CHAPTER III

RESULTS

3.1 Purification of alanine dehydrogenase

3.1.1 Preparation of crude enzyme and enzyme production

Crude enzyme solution of *E. coli* BL21(DE3) harboring pETAlaDH was prepared from 1,000 ml of LB medium supplemented with 100 μg/ml ampicilin, as described in section 2.8. The crude enzyme contained 15,100 units of total activity, 727 mg of total protein and 20.77 unit/mg protein of specific activity (Table 3.1).

3.1.2 Ammonium sulfate precipitation

Crude enzyme was further purified by ammonium sulfate precipitation as mentioned in section 2.14.2.1. The enzymes was precipitated in the range of 20-40% saturated ammonium sulfate. The precipitated proteins were then dissolved with 10 mM KPB, pH 7.4 and dialyzed against the same buffer. In this step, the enzyme was purified 1.13 fold with 40.39% recovery.

3.1.3 DEAE-Toyopearl column chromatography

The enzyme from section 3.1.2 was applied into DEAE-Toyopearl column as described in section 2.11.2.2. The chromatographic profile was shown in Figure 3.1. The bound proteins were then eluted with linear salt gradient of 0-1.0 M potassium chloride solution. The enzyme was eluted at 0.1 M potassium chloride solution as indicated in profile. The fractions with alanine dehydrogenase activity

Table 3.1 Purification of alanine dehydrogenase

Purification step	Total activity (unit)	Total protein (mg)	Specific activity (Unit/mg protein)	% recovery	Purification fold
Crude enzyme	15,100	727	20.77	100	1
20-40% Ammonium sulfate precipitation	6,100	261	23.37	40.39	1.13
DEAE-Toyopearl column	5,980	104	57.50	39.60	2.77
Blue Sepharose column	1,490	22	67.73	9.87	3.26

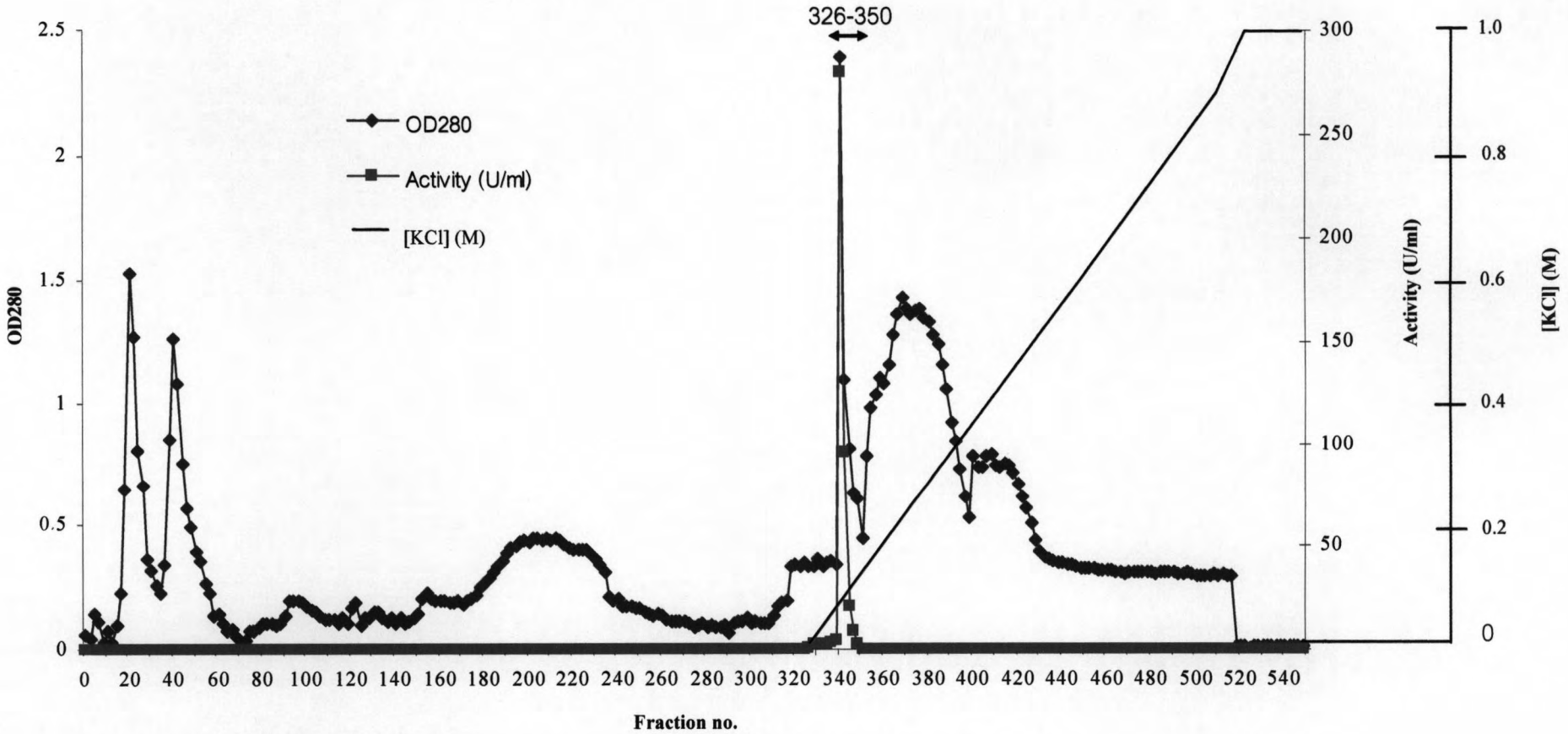

Figure 3.1 Purification of alanine dehydrogenase by DEAE-Toyopearl column

The enzyme solution was applied to DEAE-Toyopearl column and washed with 10 KPB buffer, pH 7.4 until A_{280} decreased to base line. Gradient elution of bound protein was made by 0-1 M KCl in the same buffer at the flow rate 1 ml/min

◆ A_{280} ■ alanine dehydrogenase activity —— [KCl] ↔ the pool fraction (no. 326-350)

were pooled and dialyzed against working buffer. The enzyme was purified about 2.77 fold with 39.60 % recovery compared with crude enzyme (Table 3.1).

3.1.4 Blue Sepharose column chromatography

The pooled active fractions from DEAE-Toyopearl column were further purified by Blue Sepharose column as described in section 2.11.2.3. The enzyme solution was then loaded into Blue Sepharose column and eluted with 0-0.2 M potassium chloride solution in KPB buffer, pH 7.4 as indicated in Figure 3.2. The fractions with alanine dehydrogenase activity were pooled and dialyzed against working buffer. In this step, the enzyme was purified about 3.26 fold with 9.87 % recovery.

The purified enzyme from this step was kept as aliquot at 4°C for alanine racemase activity assay.

3.2 Determination of enzyme purity and protein pattern by SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The enzyme from each step of purification was analyzed for purity and protein by SDS-PAGE as described in section 2.15. The purity of enzyme increased in each step. The enzyme from final step still had few contaminants (Figure 3.3), however, no alanine racemase was detected when the enzyme was assayed as described in section 2.10.0. The molecular weight of alanine dehydrogenase was estimated to be 40,000 Dalton.

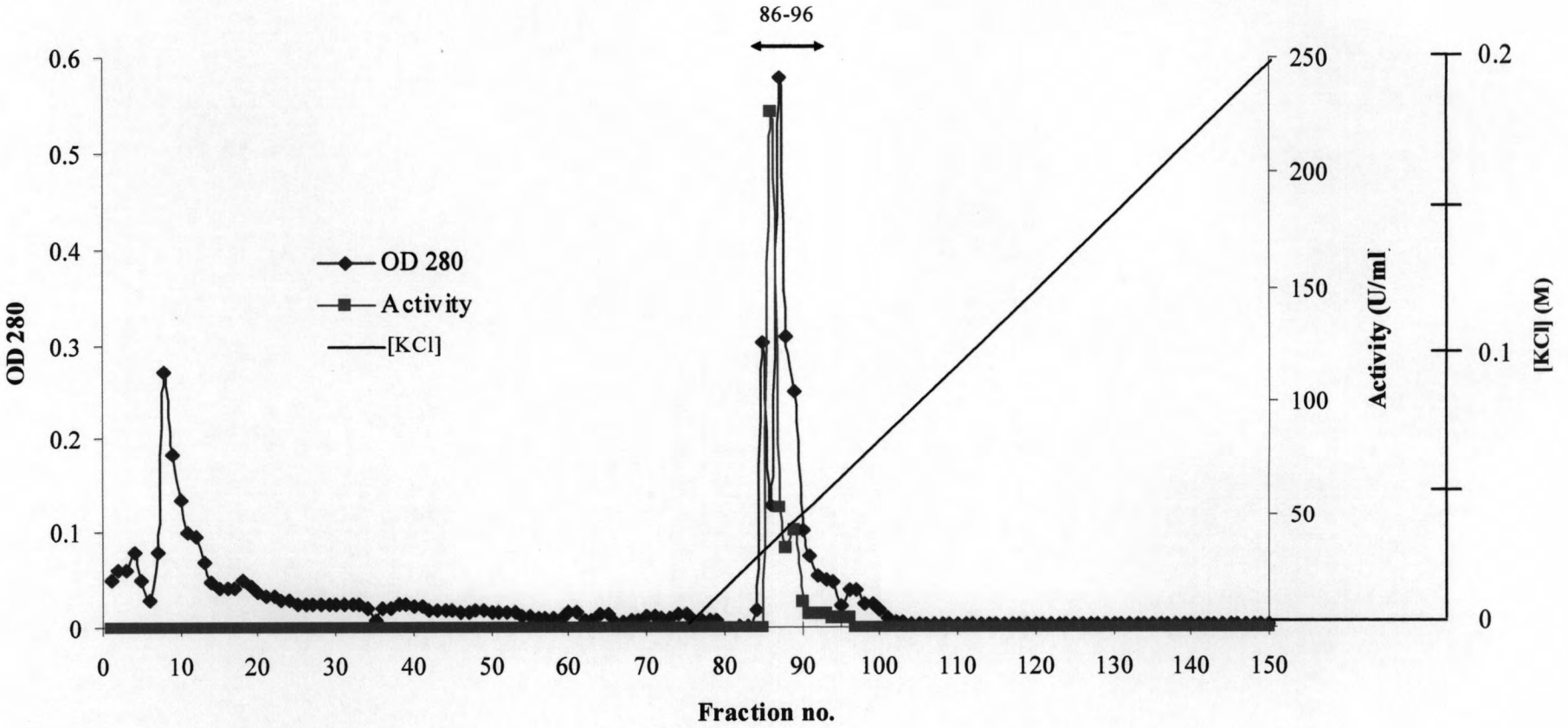

Figure 3.2 Purification of alanine dehydrogenase by Blue Sepharose column

The enzyme solution was applied to Blue Sepharose column and washed with 10 M KPB buffer, pH 7.4 until A_{280} decreased to base line. Gradient elution of bound protein was made by 0-0.2 M KCl in the same buffer at the flow rate 1 ml/min

◆ A_{280} ■ alanine dehydrogenase activity —— [KCl] ↔ the pool fraction (no. 86-96)

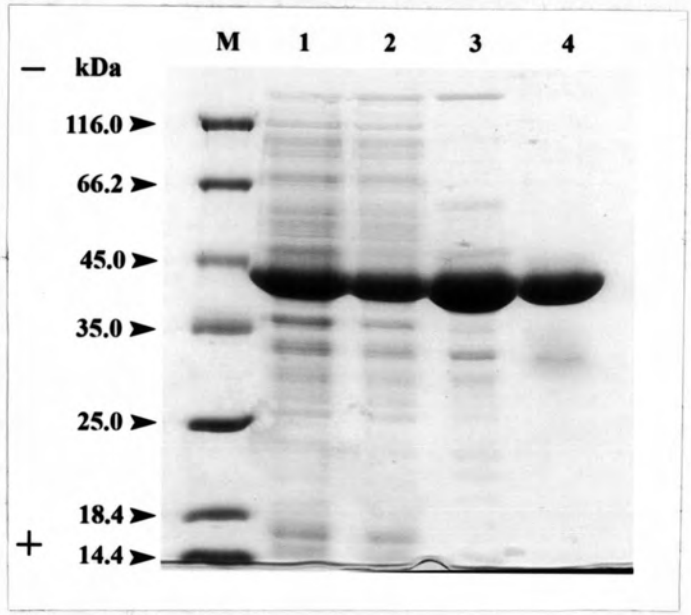

Figure 3.3 SDS-polyacrylamide gel electrophoresis of alanine dehydrogenase

Lane M = molecular weight marker protein

Lane 1 = crude enzyme

Lane 2 = 20-40% ammonium sulfate precipitation

Lane 3 = DEAE-Toyopearl column

Lane 4 = Blue Sepharose column

The fifteen micrograms of protein was loaded in each lane.

3.3 Creation of *alr* gene knockout mutant

3.3.1 Construction of pACD4K-C-Alr

To target the intron to *alr* gene, the L1.LtrB intron sequence was modified base on the sequence of *alr* gene from *E. coli* CFT073 (accession no. NC 004431) by using the InGex Intron Prediction Program. The program predicted two intron insertion sites across the 1,080 bp *alr* gene (Table 3.2). The group 1 primers inserted into position 636/637 was chosen with E-value of 0.060. The 350 bp PCR product from those primers was purified by agarose gel electrophoresis then digested with, *Bsr*GI and *Hin*dIII , before ligated into pACD4K-C.

3.3.2 Confirmation of *alr* gene inactivation

The constructed plasmid pACD4K-C-Alr was transformed into *E. coli* BL21(DE3) as described in section 2.15.2. The kanamycin resistance clone was randomly picked for determination of *alr* gene disruption.

3.3.2.1 PCR analysis

As described in section 2.17.3.1, the eighty-six colonies were picked for PCR analysis with Alr For and Alr Rev primers. As shown in Figure 3.4, size of *alr* gene amplified by using *alr* gene specific primer was 1,080 bp (Lane 1). Only one colony shown an expected 3,368 bp PCR product (lane 2) that meant the *alr* gene was inactivated by insertion of 2,288 bp intron. To confirm that T7 system still existed, all mutant colonies were amplified with T7 RNA polymerase gene specific primers, T7 For and T7 Rev primers. All of them showed an expected

Table 3.2 All predicted primers for *alr* gene inactivation from InGex Intron Prediction Program

Group	Insertion site	Primer	Score	E-value
1	636/637	IBS 5'AAAAAAGCTTATAATTATCCTTACGTCCCGGGATCGTGCGCCCAGATAGGGTG 3' EBS1d 5'AGATTGTACAAATGTGGTGATAACAGATAAGTCGGGATCATTAACTTACCTTTCTTTGT 3' EBS2 5' TGAACGCAAGTTTCTAATTTCGGTTGGACGTCGATAGAGGAAAGTGTCT 3'	8.81	0.060
2	174/175	IBS 5' AAAAAAGCTTATAATTATCCTTATTTGGCGTTGCCGTGCGCCCAGATAGGGTG 3' EBS1d 5' CAGATTGTACAAATGTGGTGATAACAGATAAGTCGTTGCCCGTAACTTACCTTTCTTTGT 3' EBS2 5' TGAACGCAAGTTTCTAATTTCGGTTCCAAATCGATAGAGGAAAGTGTCT 3'	5.75	0.491

The green letters showed *Hin*dIII restriction site

The blue letters showed *Bsr*GI restriction site

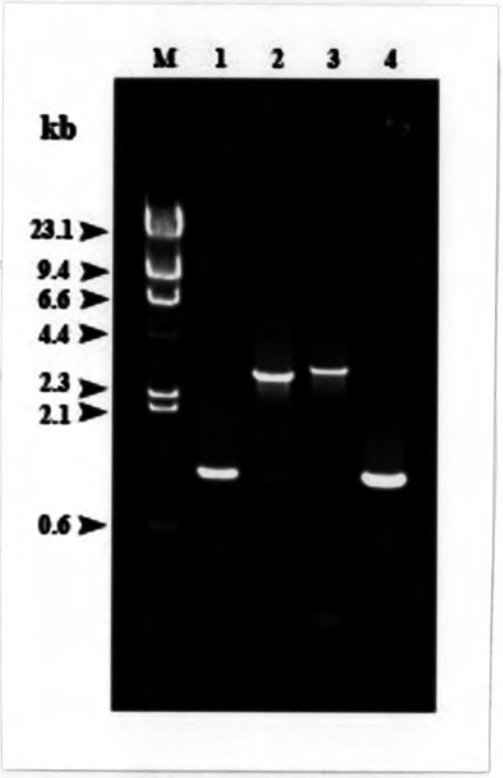

Figure 3.4 PCR analysis of the *alr* gene knockout mutant

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = amplified product of *E. coli* BL21(DE3) by using *alr* gene specific primers

Lane 2 = amplified product of *alr* gene knockout mutant *alr* gene by using *alr* gene specific primers

Lane 3 = amplified product of *alr* gene knockout mutant by using T7 RNA polymerase gene specific primers

Lane 4 = amplified product of *alr* gene knockout mutant using dadX gene specific primers

3,557 bp PCR product of T7 RNA polymerase gene (Lane 3). Since, *dadX* gene also encodes for alanine racemase and it has 45% similarity to *alr* gene, its intactness was confirmed by PCR amplification using *dadX* gene specific primers. The result showed that *dadX* gene of *alr* gene mutants still consisted of 1,071 bp.

3.3.2.2 Southern blot analysis

The alr gene knockout mutant from section 3.3.2.1 was confirmed for single intron insertion into its chromosomal DNA by Southern blot analysis as described in section 2.17.3.2. The chromosomal DNA of *alr* gene knockout mutant was digested with *Bam*HI, *Hin*dIII and *Xho*I, respectively. The probe was amplified by using the primers, Alr IBS and Alr EBS1d primers. The digested chromosomal DNA of *alr* gene knockout mutant showed a single band in all of restriction enzyme digestion. While, the chromosomal DNA of wild type showed no signal of intron insertion (Figure 3.5).

3.4 Creation of *dadX* gene knockout mutant

3.4.1 Construction of pACD4K-C-Dad

The sequence of *dadX* gene from *E. coli* CFT073 (accession no. L02948) was submitted into InGex Intron Prediction Program for construction of retargeting primers use in *dadX* gene disruption. The program generated four group of primers. The group 2 primers, Dad IBS, Dad EBS1d and Dad EBS2, gave the insertion into position 501/502 was selected with E-value of 0.070 (Table 3.3). The 350 bp PCR product from those primers was purified by agarose gel electrophoresis then digested

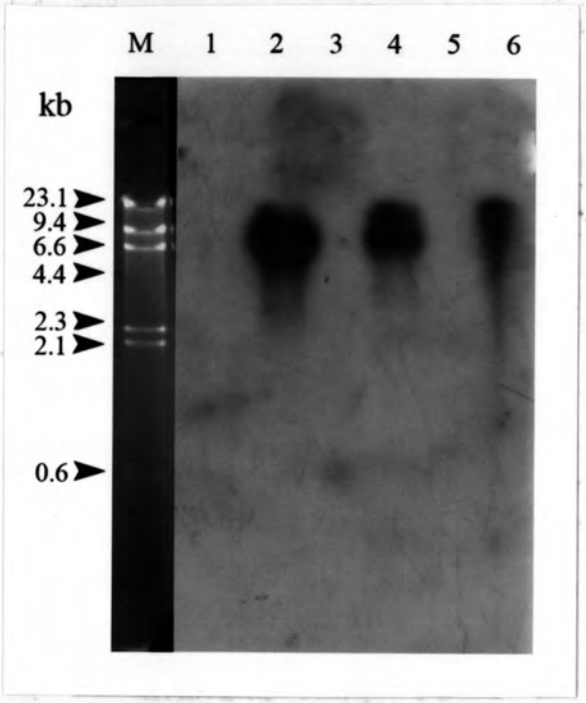

Figure 3.5 Southern blot analysis of chromosomal DNA of *alr* gene knockout mutant

Lane 1 = *Bam*HI digested DNA from *E. coli* BL21(DE3)

Lane 2 = *Bam*HI digested DNA from *alr* gene knockout mutant

Lane 3 = *Hin*dIII digested DNA from *E. coli* BL21(DE3)

Lane 4 = *Hin*dIII digested DNA from *alr* gene knockout mutant

Lane 5 = *Xho*I digested DNA from *E. coli* BL21(DE3)

Lane 6 = *Xho*I digested DNA from *alr* gene knockout mutant

The 350 bp PCR product for generation of *alr* gene knockout mutant was used as a probe.

Table 3.3 All predicted primers for *dadX* gene inactivation from InGex Intron Prediction Program

Group	Insertion site	Primer	Score	E-value
1	501/502	IBS 5' AAAAAAGCTTATAATTATCCTTAGAACACCCTGATGTGCGCCCAGATAGGGTG 3' EBS1d 5' CAGATTGTACAAATGTGGTGATAACAGATAAGTCCCTGATGGTAACTTACCTTTCTTTGT 3' EBS2 5' TGAACGCAAGTTTCTAATTTCGATTTGTTCTCGATAGAGGAAAGTGTCT 3'	2.59	0.070
2	393/394	IBS 5' AAAAAAGCTTATAATTATCCTTAATGAACCGGTTGGTGCGCCCAGATAGGGTG 3' EBS1d 5' CAGATTGTACAAATGTGGTGATAACAGATAAGTCCGGTTGGGTAACTTACCTTTCTTTGT 3' EBS2 5' TGAACGCAAGTTTCTAATTTCGATTTTCATTCGATAGAGGAAAGTGTCT 3'	6.96	0.232
3	693/694	IBS 5' AAAAAAGCTTATAATTATCCTTAGGATTCCGTCCGGTGCGCCCAGATAGGGTG 3' EBS1d 5' AGCTTTGTACATAATTATCCTTAGGATTCCGTCCGGTGCGCCCAGATAGGGTGTAGGGTG 3' EBS2 5' TGAACGCAAGTTTCTAATTTCGATTAATCCTCGATAGAGGAAAGTGTCT 3'	6.39	0.340

Table 3.3 All predicted primers for *dadX* gene inactivation from InGex Intron Prediction Program (continued)

Group	Insertion site	Primer	Score	E-value
4	167/168	**IBS 5' AAAAAAGCTTATAATTATCCTTACACC GCTGGCTTGTGCGCCCAGATAGGGTG 3'** **EBS1d 5' CAGATTGTACAAATGTGGTGATAACA GATAAGTCTGGCTTTGTAACTTACCTTT CTTTGT 3'** **EBS2 5' TGAACGCAAGTTTCTAATTTCGGTTCG GTGTCGATAGAGGAAAGTGTCT 3'**	6.22	0.374

The green letters showed *Hin*dIII restriction site

The blue letters showed *Bsr*GI restriction site

with *Bsr*GI and *Hin*dIII , before ligated into pACD4K-C to gave a pACD4K-C-Alr.

3.4.2 Confirmation of *dadX* gene inactivation

After pACD4K-C-Alr was transformed into *E. coli* BL21(DE3) as described in 2.17.2. The kanamycin resistant clones were checked for *dadX* gene inactivation.

3.4.2.1 PCR analysis

As mentioned in section 2.18.3.1, the kanamycin resistant colonies were picked for PCR analysis with *dadX* gene specific primers, Dad For and Dad Rev. The calculated size of *dadX* gene by using *dadX* gene specific primers was 1,071 bp while the size of intron was 2,288 bp.

From 86 antibiotic resistant clones, only one colony showed expected 3,359 bp PCR product as displayed in Figure 3.6, lane 2 while the wild type colony gave the 1,071bp of *dadX* gene (Lane 1). T7 RNA polymerase gene (3,557 bp) and *alr* gene (1,080 bp) of dadX gene knockout mutant were still intact (Lane 3 and Lane 4).

3.4.2.2 Southern blot analysis

The mutant from section 2.18.3.1 was confirmed for single intron insertion into chromosomal DNA of *E. coli* BL21(DE3) by Southern blot analysis. The chromosomal DNA of *dadX* gene knockout mutant was digested with *Bam*HI, *Hin*dIII and *Kpn*I, respectively. The probe was amplified by using

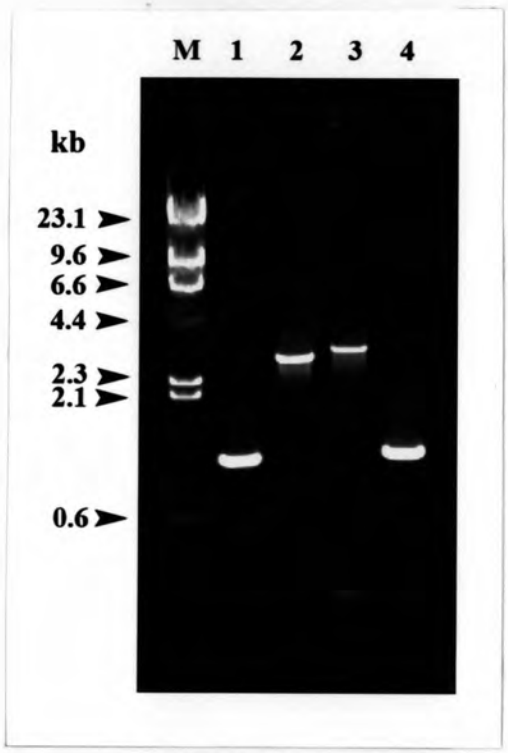

Figure 3.6 PCR analysis of the *dadX* gene knockout mutant

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = amplified product of *E. coli* BL21(DE3) by using *dadX* gene specific primers

Lane 2 = amplified product of *dadX* gene knockout mutant by using *dadX* gene specific primers

Lane 3 = amplified product of *dadX* gene knockout mutant by using T7 RNA polymerase gene specific primers

Lane 4 = amplified product of *alr* gene knockout mutant using *dadX* gene specific primers

Dad IBS and Dad EBS1d primers. The chromosomal DNA of *dadX* gene knockout mutant showed single band of about 9 kb when digested with *Hin*dIII (Figure 3.7, Lane 4). While *Bam*HI and *Kpn*I digested chromosomal DNA gave one more high molecular weight band which should be uncomplete digested DNA fragment (Lane 2 and 6). In contrast, the chromosomal DNA of wild type showed no signal of intron insertion(Lane 1, 3 and 5).

3.5 Creation of *dadX* and *alr* genes knockout mutant

3.5.1 Construction of pACD4K-C-loxP-Dad and creation of *dadX* gene knockout mutant

The primers from 3.1.1 was used for construction of 350 bp retargeting PCR product using *dadX* gene disruption. The 350 bp PCR product from those primers was purified by agarose gel electrophoresis then digested with restriction enzymes, *Bsr*GI and *Hin*dIII, before ligated into pACD4K-C-loxP to give a pACD4K-C-loxP-Dad.

The method in section 2.19.2 was used for creation of *dadX* gene knockout mutant by pACD4K-C-Dad.

3.5.2 Confirmation of *dadX* gene inactivation (first gene inactivation)

3.5.2.1 PCR analysis

The *dadX* gene specific primers, Dad For and Dad Rev and T7 gene specific primers, T7 For and T7 Rev, were used for confirmation of intron insertion into *dadX* gene and the intactness of T7 RNA polymerase gene in *E. coli* BL21(DE3), repectively. The eighty-six colony from section 3.3.1 were random

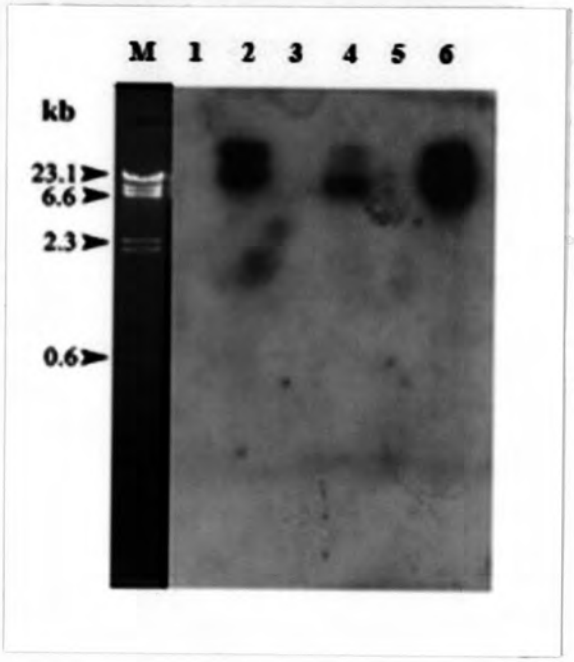

Figure 3.7 Southern blot analysis of chromosomal DNA of *dadX* gene knockout mutant

Lane 1 = *Bam*HI digested DNA from *E. coli* BL21(DE3)

Lane 2 = *Bam*HI digested DNA from *alr* gene knockout mutant

Lane 3 = *Hin*dIII digested DNA from *E. coli* BL21(DE3)

Lane 4 = *Hin*dIII digested DNA from *alr* gene knockout mutant

Lane 5 = *Kpn*I digested DNA from *E. coli* BL21(DE3)

Lane 6 = *Kpn*I digested DNA from *alr* gene knockout mutant

The 350 bp PCR product for generation of *dadX* gene knockout mutant was used as a probe.

picked. The 3,426 bp of PCR product (Figure 3.8, Lane 1), that come from 1,071 bp of *dadX* gene and 2,355 bp of intron, showed a successful intron insertion into *dadX* gene. The 3,557 bp PCR product in Lane 2 showed that T7 RNA polymerase gene still existed in mutant chromosome. Seven colonies of mutant were obtained in this step.

3.5.2.2 Southern blot analysis

The representative colony of *dadX* gene knockout mutant from section 3.3.2.1 was picked for chromosomal DNA extraction. The DNA of mutant was digested with *Bam*HI, *Hin*dIII and *Kpn*I. The probe was generated from method in section 2.19.3 by using a Dad IBS and Dad EBS1d primers. All restriction enzyme digested DNA of *dadX* gene knockout mutant showed a single band whereas no signal was detected in restriction enzyme digested DNA from *E. coli* BL21(DE3) wild type (Figure 3.9).

3.5.2.3 Excision of kanamycin resistance gene cassette by the p706-Cre from *dadX* gene knockout mutant

As described in the section 2.19.4, the kanamycin resistance gene cassette (1,367 bp) was removed from intron that inserted in *dadX* gene of *E. coli* BL21(DE3). Thus, when using Dad For and Dad Rev primers for PCR amplification, the PCR product was decreased to 2,059 bp (Figure 3.10, Lane 3)

Figure 3.8 PCR analysis of the *dadX* gene knockout mutant by pACD4K-C-loxP-Dad

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = amplified product of *dadX* gene knockout mutant by using *dadX* gene specific primers

Lane 2 = amplified product of *dadX* gene knockout mutant by using T7 RNA polymerase gene specific primers

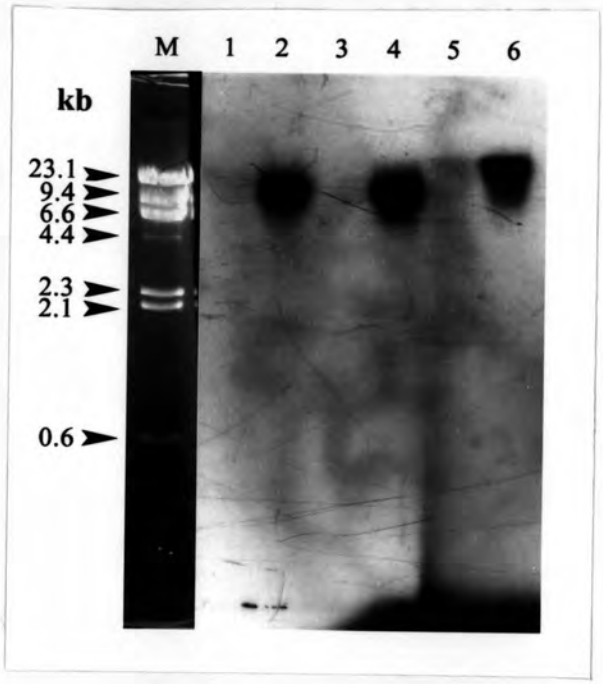

Figure 3.9 Southern blot analysis of chromosomal DNA of *dadX* gene knockout mutant from pACD4K-C-loxP-Dad

Lane 1 = *Bam*HI digested DNA from *E. coli* BL21(DE3)

Lane 2 = *Bam*HI digested DNA from *dadX* gene knockout mutant

Lane 3 = *Hin*dIII digested DNA from *E. coli* BL21(DE3)

Lane 4 = *Hin*dIII digested DNA from *dadX* gene knockout mutant

Lane 5 = *Kpn*I digested DNA from *E. coli* BL21(DE3)

Lane 6 = *Kpn*I digested DNA from *dadX* gene knockout mutant

The 350 bp PCR product for generation of *dadX* gene knockout mutant was used as a probe.

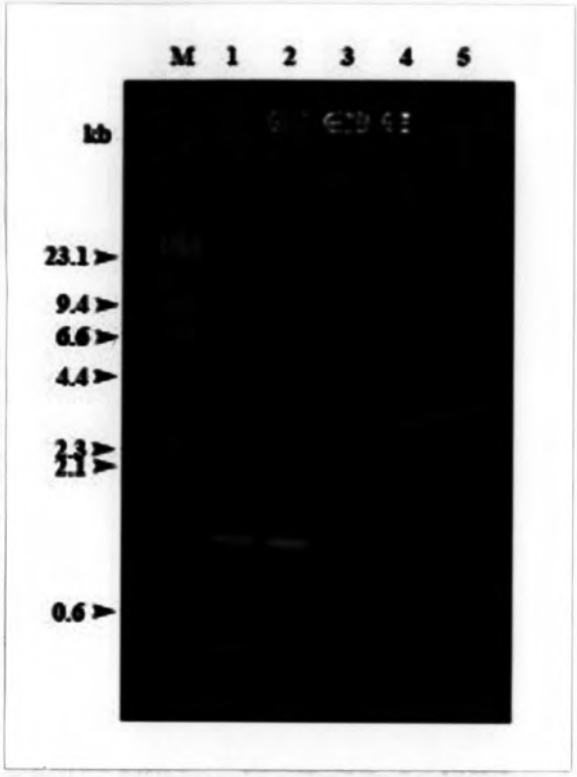

Figure 3.10 PCR analysis of the *dadX* and *alr* genes knockout mutant

Lane M = λ/*Hin*dIII standard DNA marker

Lane 1 = amplified product of *E. coli* BL21(DE3) wild type using *alr* gene specific primers

Lane 2 = amplified product using *dadX* gene specific primers in *E. coli* BL21(DE3) wild type

Lane 3 = amplified product using *dadX* gene specific primers in *dadX* with *alr* gene knockout mutant

Lane 4 = amplified product using *alr* gene specific primers in *dadX* and *alr* genes knockout mutant

Lane 5 = amplified product using *T7* RNA polymerase gene specific primers in *dadX* gene knockout mutant

3.5.2.4 Construction of pACD4K-C-loxP-Alr and creation of *dadX* and *alr* genes knockout mutant

The same primers from section 3.2.1 was used for construction of 350 bp retargeting PCR product for *alr* gene inactivation. The 350 bp PCR product from those primers was purified by agarose gel electrophoresis then digested with *Bsr*GI and *Hin*dIII , before ligated into pACD4K-C-loxP to gave a pACD4K-C-loxP-Alr.

The method in section 2.19.6 was used for creation of *dadX* and *alr* genes knockout mutant by pACD4K-C-loxP-Alr.

3.5.2.5 Confirmation of *dadX* and *alr* genes knockout mutant

3.5.2.5.1 PCR analysis

As described in section 2.18.3.1, the kanamycin resistance colonies were picked for PCR analysis with *alr* gene specific primers, Alr For and Alr Rev and *dadX* gene specific primers, Dad For and Dad Rev. In Figure 3.10, Lane 3 showed the 2,059 bp PCR product of *dadX* gene by using *dadX* gene specific primers, which *dadX* gene was inactivated by intron insertion and the kanamycin resistance gene cassette was removed from intron. Lane 4 showed 3,435 bp PCR product, which *alr* gene (1,080 bp) was inactivated by intron (2,355 bp) insertion. The *dadX* gene knockout mutant still contained T7 system since the PCR product, amplified with T7 gene specific primers, T7 For and T7 Rev, was 3,557 bp.

3.5.2.5.2 Southern blot analysis

The mutant from section 3.3.2.5.1 was confirmed for single intron insertion into chromosomal DNA by Southern blot analysis. The chromosomal DNA of *dadX* and *alr* genes knockout mutant was digested with *Bam*HI and *Hin*dIII. The probe was amplified by using the primers, Dad IBS and Dad EBS1d. Both *Bam*HI and *Hin*dIII digested chromosomal DNA of *dadX* gene knockout mutant showed single hybridizing band, while chromosomal DNA of wild type showed no signal of intron insertion (Figure 3.11).

3.6 Growth of *E. coli* BL21(DE3) and mutants

The determination of growth rate constant was performed as described in section 2.20. The growth rate constant of wild type, *alr* gene knockout mutant and *dadX* gene knockout mutant were 1.39, 0.69 and 1.39 h^{-1}, respectively (Figure 3.12). The growth rate constant of *dadX* and *alr* genes knockout mutant could not be determined because the mutant showed slow growth rate in LB broth supplemented with 10 mM D-alanine.

3.7 Alanine racemaes activity of *E. coli* BL21(DE3) and mutants

The *alr* gene knockout mutant, *dadX* gene knockout mutant and *dadX* and *alr* genes knockout mutant were analyzed for alanine racemase activity. The crude enzyme from *alr* gene knockout mutant and *dadX* gene knockout mutant had alanine racemase specific activity of 1.88 and 5.47 unit/mg protein (Table 3.4) which were 3.3 and 1.1 fold lower than that of *E. coli* BL21(DE3) wild type. In this experiment, the alanine

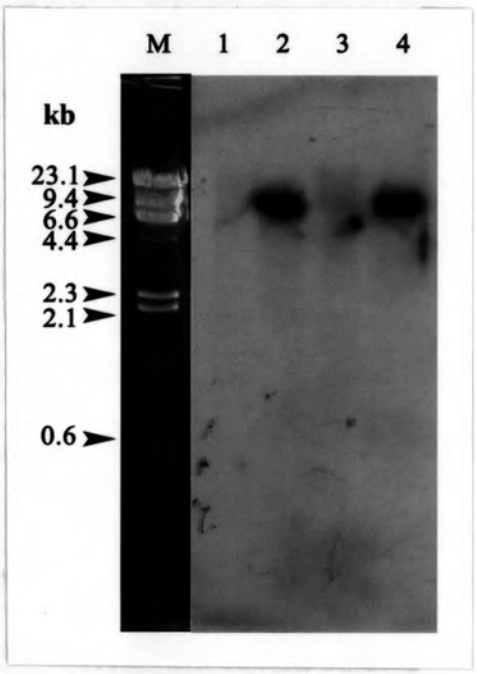

Figure 3.11 Southern blot analysis of chromosomal DNA of *dadX* and *alr* genes knockout mutant from pACD4K-C-loxP-Alr

Lane 1 = *Bam*HI digested DNA from the *E. coli* BL21(DE3)

Lane 2 = *Bam*HI digested DNA from the *dadX* and *alr* genes knockout mutant

Lane 3 = *Hin*dIII digested DNA from the *E. coli* BL21(DE3)

Lane 4 = *Hin*dIII digested DNA from the *dadX* and *alr* genes knockout mutant

The 350 bp PCR product for generation of *dadX* gene knockout mutant was used as a probe.

a) *E. coli* BL21(DE3)

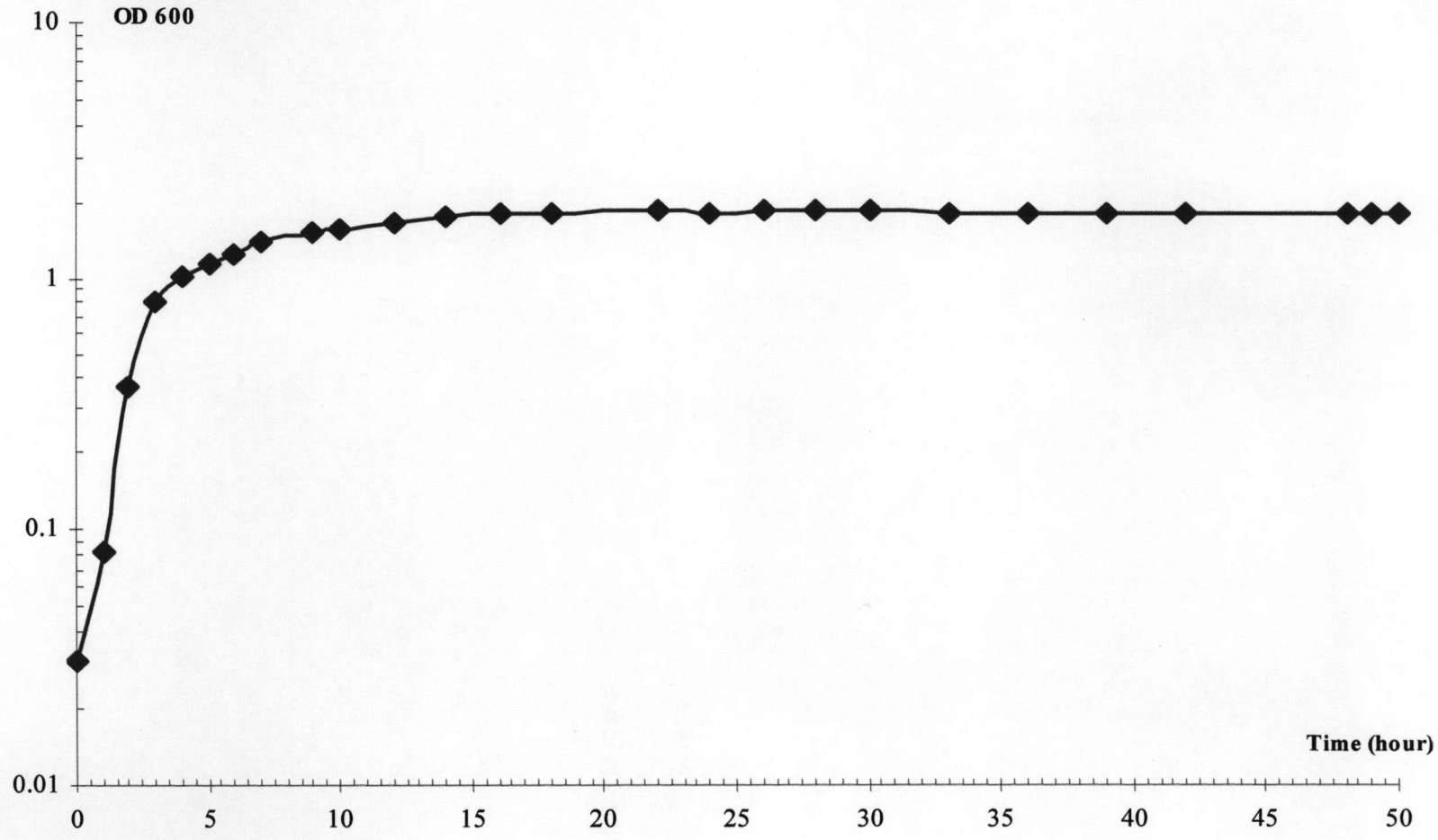

Figure 3.12 The growth curve of *E. coli* BL21(DE3) and mutants

b) *alr* gene knockout mutant

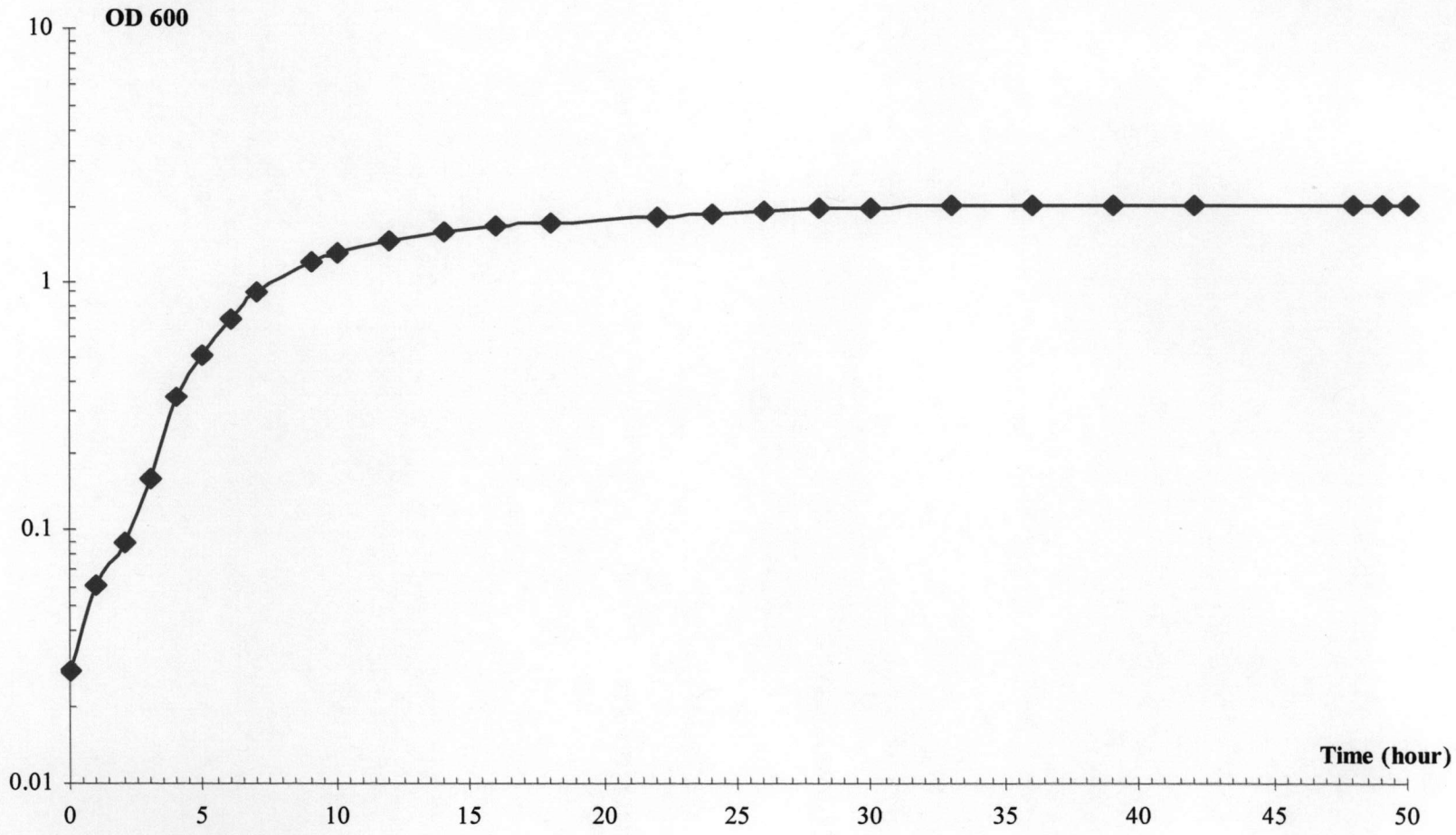

Figure 3.12 The growth curve of *E. coli* BL21(DE3) and mutants (continued)

c) *dadX* gene knockout mutant

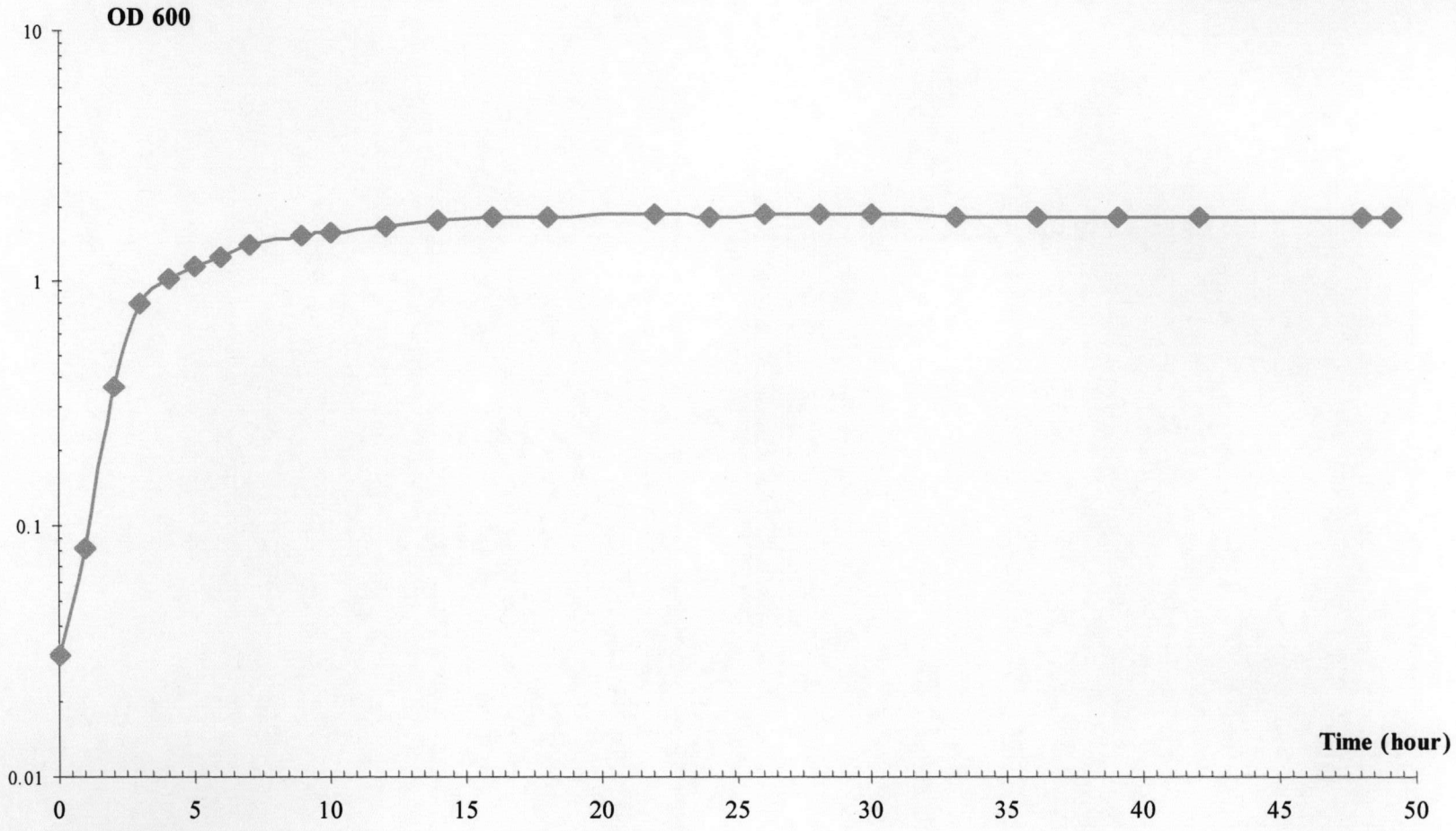

Figure 3.12 The growth curve of *E. coli* BL21(DE3) and mutants (continued)

Table 3.4 Alanine racemase activity from crude extract of *E. coli* BL21(DE3) and mutants

Mutant	Total activity (U) [a]	Total protein (mg)	Specific activity (U/mg protein)
E. coli BL21(DE3)	352	55.3	6.36
alr gene knockout mutant	80	42.6	1.88
dadX gene knockout mutant	246	45.0	5.47
dadX and *alr* genes knockout mutant	ND[b]	ND	ND

[a] Total activity from 200 ml of LB medium as described in section 2.11

[b] not determined

racemase activity of *dadX* and *alr* genes knockout mutant could not be assayed due to the mutant grew very slowly in LB medium supplemented with 10 mM D-alanine since OD_{600} of mutant still 0.2 after cultivation at 37°C with shaking for 48 hr.

3.8 Stability of intron in *E. coli* BL21(DE3) and mutants

The 1st, 5th, 10th, 15th, and 20th subcultured colonies from *alr* gene knockout mutant and *dadX* gene knockout mutant were picked up to culture and then the introns was detected by colony PCR with each specific gene prime pair. The result showed that introns were stable although mutants were subcultured for 20 times. The experiment of *dadX* with *alr* gene knockout mutant had low growth rate in LB broth supplemented with 10 mM D-alanine. Moreover, the mutant could not survive on LB agar.