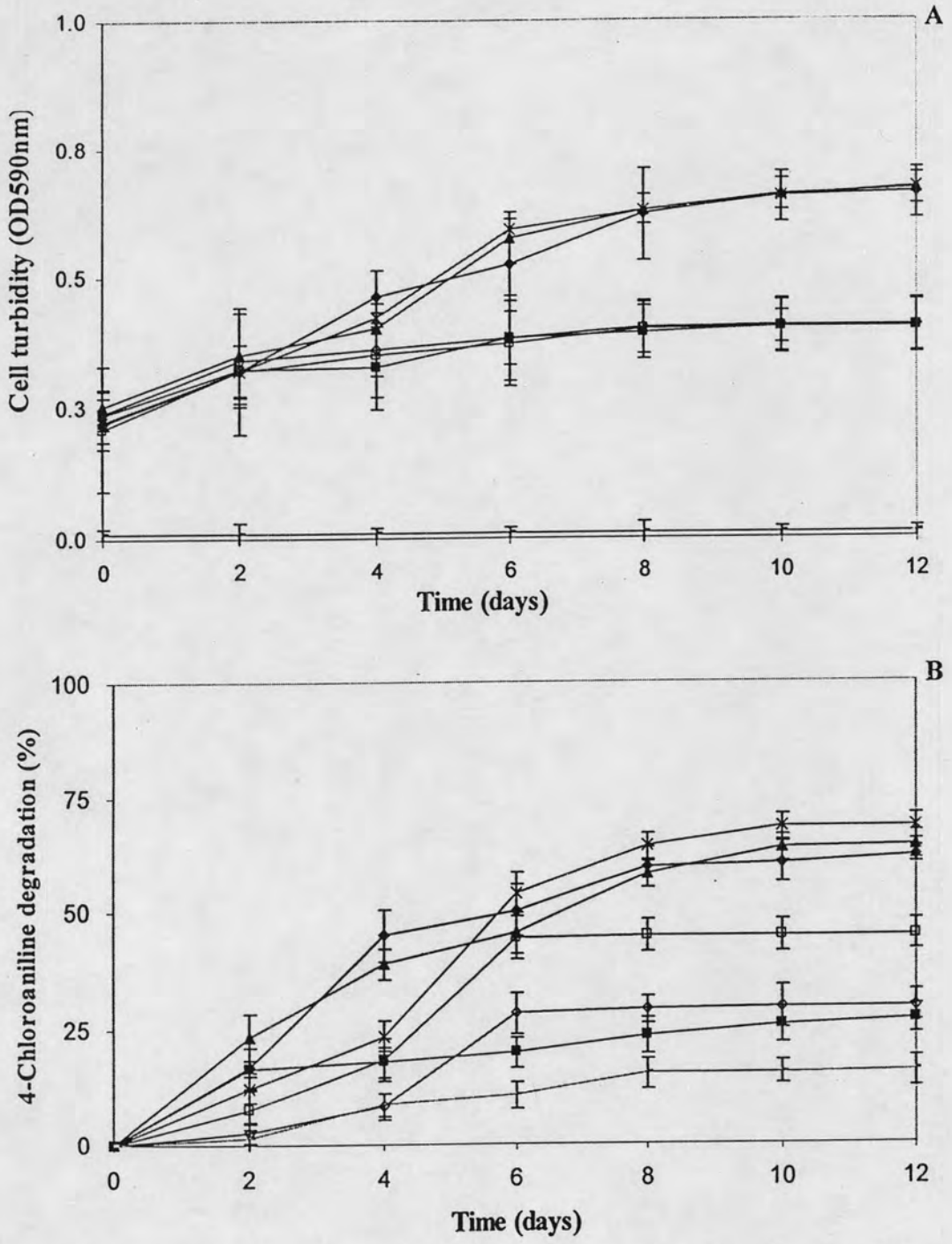

Figure 4.8 Growth (A) and 4-chloroaniline degradation (B) of *Acinetobacter baumannii* (4CA-2) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with additional carbon or nitrogen source: (4CA-2) with no additional carbon or nitrogen source (—◆—), 4 mM succinate (—■—), 4 mM citrate (—▲—),4 mM NH_4Cl (—×—),4 mM $NaNO_3$ (—◇—), 1 mM of aniline (—□—) and control (— —) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

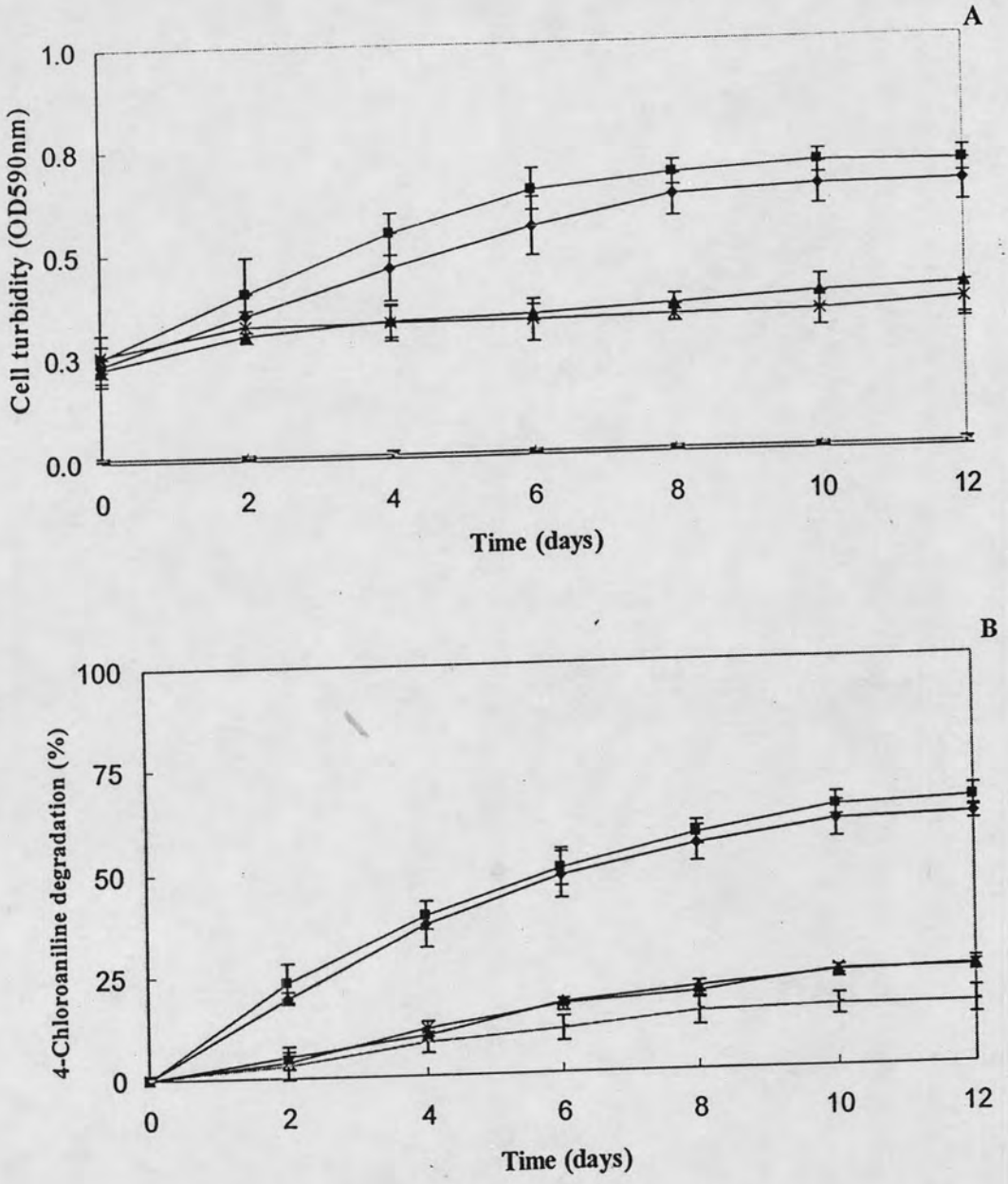

Figure 4.9 Growth (A) and 4-chloroaniline degradation (B) of *Acinetobacter baumannii* (4CA-2) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with various concentration of citrate as an additional carbon source: (4CA-2) with no additional carbon or nitrogen source (—◆—), 4 mM citrate (—■—), 8 mM citrate (—▲—), 18 mM citrate (—✕—) and control (— —) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

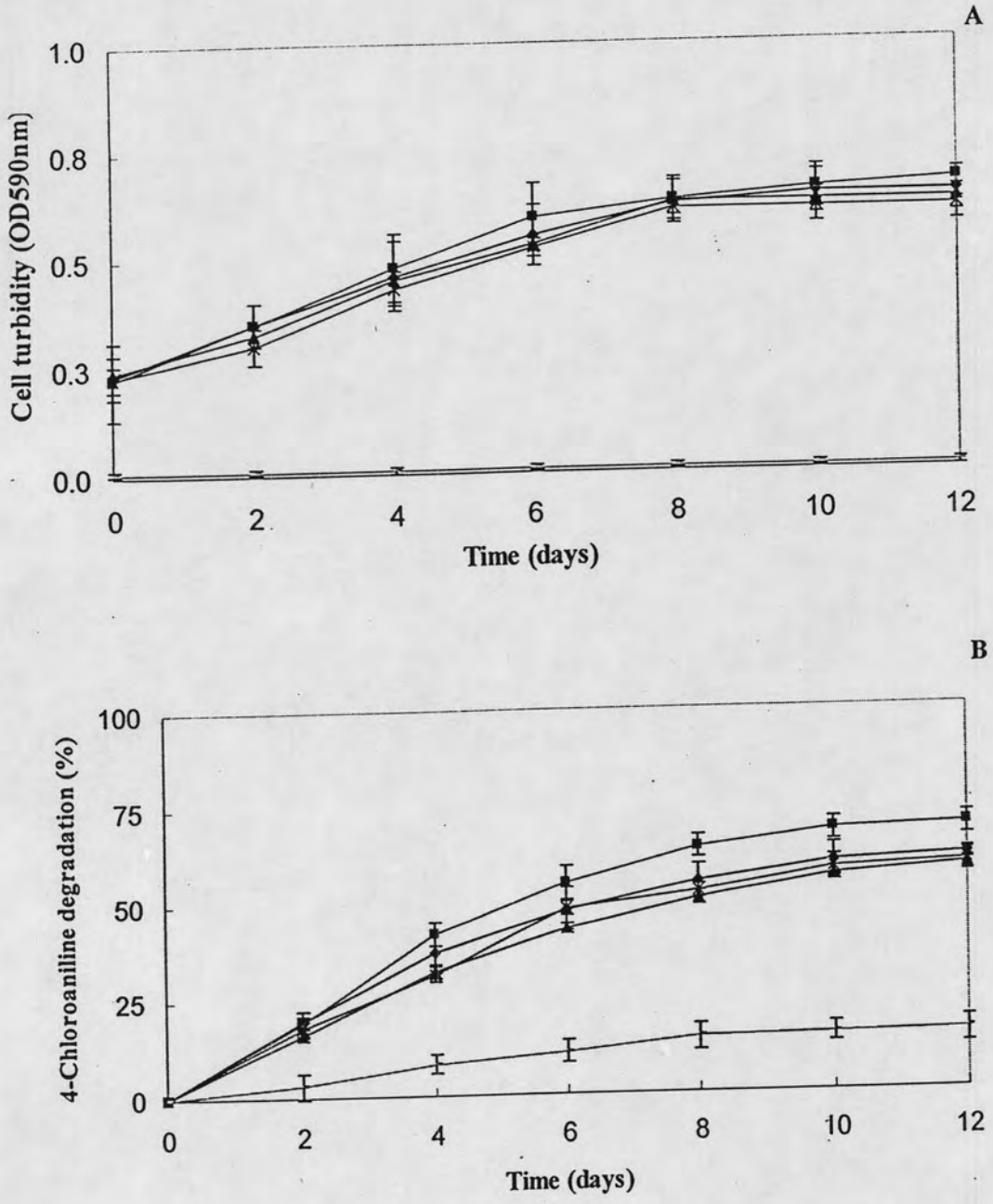

Figure 4.10 Growth (A) and 4-chloroaniline degradation (B) of *Acinetobacter baumannii* (4CA-2) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with various concentration of NH_4Cl as an additional nitrogen source: (4CA-2) with no additional carbon or nitrogen source (—◆—),4 mM NH_4Cl (—■—), 8 mM NH_4Cl (—▲—), 18 mM NH_4Cl (—✕—), and control (— —) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means
(n=3).

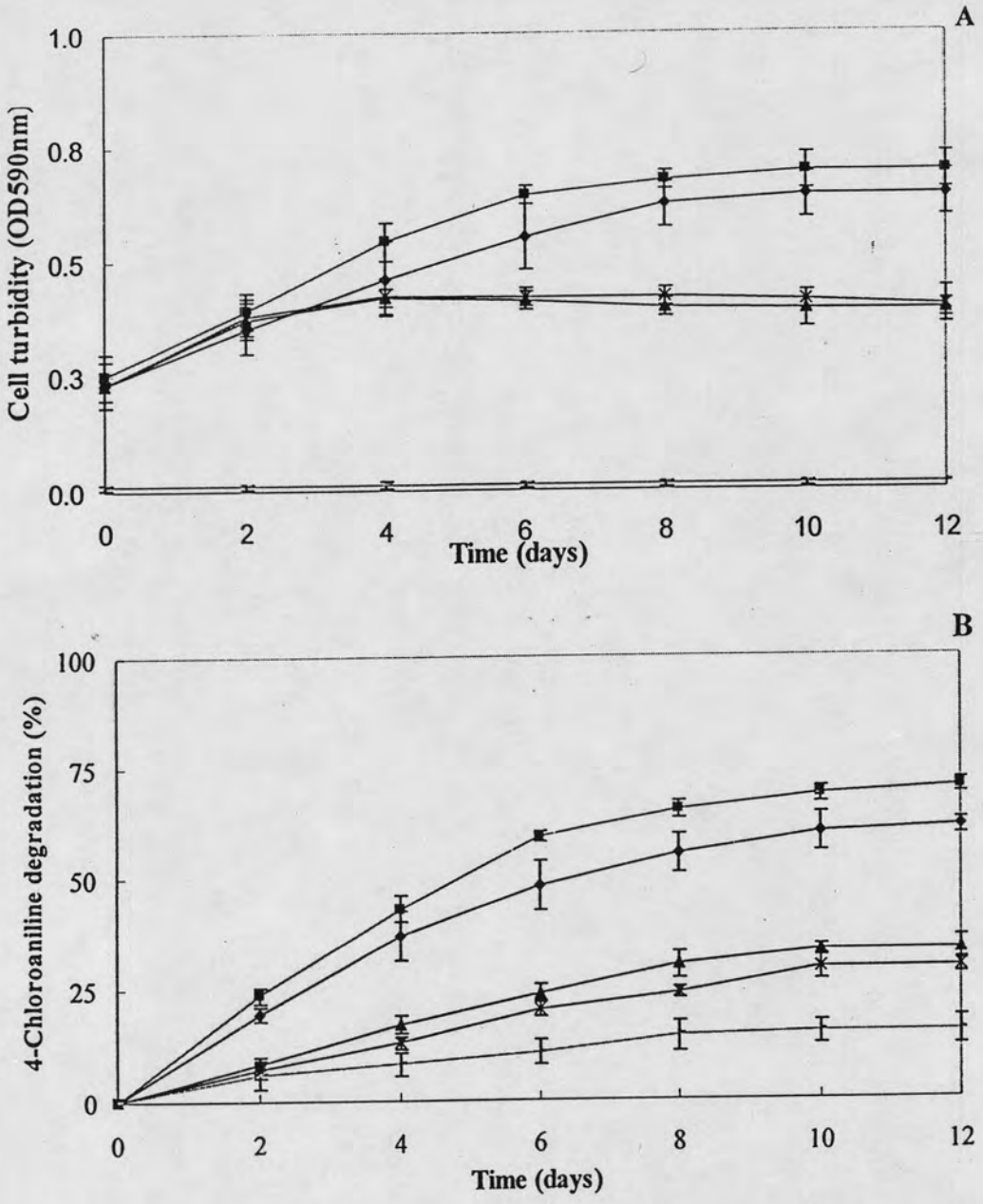

Figure 4.11 Growth (A) and 4-chloroaniline degradation (B) of *Acinetobacter baumannii* (4CA-2) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with a combination of chosen carbon and nitrogen source: (4CA-2) with no additional carbon or nitrogen source (—◆—),4 mM citrate + 4 mM NH_4Cl (—■—), 8 mM citrate + 8 mM NH_4Cl (—▲—),18 mM citrate + 18 mM NH_4Cl (—✕—), and control (——) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

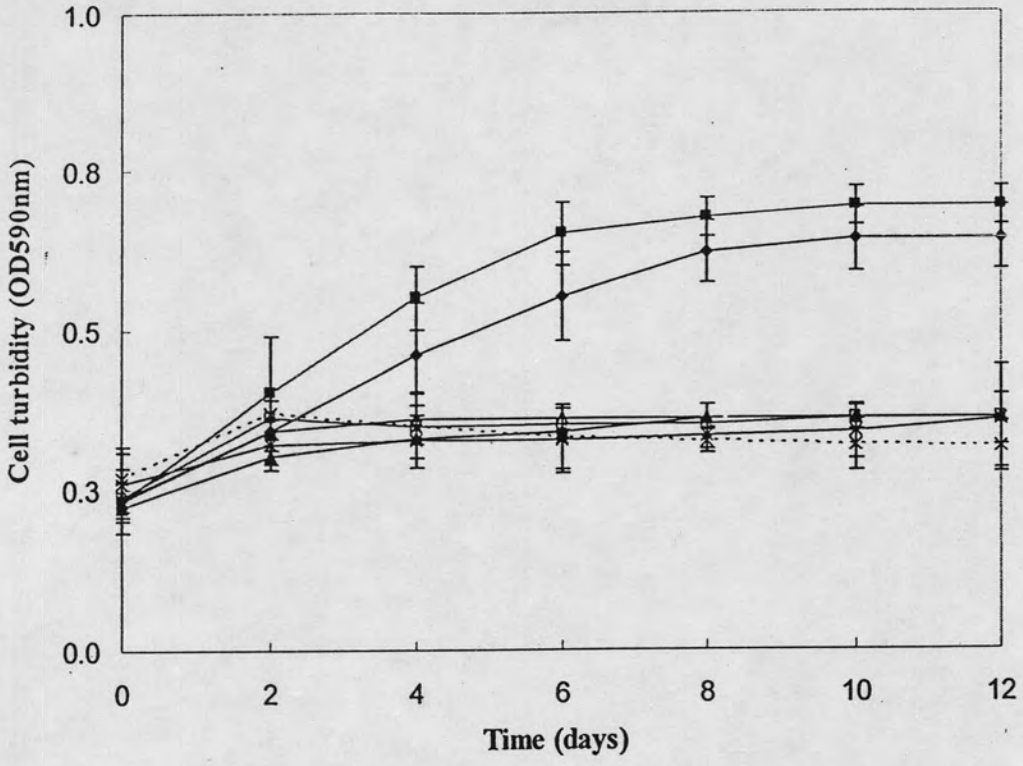

Figure 4.12 Growth of *Acinetobacter baumannii* (4CA-2) in mineral medium with 25 ppm (0.2 mM) 4-chloroaniline (solid line) and in the absence of 4-chloroaniline (dash line) (—◆—); supplemented with 4 mM citrate (—■—, —□—), 8 mM citrate (—▲—, —△—) and 18 mM citrate (—✕—,--✕--). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3)

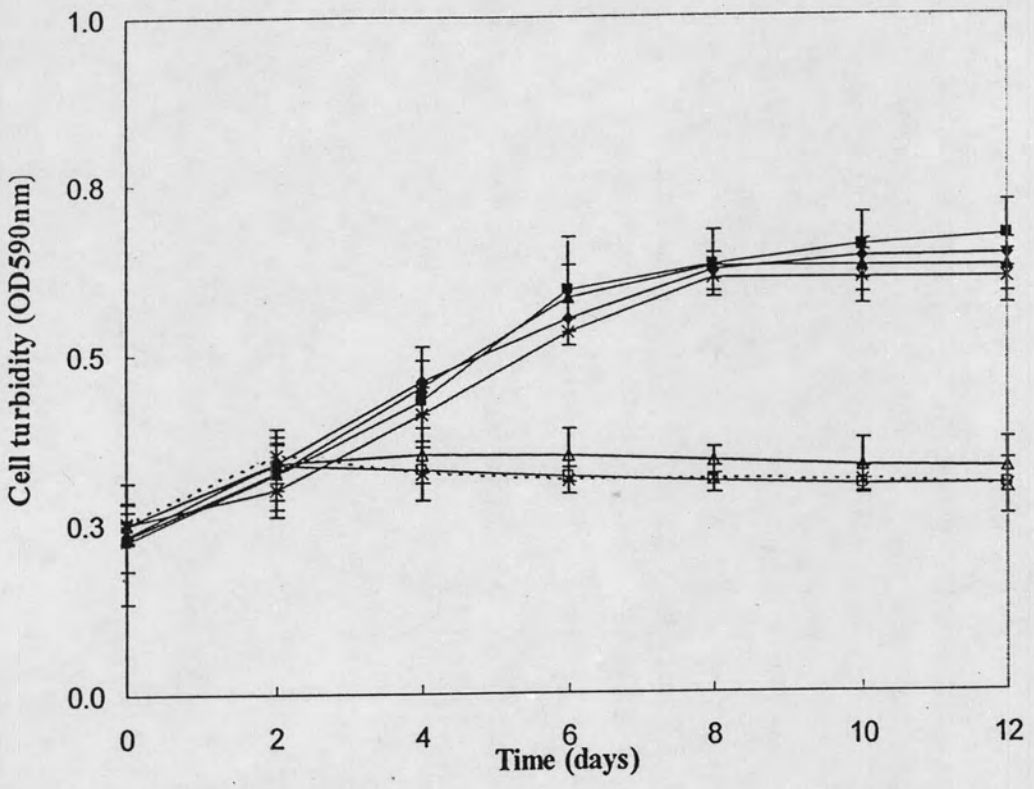

Figure 4.13 Growth of *Acinetobacter baumannii* (4CA-2) in mineral medium with 25 ppm (0.2 mM) 4-chloroaniline (solid line) and in the absence of 4-chloroaniline (dash line) (—◆—); supplemented with 4 mM NH_4Cl (—■—, —□—),8 mM NH_4Cl (—▲—, —△—) and 18 mM NH_4Cl (—×—,--×--). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3)

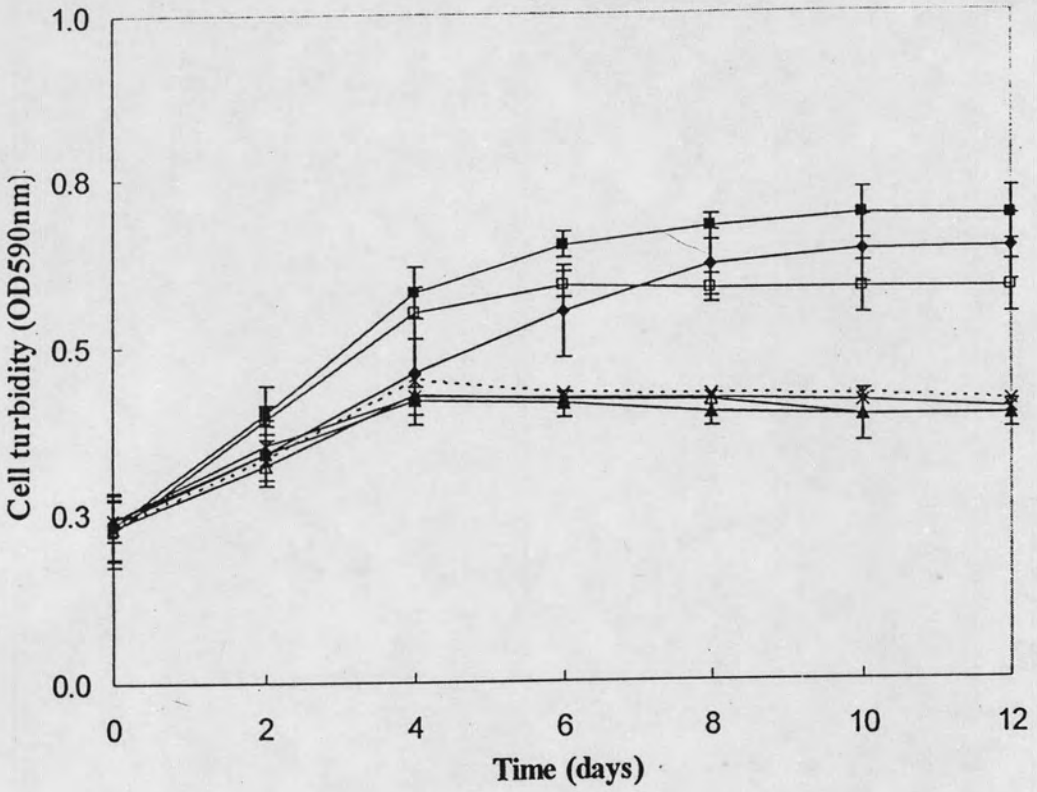

Figure 4.14 Growth of *Acinetobacter baumannii* (4CA-2) in mineral medium with 25 ppm (0.2 mM) 4-chloroaniline (solid line) and in the absence of 4-chloroaniline (dash line) (—◆—); supplemented with 4 mM citrate + 4 mM NH_4Cl (—■—, —□—),8 mM citrate + 8 mM NH_4Cl (—▲—, —△—) and 18 mM citrate + 18 mM NH_4Cl (—×—,--×--)The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3)

Table 4.13 Biodegradation ability of *Pseudomonas putida* (4CA-16) in the additional of carbon source

Degradation condition	**Growth rate (h^{-1})**	**% Total degradation (12 days)**	**Specific degradation nmole (min. mg.protein)$^{-1}$ within 4 days**
Culture medium + 4CA	0.057 ± 0.0012	59.82 ± 0.71	**13.6 ± 2.50**
4 mM citrate + 4CA	**0.065 ± 0.010**	**68.24 ± 2.55**	14.20 ± 1.85
8 mM citrate + 4CA	0.043 ± 0.010	26.75 ± 4.22	**3.51 ± 0.85**
18 mM citrate + 4CA	0.037 ± 0.009	32.91 ± 3.38	**5.62 ± 1.22**
4 mM succinate + 4CA	**0.065 ± 0.009**	**68.31 ± 2.87**	16.14 ± 2.25
8 mM succinate + 4CA	0.041 ± 0.008	40.00 ± 4.22	**7.76 ± 1.55**
18 mM succinate + 4CA	0.031 ± 0.006	36.15 ± 3.38	**6.42 ± 1.55**
4 mM citrate	0.008 ± 0.002	-	-
8 mM citrate	0.037 ± 0.006	-	-
18 mM citrate	0.044 ± 0.009		-
4 mM succinate	0.015 ± 0.003	-	-
8 mM succinate	0.020 ± 0.005	-	-
18 mM succinate	**0.021 ± 0.006**	-	-

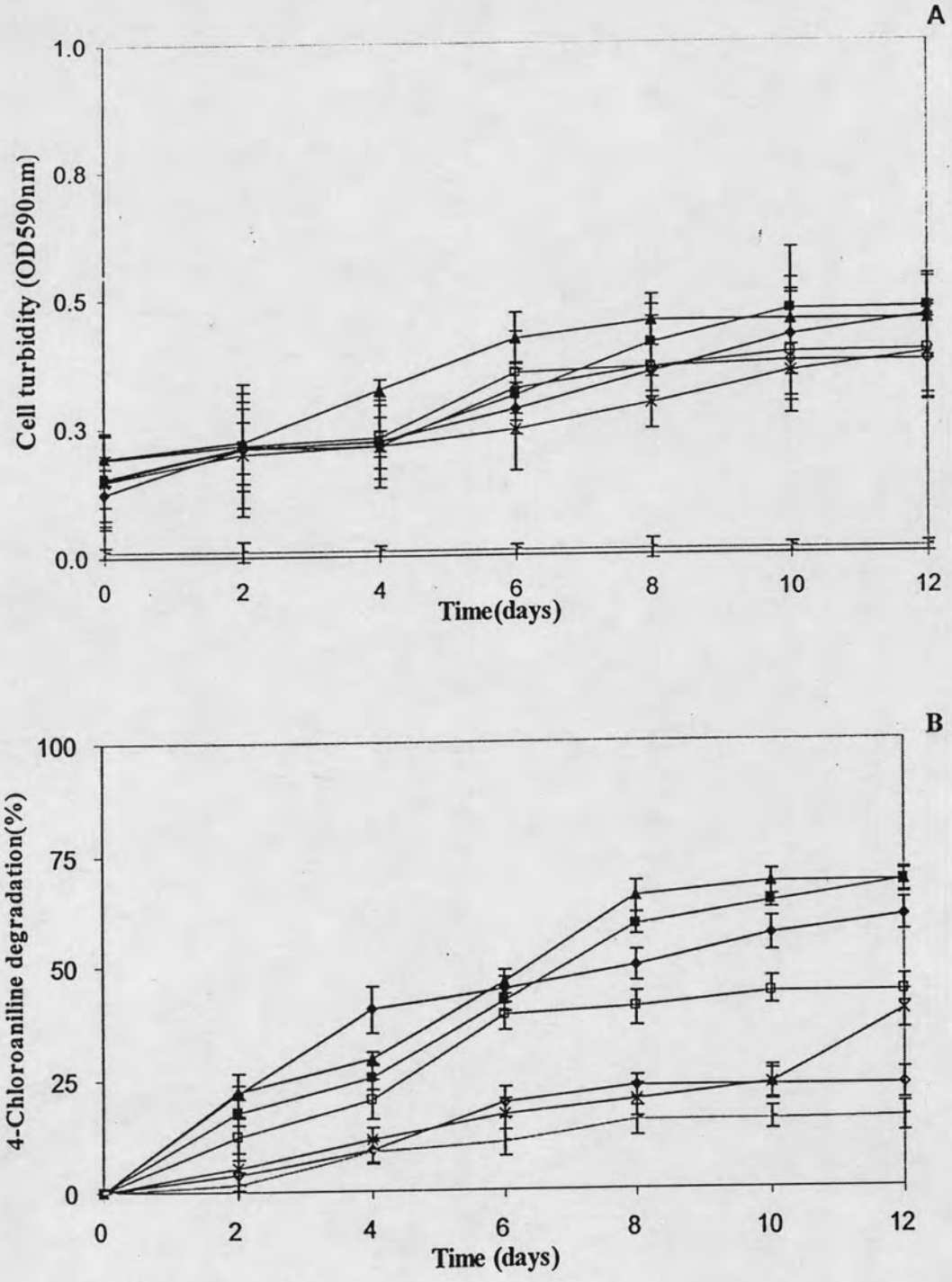

Figure 4.15 Growth (A) and 4-chloroaniline degradation (B) of *Pseudomonas putida* (4CA-16) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with additional carbon or nitrogen source: (4CA-16) with no additional carbon or nitrogen source (—◆—), 4 mM succinate (—■—), 4 mM citrate (—▲—), 4 mM NH_4Cl (—✕—), 4 mM $NaNO_3$ (—◇—), 1 mM of aniline (—□—) and control (——) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

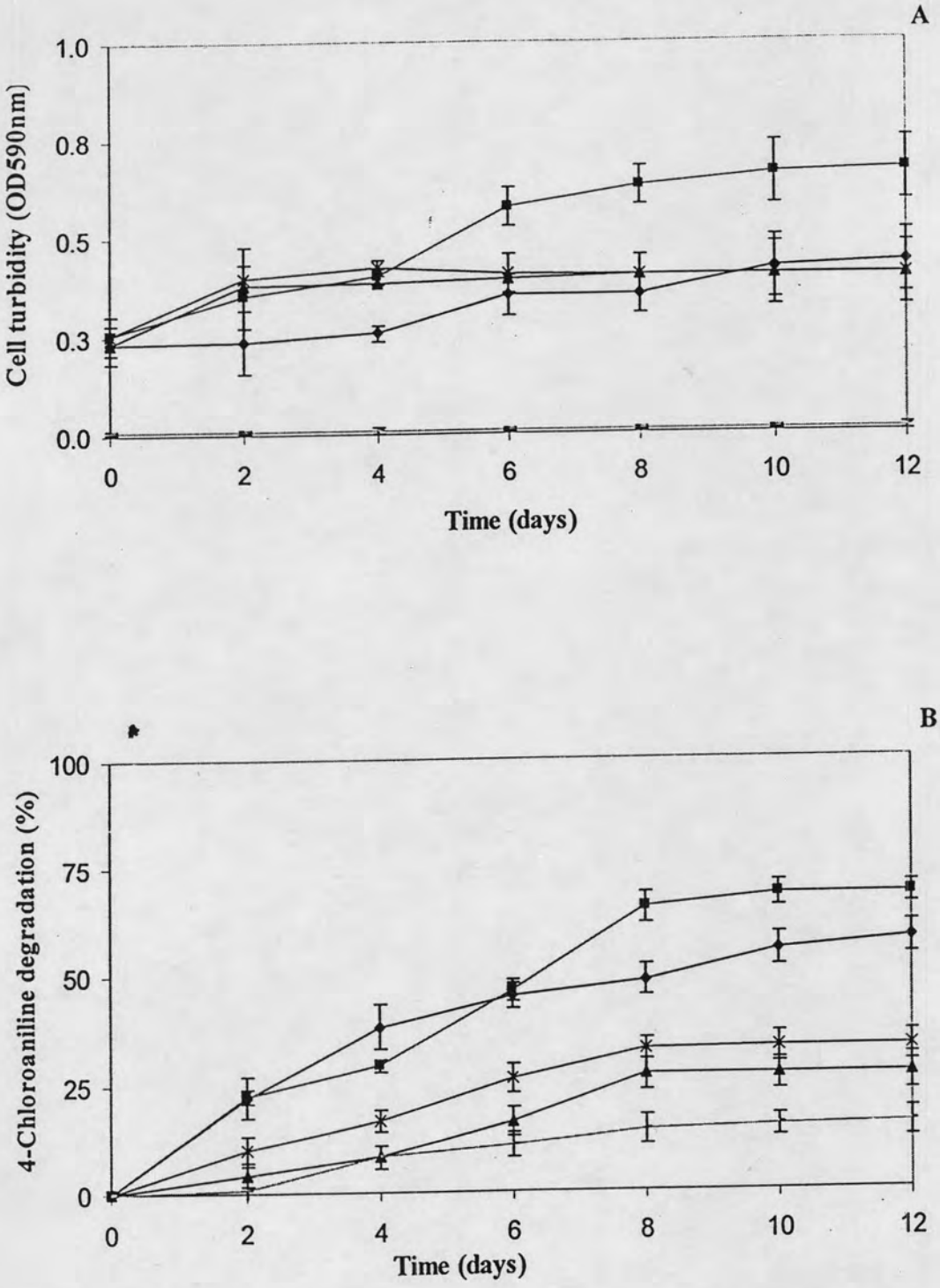

Figure 4.16 Growth (A) and 4-chloroaniline degradation (B) of *Pseudomonas putida* (4CA-16) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with various concentration of citrate as an additional carbon source: (4CA-16) with no additional carbon or nitrogen source (—◆—), 4 mM citrate (—■—), 8 mM citrate (—▲—), 18 mM citrate (—✕—) and control (——) representing abiotic degradation. The data are means from three independent experiment with vertical bars representing standard errors of the means (n=3).

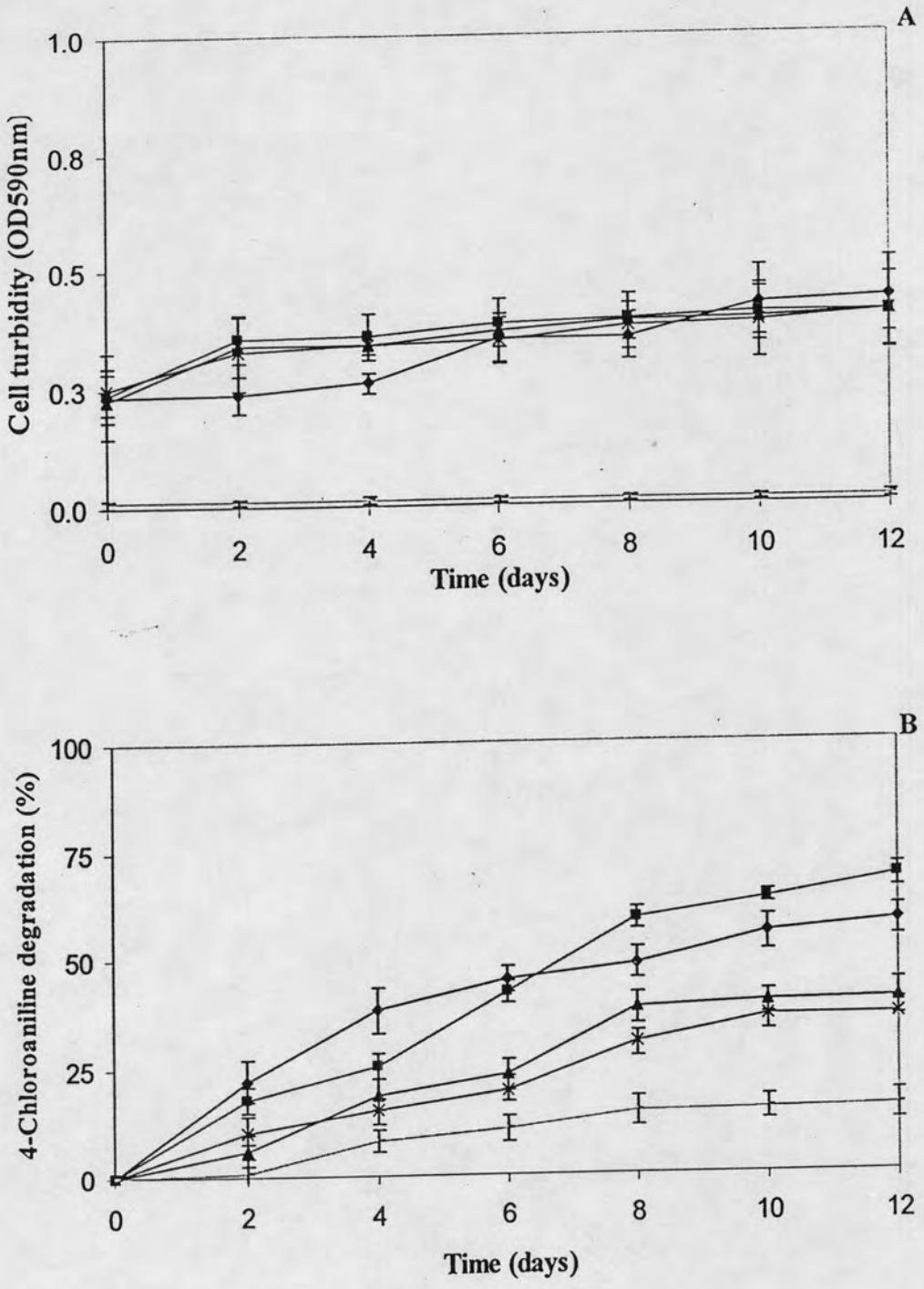

Figure 4.17 Growth (A) and 4-chloroaniline degradation (B) of *Pseudomonas putida* (4CA-16) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with various concentration of succinate as an additional carbon source: (4CA-16) with no additional carbon or nitrogen source (—◆—), 4 mM succinate (—■—), 8 mM succinate (—▲—), 18 mM succinate (—✕—) and control (——) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

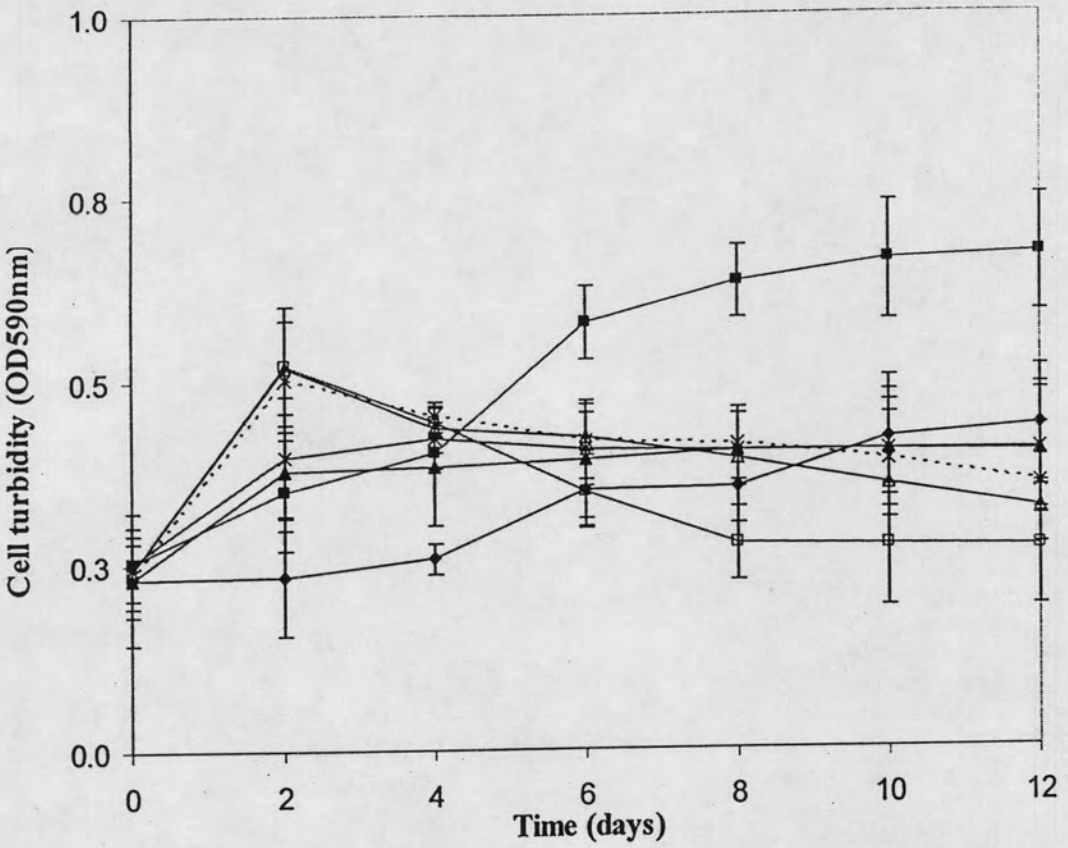

Figure 4.18 Growth of *Pseudomonas putida* (4CA-16) in mineral medium in the presence of 25 ppm (0.2 mM) of 4-chloroaniline (solid line) and in the absence of 4-chloroaniline (dash line) (—◆—); supplemented with 4 mM citrate (—■—, —□—), 8 mM citrate (—▲—, —△—) and 18 mM citrate (—✕—,--✕--). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3)

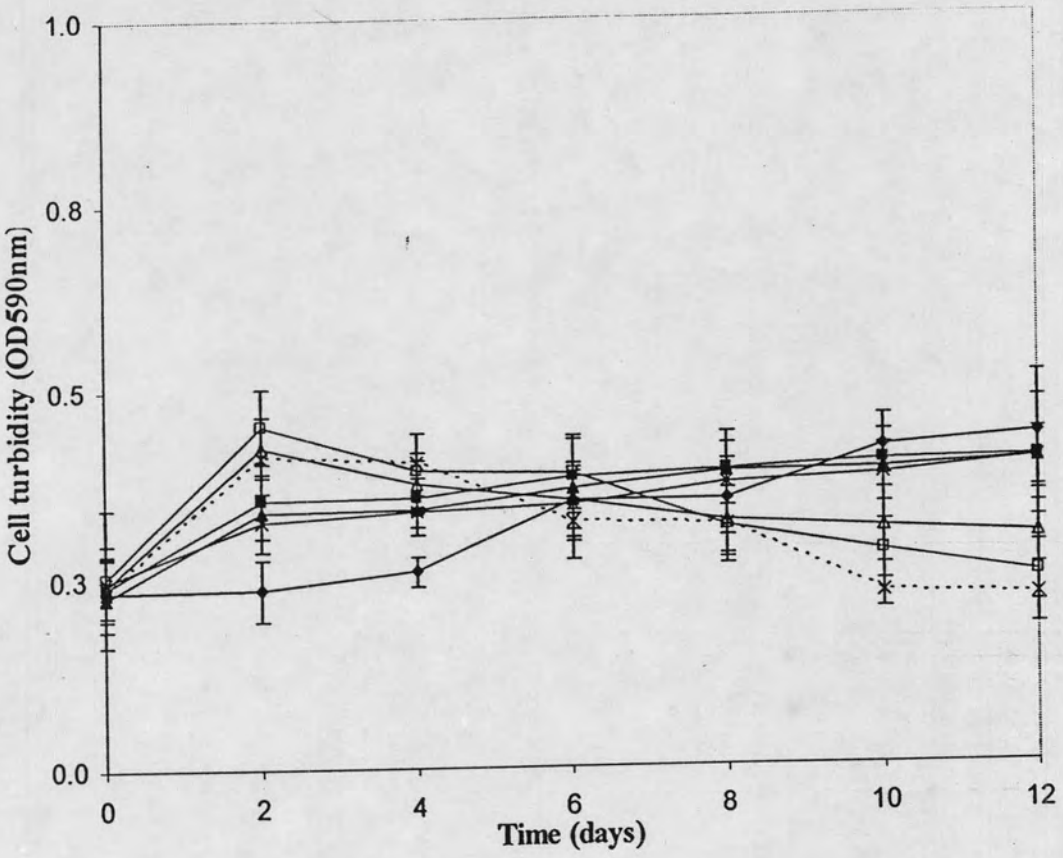

Figure 4.19 Growth of *Pseudomonas putida* (4CA-16) in mineral medium in the presence of 25 ppm (0.2 mM) of 4-chloroaniline (solid line) and in the absence of 4-chloroaniline (dash line) (—◆—); supplemented with 4 mM succinate (—■—, —□—), 8 mM succinate (—▲—, —△—) and 18 mM succinate (—✕—,--✕--). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3)

Table 4.14 Biodegradation ability of *Klebsiella pneumoniae* (4CA-17) in the various concentration of aniline as an additional carbon and nitrogen source

Degradation condition	Growth rate (h^{-1})	% Total degradation (12 days)	Specific degradation nmole (min. mg.protein)$^{-1}$ within 4 days
Culture medium	0.053 ± 0.010	62.82 ± 3.87	**19.0 ± 2.30**
1 mM Aniline + 4CA	**0.064 ± 0.011**	**68.38 ± 3.31**	21.11 ± 2.50
2 mM Aniline + 4CA	0.051 ± 0.010	**67.19 ± 3.87**	**16.14 ± 2.55**
4 mM Aniline + 4CA	0.050 ± 0.010	62.32 ± 3.31	**14.65 ± 2.87**
8 mM Aniline + 4CA	0.001 ± 0.000	10.14 ± 2.87	-
16 mM Aniline+ 4CA	0.001 ± 0.000	10.06 ± 3.50	-
1 mM Aniline	0.050 ± 0.011	-	-
2 mM Aniline	0.057 ± 0.011	-	-
4 mM Aniline	0.040 ± 0.008	-	-
8 mM Aniline	0.000 ± 0.000	-	-
16 mM Aniline	**0.000 ± 0.000**	-	-

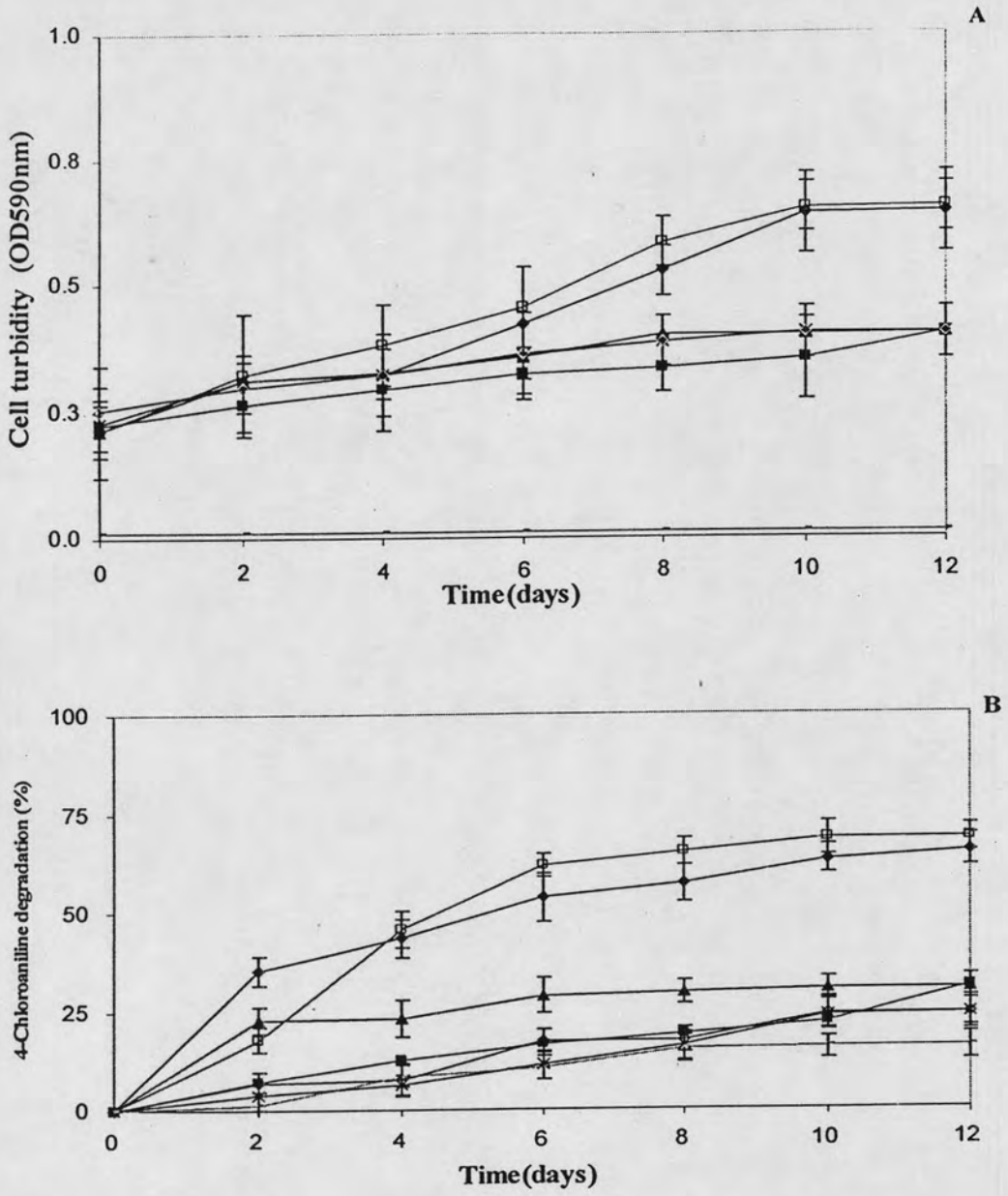

Figure 4.20 Growth (A) and 4-chloroaniline degradation (B) of *Klebsiella pneumoniae* (4CA-17) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with additional carbon or nitrogen source: (4CA-17) with no additional carbon or nitrogen source (—◆—), 4 mM succinate (—■—), 4 mM citrate (—▲—), 4 mM NH_4Cl (—×—), 4 mM $NaNO_3$ (—◇—), 1 mM of aniline (—□—) and control (——) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

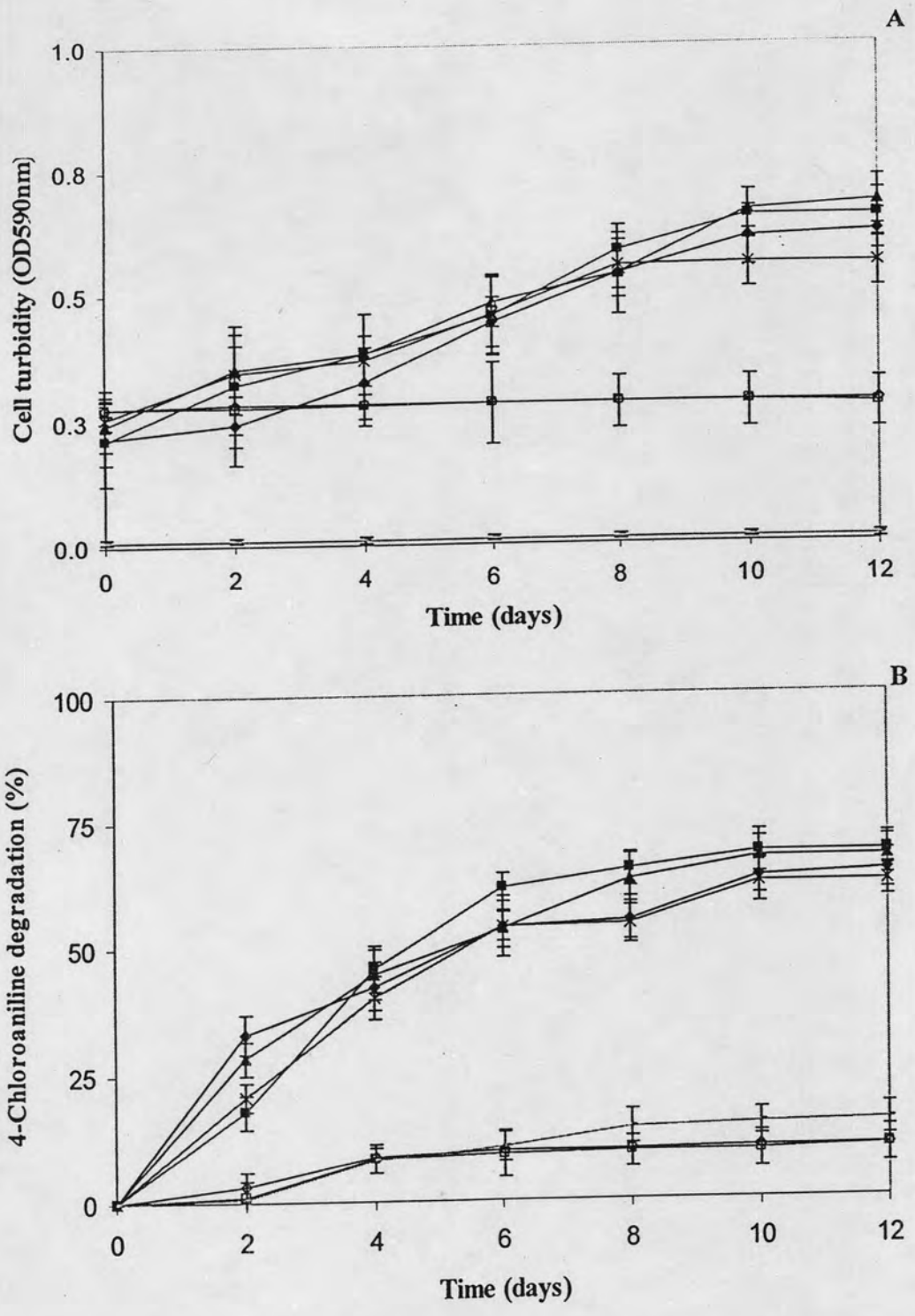

Figure 4.21 Growth (A) and 4-chloroaniline degradation (B) of *Klebsiella pneumoniae* (4CA-17) which was grown in 25 ppm (0.2 mM) 4-chloroaniline supplemented with the various concentration of aniline as an additional carbon and nitrogen source: (4CA-17) with no additional carbon or nitrogen source (—◆—), 1 mM aniline (—■—), 2 mM aniline (—▲—), 4 mM aniline (—✕—), 8 mM aniline(—◇—), 16 mM aniline (—□—) and control (——) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

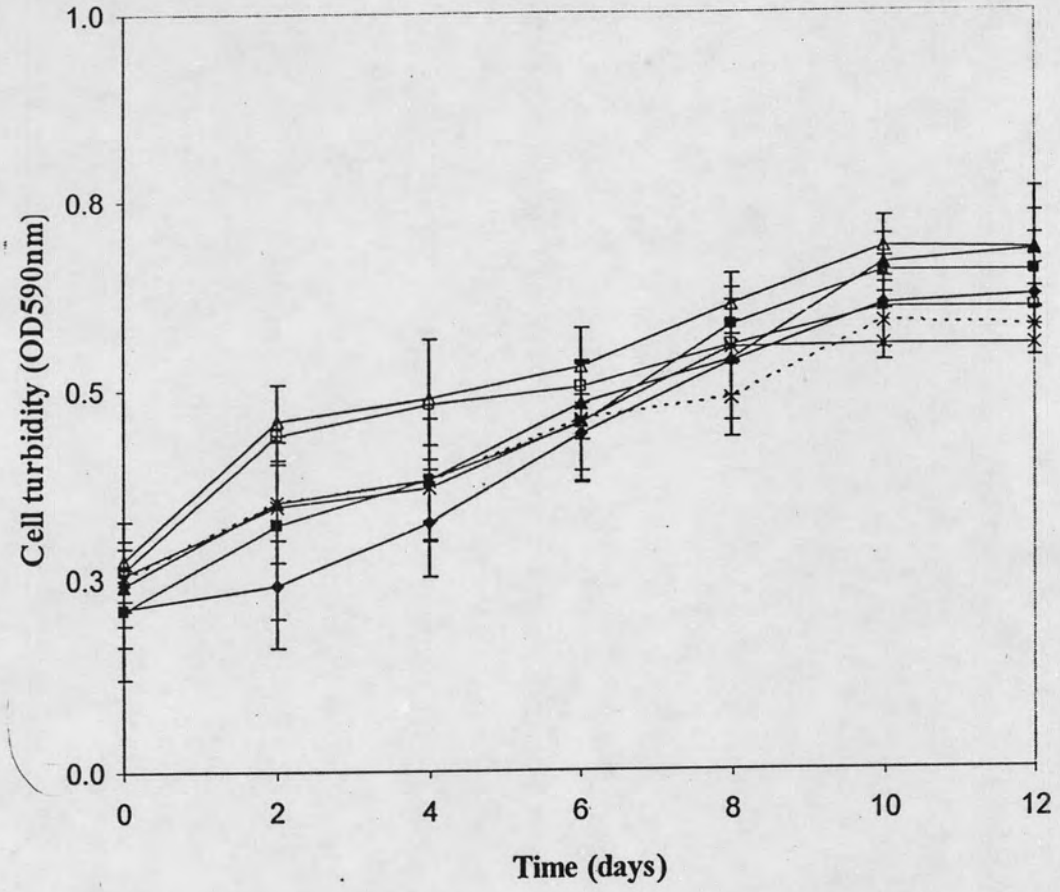

Figure 4.22 Growth of *Klebsiella pneumoniae* (4CA-17) in mineral medium in the presence of 25 ppm (0.2 mM) of 4-chloroaniline (solid line) and in the absence of 4-chloroaniline (dash line) (—◆—); supplemented with 1 mM aniline (—■—, —□—), 2 mM aniline (—▲—, —△—) and 4 mM aniline (—✕—,--✕--). The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

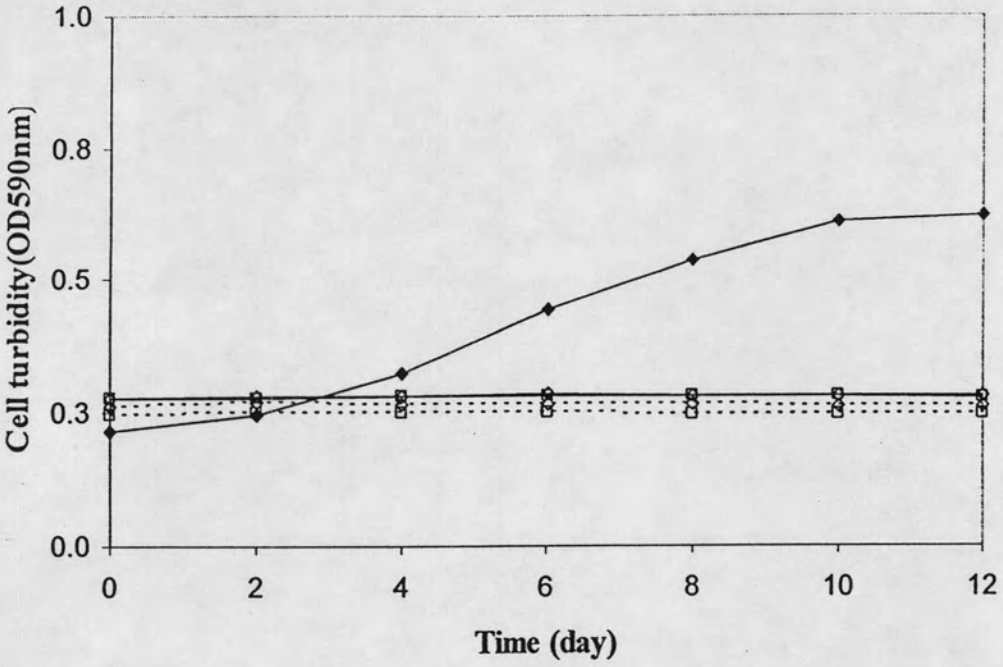

Figure 4.23 Growth of *Klebsiella pneumoniae* (4CA-17) in mineral medium in the presence of 25 ppm (0.2 mM) of 4-chloroaniline (solid line) and in the absence of 4-chloroaniline (dash line) (—◆—); supplemented with 8 mM aniline (—◆—,—◇—), 16 mM aniline (—□—,- -□- -)The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

Table 4.15 Biodegradation ability of the three bacterial isolates in optimum conditions

Bacterial name	**Degradation condition (in the presence of 25 ppm (0.2 mM) 4CA)**	**Growth rate (h^{-1})**	**% Total degradation (12 days)**	**Specific degradation nmole (min. mg.protein)$^{-1}$ within 4 days**
4CA-2	Culture medium	0.086 ± 0.011	61.00 ± 1.68	**8.70 ± 1.60**
	4 mM citrate	0.088 ± 0.011	64.06 ± 2.95	**9.51 ± 3.15**
	4 mM NH_4Cl	0.088 ± 0.009	68.21 ± 2.95	**7.67 ± 2.50**
	4 mM citrate + 4 mM NH_4Cl	0.091 ± 0.009	70.16 ± 1.56	**9.25 ± 2.87**
4CA-16	Culture medium	0.057 ± 0.012	59.82 ± 0.71	**13.6 ± 2.50**
	4 mM citrate	0.065 ±0.009	68.24 ± 2.55	**14.20 ± 1.85**
	4 mM succinate	0.065 ± 0.010	68.31 ± 2.87	**16.14 ± 2.25**
4CA-17	Culture medium	0.053 ± 0.011	62.82 ± 3.87	**19.0 ± 2.30**
	1mM Aniline	0.064 ± 0.010	68.38 ± 3.31	**21.11 ± 2.50**
	2mM Aniline	**0.051 ± 0.010**	**67.19 ± 3.87**	**16.14 ± 2.55**

Culture medium = mineral medium containing 0.1% yeast extract and 25 ppm 4-chloroaniline

4.4 Effect of 4-chloroaniline concentration on growth and biodegradability of 4-chloroaniline-degrading bacteria

4-Chloroaniline degradation ability of 4-chloroaniline-degraing bacteria was so far studied when the concentration of 4-chloroaniline was fixed at 25 ppm (0.2 mM). Further investigation was to determine if the isolates were able to tolerate and degrade 4-chloroaniline at higher concentrations. Cells were subcultured into mineral medium supplied with different concentrations of 4-chloroaniline ranging from 25 ppm (0.2 mM), 50 ppm (0.4 mM), 100 ppm (0.8 mM), 150 ppm (1.2 mM) to 200 ppm (1.6 mM). First, cells were precultured in mineral medium containing 0.1% yeast extract supplemented with 25 ppm (0.2 mM) 4-chloroaniline to the OD of 0.35 ± 0.08 (2 days). Then, Five % inoculum was added into 100 ml mineral medium containing different concentration of 4-chloroaniline as indicated. The results are displayed in Figure 4.24- Figure 4.27 and can be concluded in Table 4.16. Slope from of each line of Figure 4.24A- Figure 4.27A were also used to calculate growth rate, while those of Figure 4.24B- Figure 4.27B were used to calculate specific degradation rate within the first 4 days when cells were grown on different concentrations of 4-chloroaniline.

When the concentration of 4-chloroaniline was increased, the total degradation and growth was decreased in all culture tested. Furthermore, at 200 ppm (1.6 mM) 4-chloroanilines cell growth was inhibited, although slight 4-chloroaniline utilization (up to 5% total degradation) was observed (Table 4.16).

Table 4.16 Biodegradation ability of the three bacterial isolates in various concentrations of 4-chloroaniline

4-Chloroaniline concentration	Bacterial isolate[f]	Growth rate $(h)^{-1}$	% Total degradation (12 days)	Specific degradation nmole (min. mg.protein)$^{-1}$ within 4 days
25 ppm (0.2 mM)	(4CA-2)	0.086 ± 0.011	61.00% ± 1.68	**8.70 ±1.60**
	(4CA-16)	0.057 ± 0.012	59.82% ± 0.71	**13.60 ±2.50**
	(4CA-17)	0.053 ± 0.010	**68.20% ± 3.87**	19.00 ±2.30
	Control [a]	-	12.75% ± 0.08	-
50 ppm (0.4 mM)	(4CA-2)	0.044 ± 0.009	40.05% ± 1.68	**16.74 ± 3.20**
	(4CA-16)	0.044 ± 0.008	48.22% ± 3.60	**30.79± 2.00**
	(4CA-17)	0.040 ± 0.010	**55.5% ± 1.68**	32.89 ± 1.50
	Control [b]	-	14.55% ± 3.31	-
100 ppm (0.8 mM)	(4CA-2)	0.031 ± 0.008	37.85% ± 1.68	**34.82 ± 1.50**
	(4CA-16)	0.039 ± 0.008	40.22% ± 3.60	**30.55 ± 3.20**
	(4CA-17)	0.033 ± 0.008	**47.85% ± 3.87**	38.04 ± 1.00
	Control [c]	-	12.55% ± 3.31	-
150 ppm (1.2 mM)	(4CA-2)	0.031 ± 0.008	37.45% ± 1.68	**52.16 ± 2.00**
	(4CA-16)	0.038 ± 0.009	37.56% ± 3.67	**54.14 ± 3.10**
	(4CA-17)	0.031 ± 0.009	**39.44% ± 3.87**	55.11 ± 2.50
	Control [d]	-	10.87% ± 3.31	-
200 ppm (1.6 mM)	(4CA-2)	0.002 ± 0.000	9.55% ± 1.68	-
	(4CA-16)	0.004 ± 0.001	6.55% ± 3.68	-
	(4CA-17)	0.002 ± 0.000	10.55% ± 3.87	-
	Control [e]	-	**5.65% ± 3.31**	-

[a,b,c,d,e] Control were abiotic controls having 25 ppm, 50 ppm, 100 ppm, 150 ppm and 200 ppm 4-Chloroaniline in mineral medium without bacterial culture, respectively.

[f](4CA-2), (4CA-16) and (4CA-17) were represented for *Acinetobacter baumannii, Pseudomonas putida* and *Klebsiella pneumoniae*, respectively.

Note: raw data is available in Appendix C.

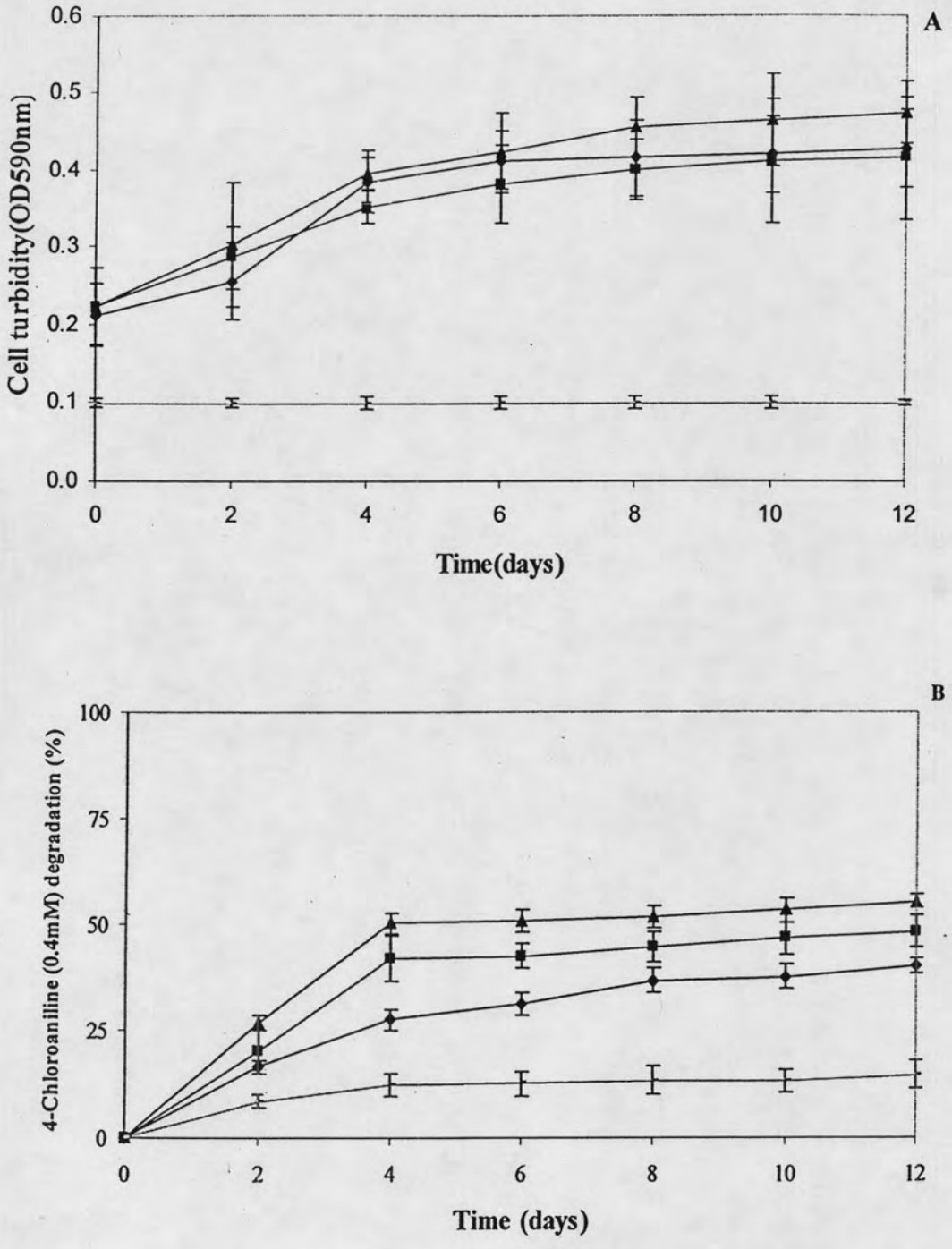

Figure 4.24 Effect of 4-chloroaniline at 50 ppm (0.4 mM) provided as carbon and nitrogen sources on (A) growth and (B) total degradation ability of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—), and control (——) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

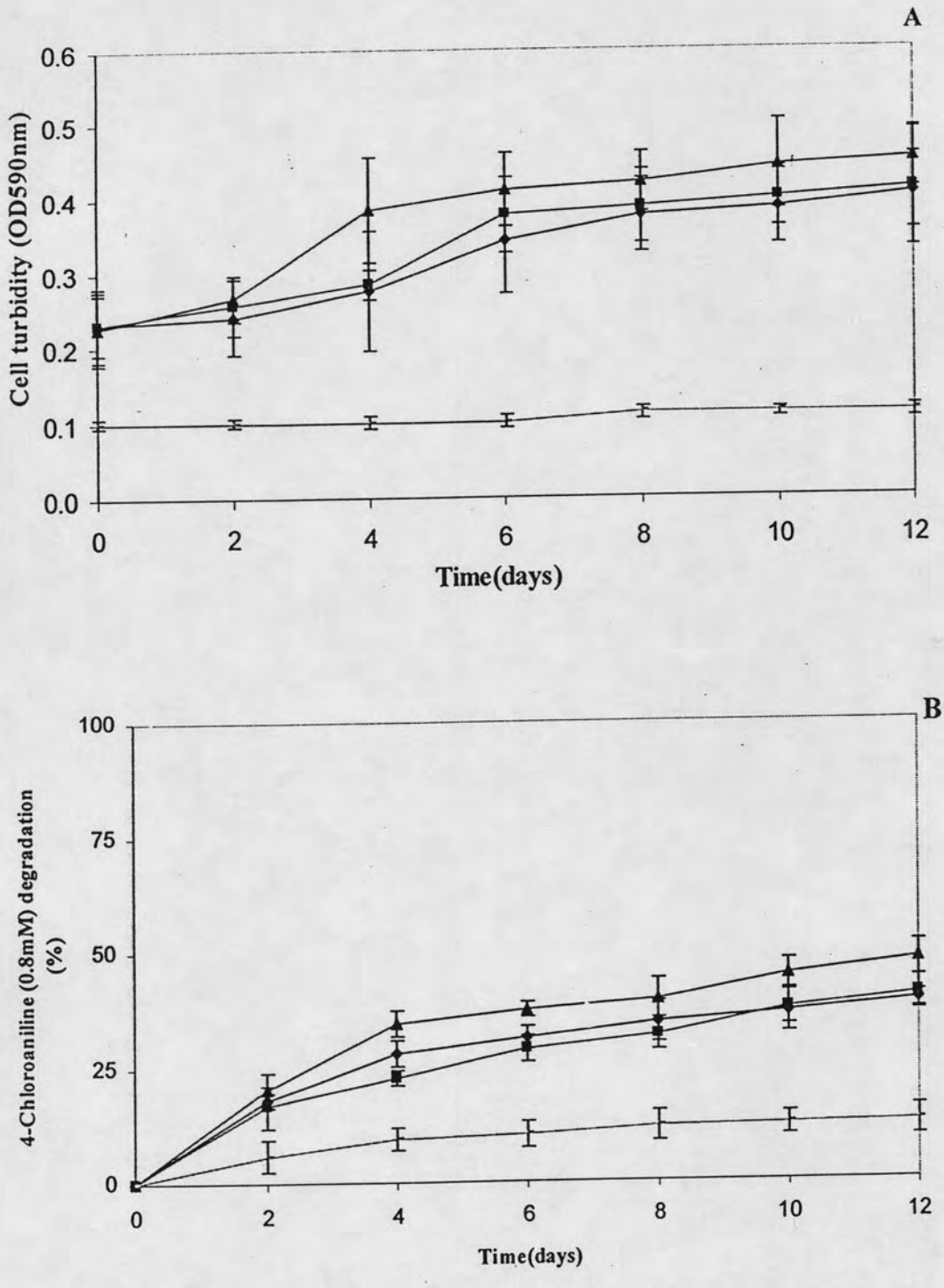

Figure 4.25 Effect of 4-chloroaniline at 100 ppm (0.8 mM) provided as carbon and nitrogen sources on (A) growth and (B) total degradation ability of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—), and control (—·—) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

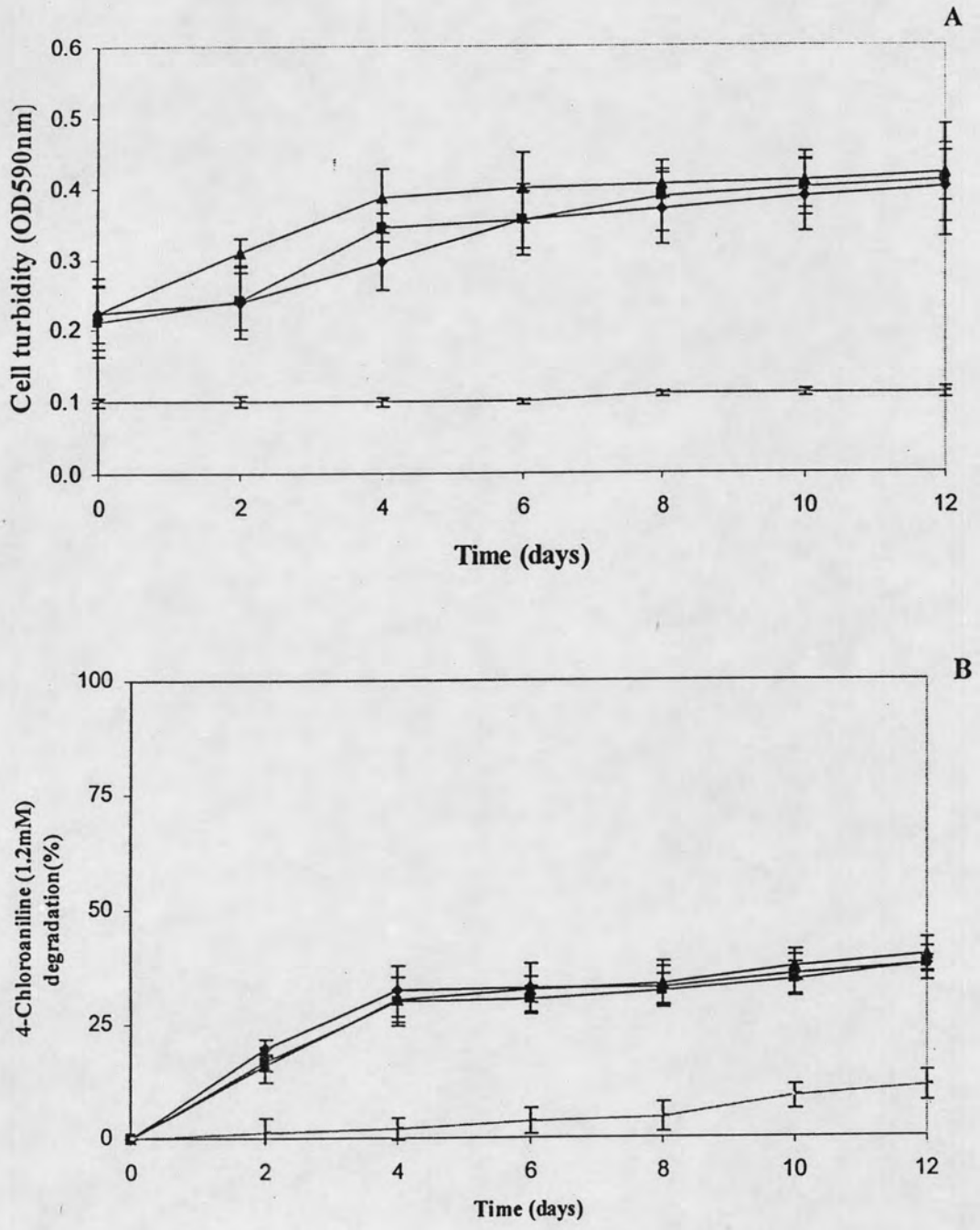

Figure 4.26 Effect of 4-chloroaniline at 150 ppm (1.2 mM) provided as carbon and nitrogen sources on (A) growth and (B) total degradation ability of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—), and control (——) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

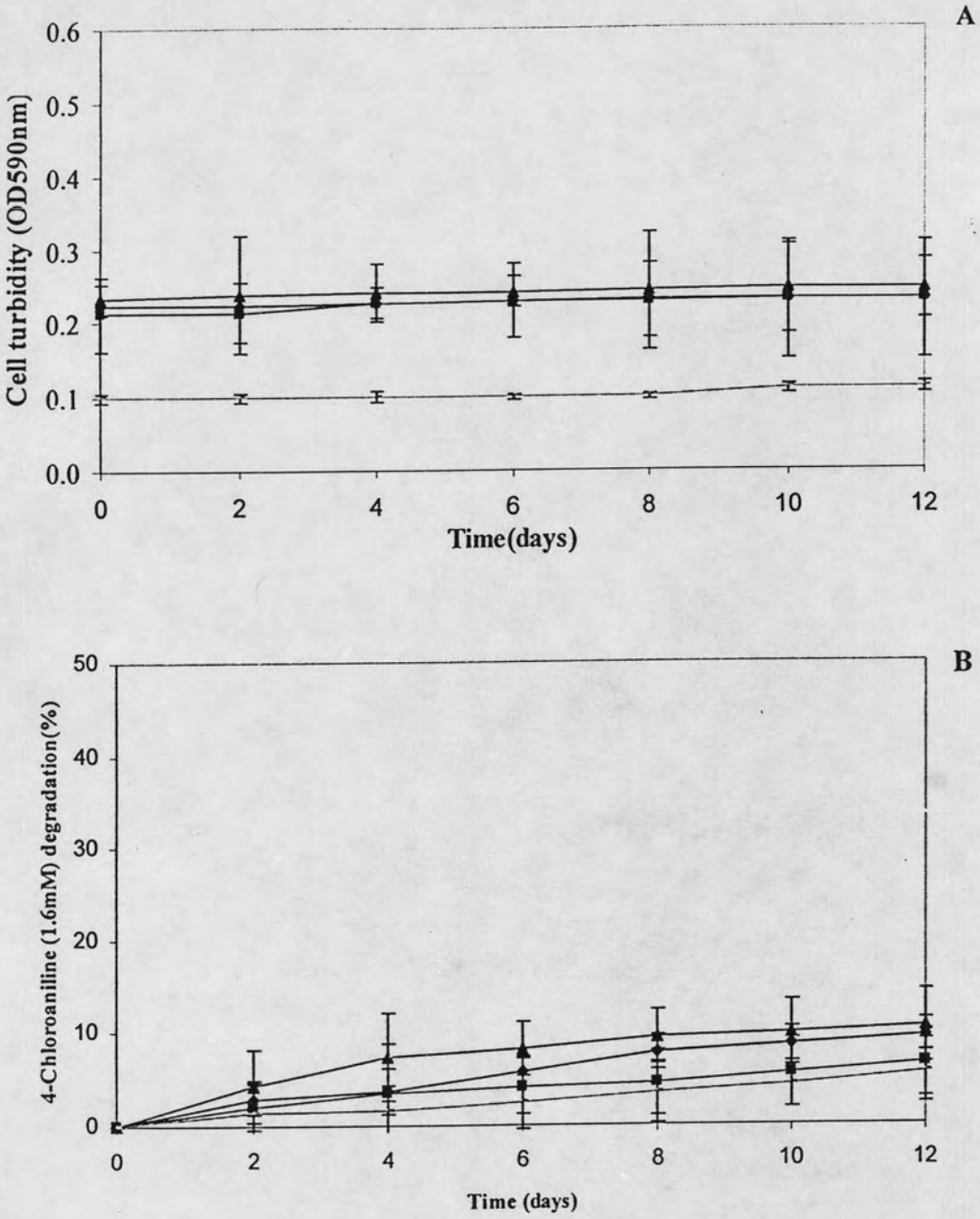

Figure 4.27 Effect of 4-chloroaniline at 200 ppm (1.6 mM) provided as carbon and nitrogen sources on (A) growth and (B) total degradation ability of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—), and control (——) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

4.5 Substrate range

Chloroanilines including 2-chloroaniline, 3-chloroaniline, 4-chloroaniline and 3,4-dichloroaniline are the intermediates of microbial degradation of phenyl urea, phenylcarbamate herbicides. As the bacterial isolates were able to degrade 4-chloroaniline to some extent, it was interested to investigate their abilities to utilize other chloroanilines as their substrate. The substrate including 2-chloroaniline, 3-chloroaniline, 4-chloroaniline were tested at 0.2 mM (25 ppm), 3,4-dichloroaniline tested at 0.15 mM (25 ppm), while aniline tested at 1 mM aniline. Cells were subcultured into mineral medium supplied to different concentrations of 25 ppm (0.2 mM) 4-chloroaniline. Then, cells were precultured in mineral medium containing 0.1% yeast extract supplemented with 25 ppm (0.2 mM) monochloroaniline or 25 ppm (0.15 mM) dichloroaniline or (1 mM) aniline. The results are showed in Figure 4.28- Figure 4.31 and can be concluded in Table 4.17. Slope from of each line of Figure 4.28B- Figure 4.31B were used to calculate specific degradation rate within the first 4 days when cells were grown on different concentration of 4-chloroaniline. Slope from of each line of Figure 4.28A- Figure 4.31A were also used to calculate growth rate.

Table 4.17 Biodegradation ability of the three bacterial isolates in substrate range of 4-chloroaniline

Concentration of substrate range	**Bacterial name[f]**	**Growth rate $(h)^{-1}$**	**% Total degradation (12days)**	**Specific degradation nmole (min. mg.protein)$^{-1}$ within 4 days**
25 ppm	(4CA-2)	0.059 ± 0.009	**64.78% ± 1.68**	12.47 ± 2.50
(0.2 mM)	(4CA-16)	0.046 ± 0.009	63.00% ± 3.60	**12.10 ± 2.87**
2-Chloroaniline	(4CA-17)	0.040 ± 0.010	58.85% ± 3.87	**11.44 ± 3.60**
	Control [b]	-	19.55% ± 3.31	-
25 ppm	(4CA-2)	0.037 ± 0.008	39.59% ± 1.68	**9.14 ± 1.50**
(0.2 mM)	(4CA-16)	0.030 ± 0.008	38.66% ± 3.60	**8.55 ± 2.87**
3-Chloroaniline	(4CA-17)	0.028 ± 0.008	**64.87% ± 1.68**	9.39 ± 3.60
	Control [b]	-	19.35% ± 3.31	-
25 ppm	(4CA-2)	0.086 ± 0.011	61.00% ± 1.68	**8.70 ±1.60**
(0.2 mM)	(4CA-16)	0.057 ± 0.012	59.82% ± 0.71	**13.60 ±2.50**
4-Chloroaniline	(4CA-17)	0.053 ± 0.0010	**68.20% ± 3.87**	19.00 ± 2.30
	Control [a]	-	12.75% ± 0.08	-
25 ppm	(4CA-2)	0.008 ± 0.002	8.55% ± 1.68	-
(0.15 mM)	(4CA-16)	0.002 ± 0.002	5.65% ± 2.87	-
3,4-Dichloroaniline	(4CA-17)	0.001 ± 0.000	9.87% ± 2.87	-
	Control [d]	-	**5.55% ± 3.11**	-
1 mM Aniline	(4CA-2)	0.028 ± 0.006	30.11% ± 1.68	**-15.73 ± 4.40**
	(4CA-16)	0.039 ± 0.008	35.66% ± 3.60	**-28.25 ± 3.87**
	(4CA-17)	0.033 ± 0.008	**45.97% ± 3.87**	-36.86 ± 3.20
	Control [e]	-	**15.65% ± 3.31**	-

[a,b,c,d,e] Control were abiotic controls having 25 ppm(0.2 mM 2-Chloroaniline, 25 ppm(0.2 mM) 3-Chloroaniline, 25 ppm(0.2 mM) 4-Chloroaniline, 25 ppm (0.15 mM) 3,4-Dichloroaniline and 1 mM aniline in mineral medium without inoculum, respectively.

[f](4CA-2), (4CA-16) and (4CA-17) were represented for *Acinetobacter baumannii, Pseudomonas putida* and *Klebsiella pneumoniae*, respectively.

Note: raw data is available in Appendix C.

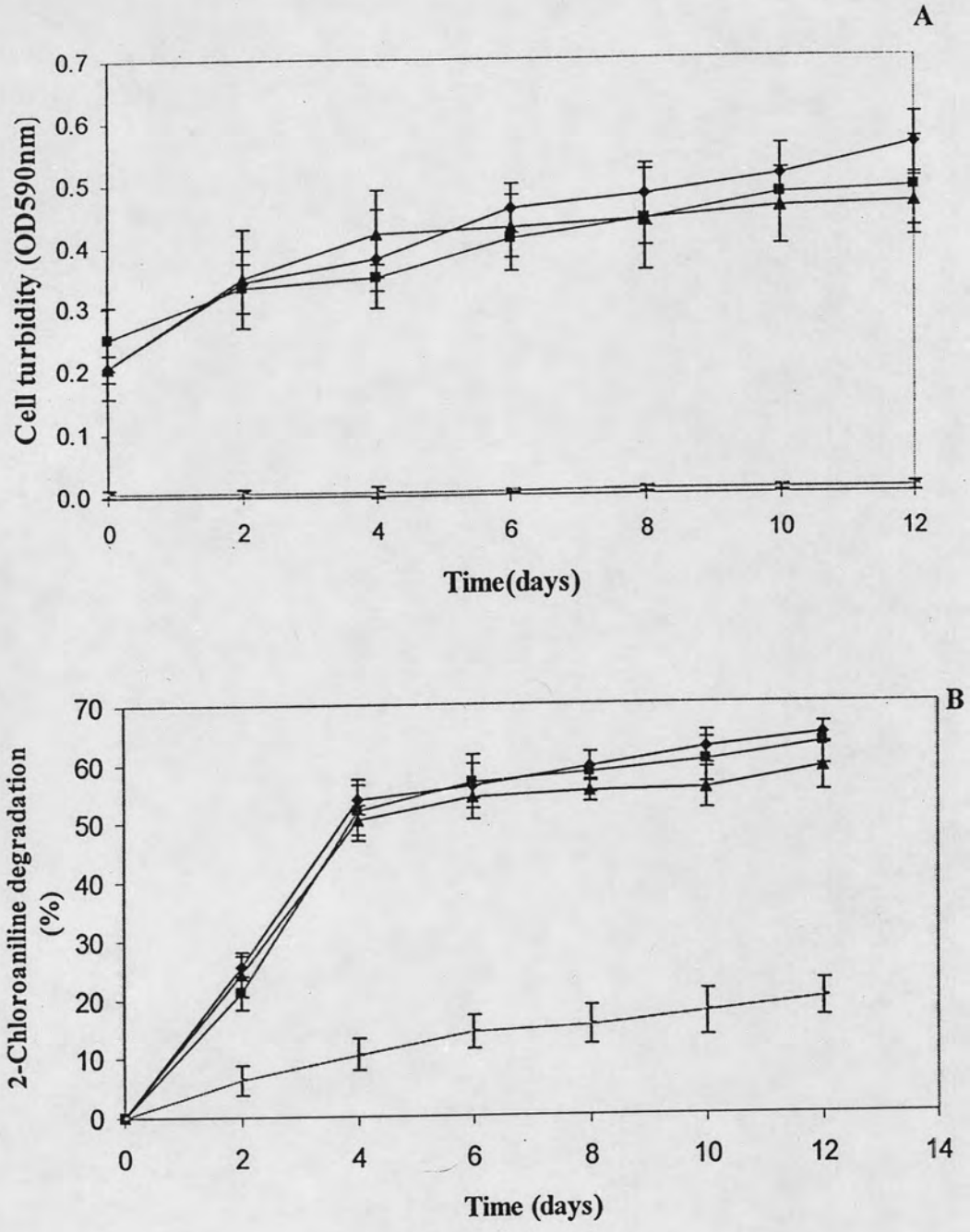

Figure 4.28 Effect of 2-chloroaniline at 25 ppm (0.2 mM) provided as carbon and nitrogen sources on (A) growth and (B) total degradation ability of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—), and control (- - -) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

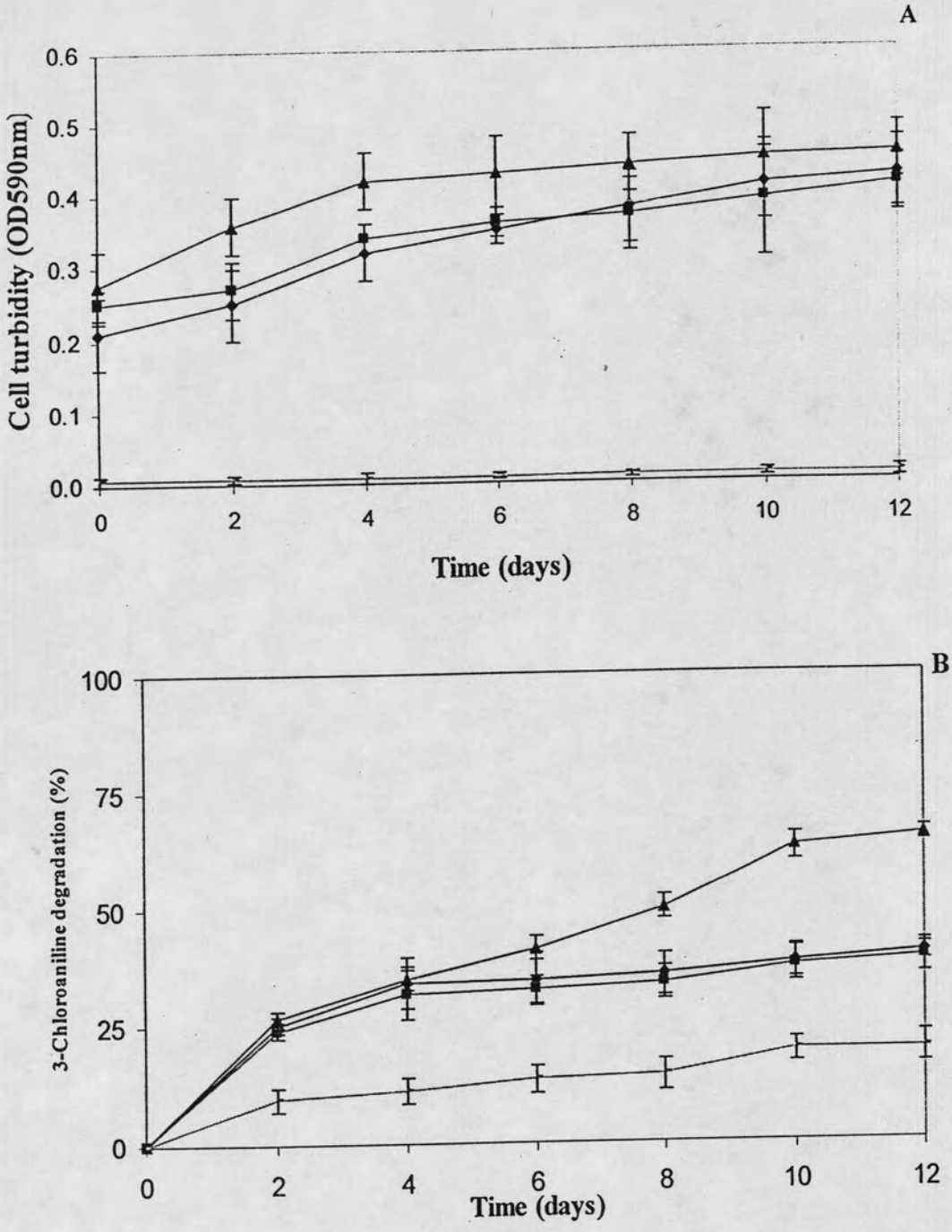

Figure 4.29 Effect of 3-chloroaniline at 25 ppm (0.2 mM) provided as carbon and nitrogen sources on (A) growth and (B) total degradation ability of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—), and control (— — —) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

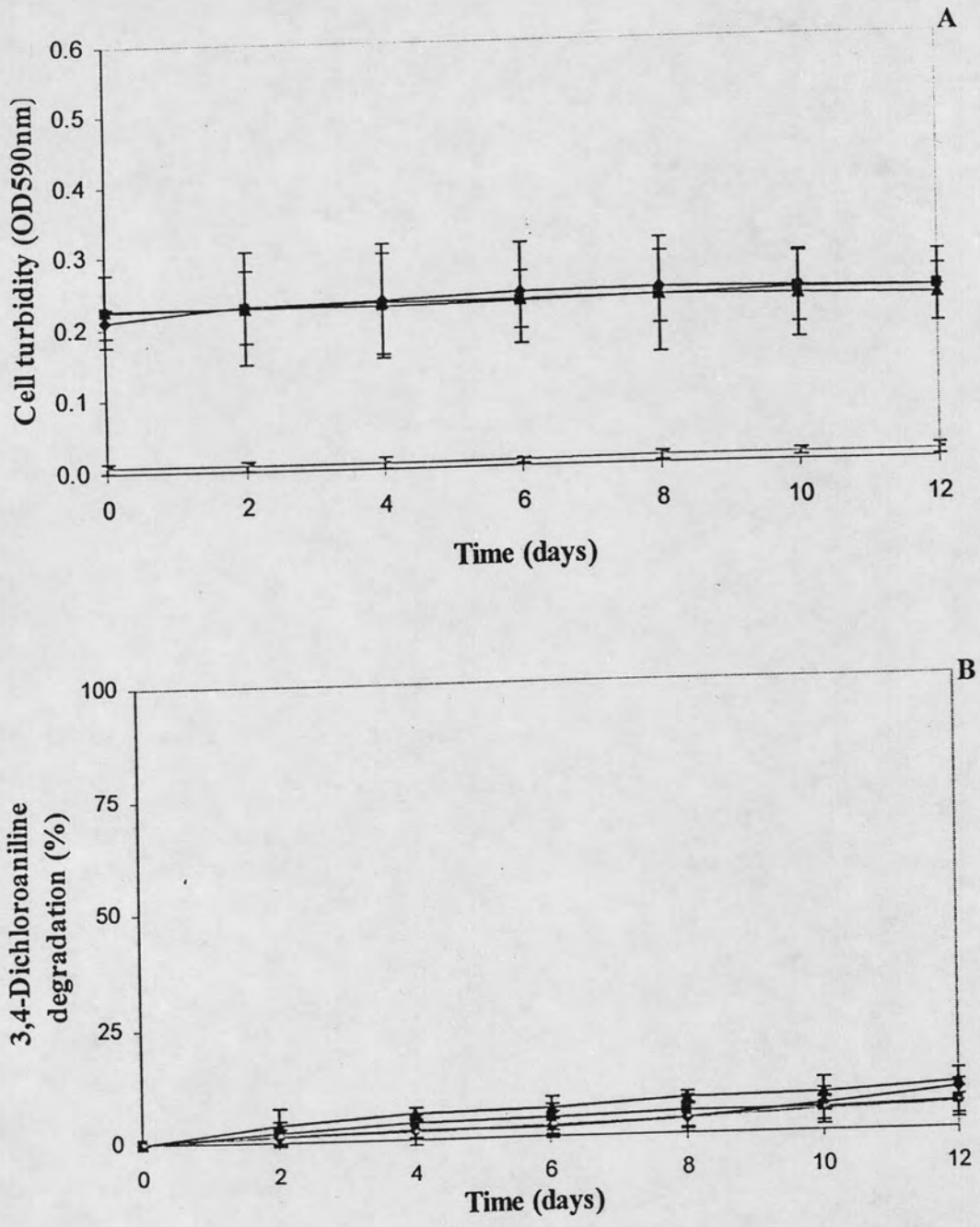

Figure 4.30 Effect of 3,4-dichloroaniline at 25 ppm (0.2 mM) provided as carbon and nitrogen sources on (A) growth and (B) total degradation ability of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—), and control (- - -) representing abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).

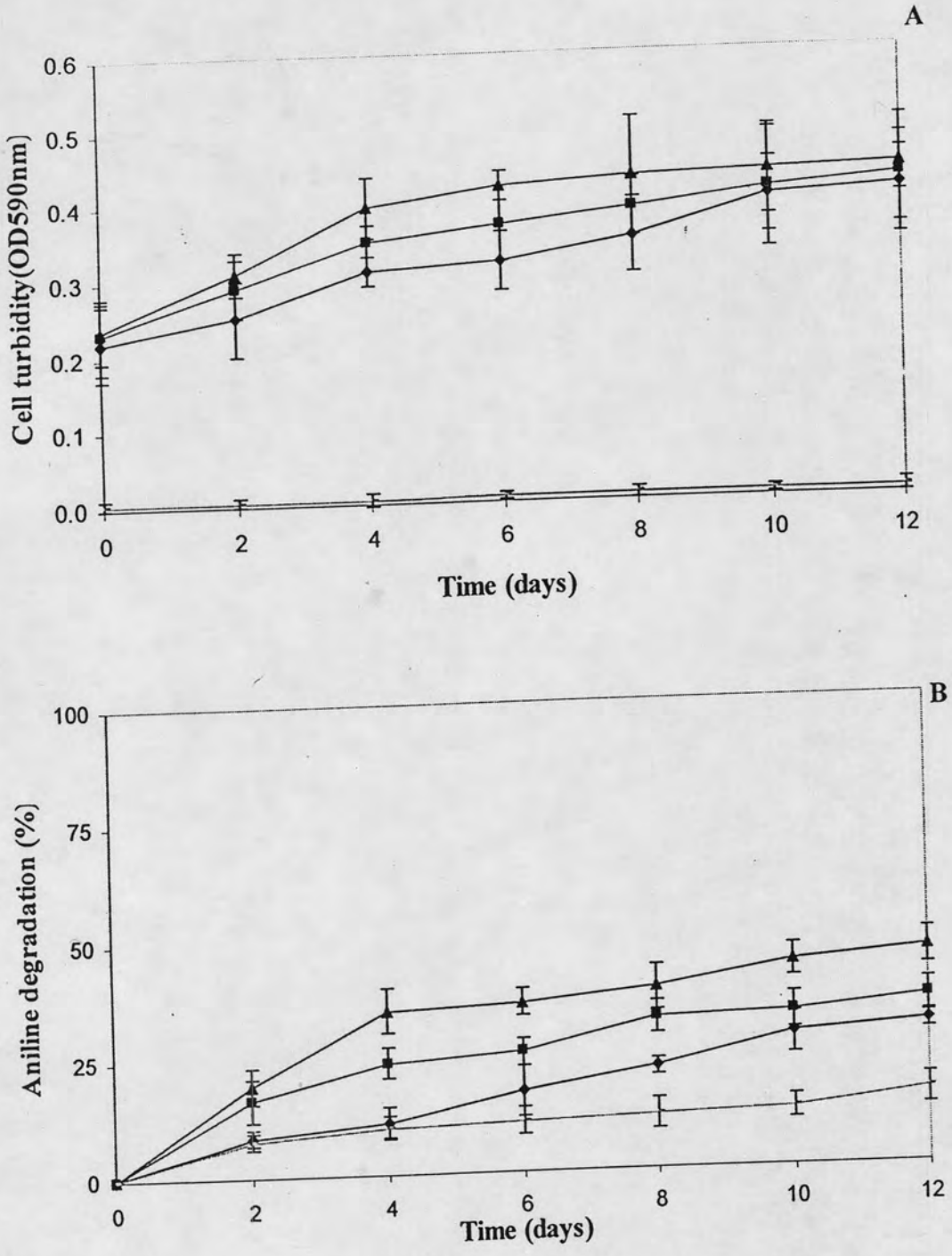

Figure 4.31 Effect of aniline at 1 mM provided as carbon and nitrogen sources on (A) growth and (B) total degradation ability of *Acinetobacter baumannii* (—◆—), *Pseudomonas putida* (—■—), *Klebsiella pneumoniae* (—▲—), and control (——) represented abiotic degradation. The data are means from three independent experiments with vertical bars representing standard errors of the means (n=3).