ALANINE PRODUCTION BY *ESCHERICHIA COLI*

THROUGH METABOLIC ENGINEERING

by

MINGTAU LEE

(Under the Direction of MARK A. EITEMAN)

ABSTRACT

Alanine production in *E. coli* mutants expressing alanine dehydrogenase was studied. In aerobic production of alanine, different agitation speeds were compared: 150 rpm, 250 rpm, 350 rpm, 500 rpm, and 750 rpm. Two *E. coli* strains were studied: PRS 181 and ALS 890. It was found that more alanine was produced at lower agitation speeds. In anaerobic production of alanine, 3 different pH levels were compared: 7.0, 7.5, and 8.0. *E. coli* strain PRS 178 was studied. It was found that pH 7.5 was the best for alanine production. These observations suggest that more glucose could be directed into aerobical production of alanine at lower agitation speeds. pH was found to be an important factor of anaerobic alanine production.

INDEX WORDS: Alanine, *E. coli*, Alanine dehydrogenase, Agitation speeds, pH

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May 2003

DEDICATION

♥

TO MY MOM AND DAD.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Alanine ($C_3H_7NO_2$) is one of the most important amino acids released by muscle, functioning as a major energy source. Alanine is also an inhibitory or calming neurotransmitter in the brain. Commercially, alanine is mainly used as a food additive (Katsumata, Hashimoto, 1996).

During early screening for glutamate-producing strains, some microorganisms accumulated alanine (Kinoshita et al., 1957). Later Iizuka and Komagata reported that alanine was produced by almost all strains tested when the medium contained glucose and ammonium chloride (Iizuka and Komagata, 1960). Sakaguchi et al. is the first group to report production of L-alanine in bacteria *Streptomyces* and *Nocardia* (Sakaguchi et al., 1959).

Originally L-alanine was thought to be generated exclusively during fermentation, but both enantiomers of alanine were produced by *Corynebacterium gelatinosum* No-7183 (Kitai et al., 1961). Several papers subsequently reported that alanine was formed as the racemate because microorganisms possess an alanine racemase.

Alanine can be synthesized from pyruvate, the glycolytic product. However, despite the isolation of alanine-producing microorganisms, L-alanine production by fermentation has not yet been realized because the product is obtained either in relatively low yield (Perry, 1967) or in the DL-form (Hirose and Yamada, 1961). Currently L-alanine can be produced in two ways: the decarboxylation of L-aspartic acid to L-alanine by microbial L-aspartic β-decarboxylase and the reductive amination of pyruvate catalyzed by L-alanine dehydrogenase (ALD).

1.1 L-aspartate β- decarboxylase

A method for the industrial production of L-alanine from L-aspartic acid by the action of microbial L-aspartate β-decarboxylase has been established (Figure 1.1) (Calton, 1992).

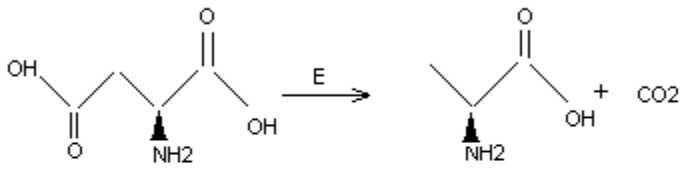
In 1948, L-aspartate β-decarboxylase was first reported in *Pseudomycobacterium* (Mardashev and Gladkova, 1948). Later this enzyme was reported in a number of microorganisms.

1.2 alanine dehydrogenase

L-alanine can also be produced by reductive amination of pyruvate catalyzed by L-alanine dehydrogenase (ALD). ALD catalyzes the reversible deamination of L-alanine to pyruvate. It occurs in vegetative cells and spores of various bacilli and cells of a few other bacteria (Uhlenbusch et al., 1991). It is a key enzyme in catabolism of L-alanine leading to intermediates of the tricarboxylic acid cycle and responsible for generation of energy during sporulation. The enzyme has been purified to homogeneity from *Bacillus* species and some other bacteria. It is used in assay of γ-glutamyltransferase as well as production of L-alanine. Thermostable ALD from the thermophilic bacterium *Bacillus stearothermophilus* is highly useful but produced in small amounts. The ALD from *Bacillus sphaericus* has a molecular weight of about 230,000 and is composed of six identical subunits. It requires NAD^+ in its catabolic reaction, which cannot be replaced by $NADP^+$. It is stable over a wide pH range (6.0-10.0) and shows maximum reactivity at about pH 10.5 for deamination and pH 9.0 for the amination reaction. The reductive amination goes through a sequential ordered ternary-binary mechanism. NADH binds first to enzyme followed by ammonia and pyruvate, and products are released in the order of L-alanine and NAD^+ (Ohashima, 1979).

L-alanine production from sugar fermentation has also been reported for the intestinal parasite *Giardia lamblia* (Paget et al., 1990), and a genetically modified bacterium *Zymomonas mobilis* (Uhlenbusch et al., 1991). In case of *Z. mobilis*, 7-8% of carbon in the glucose utilized was recovered in alanine by the recombinant strain. Later *Salmonella typhimurium* (Wasserman

Figure 1.1. Production of L-alanine from L-aspartic acid.



C4H7NO4

L-aspartate

C3H7NO2

L-alanine

E: L-aspartate B-decarboxylase

et al., 1983) was studied. Pyruvate was aminated by a transamination reaction coupled to alanine aminotransferase.

Researchers in Tokyo Research Laboratories recently isolated an *Arthrobacter oxydans* HAP-1 strain that produces a higher yield of DL-alanine than any of the alanine-producing microorganisms reported previously, 75.6 g/L L-alanine (Hashimoto, 1998). In 1998, they revealed that the alanine overproduction in strain HAP-1 was caused by L-alanine dehydrogenase (ALD), which functions to preferentially catalyze the amination reaction in cells.

1.3 Naturally occuring alanine producing microorganisms

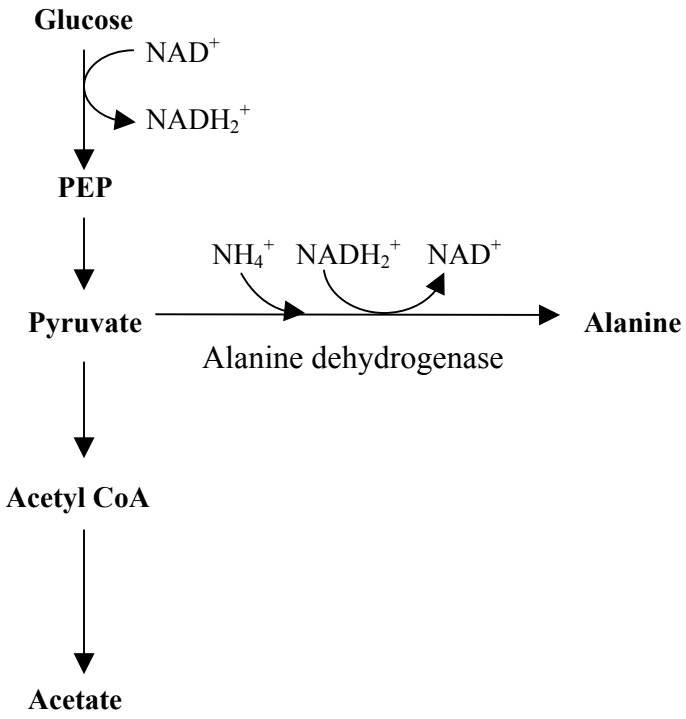
Sakaguchi et al. were the first group to report production of L-alanine using the bacteria, *Streptomyces* and *Nocardia* (Sakaguchi et al., 1959). Bacterial production was studied further in detail in *E. coli* (Wild, 1985) and *Salmonella typhimurium* (Wasserman, 1983). In these bacteria, L-alanine is synthesized by transamination of pyruvate by glutamate or valine (Tan-Wilson, 1983). Isomerized by alanine racemase (AR) to form D-alanine, an essential component of peptidoglycan. *Arthrobacter oxydans* can produce DL-alanine from glucose by reductive amination of pyruvate catalyzed by ALD.

1.4 Recombinant Microorganisms

Arthrobacter oxydans can produce DL-alanine from glucose by reductive amination of pyruvate catalyzed by ALD. This bacterium was found to grow on either D- or L-alanine as a sole carbon source with lower growth rates than on glucose. The effects of an AR inhibitor on the accumulation and/or consumption of D- or L-alanine by the bacterium indicated that D-alanine was formed by isomerization of L-alanine and degraded through the reverse reaction. D-alanine non-utilizing mutants were derived from strain HAP-1 by a staining method using the chromogenic agent 2,3,5-triphenyl tetrazolium chloride. The mutants obtained were of two kinds: ALD-deficient and AR-deficient. The former lost the ability to grow on both D- and L-alanine, indicating that the ALD of strain HAP-1 functions not only in L-alanine synthesis but also in L-alanine degradation. The latter could utilize L-alanine as a carbon source but not D-

alanine. Furthermore, they required L-alanine for growth in minimum nutrient medium. An AR-deficient mutant, DAN75, produced 75.6 g/L of L-alanine with high optical purity (97% *e.e.*, enantiomeric excess) in a fed-batch cultivation (glucose was fed to 15% in total) using a jar-fermentor. The L-alanine titer was almost equal to sum of D- and L-alanine produced by the parental strain. The production rate of DAN 75, as well as that of the parental strain HAP-1, slowed down late in the cultivation. Examination of the influence of exogenously added alanine showed that this reduced production was caused by a deceleration in the glucose consumption rate arising from inhibition by alanine produced (Hashimoto, 1998). Some researchers in Japan found an *Arthrobacter* strain that can produce 80.8 g/L L-alanine after 100 hours culture (Hashimoto, 1993). *E. coli* has also been used as a host to produce L-alanine, but the production was only 2.9 g/L. The gene expressing L-alanine dehydrogenase is from *Arthrobacter sp*. HAP1 (Katsumata and Hashimoto, 1996). The pathway to produce alanine in *E. coli* is shown in Figure 1.2.

Figure 1.2. Metabolic pathway in *E. coli*


Glucose
NAD+
NADH2+
PEP
NH4+
NADH2+
NAD+
Pyruvate
Alanine
Alanine dehydrogenase
Acetyl CoA
Acetate

Reference:

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CHAPTER 2

AEROBIC PRODUCTION OF ALANINE BY *ESCHERICHIA COLI*

INTRODUCTION

Alanine ($C_3H_7NO_2$) is the smallest chiral amino acid. It is released by muscle, functioning as a major energy source and an inhibitory neurotransmitter in the brain (Katsumata and Hashimoto, 1996). Commercially, alanine is used as a food additive (Katsumata and Hashimoto, 1996). An enzymatic or fermentation process which can generate optically active L- or D- alanine may be economically advantageous in comparison with chemical syntheses that require chiral separation. Presently, L-alanine is produced from L-aspartate via the enzyme L-aspartic-β-decarboxylase (Chibata et al., 1969).

Several microbial processes have been studied to generate alanine. For example, L-alanine may be generated by a strain of the genus *Arthrobacter* with a yield of 35% from glucose (Katsumata et al., 1991), while 7.5 g/L of L-alanine was anaerobically produced by *Zymomonas* into which the gene coding for *Bacillus* L-alanine dehydrogenase was introduced (Uhlenbusch et al., 1991). Katsumata and Hashimoto (1996) described L-alanine production using strains belonging to the genera *Escherichia*, *Corynebacterium* and *Brevibacterium*. These researchers produced 75.6 g/L of L-alanine with high optical purity (97% e.e.) in 120 hours using the alanine racemase deficient mutant *Arthrobacter oxydans* DAN 75. When 2 g/L D-alanine was initially added, DAN 75 produced significant alanine when the culture reached stationary phase

(Hashimoto and Katsumata, 1998). *Escherichia coli* has also been used to produce L-alanine, but the production was only 2.9 g/L (Katsumata and Hashimoto, 1998).

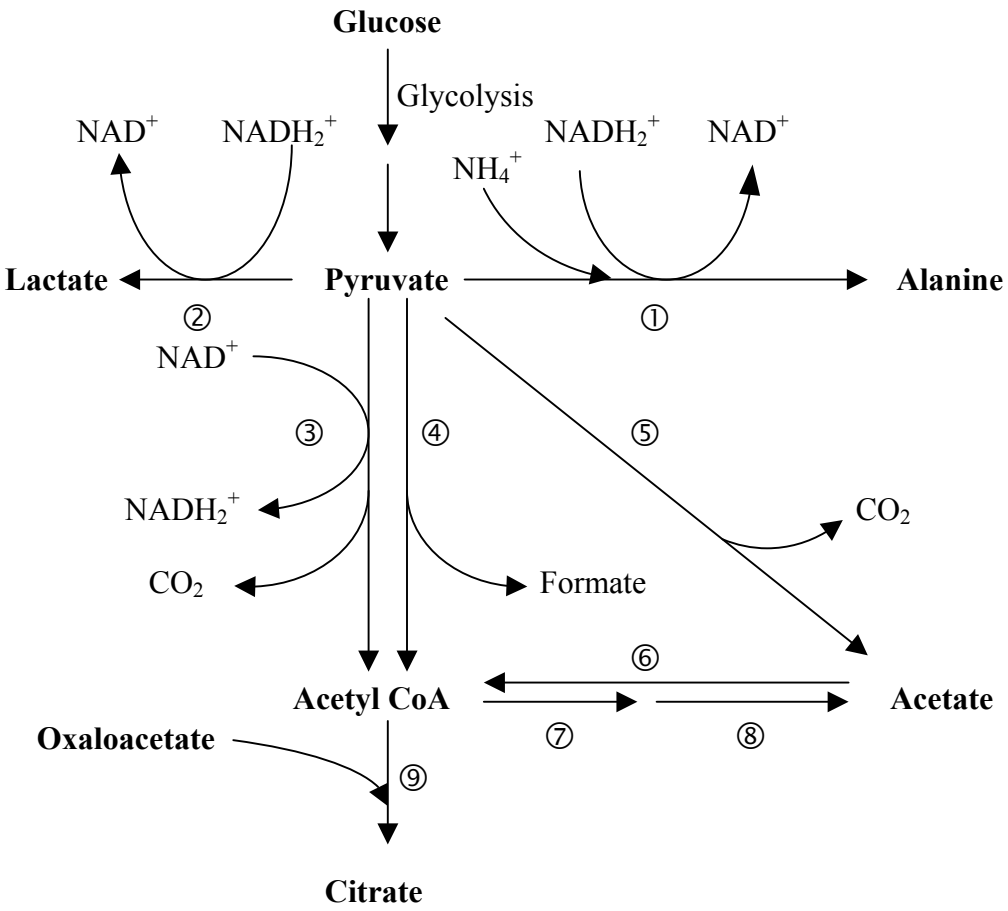
The key metabolic steps in *Escherichia coli* influencing alanine synthesis are shown in Figure 2.1. Glucose is metabolized to pyruvate through glycolysis. A common approach for the production of alanine by any organism has been to overexpress alanine dehydrogenase, a single enzymatic step converting pyruvate to alanine. However, several enzymes compete with alanine dehydrogenase for the substrate pyruvate. Lactate dehydrogenase is allosteric for pyruvate (Futai and Kimura, 1977), and since this enzyme like alanine dehydrogenase uses NADH, lactate dehydrogenase would likely be an unavoidable competitor to alanine production. Pyruvate formate lyase is active only during anaerobic conditions (Knappe and Sawers, 1990) and therefore would not compete with alanine dehydrogenase if oxygen was present. The pyruvate dehydrogenase complex is the primary route of pyruvate assimilation under aerobic growth. Recently, a mutant in the *aceF* gene encoding for the dihydrolipoyl transacetylase component of this complex was able to accumulate about 40 g/L pyruvate when the medium was supplemented with acetate (Tomar et al., 2003). Acetate is necessary for the *aceF* mutant to generate acetyl CoA required for the synthesis of biomass via the enzyme acetyl CoA synthetase.

The objective of this research was to study alanine production in *E. coli aceF* mutants overexpressing alanine dehydrogenase. Because lactate dehydrogenase would appear to be an unavoidable competitor, we also constructed *aceF ldhA* double mutants. High oxygenation would tend to reduce the availability of NADH for alanine dehydrogenase, while the absence of oxygen would permit the expression of pyruvate formate lyase and hence the redirection of pyruvate from alanine. Thus, the focus of the study is to understand how oxygenation impacts alanine formation.

MATERIALS AND METHODS

Construction of pTrc99A-*alaD*. The *Bacillus sphaericus* alanine dehydrogenase gene (*alaD*) was amplified using the polymerase chain reaction (PCR). *Pfu* DNA polymerase was used instead of *Taq* DNA polymerase, and the pBm2O*alaD* plasmid served as the DNA template. Primers were designed based on the published *B. sphaericus alaD* gene sequence (Kuroda et al., 1990) and contained a *Bam*H

Figure 2.1. *E. coli* metabolic pathways involved in alanine synthesis.



① Alanine dehydrogenase
② Lactate dehydrogenase
③ Pyruvate dehydrogenase complex
④ Pyruvate formate lyase
⑤ Pyruvate oxidase
⑥ Acetyl CoA synthetase
⑦ Phosphate acetyltransferase
⑧ Acetate kinase
⑨ Citrate synthase

I (GGATCC) restriction site and Shine-Dalgarno sequence at the beginning of the amplified fragment and a *Hin*d III (AAGCTT) restriction site at the end of the amplified fragment; forward primer 5'' TAC TAT GGA TCC AGG AGG AAC AGC TAT GAA GAT TGG TAT TCC AAA GGA AAT TAA AAA C 3''; reverse primer 5'' ATA GCG ATC GAT AGC GGT AAG CTT ATT ATT GGA TTA ATT CAT CCA CAT TCA CAT ATG 3'' (the *Bam*H I, Shine-Dalgarno, ATG start, and *Hin*d III sites are underlined). The resulting 1.2 kb PCR product was gel isolated, restricted with *Bam*H I and *Hin*d III and ligated into the pTrc99A expression vector which had been restricted with the same two enzymes.

Strains and Plasmids. *E. coli* CGSC6162 (F^+ *aceF*10 *fadR*200 *tyrT*58 (AS) *adhE*80 *mel*-1) was the parent strain used in this study. The *ldhA* mutant was derived from CGSC6162 by P1 phase transduction using *E. coli* strain NZN111 as the donor (Bunch et al., 1997). The organisms used to study the production of alanine were designated PRS181 (CGSC6162 pTrc99A-*alaD*) and ALS 890 (CGSC6162 *ldhA*::Kan pTrc99A-*alaD*).

Media and growth conditions. Cells were first grown in an agitated 20 mL screw-top test tube composed of (pH=7.0): 25% LB (2.5 g/L tryptone, 2.5 g/L NaCl, 1.25 g/L yeast extract), 15.0 g/L glucose, 3.0 g/L acetic acid and 6.0 g/L succinic acid. After 3h of growth in this medium, 10 mL were used to inoculate a 250 mL baffled shake flask with 100 mL of medium composed of (pH 7.0): 15.0 g/L glucose, 6.0 g/L succinic acid, 3.0 g/L acetic acid, 10.0 g/L tryptone, 2.5 g/L yeast extract, 3.0 g/L KH_2PO_4, 6.0 g/L Na_2HPO_4, 6.0 g/L NH_4Cl, 0.14 g/L $CaCl_2 \bullet 2H_2O$ and 0.25 g/L $MgSO_4 \bullet 7H_2O$. Cells were grown at 250 rpm agitation (19 mm radius of orbit) for 6 h and then used to inoculate a fermenter of the same composition except that it contained 40.0 g/L glucose. Fermentations of 1.5 L volume used a BioFlow 2000 fermenter (New Brunswick Scientific Company, New Brunswick, NJ). Air was supplied continuously at 1.0 L/min with agitation as described in text. The pH was controlled at 7.0 using 20% NaOH and 20% H_2SO_4. After 4.0 h of growth in the fermenter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. All media were supplemented with ampicillin at 100 mg/L and were maintained at 37 °C. An initial examination of the

effect of NH_4Cl on growth rate used the identical initial media composition as in shake flasks but used four different concentrations of NH_4Cl.

Analyses. Cell growth was monitored by measuring the optical density (OD) at 550 nm (DU-650 UV-VIS spectrophotometer, Beckman Instruments, San Jose, CA). Samples were centrifuged (10,000×g for 10 minutes at 25 °C), and the supernatant analyzed for glucose, succinate, lactate, acetate, pyruvate by high pressure liquid chromatography (HPLC) using a previously described method (Eiteman and Chastain, 1997). Alanine was analyzed by HPLC using an Aminex HPX-87C Carbohydrate column with 0.1 M $Ca(NO_3)_2$ effluent and a refractive index detector (Waters 2410, Millipore Corp., Milford, MA).

Enzyme assays. Cell-free extracts were prepared by washing the cell pellet at 4 °C twice with 10 mM potassium phosphate buffer (pH 7.0). B-PER II Reagent (Pierce, Rockford, IL) was used to lyse the cells, cell debris were removed by centrifugation (20,000×g for 20 minutes at 4 °C) and the cell-free extract used for measuring alanine dehydrogenase activity (Ohashima and Soday, 1979). One unit of enzyme activity is the quantity of enzyme required to produce 1.0 μmole of pyruvate in one minute. Total protein in the cell-free extract was determined using bovine serum albumin as the standard (Lowry et al., 1951).

RESULTS

Ammonium toxicity. Ammonium is required as a substrate by alanine dehydrogenase to produce alanine. The ammonium concentration should be as high as possible so that this substrate does not become limiting to the enzymatic reaction. However, high ammonium concentrations may be inhibitory to the growth of *E. coli*. We therefore first examined the effect of ammonium on *E. coli* growth by comparing four initial NH_4Cl concentrations, and Figure 2.2 shows the results. Based on the triplicate test results of specific growth rate μ, an ANOVA table was constructed to determine if NH_4Cl concentration affected the growth of *E. coli*. Using the confidence interval of 90% (α=0.1), the conclusion is that cell growth was not affected by NH_4Cl concentration in the range of 2 g/L to 8 g/L.

Effect of agitation rate on alanine production. We next compared the products formed during aerobic production of alanine in *E. coli* PRS 181 with agitation speeds of 750 rpm, 500 rpm, 350 rpm, and 250 rpm.

At a continuous agitation of 750 rpm, the rate of glucose consumption was 1.95 g/Lh with an alanine productivity of 0.26 g/Lh and mass yield of 0.13 (Figure 2.3). Pyruvate accumulated at 1.21 g/Lh with a yield of 0.62. Lactate accumulated at 0.10 g/Lh with a yield of 0.05. The final molar ratio of alanine:lactate was 2.7:1. The optical density at 14 h was 12.3.

At a continuous agitation of 500 rpm, the rate of glucose consumption was 1.44 g/Lh with an alanine productivity of 0.17 g/Lh and mass yield of 0.12 (Figure 2.4). Pyruvate accumulated at 1.00 g/Lh with a yield of 0.70. Lactate accumulated at 0.06 g/Lh with a yield of 0.04. The final molar ratio of alanine:lactate was 2.9:1. The optical density at 14 h was 10.9.

At a continuous agitation of 350 rpm, the rate of glucose consumption was 1.93 g/Lh with an alanine productivity of 0.33 g/Lh and mass yield of 0.17 (Figure 2.5). Pyruvate accumulated at 0.37 g/Lh with a yield of 0.19. Lactate accumulated at 0.74 g/Lh with a yield of 0.38. The final molar ratio of alanine:lactate was 1:2.2. The optical density at 14 h was 8.6.

At a continuous agitation of 250 rpm, the rate of glucose consumption was 0.88 g/Lh with an alanine productivity of 0.28 g/Lh and mass yield of 0.32 (Figure 2.6). Pyruvate accumulated at 0.11 g/Lh with a yield of 0.13. Lactate accumulated at 0.62 g/Lh with a yield of 0.71. The final molar ratio of alanine:lactate was 1:2.3. The optical density at 14 h was 6.1.

Figure 2.2. Effect of ammonium chloride on the growth of *E. coli*.

◇: 2 g/L, □: 4 g/L, ▲: 6 g/L, ∗: 8 g/L

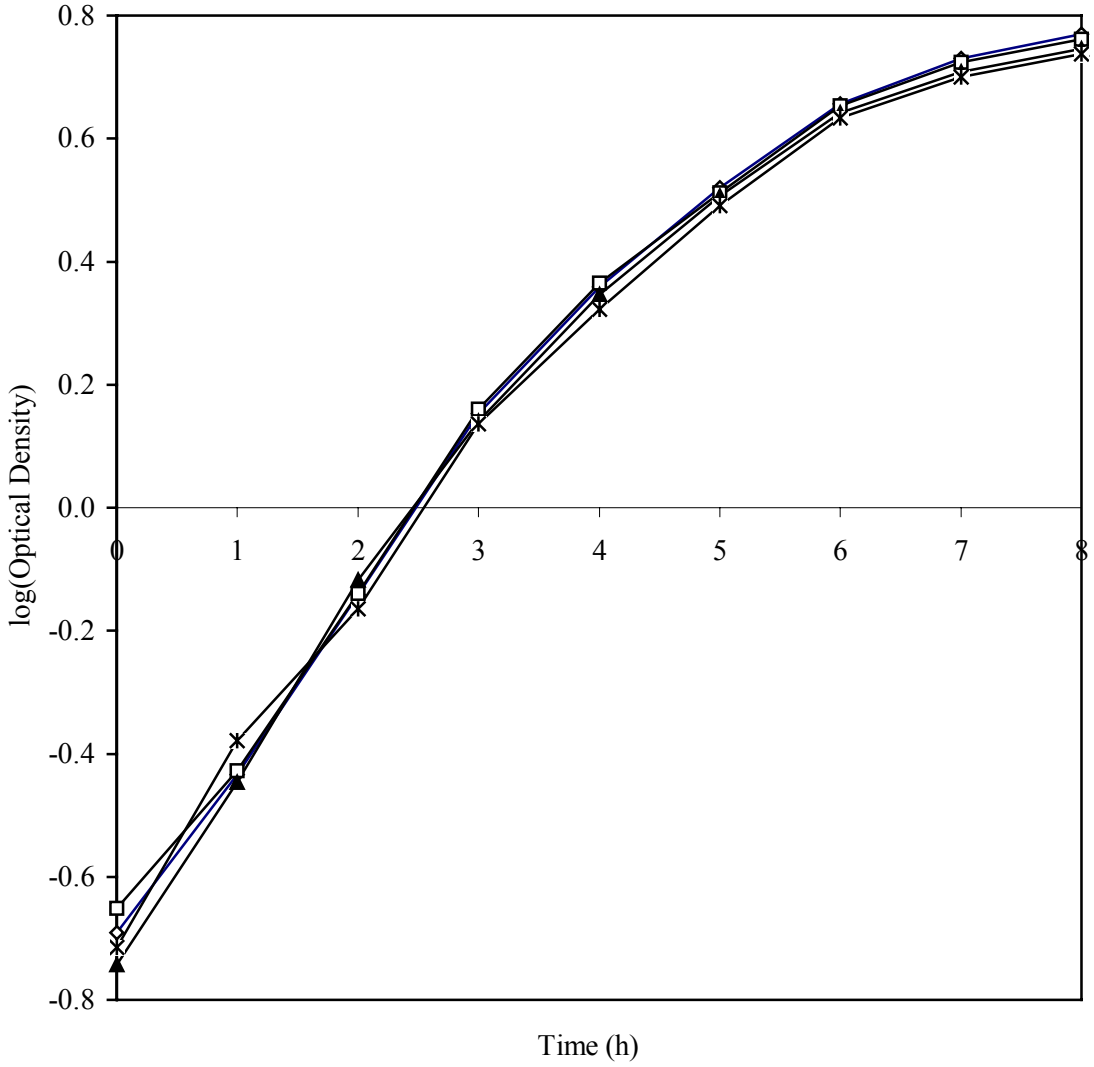

0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
0
1
2
3
4
5
6
7
8
log(Optical Density)
Time (h)

Figure 2.3.1. Example fermentation of *E. coli* PRS 181 at constant agitation of 750 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

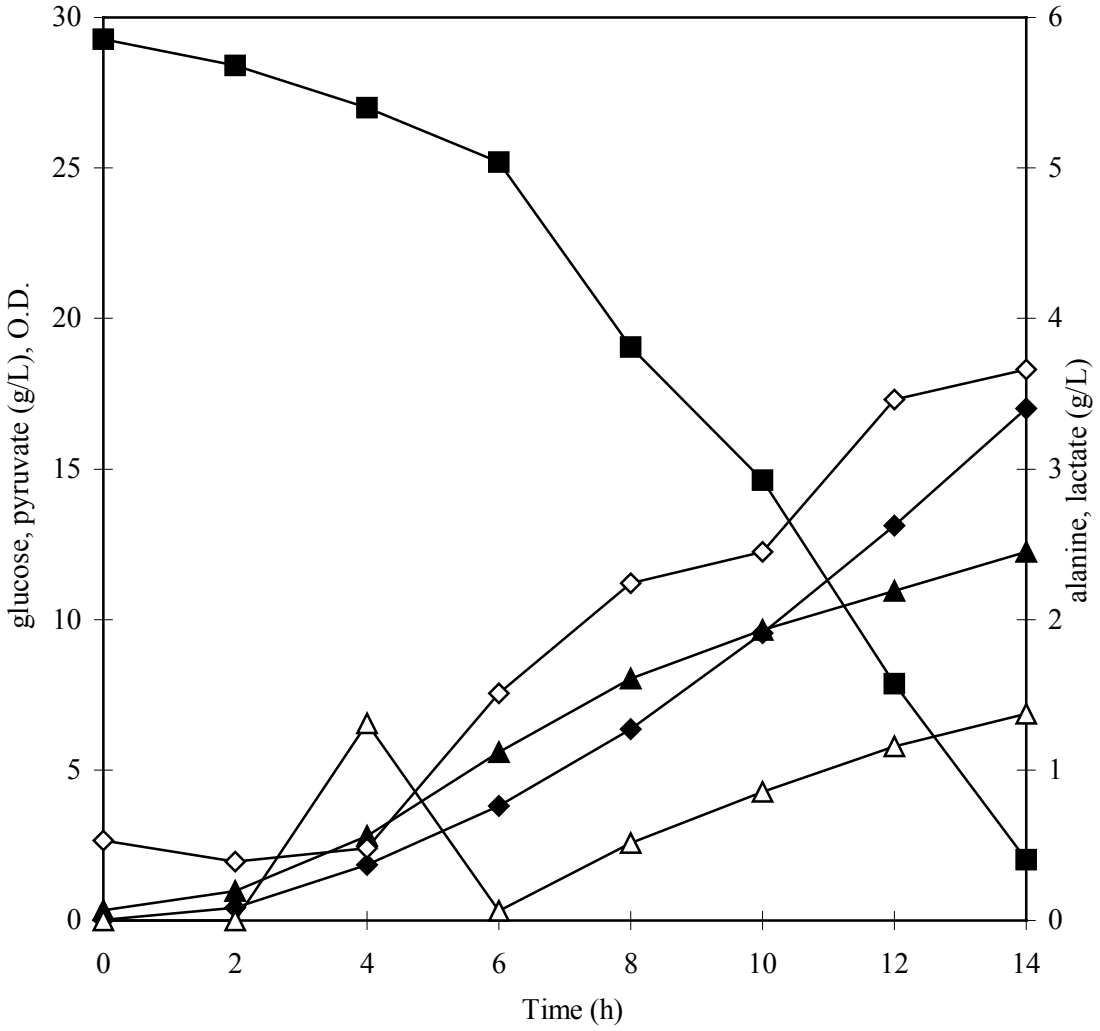

glucose, pyruvate (g/L), O.D.
alanine, lactate (g/L)
Time (h)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30
0
1
2
3
4
5
6

Figure 2.3.2. Example fermentation of *E. coli* PRS 181 at constant agitation of 750 rpm.

□: succinate, ∗: acetate

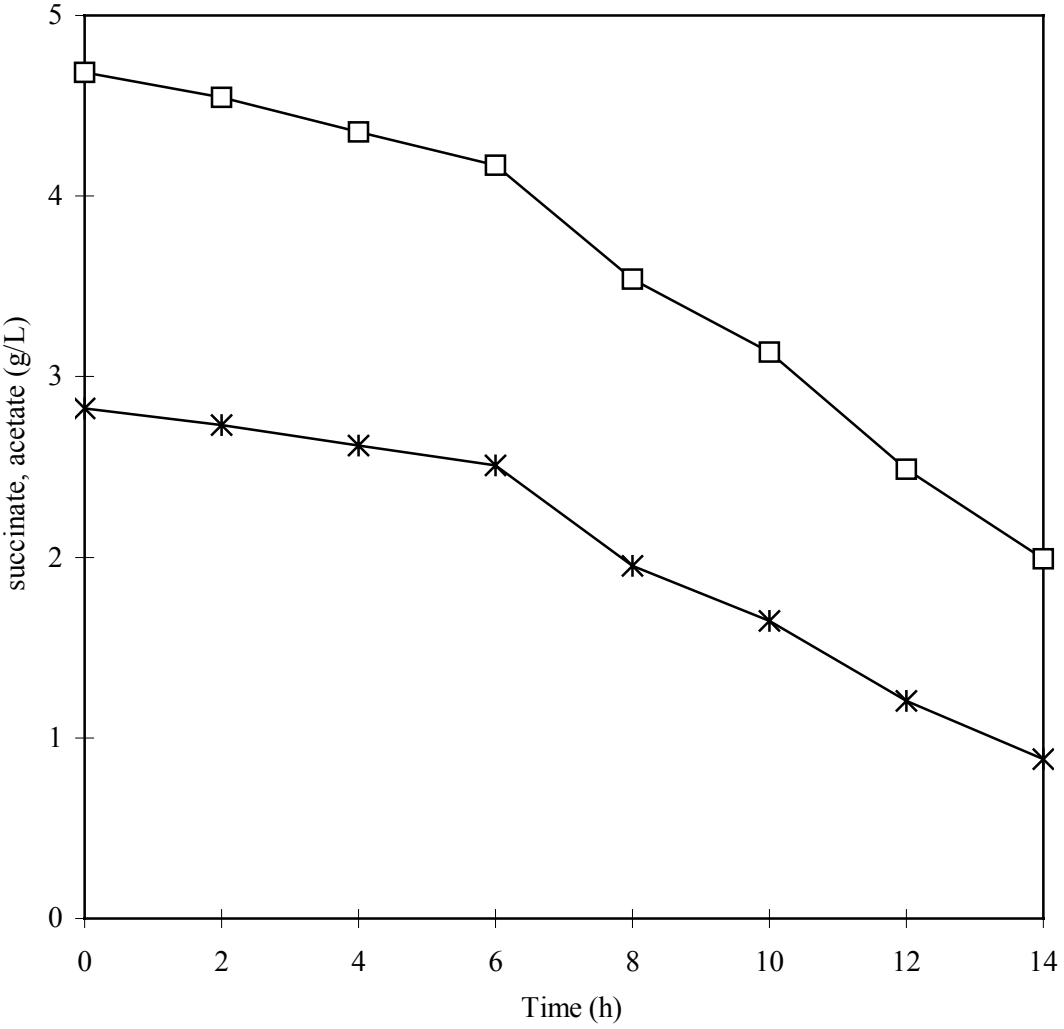

5
4
3
2
1
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
Time (h)

Figure 2.4.1. Example fermentation of *E. coli* PRS 181 at constant agitation of 500 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

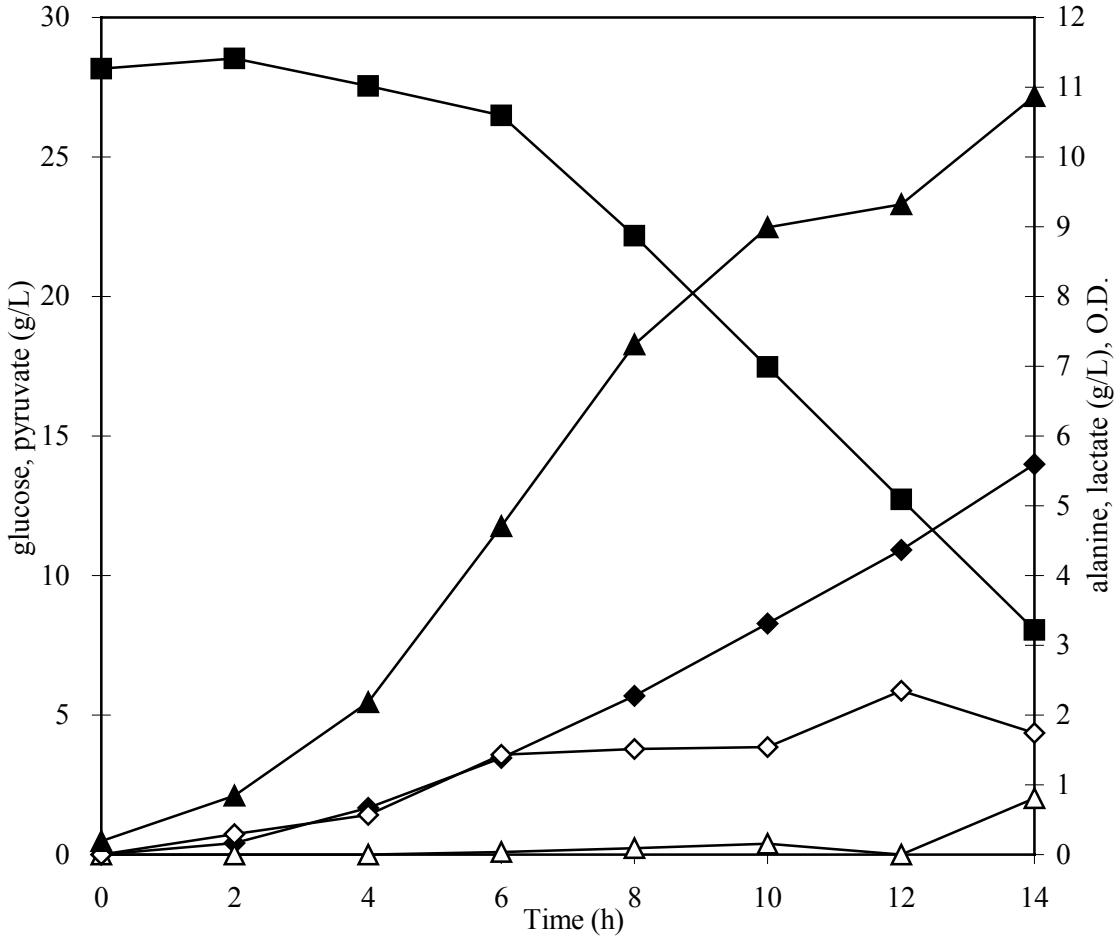

glucose, pyruvate (g/L)
alanine, lactate (g/L), O.D.
Time (h)
0
5
10
15
20
25
30
0
1
2
3
4
5
6
7
8
9
10
11
12
0
2
4
6
8
10
12
14

Figure 2.4.2. Example fermentation of *E. coli* PRS 181 at constant agitation of 500 rpm.

□: succinate, ∗: acetate

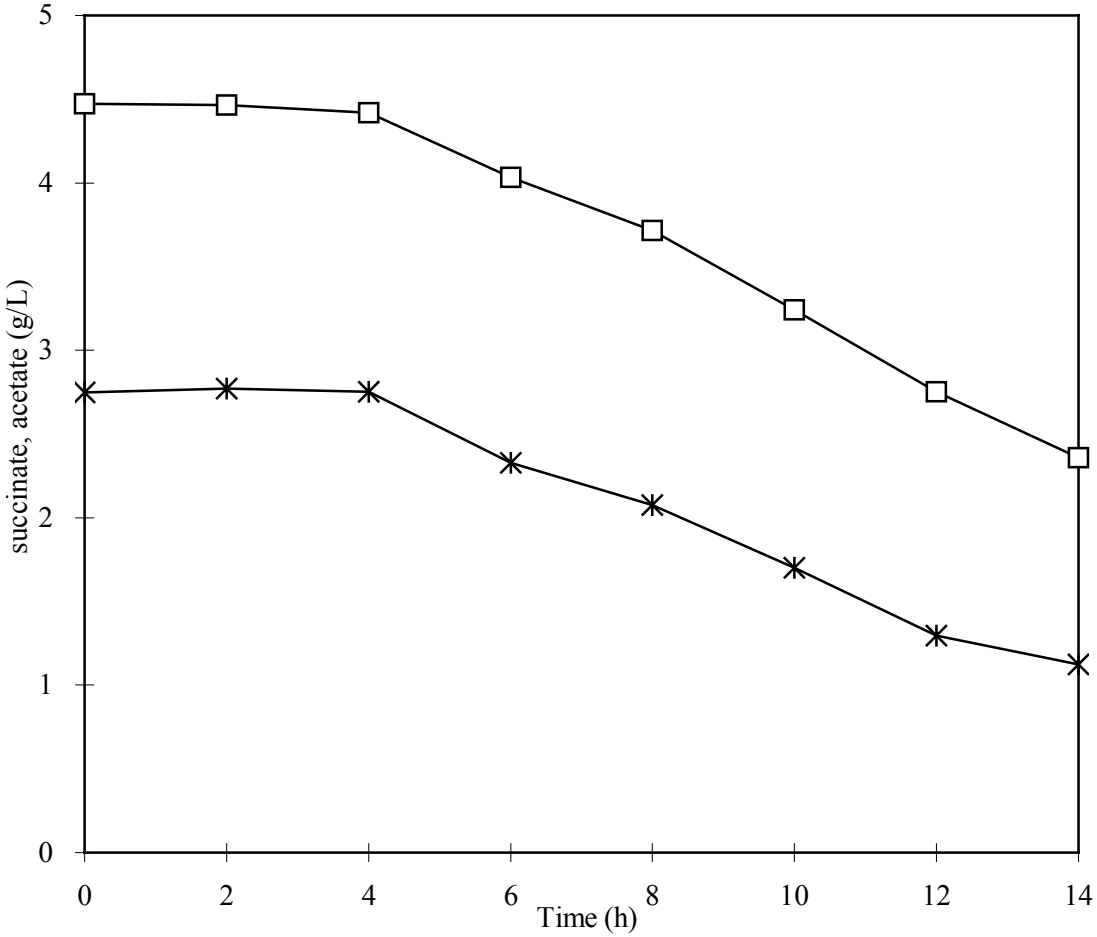

5
4
3
2
1
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
Time (h)

Figure 2.5.1. Example fermentation of *E. coli* PRS 181 at constant agitation of 350 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

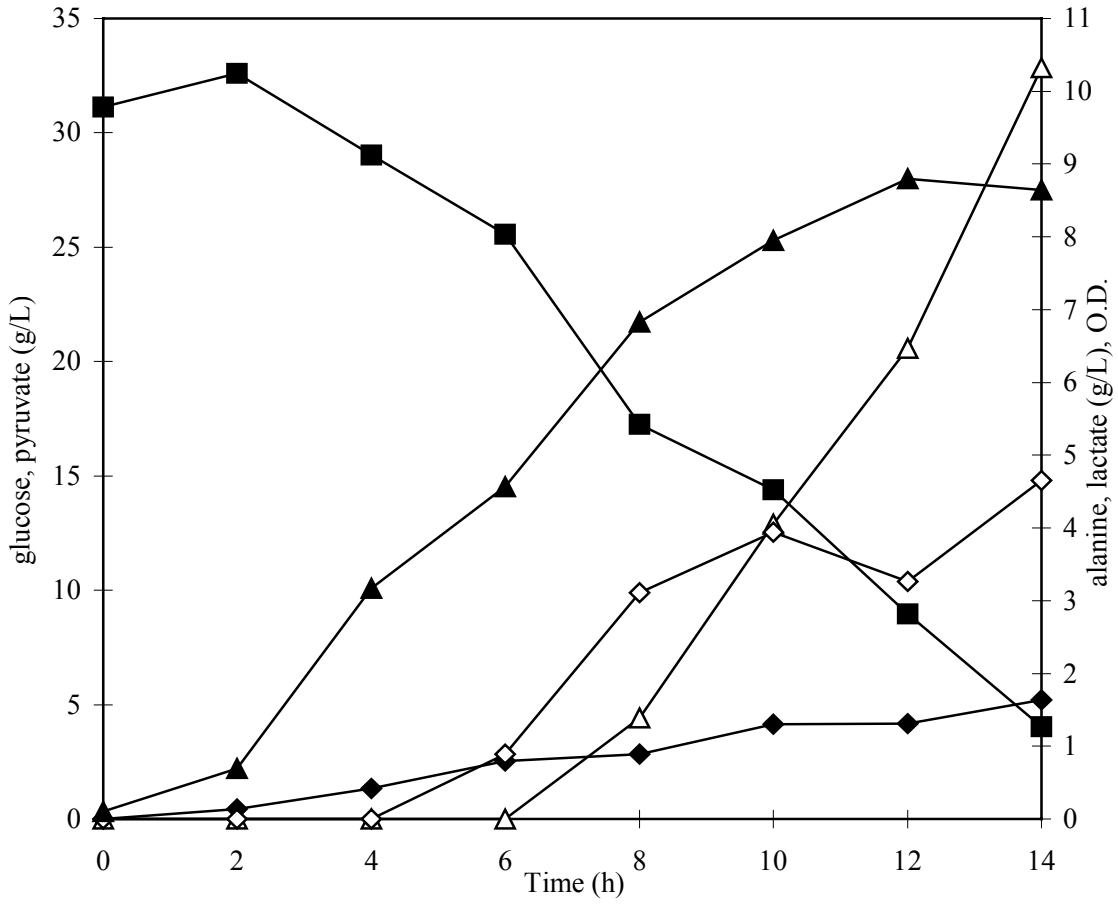

glucose, pyruvate (g/L)
alanine, lactate (g/L), O.D.
Time (h)
0
5
10
15
20
25
30
35
0
1
2
3
4
5
6
7
8
9
10
11
0
2
4
6
8
10
12
14

Figure 2.5.2. Example fermentation of *E. coli* PRS 181 at constant agitation of 350 rpm.

□: succinate, ∗: acetate, ◆: D.O.

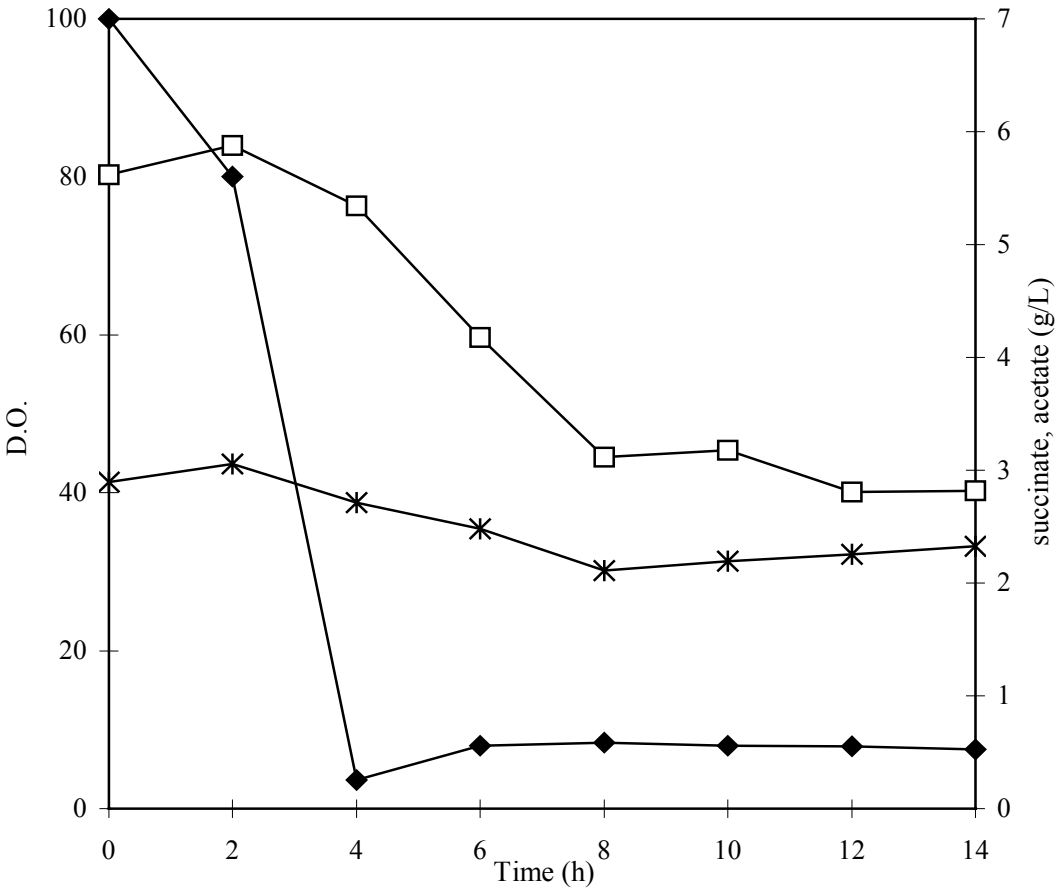

100
80
60
40
20
0
D.O.
7
6
5
4
3
2
1
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
Time (h)

Figure 2.6.1. Example fermentation of *E. coli* PRS 181 at constant agitation of 250 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

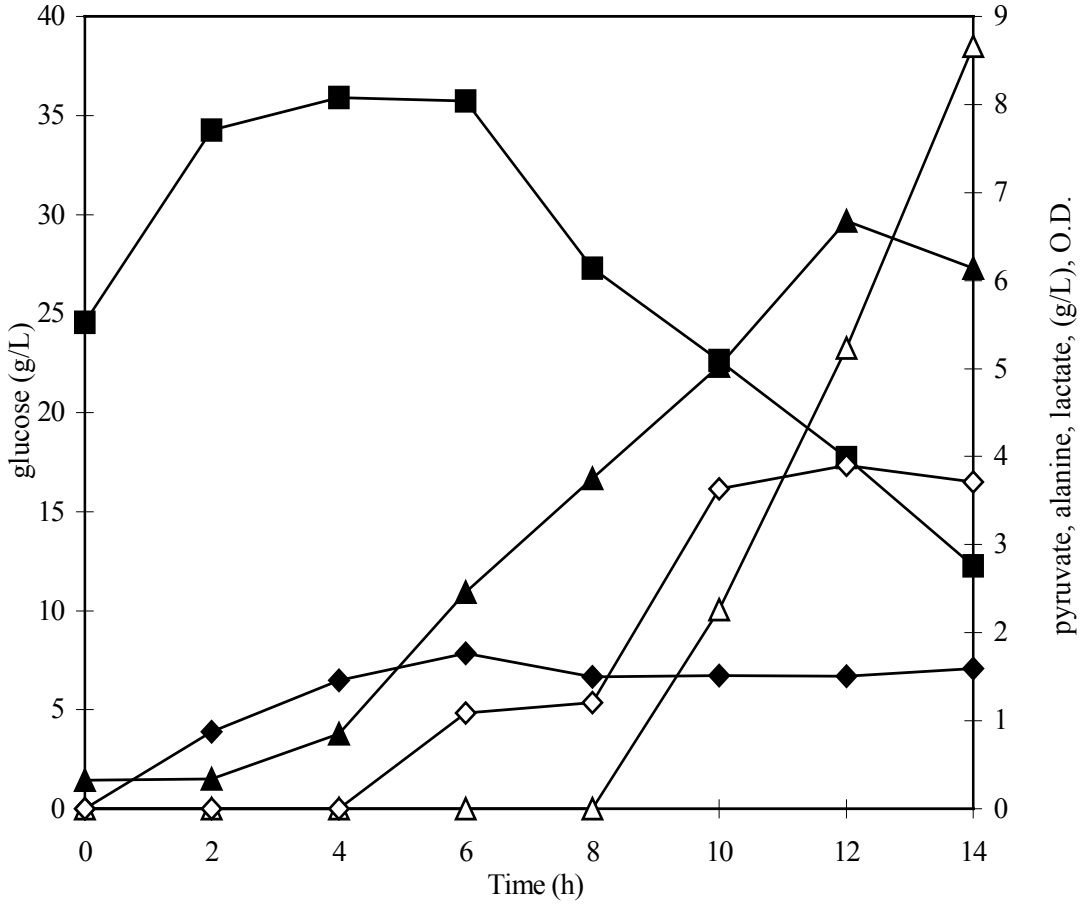
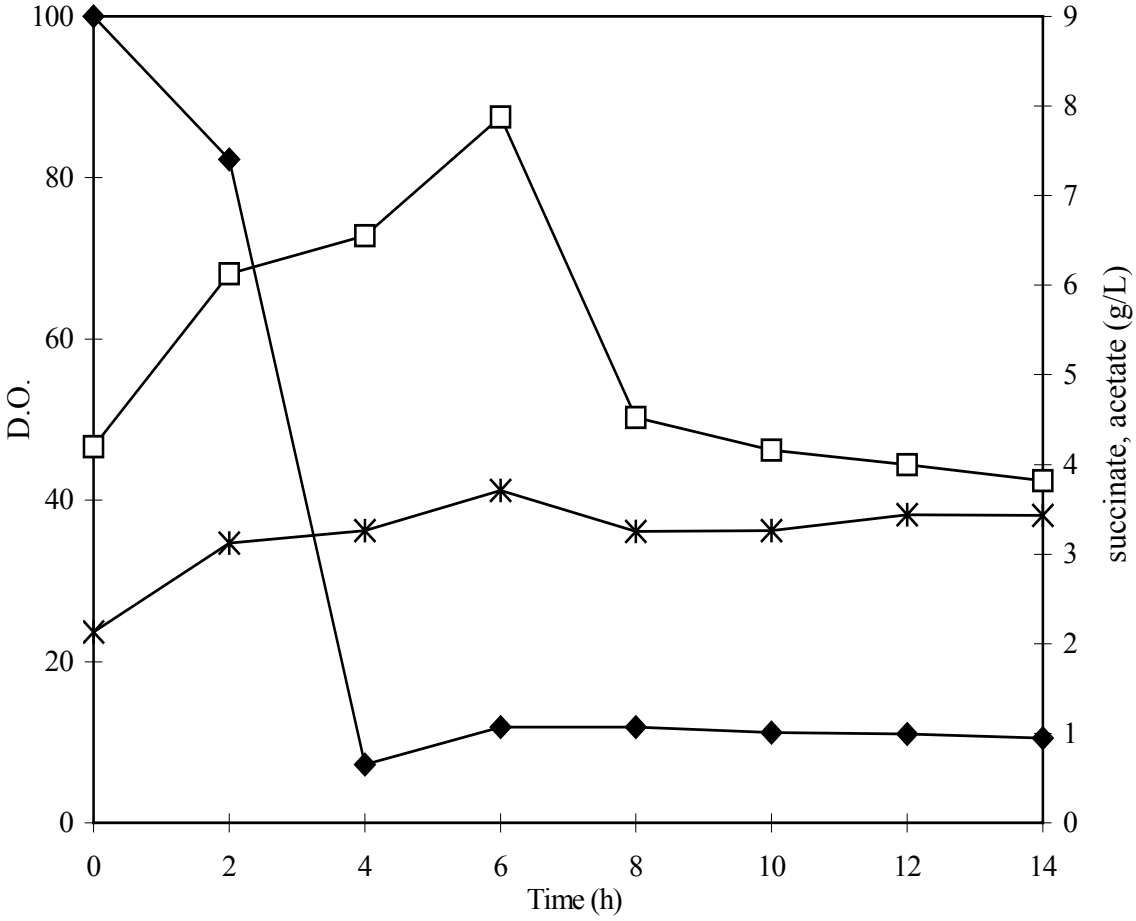

glucose (g/L)
pyruvate, alanine, lactate, (g/L), O.D.
Time (h)
0
5
10
15
20
25
30
35
40
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
14

Figure 2.6.2. Example fermentation of *E. coli* PRS 181 at constant agitation of 250 rpm.

◆: D.O., □: succinate, ∗: acetate


100
80
60
40
20
0
D.O.
9
8
7
6
5
4
3
2
1
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
Time (h)

Products yields in aerobic production of alanine in *E. coli* are summarized in Table 2.1. Less than 100% of the glucose carbon was converted to the three products pyruvate, lactate and alanine in all cases. The lowest agitation rate resulted in the lowest carbon recovery.

Batch fermentation using ALS 890. The products formed during aerobic growth of *E. coli* ALS 890, a *ldhA* mutant were next studied. Agitation speeds of 250 rpm and 350 rpm were compared. As in the previous study, growth in a screw top test tube preceded growth in a baffled shake flask, which was then used to inoculate a fermentor containing 40.0 g/L glucose.

At a continuous agitation of 250 rpm, the rate of glucose consumption was 0.95 g/Lh with an alanine productivity of 0.58 g/Lh and mass yield of 0.61 (Figure 2.7). Pyruvate accumulated at 0.29 g/Lh with a yield of 0.31. A small amount of lactate accumulated (less than 0.2 g/L). The optical density at 16 h was 6.8.

At a continuous agitation of 350 rpm, the rate of glucose consumption was 1.17 g/Lh with an alanine productivity of 0.49 g/Lh and mass yield of 0.42 (Figure 2.8). Pyruvate accumulated at 0.46 g/Lh with a yield of 0.39. A small amount of lactate again accumulated. The optical density at 16 h was 7.2.

Products yields in aerobic production of alanine in *E. coli* ALS 890 are summarized in Table 2.2. For both agitation rates nearly 100% of the carbon from glucose was accounted in the three principal fermentation products.

Fed batch fermentation using ALS 890. Lactate production was essentially eliminated by using ALS 890. Since both glucose and ammonium are required for alanine, a fed batch fermentation was simulated to determine whether addition of these compounds during the course of a fermentation would increase alanine production. At 14 h, 60mL of 500 g/L glucose and 50mL of 150 g/L NH_4Cl were added.

At a continuous agitation of 250 rpm, the rate of glucose consumption was 1.58 g/Lh with an alanine productivity of 0.63 g/Lh and mass yield of 0.40 (Figure 2.9). Pyruvate accumulated at 0.61 g/Lh with a yield of 0.39. Glucose concentration at 14 hours before addition

Table 2.1. Mass yields of products during aerobic production of alanine in *E. coli* at four different continuous agitation rates.

Agitation Speed (rpm)	Mass Yields (g product/g glucose)			% of glucose carbon in these 3 products
	Pyruvate	Lactate	Alanine	
750	0.67	0.06	0.09	84.0
500	0.70	0.04	0.12	87.0
350	0.19	0.38	0.17	75.2
250	0.03	0.39	0.18	60.6

Figure 2.7.1. Example fermentation of *E. coli* ALS 890 at constant agitation of 250 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

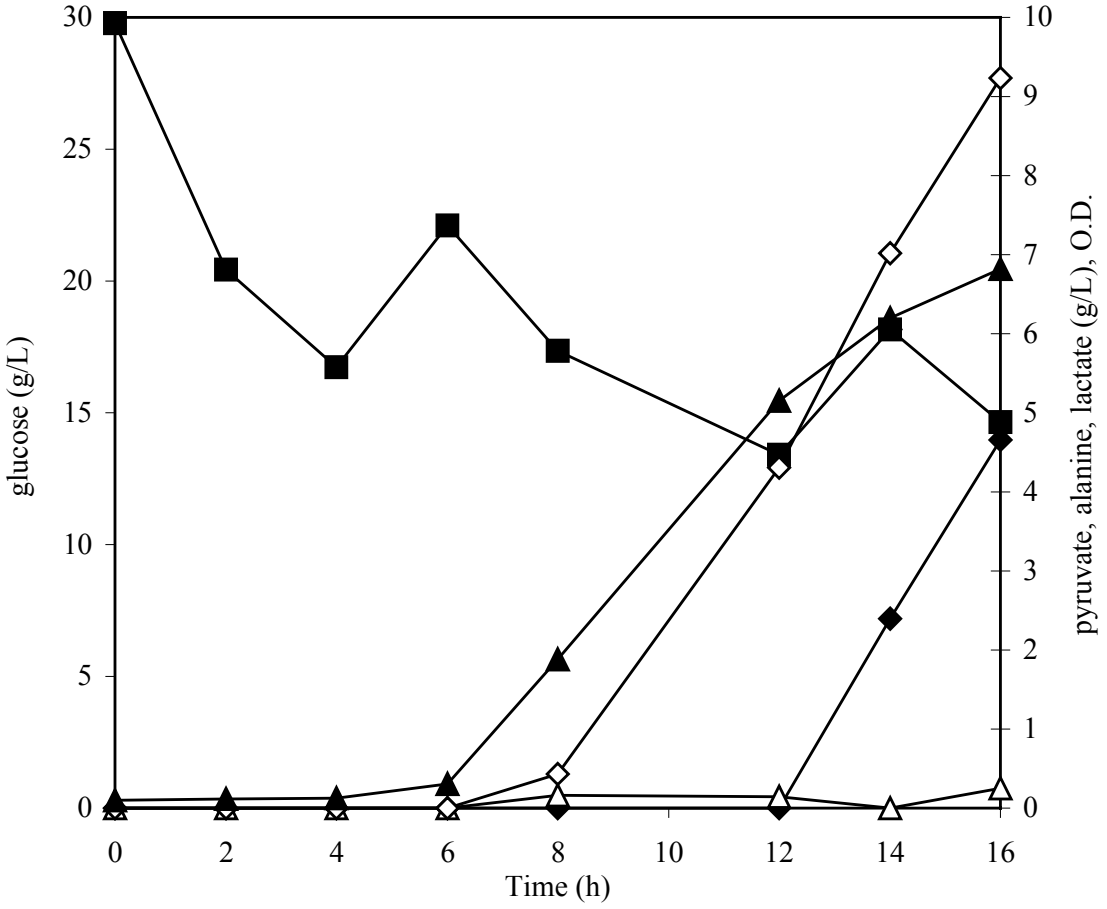

glucose (g/L)
pyruvate, alanine, lactate (g/L), O.D.
Time (h)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30
0
1
2
3
4
5
6
7
8
9
10

Figure 2.7.2. Example fermentation of *E. coli* ALS 890 at constant agitation of 250 rpm.

◆: D.O., □: succinate, ∗: acetate

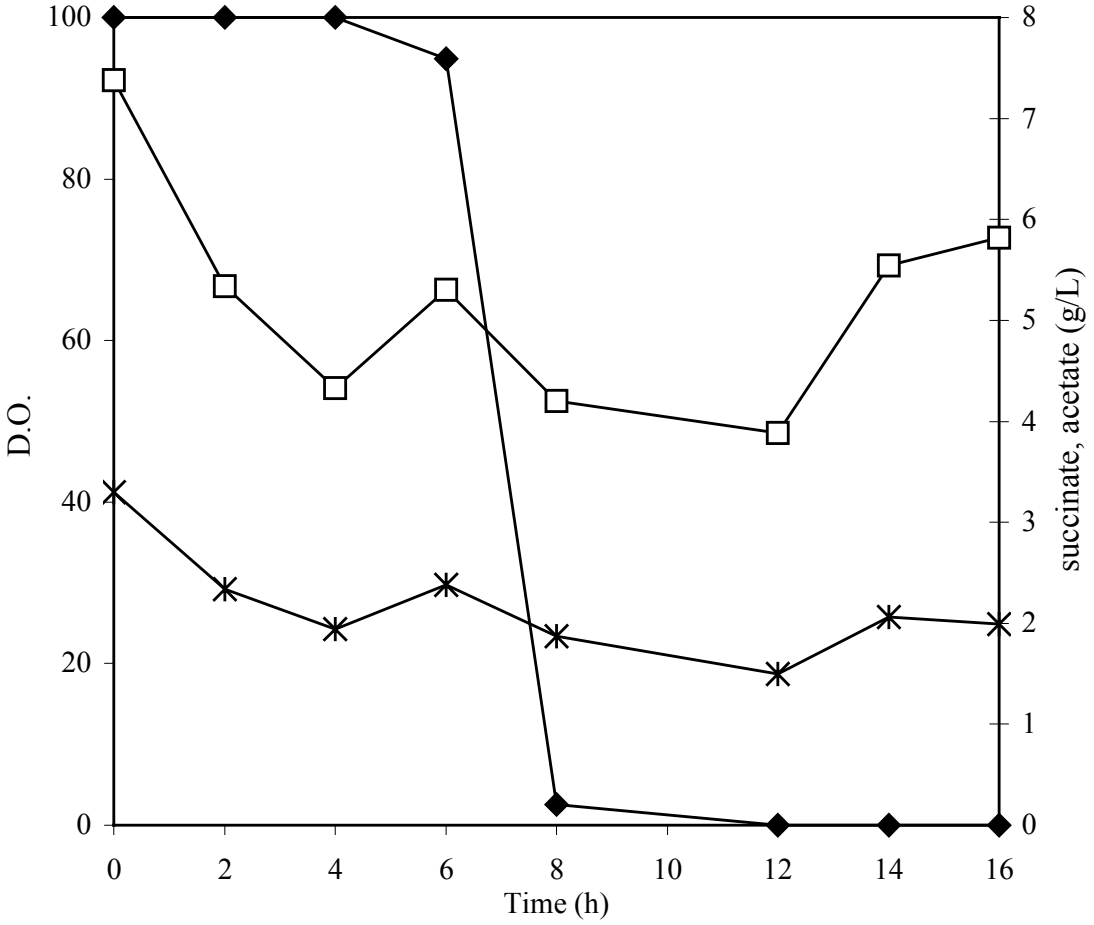

100
80
60
40
20
0
D.O.
8
7
6
5
4
3
2
1
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
16
Time (h)

Figure 2.8.1. Example fermentation of *E. coli* ALS 890 at constant agitation of 350 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

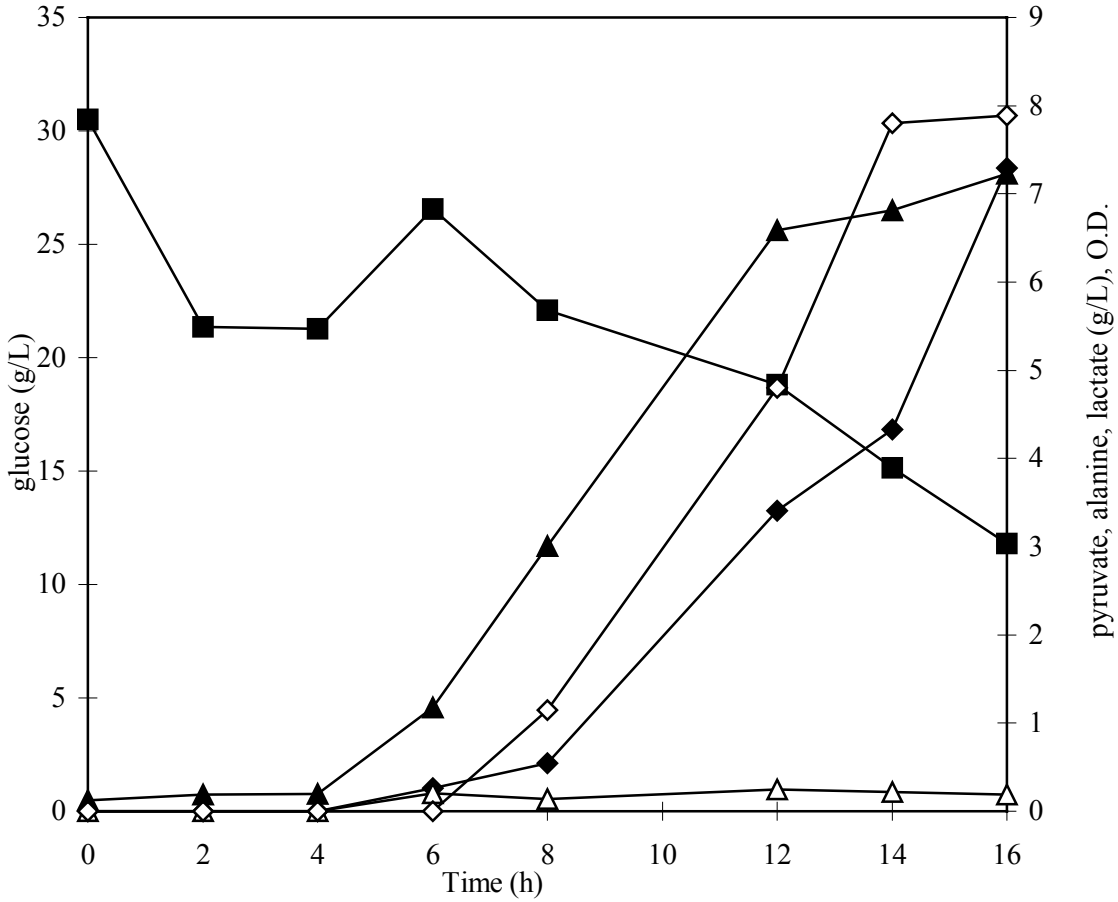

35
30
25
20
15
10
5
0
glucose (g/L)
9
8
7
6
5
4
3
2
1
0
pyruvate, alanine, lactate (g/L), O.D.
0
2
4
6
8
10
12
14
16
Time (h)

Figure 2.8.2. Example fermentation of *E. coli* ALS 890 at constant agitation of 350 rpm.

◆: D.O., □: succinate, ∗: acetate

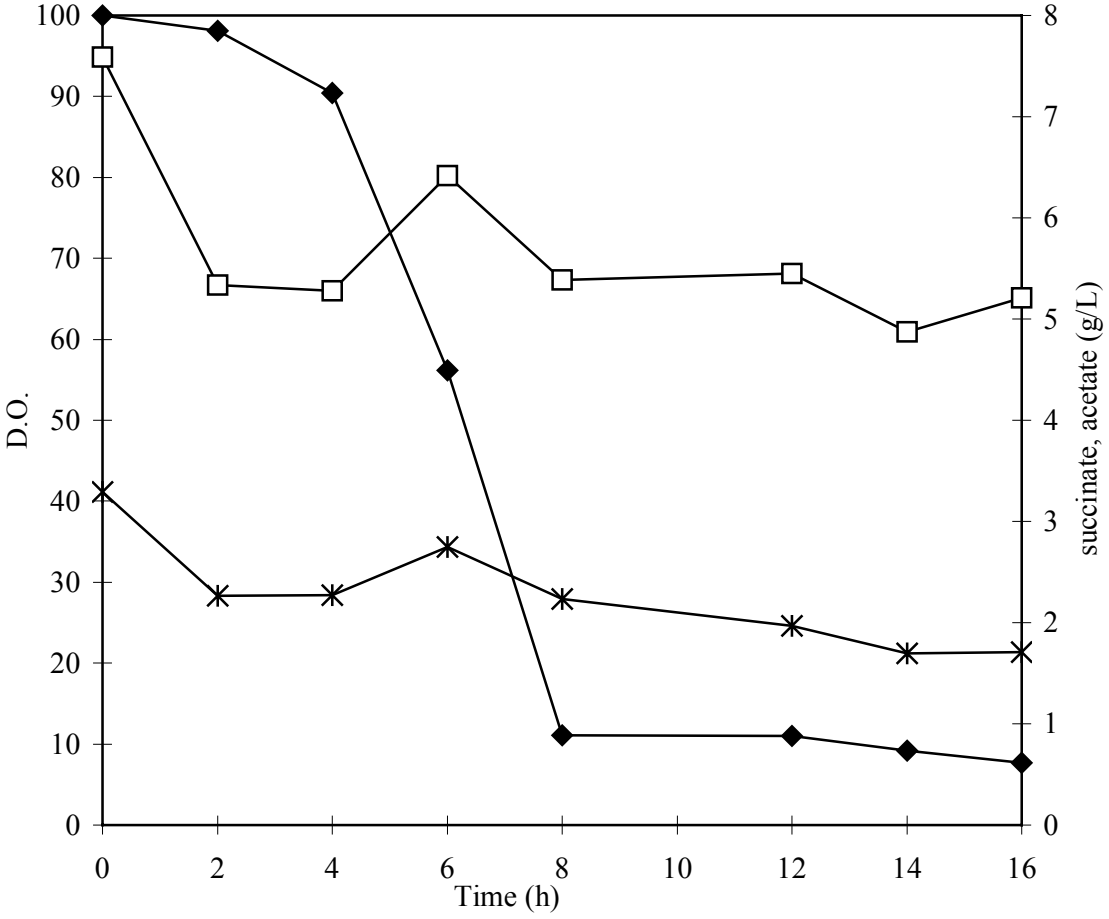

100
90
80
70
60
50
40
30
20
10
0
D.O.
8
7
6
5
4
3
2
1
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
16
Time (h)

Table 2.2. Mass yields of products during aerobic production of alanine in *E. coli* at two different continuous agitation speeds.

Agitation Speed (rpm)	Mass Yields (g product/g glucose)			% of glucose carbon in these 3 products
	Pyruvate	Lactate	Alanine	
250	0.28	0.02	0.78	97.4
350	0.39	0.01	0.55	91.7

Figure 2.9.1. Example fermentation of *E. coli* ALS 890 at constant agitation of 250 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

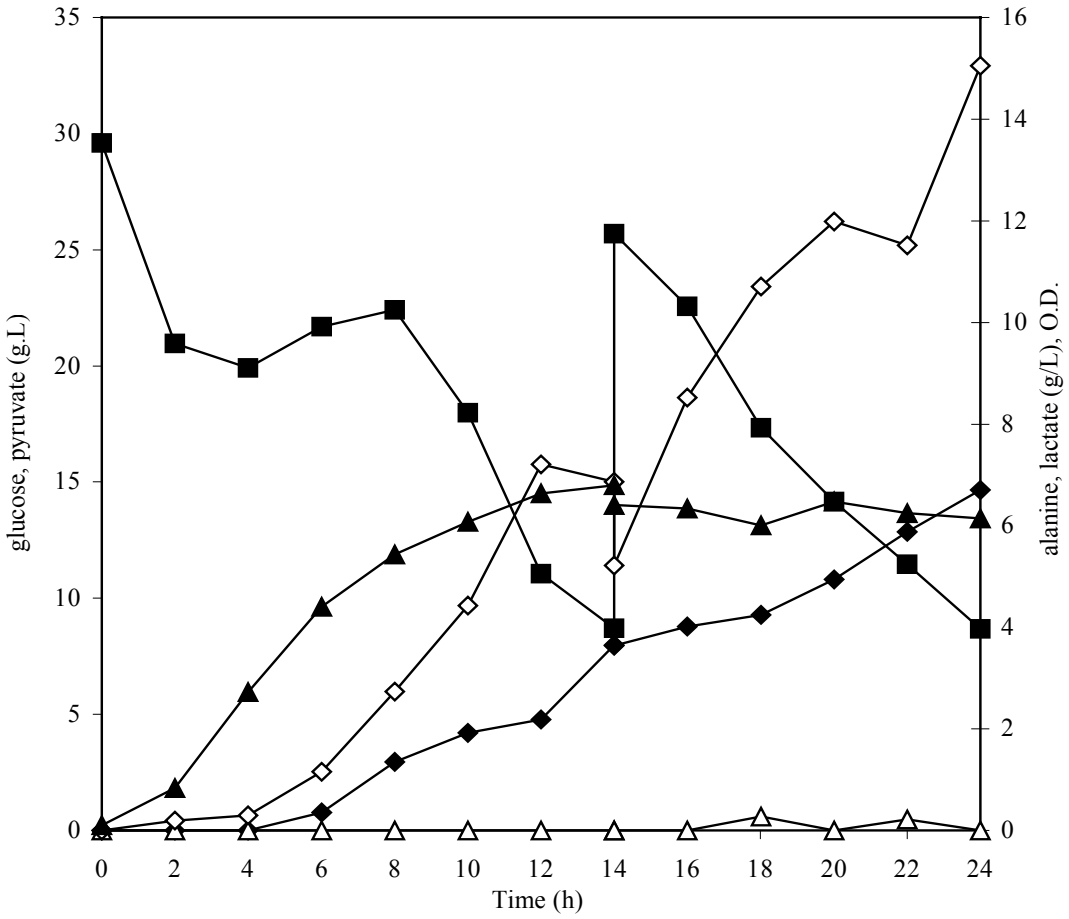
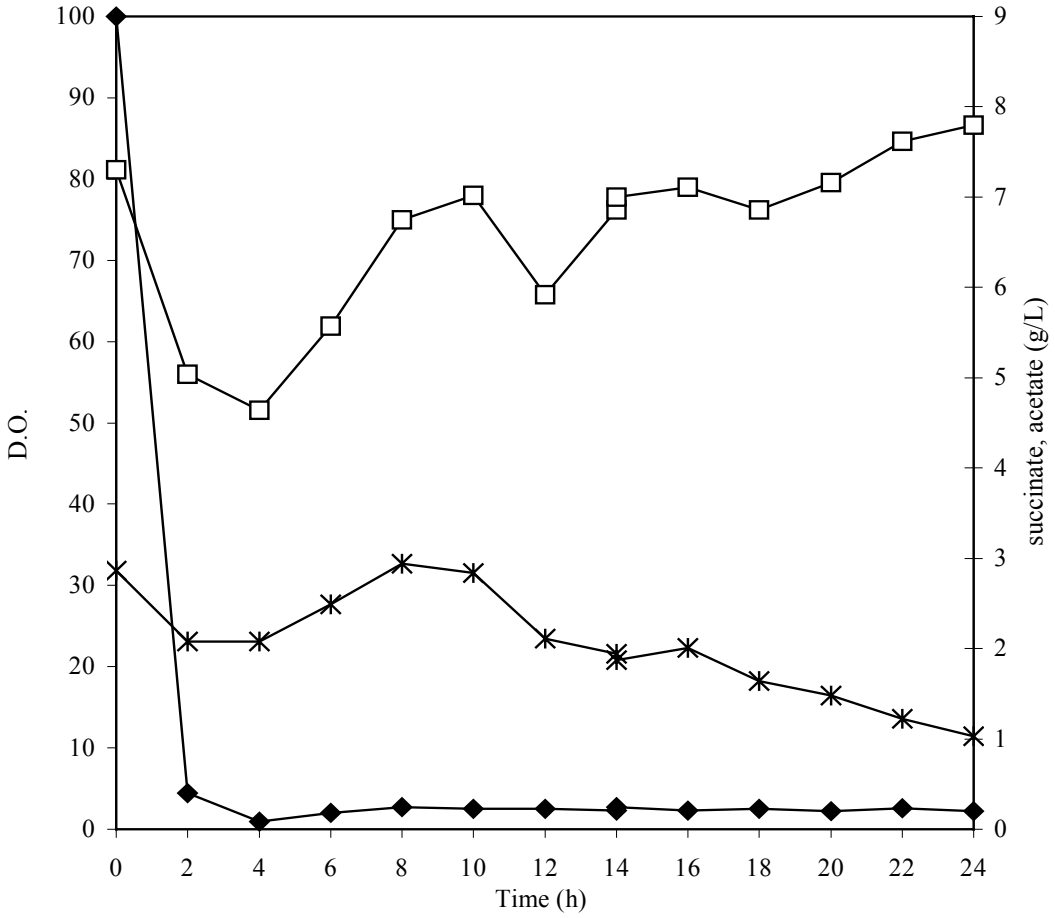

glucose, pyruvate (g.L)
alanine, lactate (g/L), O.D.
Time (h)
0
5
10
15
20
25
30
35
0
2
4
6
8
10
12
14
16
0
2
4
6
8
10
12
14
16
0
2
4
6
8
10
12
14
16
18
20
22
24

Figure 2.9.2. Example fermentation of *E. coli* ALS 890 at constant agitation of 250 rpm.

◆: D.O., □: succinate, ∗: acetate


D.O.
succinate, acetate (g/L)
Time (h)
100
90
80
70
60
50
40
30
20
10
0
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
18
20
22
24

of glucose was 8.70 g/L. Glucose concentration at 24 hours was 8.7 g/L. The optical density at 14 h was 6.8, while at 24 h it was 6.1.

At a continuous agitation of 350 rpm, the rate of glucose consumption was 1.72 g/Lh with an alanine productivity of 0.51 g/Lh and mass yield of 0.30 (Figure 2.10). Pyruvate accumulated at 0.86 g/Lh with a yield of 0.50. Glucose concentration at 14 hours before addition of glucose was 3.39 g/L. Glucose concentration at 24 h was 1.0 g/L. The optical density at 14 h was 8.9, while at 24 h it was 7.3.

Products yields in aerobic production of alanine in *E. coli* are summarized in Table 2.3. For both agitation rates, less than 80% of the glucose carbon could be accounted in the 3 principal fermentation products.

Fed batch fermentation using ALS 890 with shift in agitation. Results obtained to this point demonstrate that high oxygenation leads to greatest cell mass while low oxygenation leads to greatest alanine production. Thus, an improved approach to generate alanine would involve an initial high oxygenation growth phase followed by a low oxygenation production phase. For these experiments, 1000 rpm was used initially, then 150 rpm, 250 rpm, or 500 rpm was used in a "non-growth" production phase (Figure 2.11). 30 g glucose and 7.5 g NH_4Cl were added at 15 h. The maximum alanine concentrations observed in these 3 fermentation were: 13.2 g/L (150 rpm), 12.0 g/L (250 rpm), and 3.9 g/L (500 rpm).

At an agitation of 150 rpm during the production phase, the rate of glucose consumption was 1.65 g/Lh with an alanine productivity of 0.53 g/Lh and mass yield of 0.32 (Figure 2.12). Pyruvate accumulated at 0.38 g/Lh with a yield of 0.23. The optical density at 15 h was 12.1 and at 25 h was 10.5.

At an agitation of 250 rpm, the rate of glucose consumption was 1.66 g/Lh with an alanine productivity of 0.46 g/Lh and mass yield of 0.28 (Figure 2.13). Pyruvate accumulated at 0.58 g/Lh with a yield of 0.35. The optical density at 15 h was 12.8 and at 25 h was 10.2.

At an agitation of 500 rpm, the rate of glucose consumption was 2.09 g/Lh with an

Figure 2.10.1. Example fermentation of *E. coli* ALS 890 at constant agitation of 350 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D., +: NH_4Cl

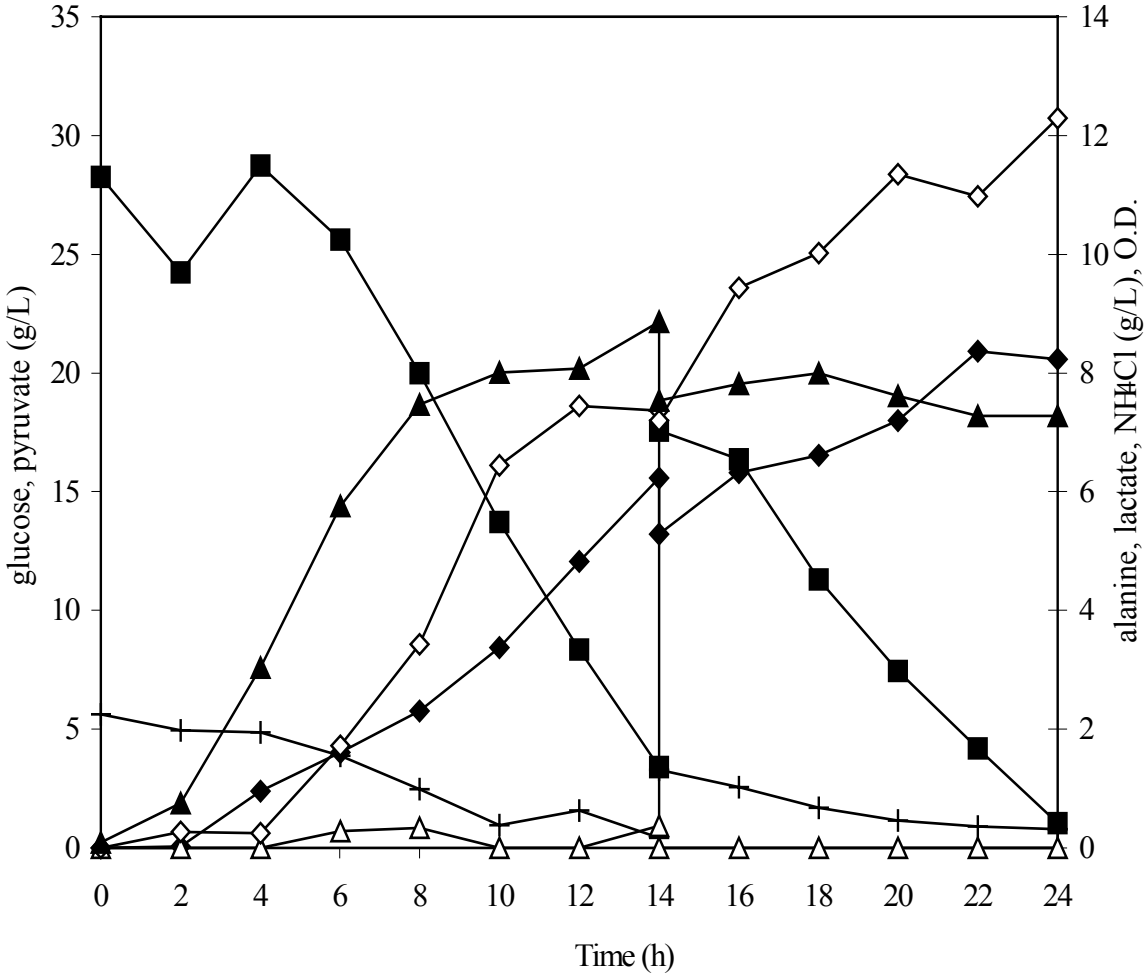

35
30
25
20
15
10
5
0
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
22
24
glucose, pyruvate (g/L)
alanine, lactate, NH4Cl (g/L), O.D.
Time (h)

Figure 2.10.2. Example fermentation of *E. coli* ALS 890 at constant agitation of 350 rpm.

◆: D.O., □: succinate, ∗: acetate

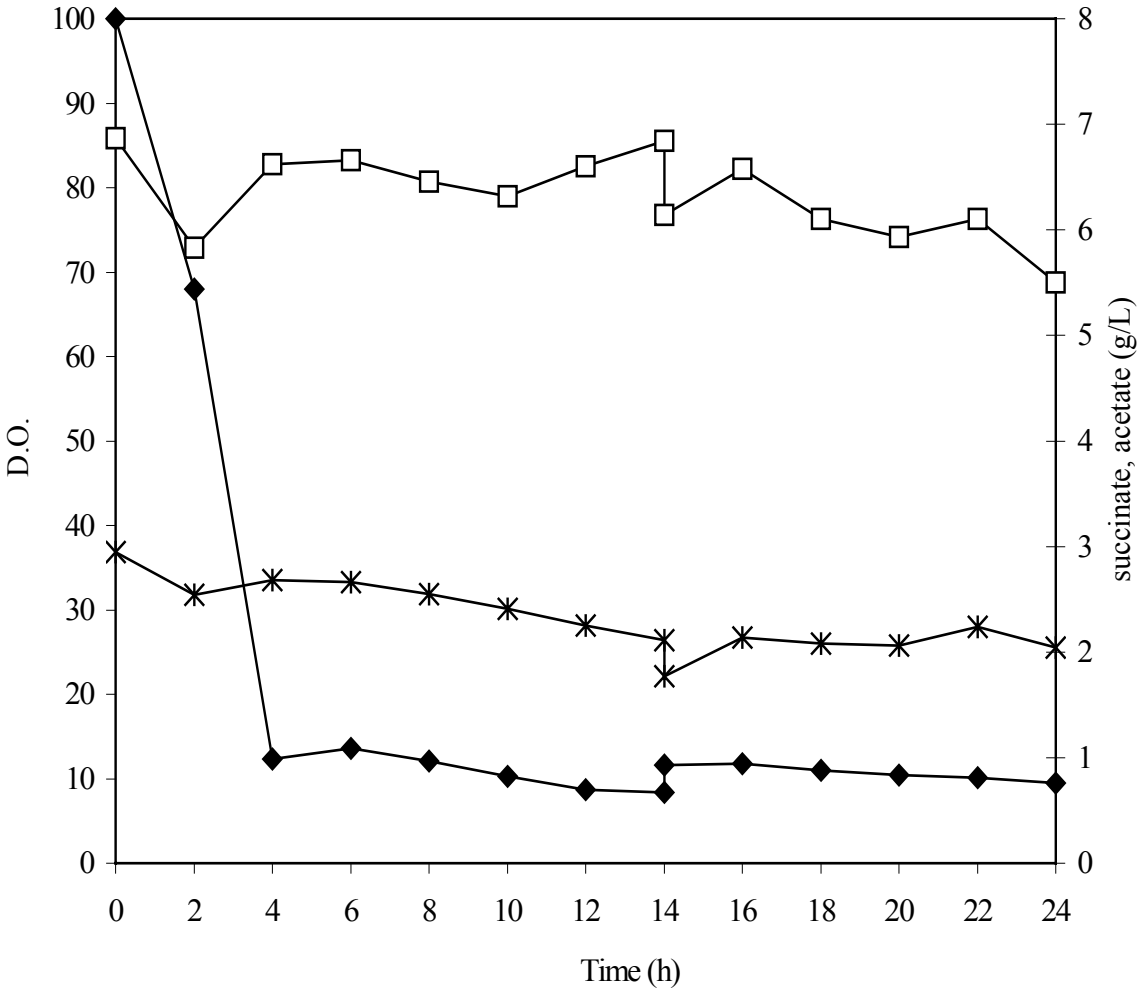

100
90
80
70
60
50
40
30
20
10
0
D.O.
8
7
6
5
4
3
2
1
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)

Table 2.3. Mass yields of products during aerobic production of alanine in *E. coli* at two different continuous agitation speeds.

Agitation Speed (rpm)	Mass Yields (g product/g glucose)			% of glucose carbon in these 3 products
	Pyruvate	Lactate	Alanine	
250	0.37	0	0.39	77.8
350	0.43	0	0.31	76.0

Figure 2.11. Fed-batch fermentation of ALS 890 with shift in agitation.

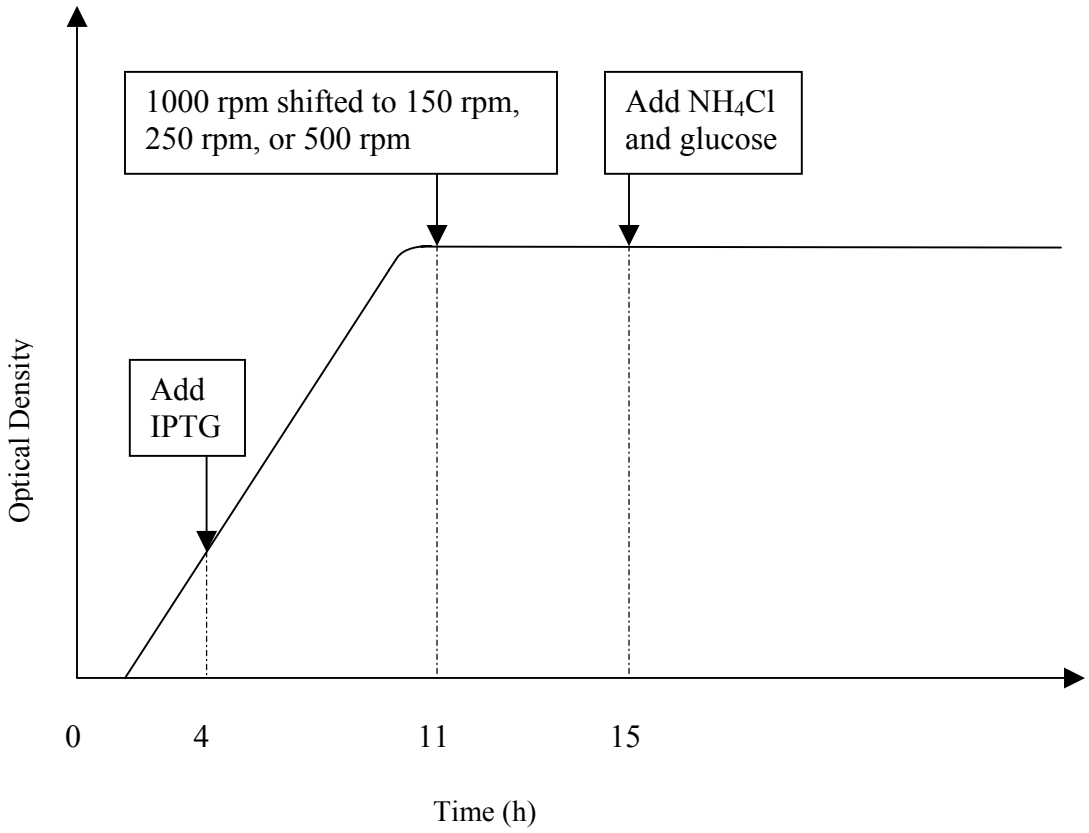

1000 rpm shifted to 150 rpm,
250 rpm, or 500 rpm
Add NH_4Cl
and glucose
Add
IPTG
Optical Density
0
4
11
15
Time (h)

Figure 2.12.1. Example fermentation of *E. coli* ALS 890 shifted at 11 h from an agitation of 1000 rpm to 150 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

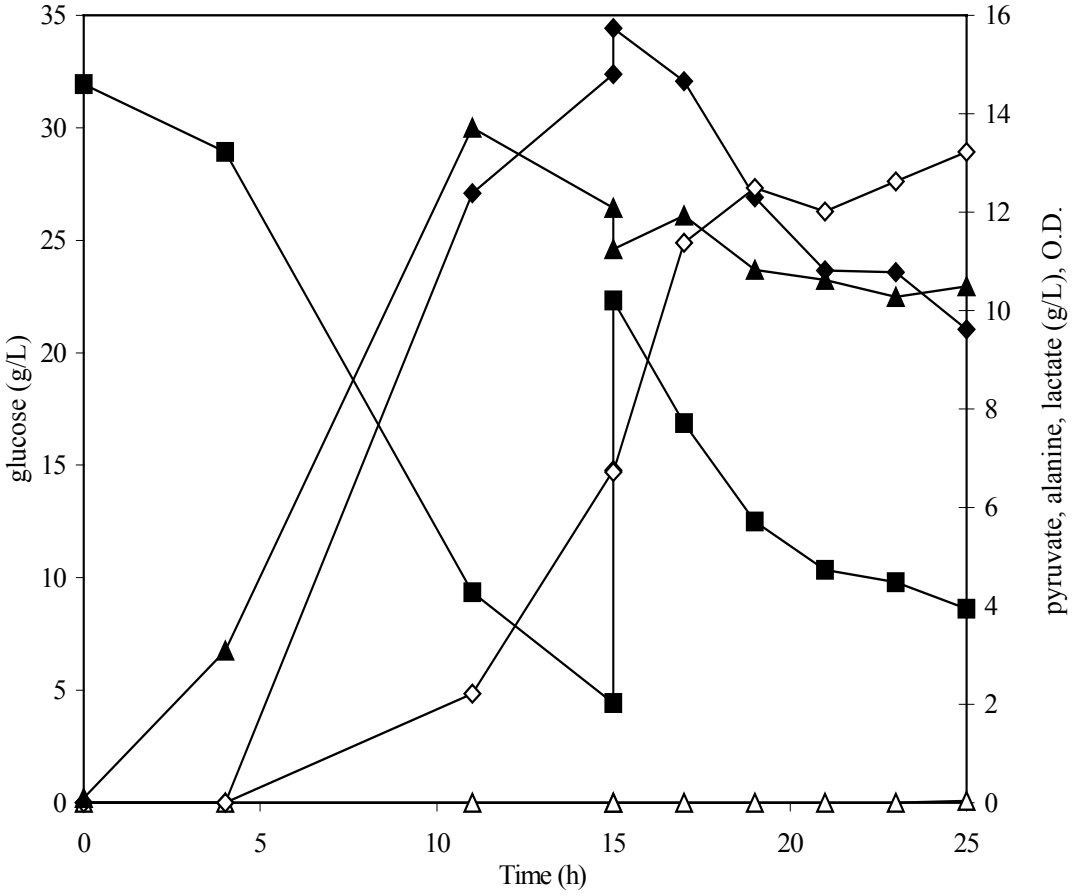

35
30
25
20
15
10
5
0
glucose (g/L)
16
14
12
10
8
6
4
2
0
pyruvate, alanine, lactate (g/L), O.D.
0
5
10
15
20
25
Time (h)

Figure 2.12.2. Example fermentation of *E. coli* ALS 890 shifted at 11 h from an agitation of 1000 rpm to 150 rpm.

◆: D.O., □: succinate, ∗: acetate

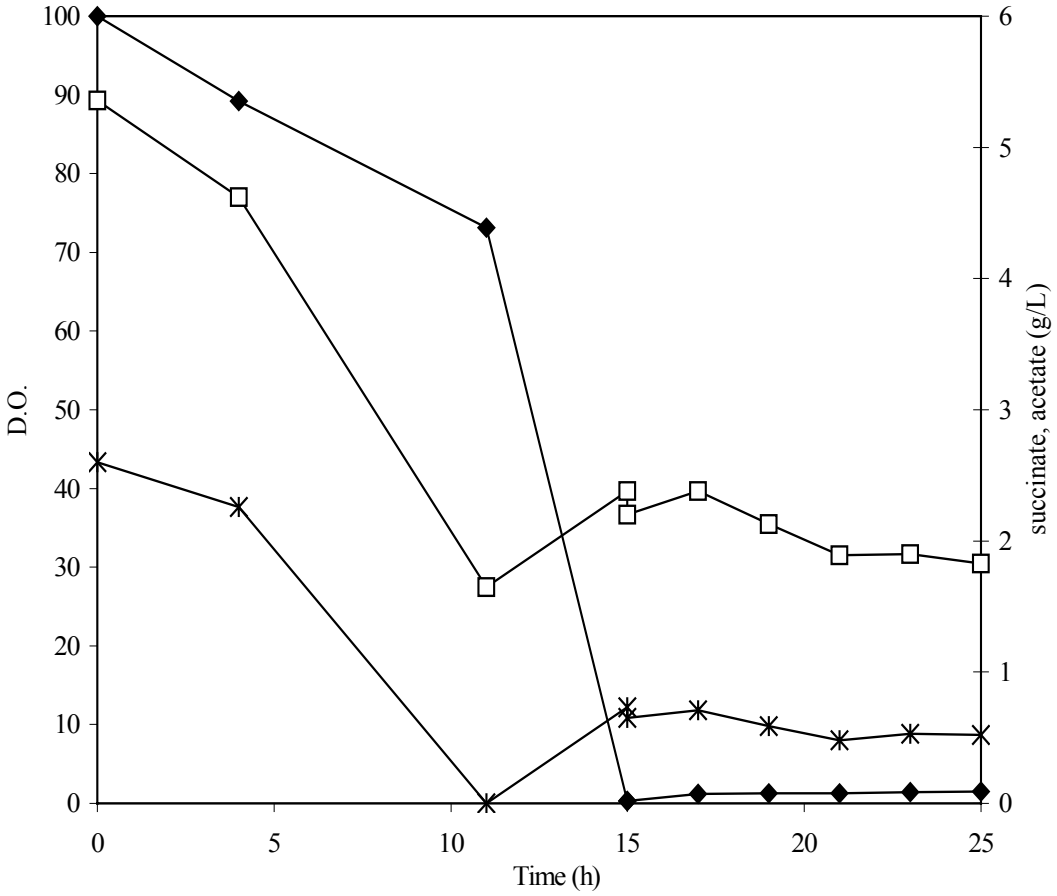

100
90
80
70
60
50
40
30
20
10
0
D.O.
6
5
4
3
2
1
0
succinate, acetate (g/L)
0
5
10
15
20
25
Time (h)

Figure 2.13.1. Example fermentation of *E. coli* ALS 890 shifted at 11 h from an agitation of 1000 rpm to 250 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

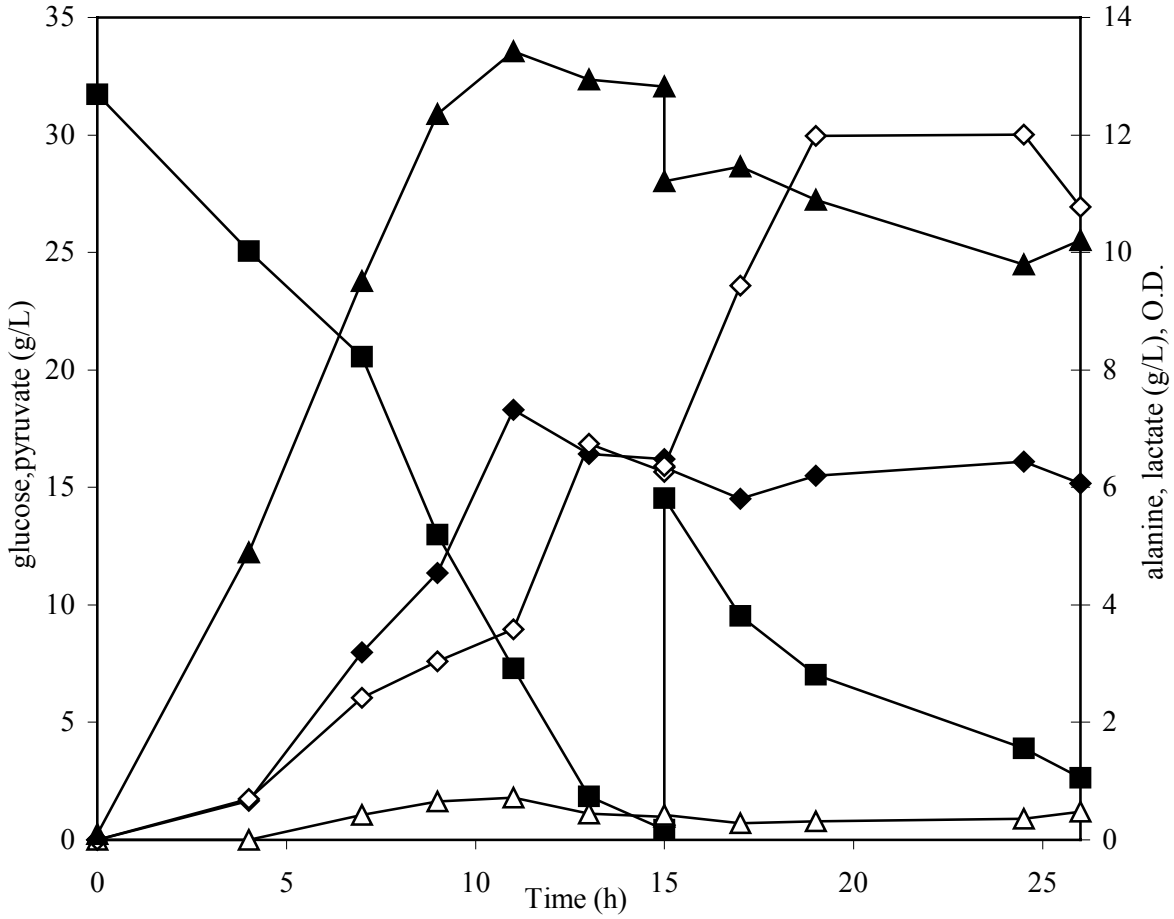
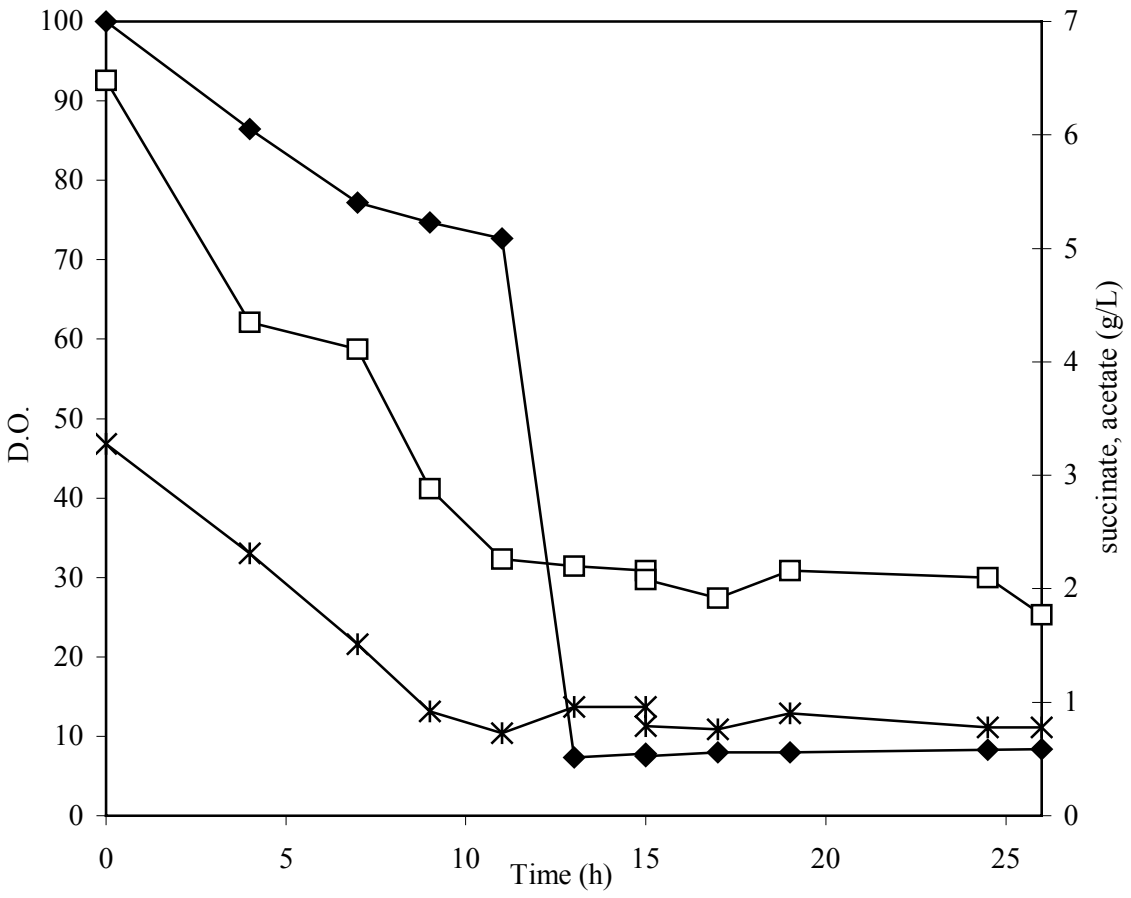

35
30
25
20
15
10
5
0
14
12
10
8
6
4
2
0
0
5
10
15
20
25
Time (h)
glucose,pyruvate (g/L)
alanine, lactate (g/L), O.D.

Figure 2.13.2. Example fermentation of *E. coli* ALS 890 shifted at 11 h from an agitation of 1000 rpm to 250 rpm.

◆: D.O., □: succinate, ∗: acetate


D.O.
succinate, acetate (g/L)
Time (h)
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
5
6
7
0
5
10
15
20
25

alanine productivity of 0.14 g/Lh and mass yield of 0.07 (Figure 2.14). Pyruvate accumulated at 0.93 g/Lh with a yield of 0.45. The optical density at 15 h was 14.4 and at 25 h was 15.1.

Products yields in the aerobic production of alanine in *E. coli* ALS 890 are summarized in Table 2.4. For these fermentations a large fraction of the glucose carbon could not be accounted in the 3 principal products.

DISCUSSION

In this study we first demonstrated that NH_4Cl does not affect cell growth rate below a concentration of 8.0 g/L. Ammonium is a necessary cosubstrate in the enzymatic step of converting pyruvate to alanine.

We then compared the effect of the agitation rate on alanine production by PRS 181. Each of these fermentations was conducted with an air flow rate of 1.0 L/min and with a single continuous agitation rate for the duration of each experiment. Pyruvate is the key intermediate in the production of alanine, and pyruvate accumulated at all four agitation rates. With decreasing rotation speed and presumably greater oxygen limitation, pyruvate mass yield decreased from about 0.67 to 0.03 (Table 2.1). Concomitant with this decrease in pyruvate yield was an increase

in lactate and alanine yield. A threshold agitation rate appeared to occur between 500 rpm and 350 rpm, in which the products switched from predominantly pyruvate to predominantly alanine and lactate. Lactate dehydrogenase has been shown to be rapidly and irreversibly inactivated by oxygen (Futai, 1977). Lactate production in this study correlated with the decrease of agitation speed, which is consistent with these previous results. Alanine production generally paralleled lactate production. However, the cells were able to generate more alanine than lactate at high agitation (oxygenation), probably because alanine dehydrogenase expression was greater under these conditions than lactate dehydrogenase. At low agitation (oxygenation) lactate generation was greater than alanine generation. Of course, both enzymes use NADH as a cofactor.

Agitation speed changes also influenced the *E. coli* cell growth. The optical density decreased at 14 h from 12.25 (750 rpm) to 6.14 (250 rpm).

Figure 2.14.1. Example fermentation of *E. coli* ALS 890 shifted at 11 h from an agitation of 1000 rpm to 500 rpm.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

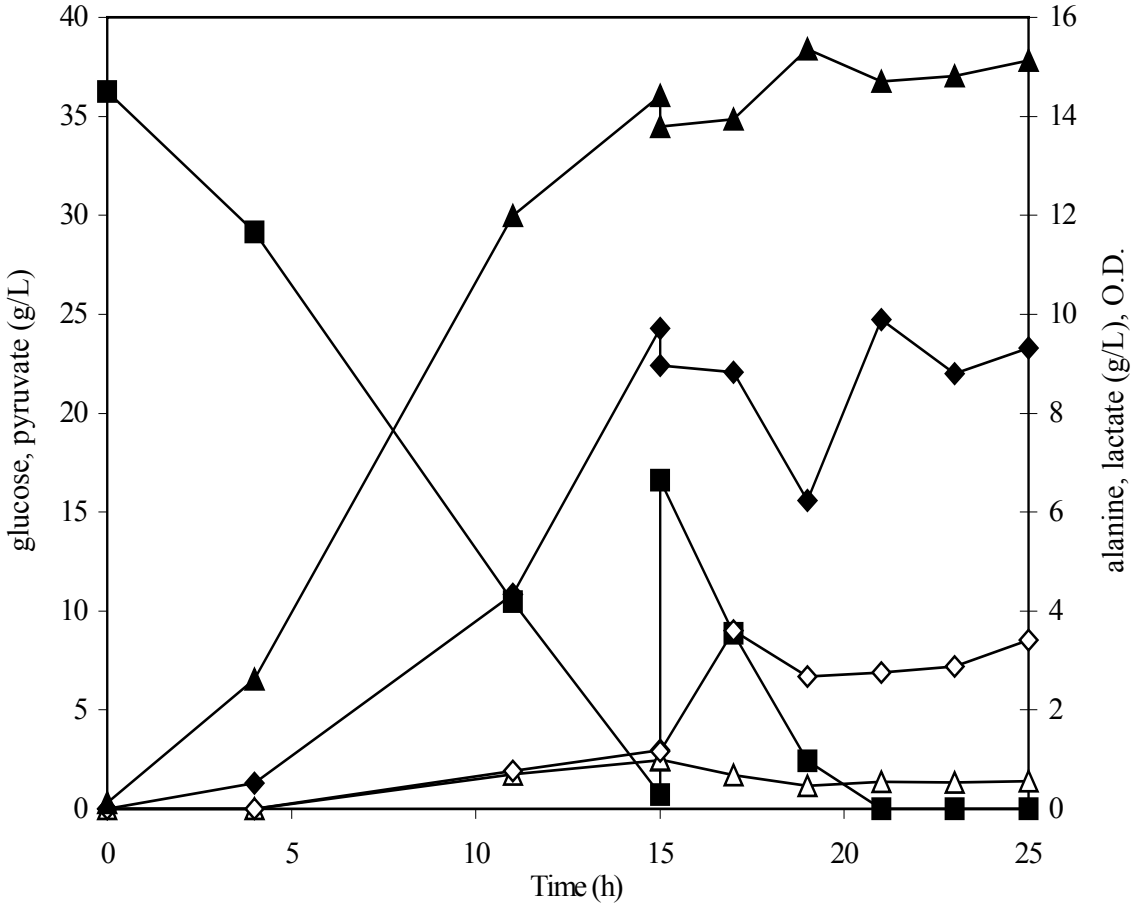

glucose, pyruvate (g/L)
alanine, lactate (g/L), O.D.
Time (h)
0
5
10
15
20
25
0
5
10
15
20
25
30
35
40
0
2
4
6
8
10
12
14
16

Figure 2.14.2. Example fermentation of *E. coli* ALS 890 shifted at 11 h from an agitation of 1000 rpm to 500 rpm.

◆: D.O., □: succinate, ∗: acetate

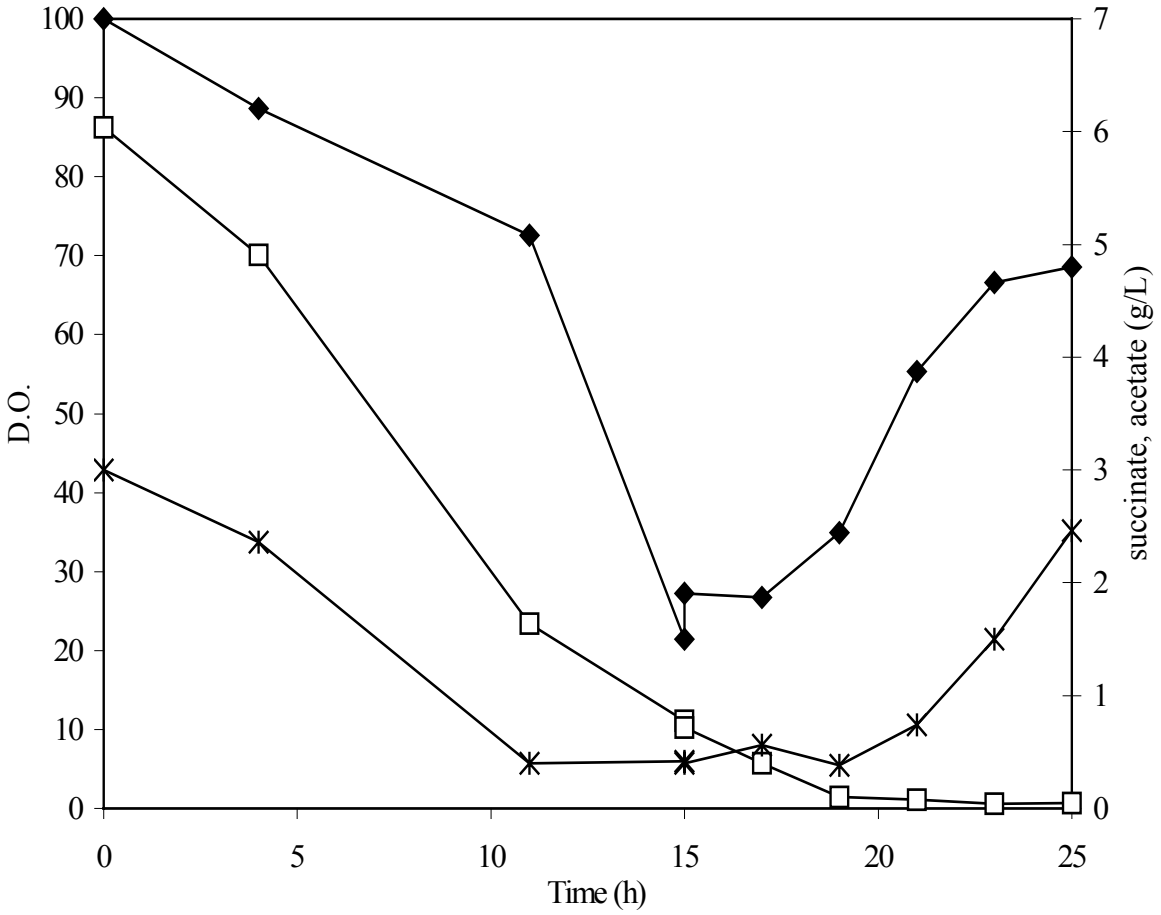

100
90
80
70
60
50
40
30
20
10
0
D.O.
7
6
5
4
3
2
1
0
succinate, acetate (g/L)
0
5
10
15
20
25
Time (h)

Table 2.4. Mass yields of products during aerobic production of alanine in *E. coli* at three different agitation speed combinations.

Non-growth Agitation (rpm)	Mass Yields (g product/g glucose)			% of glucose carbon in these 3 products
	Pyruvate	Lactate	Alanine	
150	0.31	0.00	0.36	68.0
250	0.36	0.02	0.42	81.3
500	0.34	0.01	0.07	42.0

ALS 890 is a lactate dehydrogenase mutant, and the effect of the agitation rate on this strain was next studied. Each of these fermentations also was conducted with an air flowrate of 1.0 L/min and with a single continuous agitation rate for the duration of each experiment. Pyruvate again accumulated at the two agitation rates. There was not much difference in the results of 250 rpm and 350 rpm. The optical density in 350 rpm is a little higher than that in 250 rpm.

Fed batch was also used to increase alanine production. Mass yields of pyruvate was increasing while mass yields of alanine decreased. Percent of glucose carbon in 3 products (pyruvate, lactate, and alanine) decreased from about 95% to 75% (Table 2.2, Table 2.3). Since cell growth was already in stationary phase, glucose and NH_4Cl were mainly used for non-growth metabolism. Glucose was used more quickly in 350 rpm than in 250 rpm. Cell grew more quickly in 350 rpm than in 250 rpm.

Fed batch with a shift in agitation rate was also used to increase alanine production. In 250 rpm, there was not much difference between fed batch and fed batch with shift in agitation (Table 2.3, Table 2.4). The maxmium alanine production in 150 rpm and 250 rpm didn't change much, while the alanine production in 500 rpm was much lower (3.92 g/L). Cells grew more quickly in 500 rpm than 150 rpm. Alanine mass yield in 500 rpm was only 0.07. Only 42 % of glucose carbon was in the 3 products: pyruvate, lactate, and alanine (Table 2.4). The probable reason is that more glucose was used in the TCA cycle to let cells grew in 500 rpm.

Reference:

1. Bunch, K. Pamela, F. Mat-Jan, N. Lee, D. P. Clark. 1997. The *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. Microbiol. 143:187-195.
2. Chibata, I., T. Kakimoto, J. Kato. 1969. U.S. patent 3,458,400. Process for producing L-alanine.
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CHAPTER 3

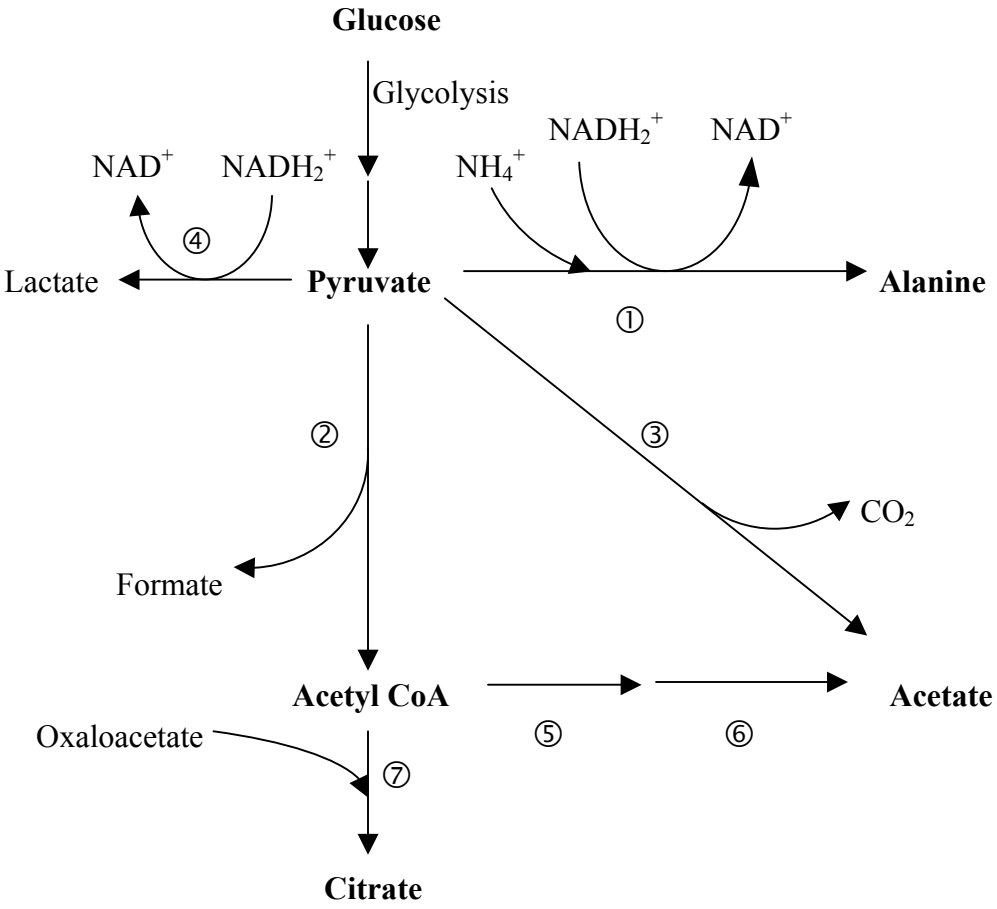
ANAEROBIC PRODUCTION OF ALANINE BY *ESCHERICHIA COLI*

INTRODUCTION

Alanine ($C_3H_7NO_2$) is the smallest chiral amino acid. It is released by muscle, functioning as a major energy source and an inhibitory neurotransmitter in the brain (Katsumata and Hashimoto, 1996). Commercially, alanine is used as a food additive (Katsumata and Hashimoto, 1996). An enzymatic or fermentation process which can generate optically active L- or D- alanine may be economically advantageous in comparison with chemical syntheses that require chiral separation. Presently, L-alanine is produced from L-aspartate via the enzyme L-aspartic-β-decarboxylase (Chibata et al., 1969). Several aerobic processes have been studied to generate alanine. For example, Katsumata and Hashimoto (1996) described L-alanine generation of 75.6 g/L in 120 hours using the alanine racemase deficient mutant *Arthrobacter oxydans* DAN 75. *Escherichia coli* produced 2.9 g/L L-alanine (Katsumata and Kashimoto, 1998). Few anaerobic processes have been studied to generate alanine. 7.5 g/L of L-alanine anaerobically produced by *Zymomonas* into which the gene coding for *Bacillus* L-alanine dehydrogenase was introduced (Uhlenbusch et al., 1991).

The key anaerobic metabolic steps in *Escherichia coli* influencing alanine synthesis are shown in Figure 3.1. Glucose is metabolized to pyruvate through glycolysis. A common approach in the production of alanine by any organism has been to overexpress alanine dehydrogenase, a single enzymatic step converting pyruvate to alanine. However, several enzymes compete with alanine dehydrogenase for the substrate pyruvate. Pyruvate formate lyase

Figure 3.1. *E. coli* anaerobic metabolic pathways involved in alanine synthesis.



① Alanine dehydrogenase
② Pyruvate formate lyase
③ Pyruvate oxidase
④ Lactate dehydrogenase
⑤ Phosphate acetyltransferase
⑥ Acetate kinase
⑦ Citrate synthase

encoded by the *pfl* gene is the primary route of pyruvate assimilation under anaerobic growth. Lactate dehydrogenase encoded by the *ldhA* gene directly competes with alanine dehydrogenase because both enzymes use $NADH_2^+$ as a cosubstrate. Recently, a *ldhA pfl* double mutant of *E. coli* (AFP111) has been used to generate succinate (Stols et al., 1997; Vemuri et al., 2002; Vemuri et al., 2002). This strain requires carbon dioxide to generate succinate via PEP carboxylase. Furthermore, *E. coli* AFP111 grows very poorly under anaerobic conditions. However, this double mutant can be grown under aerobic conditions to generate biomass followed by an anaerobic phase in which cells do not grow (Nghiem et al., 1999). *E. coli* AFP111 overexpressing alanine dehydrogenase may be a suitable means to generate alanine during this secondary anaerobic phase because it lacks activity in both of the key competing enzymes, lactate dehydrogenase and pyruvate formate lyase.

The objective of this research was to study anaerobic alanine production in *E. coli* AFP111 overexpressing alanine dehydrogenase.

MATERIALS AND METHODS

Construction of pTrc99A-*alaD*. The *Bacillus sphaericus* alanine dehydrogenase gene (*alaD*) was amplified using the polymerase chain reaction (PCR). *Pfu* DNA polymerase was used instead of *Taq* DNA polymerase and the pBm2O*alaD* plasmid served as the DNA template. Primers were designed based on the published *B. sphaericus alaD* gene sequence (Kuroda et al., 1990) and contained a *Bam*H I (GGATCC) restriction site and Shine-Dalgarno sequence at the beginning of the amplified fragment and a *Hin*d III (AAGCTT) restriction site at the end of the amplified fragment; forward primer 5'' TAC TAT GGA TCC AGG AGG AAC AGC TAT GAA GAT TGG TAT TCC AAA GGA AAT TAA AAA C 3''; reverse primer 5'' ATA GCG ATC GAT AGC GGT AAG CTT ATT ATT GGA TTA ATT CAT CCA CAT TCA CAT ATG 3'' (the *Bam*H I, Shine-Dalgarno, ATG start, and *Hin*d III sites are underlined). The resulting 1.2 kb PCR product was gel isolated, restricted with *Bam*H I and *Hin*d III and ligated into the pTrc99A expression vector which had been restricted with the same two enzymes.

Strains and Plasmids. *E. coli* APF111 ($F^+\lambda^-$ *rpoS*396(Am) *rph*-1 Δ*pflAB*::Cam *ldhA*::Kan *ptsG*) was the parent strain used in this study. The organism used to study the production of alanine was designated PRS178 (AFP111 pTrc99A-*ald*).

Media and growth conditions. Cells were first grown in an agitated 20 mL screw top test tube composed of (pH=7.0): 2.5 g/L tryptone, 2.5 g/L NaCl, 1.25 g/L yeast extract, 15.0 g/L glucose. After 3h of growth 10 mL cells were used to inoculate a 100 mL volume in a 250 mL baffled shake flask composed of (pH 7.0): 15.0 g/L glucose, 10.0 g/L tryptone, 2.5 g/L yeast extract, 0.3 g/L KH_2PO_4, 0.6 g/L Na_2HPO_4, 6.0 g/L NH_4Cl, 0.14 g/L $CaCl_2{\cdot}2H_2O$ and 0.25 g/L $MgSO_4{\cdot}7H_2O$. Cells were grown at 250 rpm agitation (19mm radius of orbit) for 6h and then used to inoculate a fermenter of the same composition except 40.0 g/L glucose. Fermentations of 1.5 L volume were conducted using a BioFlow 2000 fermenter (New Brunswick Scientific Company, New Brunswick, N.J.). Air was supplied initially at 1.0 L/min. The pH was controlled at 7.0 using 20% NaOH and 20% H_2SO_4. After 4.0 h of growth, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. After 6.0 h of growth, air was replaced by nitrogen at 0.2 L/min with 250 rpm agitation and the pH controlled at either 7.0, 7.5 or 8.0. After 14.0 h of growth, 30 g glucose and 7.5 g NH_4Cl (in 110 mL water) were added. All media were grown at 37 °C and supplemented with ampicillin at 100 mg/L.

Analyses. Cell growth was monitored by measuring the optical density (OD) at 550 nm (DU-650 UV-VIS spectrophotometer, Beckman Instruments, San Jose, CA). Samples were centrifuged (10,000×g for 10 minutes at 25 °C), and the supernatant analyzed for glucose, and organic acids by high pressure liquid chromatography (HPLC) using a previous method (Eiteman and Chastain, 1997). Alanine was analyzed by HPLC using an Aminex HPX-87C Carbohydrate column with 0.1 M $Ca(NO_3)_2$ effluent and a refractive index detector (Waters 2410, Millipore Corp., Milford, M.I.).

Enzyme assays. Cell-free extracts were prepared by washing the cell pellet at 4 °C twice with 10 mM potassium phosphate buffer (pH 7.0). B-PER II Reagent (Pierce, Rockford, IL) was used to lyse the cells, cell debris were removed by centrifugation (20,000×g for 20 minutes at 4 °C) and the cell-free extract used for measuring alanine dehydrogenase activity. One unit of enzyme activity is the quantity of enzyme required to produce 1.0 μmole of pyruvate in one minute. Total protein in the cell-free extract was determined using bovine serum albumin as the standard (Lowry et al., 1951).

RESULTS

Effect of pH on alanine production. We compared the products formed during fermentations of *E. coli* PRS 178 with the pH during the production phase controlled either at 7.0, 7.5 or 8.0.

At a pH of 7.0 during the production phase, the rate of glucose consumption was 0.96 g/Lh with an alanine productivity of 0.35 g/Lh and mass yield of 0.36 (Figure 3.2). The optical density at 14 hours was 2.0.

At a pH of 7.5, the rate of glucose consumption was 0.59 g/Lh with an alanine productivity of 0.27 g/Lh and mass yield of 0.46 (Figure 3.3). The optical density of 14 h was 1.7.

At a pH of 8, the rate of glucose consumption was 0.94 g/Lh with an alanine productivity of 0.19 g/Lh and mass yield of 0.20 (Figure 3.4). The optical density of 14 h was 1.9.

DISCUSSION

In this study we compared the effect of pH on alanine production by PRS 178. Fed batch with shift in agitation and pH was used to increase alanine production. Alanine mass yield in pH 7.5 was the highest among 3 pH conditions. The probable reason is that pH change affected enzymes activity related to alanine production in the cells.

From the results obtained in this thesis, anaerobic alanine production was much lower than aerobic alanine production. However, the highest O.D. (about 3) in anaerobic production was much lower than the highest O.D. (about 14) in aerobic production. In consideration of this, anaerobic alanine production still have potential to gain more alanine if O.D. can be increased a lot. Higher O.D. is possible to reach if medium and other culture conditions are changed.

Figure 3.2.1. Example fermentation of *E. coli* PRS 178 shifted at 6 h.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

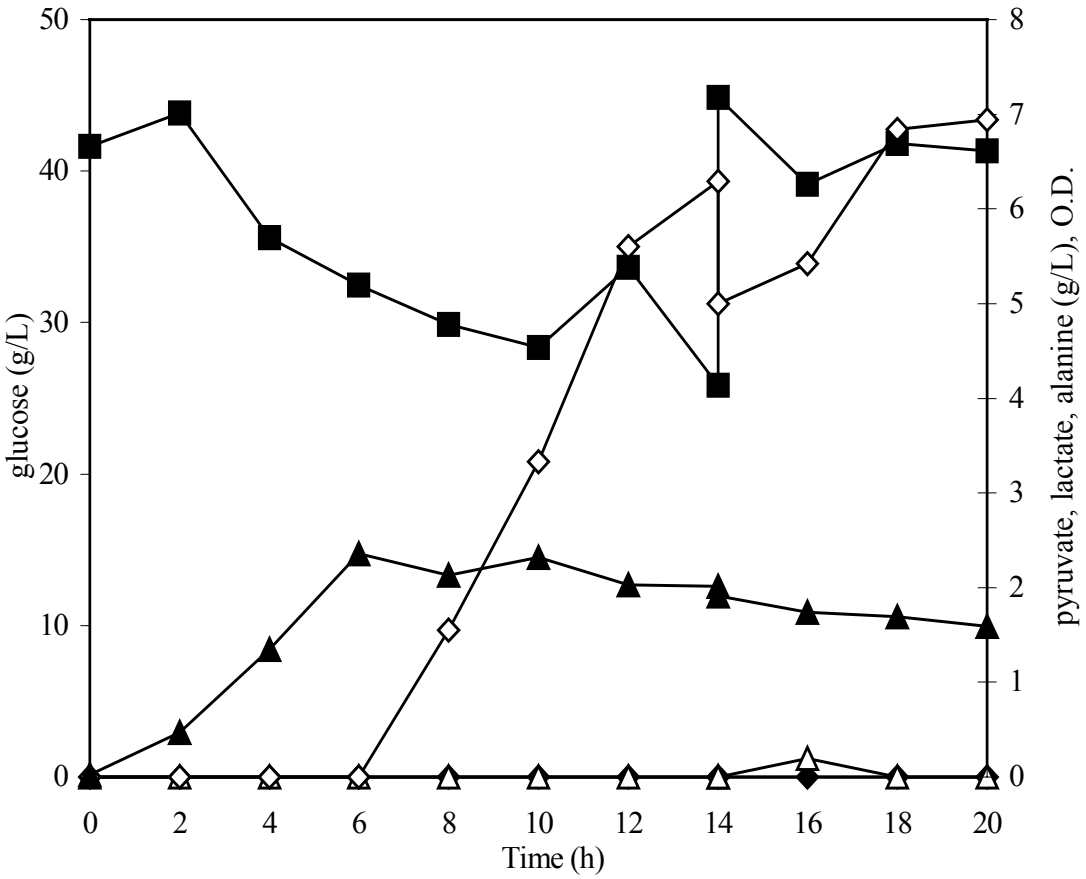

50
40
30
20
10
0
glucose (g/L)
8
7
6
5
4
3
2
1
0
pyruvate, lactate, alanine (g/L), O.D.
0
2
4
6
8
10
12
14
16
18
20
Time (h)

Figure 3.2.2. Example fermentation of *E. coli* PRS 178 shifted at 6 h.

◆: D.O., □: succinate, ∗: acetate

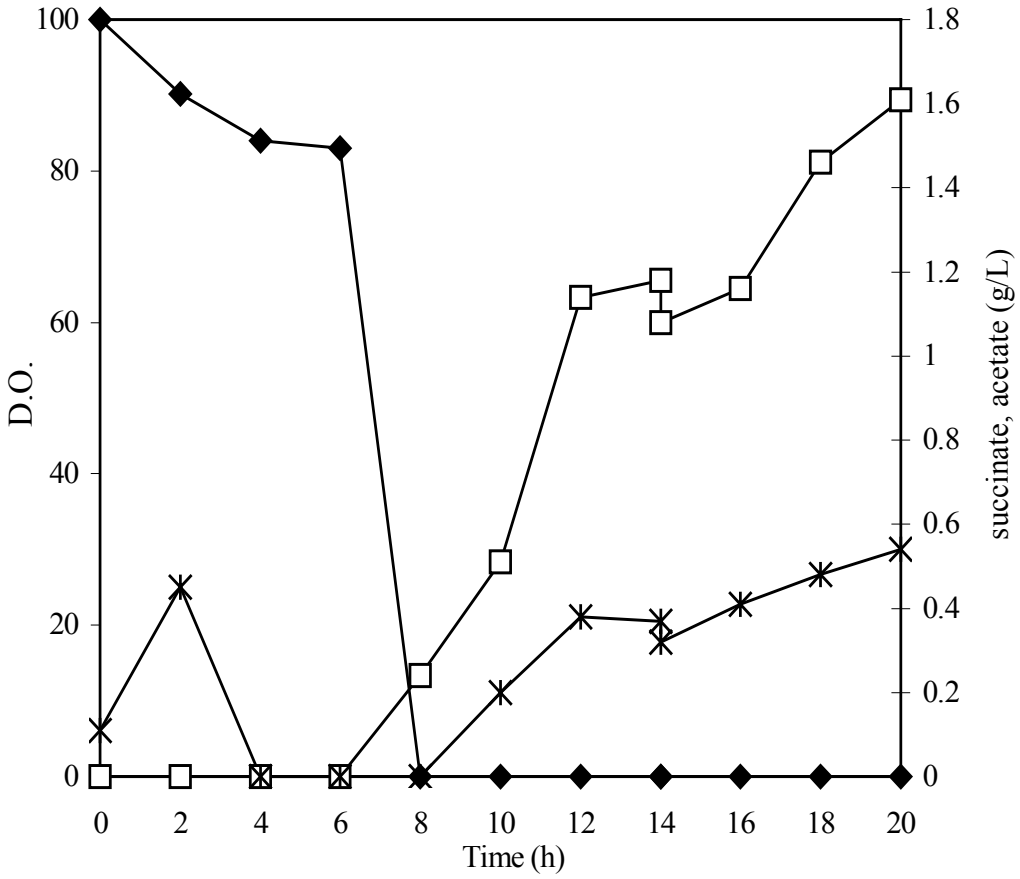

100
80
60
40
20
0
D.O.
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
16
18
20
Time (h)

Figure 3.3.1. Example fermentation of *E. coli* PRS 178 shifted at 6 h from 7.0 to 7.5.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

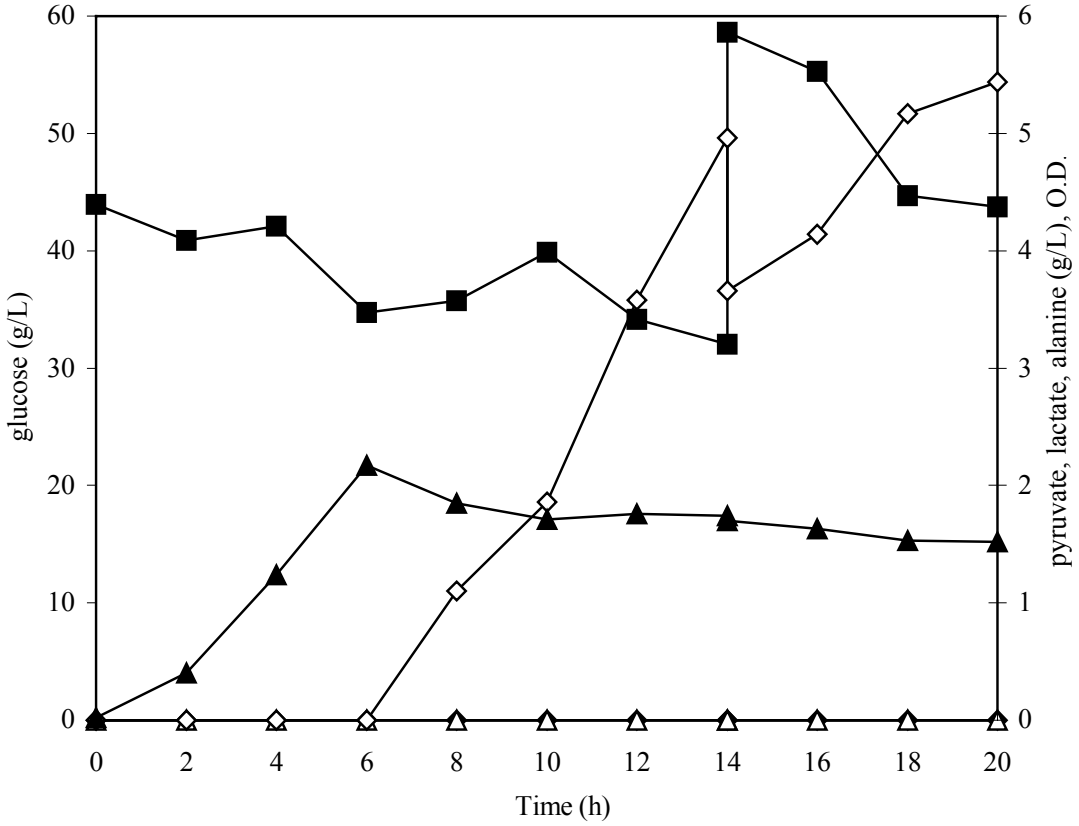

glucose (g/L)
pyruvate, lactate, alanine (g/L), O.D.
Time (h)
0
10
20
30
40
50
60
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14
16
18
20

Figure 3.3.2. Example fermentation of *E. coli* PRS 178 shifted at 6 h from 7.0 to 7.5.

◆: D.O. □: succinate, ∗: acetate

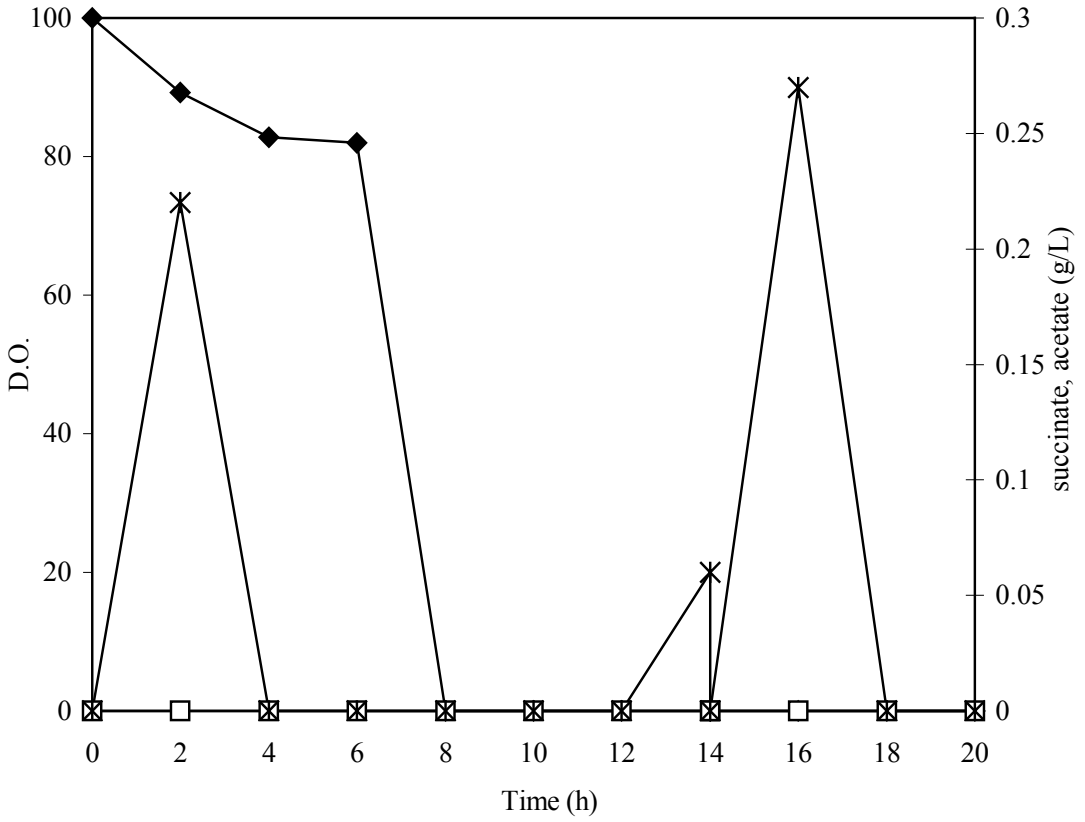

100
80
60
40
20
0
D.O.
0.3
0.25
0.2
0.15
0.1
0.05
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
16
18
20
Time (h)

Figure 3.4.1. Example fermentation of *E. coli* PRS 178 shifted at 6 h from 7.0 to 8.0.

■: glucose, ◆: pyruvate, Δ: lactate, ◇: alanine, ▲: O.D.

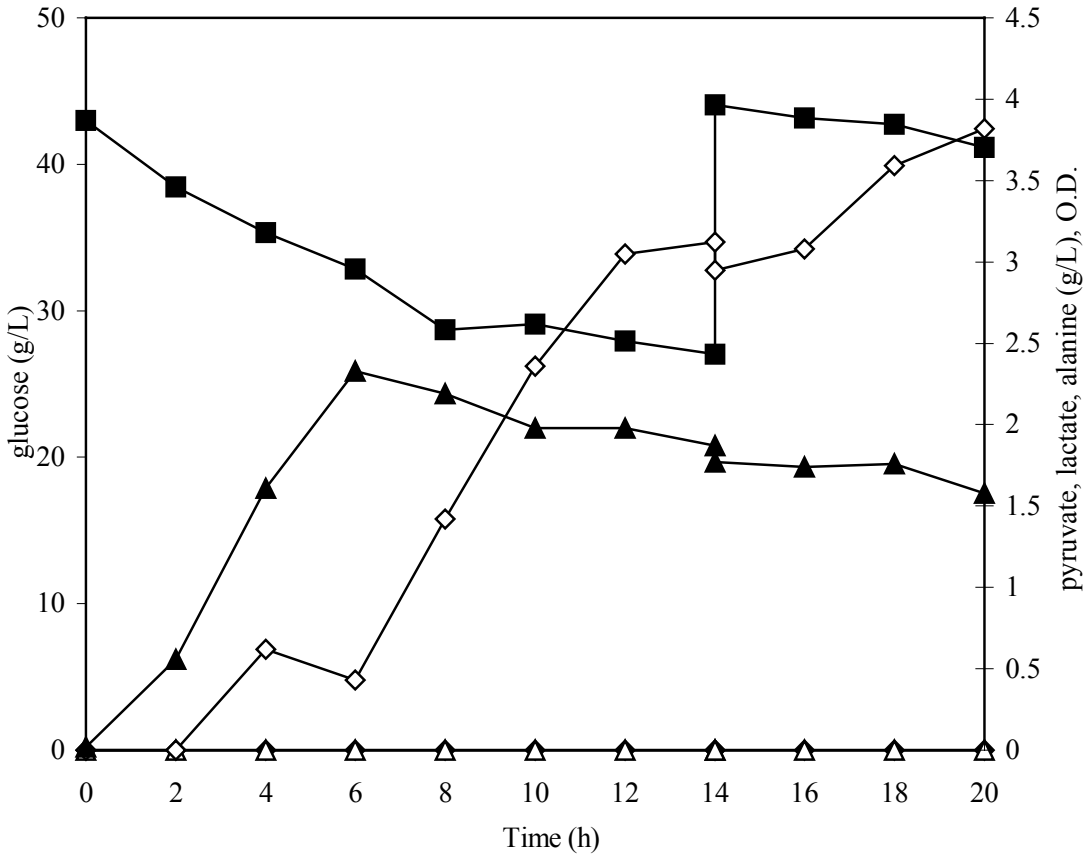
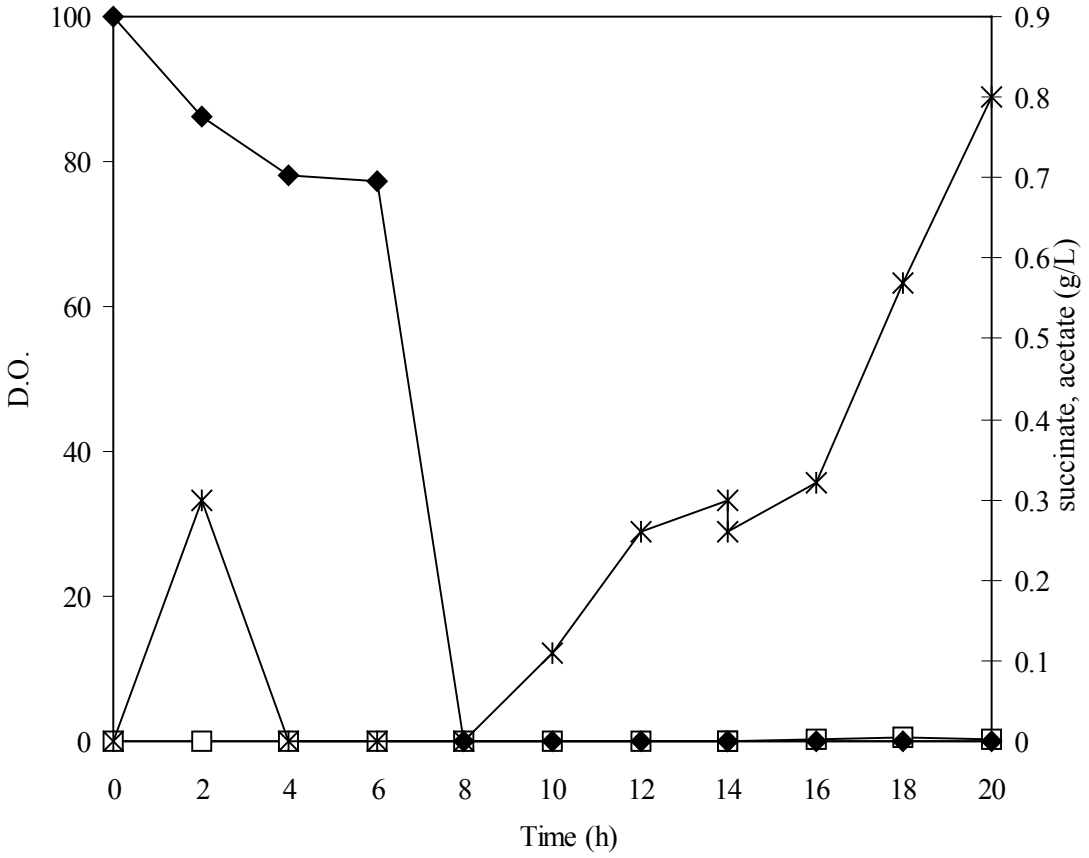

50
40
30
20
10
0
glucose (g/L)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
pyruvate, lactate, alanine (g/L), O.D.
0
2
4
6
8
10
12
14
16
18
20
Time (h)

Figure 3.4.2. Example fermentation of *E. coli* PRS 178 shifted at 6 h from 7.0 to 8.0.

◆: D.O., □: succinate, ∗: acetate


100
80
60
40
20
0
D.O.
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
succinate, acetate (g/L)
0
2
4
6
8
10
12
14
16
18
20
Time (h)

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