**Alanine production in an $H^+$-ATPase- and lactate dehydrogenase-defective mutant of *Escherichia coli* expressing alanine dehydrogenase**

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Key words: alanine production; *Escherichia coli*; $H^+$-ATPase; alanine dehydrogenase

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**Abstract**

Previously we reported that pyruvate production was markedly improved in TBLA-1, an $H^+$-ATPase-defective *Escherichia coli* mutant derived from W1485*lip2*, a pyruvate-producing *E. coli* K-12 strain. TBLA-1 produced more than 30 g/L pyruvate from 50 g/L glucose by jar-fermentation, while W1485*lip2* produced only 25 g/L pyruvate (Yokota et al. 1994b). In this study, we tested the ability of TBLA-1 to produce alanine by fermentation. The alanine dehydrogenase (ADH) gene from *Bacillus stearothermophilus* was introduced into TBLA-1, and direct fermentation of alanine from glucose was carried out. However, a considerable amount of lactate was also produced. In order to reduce lactate accumulation, we knocked out the lactate dehydrogenase gene (*ldhA*) in TBLA-1. This alanine dehydrogenase-expressing and lactate dehydrogenase-defective mutant of TBLA-1 produced 20 g/L alanine from 50 g/L glucose after 24 h of fermentation. The molar conversion ratio of glucose to alanine was 41%, which is the highest level of alanine production reported to date. This is the first report to show that an $H^+$-ATPase-defective mutant of *E. coli* can be used for amino acid production. Our results further indicate that $H^+$-ATPase-defective mutants may be used for fermentative production of various compounds, including alanine.

## Introduction

L-Alanine is an important amino acid in terms of its applications in the pharmaceutical, food, and cosmetic industries (Leutenberger 1996). Though it is largely produced for commercial use by chemical synthesis, various microbial approaches for alanine production exist. Alanine is the end product of glycolysis in some organisms (Chico et al. 1978; Örlygsson et al. 1995), especially in thermophilic bacteria and archaea (Kengen et al. 1994; Kobayashi et al. 1995; Rovot et al. 1996). In addition, there are reports of alanine production directly from glucose. For example, *Arthrobacter oxydans* can produce alanine from glucose. Hashimoto and Katsumata isolated *A. oxydans* strain DAN 75 lacking alanine racemase, and this strain accumulated 75.6 g/L L-alanine in 120 h (Hashimoto and Katsumata 1998).

Other means of alanine production have also been documented. Most *Bacillus* species produce alanine dehydrogenase (ADH), which they use for spore germination (Freese et al. 1964). The introduction of this enzyme into another microorganism is a powerful approach in alanine production. Uhlenbusch et al. (1991) cloned the ADH gene from *B. sphaericus* into *Zymomonas mobilis*, an ethanol producer, and reported that this strain produced 7.5 g/L alanine. Galkin et al. (1997) attempted to produce alanine in *E. coli* using the plasmid pFDHAlaDH, which contains the ADH gene from *B.*

*stearothermophilus* and the formate dehydrogenase (FDH) gene from *Mycobacterium vaccae*. ADH utilizes pyruvate, $NH_4^+$, and NADH as substrates, and in this system, the NADH produced by FDH was used to produce alanine. However, these two studies did not rely on direct fermentation from glucose. Instead, Uhlenbusch et al. (1991) used a two-step procedure. In the first step, recombinant *Zymomonas* cells were grown in liquid medium at pH 5.5 to the late exponential phase, and then the cells were washed and transferred to fresh medium at pH 7.0. In comparison, in the alanine production system reported by Galkin et al. (1997), pyruvate was added to the production medium as a substrate. Thus, these systems involved conversion rather than fermentation.

Lee et al. (2004) reported the aerobic production of alanine by *E. coli aceF* (which encodes one of the pyruvate dehydrogenase complex genes) and *ldhA* (which encodes the fermentative lactate dehydrogenase [LDH] gene) mutants expressing the *B. sphaericus* ADH gene. In their report, *E. coli* was first grown under aerobic conditions, and then incubated under oxygen-limited conditions to induce alanine production. The cells were fed three times with glucose and $NH_4Cl$, and 32 g/L alanine was produced from 100 g/L total glucose. In addition to these reports, lactic acid bacteria have also been used for alanine production (Hols et al. 1999).

Unlike these studies, we attempted alanine fermentation under completely aerobic

conditions in a single step using an $H^+$-ATPase-defective mutant of *E. coli*. The sugar metabolism (both uptake and glycolytic flux) of this mutant is enhanced by its decreased cellular energy level (Yokota et al. 1997; Noda et al. 2006). An enhanced sugar metabolism is believed to be useful for improving fermentative production. For instance, *E. coli* TBLA-1, an $H^+$-ATPase-defective mutant of the pyruvate-producing *E. coli* strain W1485*lip2*, showed increased pyruvate production (Yokota et al. 1994b). Causey et al. (2004) also reported that a mutation in $H^+$-ATPase stimulated pyruvate production in *E. coli*. In this study, we introduced two genetic alternations into the pyruvate-producing *E. coli* strain TBLA-1 to allow alanine fermentation. Alanine is synthesized from pyruvate, $NH_4^+$, and NADH by ADH in a one-step reaction. In $H^+$-ATPase-defective cells, the intracellular concentration of NADH, which is produced by glycolysis, is higher than in normal cells (Yokota et al. 1997; Noda et al. 2006). Therefore, we introduced the ADH gene into TBLA-1 by transformation. LDH, which also use NADH and pyruvate as substrates for lactate formation, seemed to compete with ADH for their common substrates. Thus, we also constructed a fermentative LDH gene (*ldhA*) knockout mutant of TBLA-1 in order to prevent lactate by-production (Fig. 1). Using these mutant strains, the direct production of alanine from glucose has been investigated in this study.

## Materials and Methods

### Bacterial strains

*E. coli* W1485*lip2* (ATCC25645; Herbert and Guest 1968) is a lipoic acid-requiring pyruvate producer (Yokota et al. 1994a). An $F_1$-ATPase-defective mutant, TBLA-1 (*atpA401*, *bgl*$^+$), was constructed by introducing a defective $F_1$-ATPase gene, *atpA401* (Butlin et al.1971), into W1485*lip2* by P1 transduction (Yokota et al. 1994b).

A defective LDH gene from a KO-collection strain, *E. coli* K-12 BW25113 *ΔldhA*, (Nara Institute of Science and Technology; Baba et al. 2006) was also introduced into TBLA-1 by P1 transduction.

### Plasmid construction

The ADH gene of *B. stearothermophilus* was obtained from pFDHAlaDH (Galkin et al. 1997) by digesting the plasmid with *Sph*I and *Eco*RI (i.e., at both ends of the *ADH* insert). Then, the ADH gene was ligated into the pUC19 vector (pMB1 replicon, high-copy number, i.e., >200 copy/cell). From this plasmid, the ADH insert was digested with *Hind*III and *Eco*RI and cloned into the *Hind*III and *Eco*RI sites of the low-copy plasmids pSTV29 (p15A replicon, low-copy number, i.e., 10-12 copy/cell, TAKARA) and pHSG576 (pSC101 replicon, low-copy number, i.e., about 5 copy/cell, National Institute

of Genetics, Mishima, Japan).

**PCR verification**

*E. coli* K-12 BW25113 *ΔldhA* has a kanamycin-resistance gene in its chromosome at the *ldhA* position (Baba et al. 2006). Therefore, following P1 transduction, the *ΔldhA* mutant of TBLA-1 should have contained the kanamycin-resistance gene at the *ldhA* position. In order to verify the *ldhA* knockout, two-step PCR was performed using our candidate knockout strains. Five primers were used: kt (CGGCCACAGTCGATGAATCC), k1 (CAGTCATAGCCGAATAGCCT), k2 (CGGTGCCCTGAATGAACTGC), ldh-F (TTCTACCGTGCCGACGTTCA), and ldh-R (GCTGTTCTGGCGTAACAGCA). At first, colony-direct PCR was done using primers kt and k2 to test for a 471-bp fragment of the kanamycin-resistance gene. Subsequently, genomic PCR was performed to show that the mutants had the kanamycin-resistance gene at the correct position. The primers ldh-F and k1 were used to test for a 1000-bp fragment, ldh-F and kt were used to test for a 1500-bp fragment, and ldh-R and k2 were used to test for a 900-bp fragment. The thermal cycler parameters were as follows: 25 cycles of 30 s at 94 °C, 10 s at 56 °C, and 1 min at 72 °C. The strain that gave amplified fragments of the appropriate lengths was selected for use as TBLA-1*ΔldhA*.

**Culture conditions**

The medium used for shaking-flask fermentation contained 20 g/L glucose, 4 g/L Polypepton (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 2 g/L NaCl, 10 g/L $(NH_4)_2SO_4$, 1 g/L $K_2HPO_4$, and 0.5 g/L $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 8.0 with NaOH and sterilized at 120 ºC. Sterilized 20 g/L $CaCO_3$, filter-sterilized 1 μg/L lipoic acid, 0.2 mM IPTG, and the appropriate antibiotics (50 mg/L ampicillin or chloramphenicol) for plasmid maintenance were also added to the medium prior to cultivation.

The medium used for jar-fermentation contained 50 g/L glucose, 4 g/L Polypepton (Nihon Pharmaceutical Co., Ltd.), 2 g/L NaCl, 10 g/L $(NH_4)_2SO_4$, 1 g/L $K_2HPO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 14.7 mg/L $CaCl_2 \cdot 2H_2O$, and one drop of the antiforming agent Adekanol LG109 (ADEKA Corp., Tokyo, Japan). The pH was adjusted to 8.0 using NaOH and sterilized at 120 ºC. Filter-sterilized 1 μg/L lipoic acid, 0.5 mM IPTG, and antibiotics (50 mg/L chloramphenicol for all of the strains and 50 mg/L kanamycin for the *ldh* mutant strains) were also added. The medium used for the seed cultures was LB-glucose, including 2 g/L glucose and the appropriate antibiotics.

**Culture method**

All cultivations were carried out at 37 °C. For shaking flask fermentation, reciprocal shaker was set at 160 strokes/min with 4.5 cm amplitude. Fresh cells from LB-glucose agar plates were inoculated into 1 mL of LB-glucose and cultured with shaking for 10 h. The entire culture broth was then transferred into 50 mL of LB-glucose in a flask and cultured with shaking for 12 h. The pre-culture broth was inoculated into 1.2 L of fermentation medium to give an initial O.D. 660 of 0.07-0.08.

**Alanine fermentation**

The jar-fermentor (BMJ-02PI, ABLE Corp., Tokyo, Japan) was controlled at 600 rpm, 1 vvm aeration, 37 ºC, and pH 6.0 with a 1.2-L working volume in a 2-L reactor. The pH was kept at 6.0 using a 14% (w/v) ammonia solution in order to supply the ammonium ions needed for alanine production.

**Measurement of enzymatic activity**

The levels of ADH and LDH activity were measured in crude cell extracts. Cells were harvested by centrifugation and washed twice with the appropriate buffer. After sonication, cell debris was removed by centrifugation at 18,000 x *g* for 10 min at 4 °C.

The supernatant was used for the assays. The protein concentration in the cell extracts was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

The levels of ADH and LDH activity were analyzed by measuring the formation rate of alanine or lactate, respectively, by monitoring the decrease in NADH concentration at 340 nm ($\varepsilon = 6.22\ M^{-1}\ cm^{-1}$) using a DU-7400 spectrophotometer (Beckmann Inc., Fullerton, CA, USA). One unit of enzymatic activity was defined as the amount of enzyme required to produce a decrease in the level of NADH of 1 μmol/min. The reaction mixture for the ADH assay (1.0-mL total volume) consisted of 750 mM $NH_4OH/NH_4Cl$ buffer (pH 9.0), 30 mM sodium pyruvate, and 0.1 mM NADH. The reaction mixture for the LDH assay consisted of 100 mM potassium phosphate buffer (pH 7.0), 30 mM sodium pyruvate, and 0.1 mM NADH in 1.0 mL of total volume. Both reactions were initiated by the addition of sodium pyruvate. ADH also needs $NH_4^+$ in addition to pyruvate and NADH as substrate, activity of ADH and LDH can be measured separately in these buffer system.

**Identification of products and glucose consumption**

The concentration of alanine in the culture supernatant was determined using a

JLC-500S amino acid analyzer (JEOL, Tokyo, Japan). The levels of various organic acids were determined by HPLC using an L-7500 system (HITACHI Inc., Tokyo, Japan) equipped with an AMINEX HPX-87 column (Bio-Rad) using 0.01 N $H_2SO_4$ as the mobile phase (0.6 mL/min). The absorption at 210 nm was monitored to determine the organic acid concentrations. The concentration of glucose remaining in the culture broth was determined using a Glucose CII Test (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Results

**Construction of the ADH-expressing plasmid**

W1485*lip2* is a pyruvate producer with a wild-type $H^+$-ATPase, whereas TBLA-1 is an isogenic strain from W1485*lip2* with a leaky $H^+$-ATPase. ADH activity of the host strains, both W1485*lip2* and TBLA-1 without plasmid, were less than the detection limit (< 0.05 μmol/min/mg protein). The ADH gene from *B. stearothermophilus* was cloned into the pUC19 vector then transformed into TBLA-1 and W1485*lip2*; however, the resulting strain, TBLA-1/pUCADH, did not carry the plasmid stably. This may have resulted from the instability of a high-copy-number plasmid in an $H^+$-ATPase-defective mutant. Thus, low-copy-number plasmids harboring the ADH gene (pSTVADH and pHSGADH) were

also constructed. TBLA-1 was transformed by these plasmids to create TBLA-1/pUCADH, TBLA-1/pSTVADH, and TBLA-1/pHSGADH. By measuring the growth, alanine production, and ADH activity of these strains (Table 1), low-copy number plasmid, pSTV29, was found to be the most suitable vector for expressing the ADH gene in an $H^+$-ATPase-defective mutant.

As shown in Table 1, ADH activity measured in vitro did not correlate with alanine productivity. In addition, alanine production in TBLA-1/pUCADH, and TBLA-1/pHSGADH were neither stable nor reproducible (data not shown). Thus, we decided to use pSTVADH in order to introduce ADH gene into *E. coli*.

**Effect of the mutation in $H^+$-ATPase**

As shown in Fig. 2A, the growth rate and the final cell density were higher in W1485*lip2*/pSTVADH than in TBLA-1/pSTVADH during cultures, although the difference became much less than than that of W1485*lip2*during those observed in the corresponding parental strains under pyruvate fermentation, production conditions than that of W1485*lip2*during as reported previously (Yokota et al. 1994b). For example, the final cell density with the ancestor strain TBLA-1 without pSTVADH was much lower (the final $OD_{590}$ = 5.7) than that of W1485*lip2* without pSTVADH (the final $OD_{590}$ = 8.5)

during pyruvate fermentation (Yokota et al. 1994b). It seemed that introduction of pSTVADH exhibited adverse effect on the growth of W1485*lip2*.

On the other hand, the glucose consumption rate was roughly the same in the two strains (Fig. 2B). This result indicates that specific glucose consumption rate (glucose consumption rate/cell) by TBLA-1/pSTVADH was higher than that by W1485*lip2*/pSTVADH. In fact, all of the available glucose was consumed about after 24 h in both cultures (Fig. 2B). Interestingly, the rate of glucose consumption was higher under ammonium ion-excess conditions (neutralizer; ammonia solution) than under ammonium ion-limited conditions (neutralizer; NaOH solution). For example, when NaOH was used as the neutralizer instead of the ammonia solution, the consumption of glucose by TBLA-1/pSTVADH was much slower (34 h). Alanine production was also remarkably increased as a result of using ammonia as the neutralizer. For example, 15 g/L alanine was produced under conditions of excess ammonium ions (Fig. 2C), while 6 g/L alanine was produced by TBLA-1/pSTVADH under ammonium ion-limited conditions (data not shown in Fig. 2C, neutralizer; NaOH solution).

As shown in Fig. 2C, TBLA-1/pSTVADH produced 15 g/L alanine while W1485*lip2*/pSTVADH, which contains a wild-type $H^+$-ATPase, produced only 11 g/L alanine. As for the pyruvate to alanine conversion ratio, TBLA-1/pSTVADH converted

56.9% of the pyruvate it produced to alanine, while W1485*lip2*/pSTVADH converted 43.8% (Table 2). These improvements may be a result of the $H^+$-ATPase mutation.

**Effect of the *ldh* mutation**

The main by-products produced by the mutant (pyruvate, acetate, and lactate) are shown in Fig. 3. A large amount of pyruvate, which is a substrate of ADH, was produced by the strains, suggesting that the conversion of pyruvate to alanine is a rate-limiting step in alanine production (Fig. 3A). Acetate was also produced by all of the strains tested, but its yield was less than 5 g/L (Fig. 3B). However, acetate production increased after the glucose in the medium was consumed (24 h). Alanine was produced during the consumption of glucose; thus, acetate production does not likely compete with alanine production. Consequently, lactate was the main competitor of alanine. TBLA-1/pSTVADH produced roughly 7.0 g/L lactate, which is a considerable amount (Fig. 3C); therefore, an *ldh* mutant version of TBLA-1/pSTVADH was constructed in order to reduce lactate accumulation.

In the resulting strain, TBLA-1*Δldh*/pSTVADH, lactate production was almost negligible (Fig. 3C) while alanine production reached 20 g/L (Fig. 2C). This result indicates that *ldh* mutation has been correctly introduced into TBLA-1, and more

pyruvate was used for alanine production in TBLA-1*Δldh*/pSTVADH cells than in TBLA-1/pSTVADH cells. The molar conversion ratio of glucose to alanine was calculated to be 40.8% (Table 2). Similarly, the conversion ratio of pyruvate to alanine was improved from 56.9% (TBLA-1/pSTVADH) to 60.7% (TBLA-1*Δldh*/pSTVADH).

Notably, TBLA-1*Δldh*/pSTVADH still produced a small amount of lactate after its glucose-consuming phase in spite of the *ldh* mutation (Fig. 3C). This lactate was likely produced by some other means than fermentation by the *ldhA*-encoded LDH. However, the production of lactate seemed to have little effect on alanine production. Even in the best alanine producer in this study, TBLA-1*Δldh*/pSTVADH, which produced a large amount of pyruvate, the conversion ratio did not exceed 61% (Fig. 3A, Table 2).

**Enzymatic activity of jar-fermented cells**

The levels of ADH and LDH activity in the alanine-producing strains are given in Table 3. These enzyme activities were measured using the cell from jar-fermentor, and thus, ADH activities of these strains were different from the data shown in Table 1. TBLA-1/pSTVADH showed higher levels of ADH and LDH activity than W1485*lip2*/pSTVADH. This result may be related to the alanine productivity of these strains. Strain TBLA-1*Δldh*/pSTVADH possessed no LDH activity, indicating that the

*ldh* mutation had been introduced correctly. Even after 36 h of cultivation, TBLA-1*Δldh*/pSTVADH still showed a high level of ADH activity. This result indicates that the transformed plasmids were maintained in the cells, even after long-term cultivation.

**Discussion**

Our results clearly indicate that the combination of an $H^+$-ATPase defect with the ADH gene from another organism is effective for alanine production. The $H^+$-ATPase-defective mutant, TBLA-1, produced 1.4-fold more alanine than its parental strain, W1485*lip2*. This improvement may be due to increased glucose consumption per cell by TBLA-1, as described previously (Yokota et al. 1994b; Noda et al. 2006). In fact, glucose consumption rate per cell by TBLA-1/pSTVADH seemed to be higher than that by W1485*lip2*/pSTVADH (Fig. 2A, B). Moreover, the conversion ratio of pyruvate to alanine in TBLA-1 was 13% higher than that in W1485*lip2*. To produce alanine via an ADH reaction, three substrates are needed: pyruvate, $NH_4^+$, and NADH. A high concentration of pyruvate was still detected in the culture supernatant of both strains when excess ammonium ion was supplied to control the pH. Thus, NADH is a limiting factor in the conversion of pyruvate to alanine. Our results suggest that the intracellular

concentration of NADH in TBLA-1/pSTVADH was higher than that in W1485*lip2*/pSTVADH. Moreover, glucose consumption of TBLA-1/pSTVADH under ammonium excess-conditions was higher than that of TBLA-1/pSTVADH under ammonium limited-conditions. ADH can recycle the excess NADH formed by the enhanced central metabolism of TBLA-1, thus the alanine production by ADH may inhibit generation of an excessive proton-motive force, which inhibits cell growth (Noda et al. 2006).

This is the first report to show that an $H^+$-ATPase-defective mutant of *E. coli* is useful for amino acid production. These results also indicate that an $H^+$-ATPase defective mutant may be used for the fermentative production of various compounds, including alanine.

As shown in Table 1, ADH activity itself did not correlate to the alanine productivity. This result may indicate that the other factors than ADH activity (i.e., intracellular concentration of NADH, $NH_4^+$ incorporation into the cell, etc.) are rate-limiting step of alanine production using these strains.

Still, a considerable amount (about 7 g/L) of lactate was produced by TBLA-1/pSTVADH (Fig. 3C). LDH competes with ADH for the substrates pyruvate and NADH; therefore, we added an *ldh* mutation to TBLA-1 in order to improve alanine

production. Improved levels of alanine production have been shown in *ldh* knockouts in *E. coli* (Lee et al. 2004) and *Lactococcus lactis* (Hols et al. 1999). Knocking out *ldh* was also effective in our system. Consequently, 1.3 times more alanine was produced by TBLA-1Δ*ldh*/pSTVADH compared with TBLA-1/pSTVADH. In TBLA-1Δ*ldh*/pSTVADH, the NADH consumed by LDH was used in the ADH-catalyzed reaction. This extra NADH may have improved the level of alanine production. In this strain, 41% of the consumed glucose was converted into alanine. To the best of our knowledge, this is the highest conversion ratio of glucose to alanine reported to date by fermentation, and it was achieved via simple batch-culture without optimization of the growth conditions. Still, glucose feeding, aeration, pH, and the medium components seemed to play a role in controlling the yield of alanine.

In contrast, a large amount of pyruvate was still detected in all of the jar-fermented cultures, and the conversion ratio of pyruvate to alanine did not exceed 61%, even for TBLA-1Δ*ldh*/pSTVADH. ADH catalyzes a reversible reaction between pyruvate and alanine. It has been suggested that the reverse reaction (i.e., alanine to pyruvate) may inhibit further pyruvate conversion. This reverse reaction may be caused by a shortage of NADH or $NH_4^+$. In TBLA-1, glycolysis was the major source of NADH, meaning that the supply of NADH was exhausted once the available glucose was entirely consumed.

$NH_4^+$ was used to control the pH of the growth medium, thus it was supplied in excess during the log phase; however, this supply decreased at the stationary phase. To overcome this, additional glucose or ammonium ion, in the form of $NH_4Cl$ or $(NH_4)_2SO_4$, may be required during fermentation, as reported by Lee et al. (2004).

Additional mutations in the acetate pathway may also increase alanine productivity, because a small amount of acetate was detected in the culture media during alanine production (Fig. 3B). In TBLA-1, the activity of pyruvate dehydrogenase is strictly limited, thus, the activity of phosphotransacetylase and acetate kinase, which produce acetate from acetyl-CoA, was not considered. In this strain, acetate was probably synthesized from pyruvate by pyruvate oxidase (Fig. 1). Thus, mutation of this enzyme may promote additional alanine production. In fact, knocking out the *E. coli* pyruvate oxidase gene (*PoxB*) increases pyruvate production (Causey et al. 2004). Accordingly, *PoxB* would be a good candidate for mutation to improve the yield of alanine.

This study recorded the highest conversion yield of alanine directly from glucose via microbial fermentation to date. In addition, this is the first report of alanine fermentation under totally aerobic conditions by *E. coli*. These results suggest that the combination of an energy-deficiency mutation with several other mutations can be useful for compound production.

**Acknowledgements**

We thank Dr. Esaki of Kyoto University for kindly providing the pFDHADH plasmid. We also thank the National BioResource Project (NIG, Japan) for providing the Δ*ldhA* mutant of *E. coli* (KO-collection). This study was supported by the Industrial Research Grant Program (no. 04A07004 to M.W.) of the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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Fig. 1 Schematic drawing of the central metabolism in *E. coli* strain TBLA-1. POX: pyruvate oxidase, LDH: lactate dehydrogenase, PDHc: pyruvate dehydrogenase complex, ACK: acetate kinase, PTA: phosphotransacetylase, ADH: alanine dehydrogenase, *acetyl CoA synthetase, UQ: ubiquinone (oxidized form), $UQH_2$: ubiquinone (reduced form). Dotted allows indicate weakened metabolic flux in TBLA-1. Bold allows indicate enhanced metabolic flux in TBLA-1/pSTVADH.

Fig. 2 Production of alanine by jar-fermentation. A, growth; B, residual glucose; C, alanine. Open circle, W1485*lip2*/pSTVADH; Open triangle, TBLA-1/pSTVADH; Open square, TBLA-1*Δldh* /pSTVADH. The data shown are representative of three independent experiments that yielded similar results.

Fig. 3 Pyruvate, acetate, and lactate accumulation during jar-fermentation. A, pyruvate; B, lactate; C, acetate. Open circle, W1485*lip2*/pSTVADH; Open triangle, TBLA-1/pSTVADH; Open square, TBLA-1*Δldh* /pSTVADH. The data shown are representative of three independent experiments that yielded similar results.

Table 1. Growth, alanine production, and ADH activity of TBLA-1 and W1485*lip2* with ADH gene-containing plasmids*

| Plasmid | Growth ($OD_{660}$) | | Alanine production (g/L) | | ADH activity (μmol/min/mg protein) | |
|---|---|---|---|---|---|---|
| | TBLA-1 | W1485*lip2* | TBLA-1 | W1485*lip2* | TBLA-1 | W1485*lip2* |
| pUCADH | 5.8 | 5.9 | 1.0 | 1.3 | 42 | 26 |
| pSTVADH | 5.5 | 7.3 | 3.6 | 2.3 | 26 | 49 |
| pHSGADH | 4.8 | 6.0 | 3.0 | 0.85 | 38 | 33 |

*The cells were cultured with shaking in flasks for 25 h.

Table 2. Comparison of production efficiency

| | TBLA-1*Δldh*/pSTVADH | TBLA-1/pSTVADH | W1485*lip2*/pSTVADH |
|---|---|---|---|
| Alanine production (g/L) | 20.0±0.53 | 14.7±1.76 | 10.9±1.01 |
| Pyruvate production (g/L) | 16.0 ±1.20 | 13.8±1.70 | 17.3±2.12 |
| Glucose consumption (g/L) | 49.5±0.32 | 49.5±0.29 | 49.4±0.42 |
| *Conversion ratio from pyruvate to alanine (%) | 60.7±2.48 | 56.9±2.83 | 43.8±2.12 |
| ** Alanine production ratio from glucose (%) | 40.8±1.14 | 30.0±2.12 | 22.3±2.04 |

The data shown in Fig. 2 (after 24 h) were used for the calculations.
*and ** represent the molar conversion ratios.

Table 3. Activity of ADH and LDH*

| Strain | Culture time (h) | ADH activity (μmol/min/mg protein) | LDH activity (μmol/min/mg protein) |
|---|---|---|---|
| W1485*lip2*/pSTVADH | 12 | 5.37±0.10 | 1.38±0.003 |
| TBLA-1/pSTVADH | 12 | 11.3±0.30 | 2.29±0.02 |
| TBLA-1*Δldh*/pSTVADH | 12 | 10.9±0.16 | <0.05 |
| | 24 | 8.31±0.05 | <0.05 |
| | 36 | 8.90±0.37 | <0.05 |

**Cells from the jar-fermentor shown in Fig. 2 were used to measure the activity level

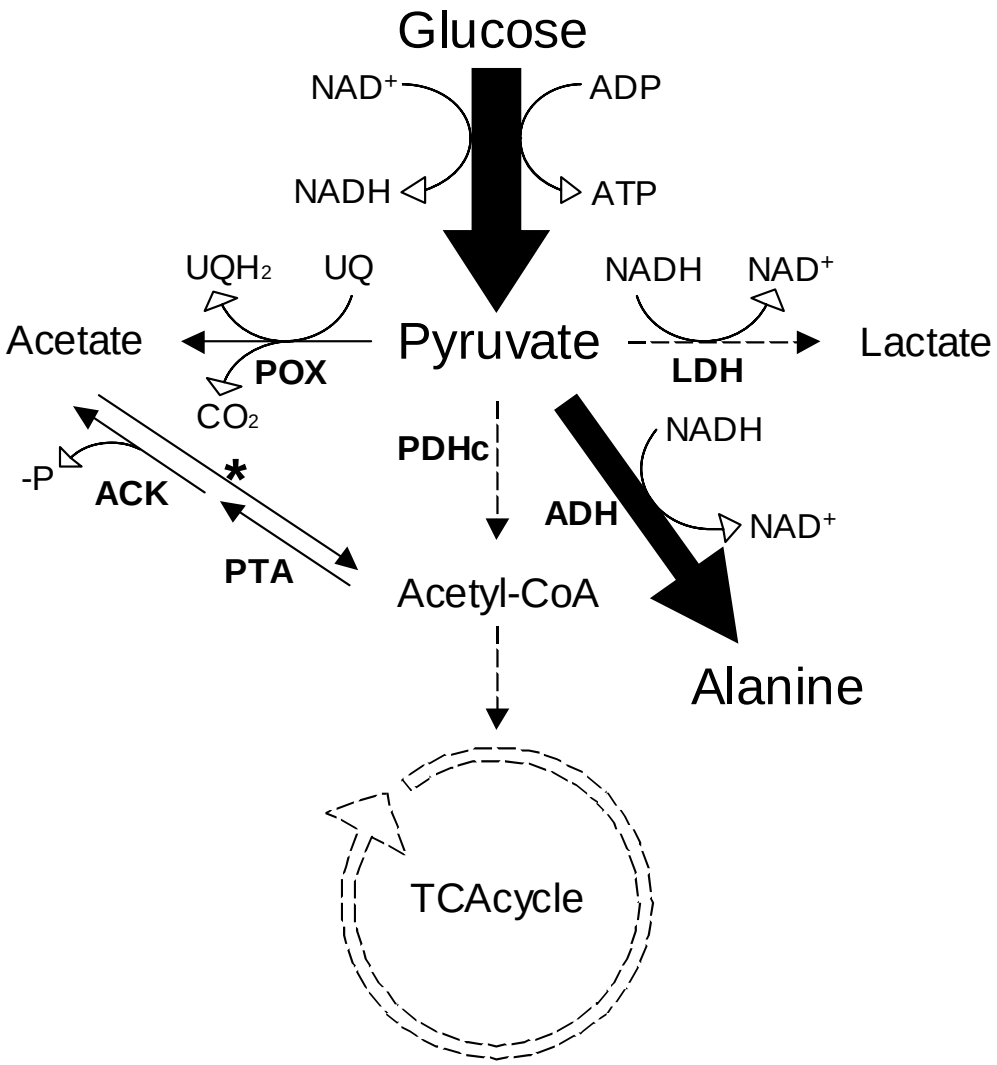


FIG. 1 WADA ET AL.

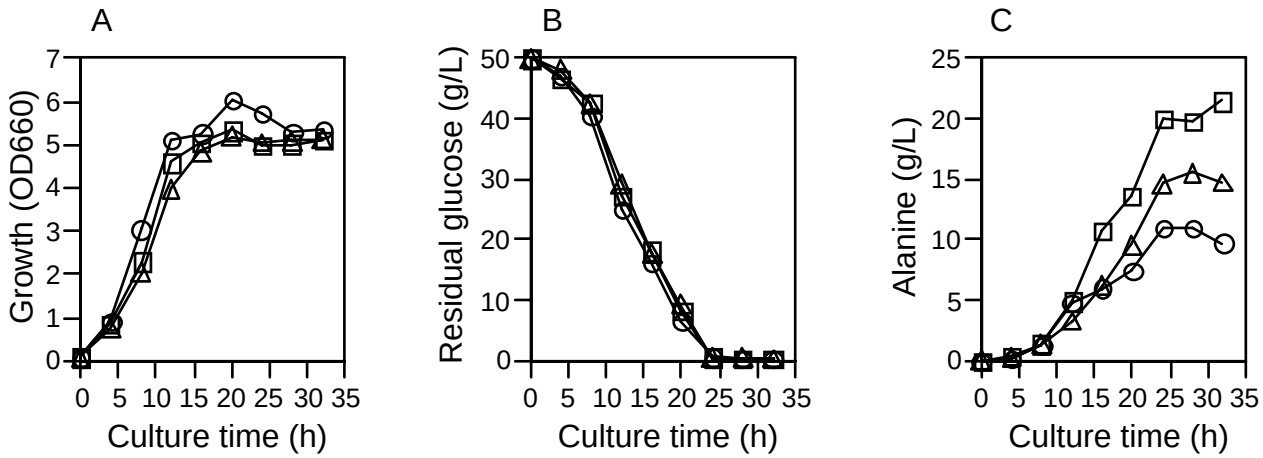


Fig. 2. Wada et al.

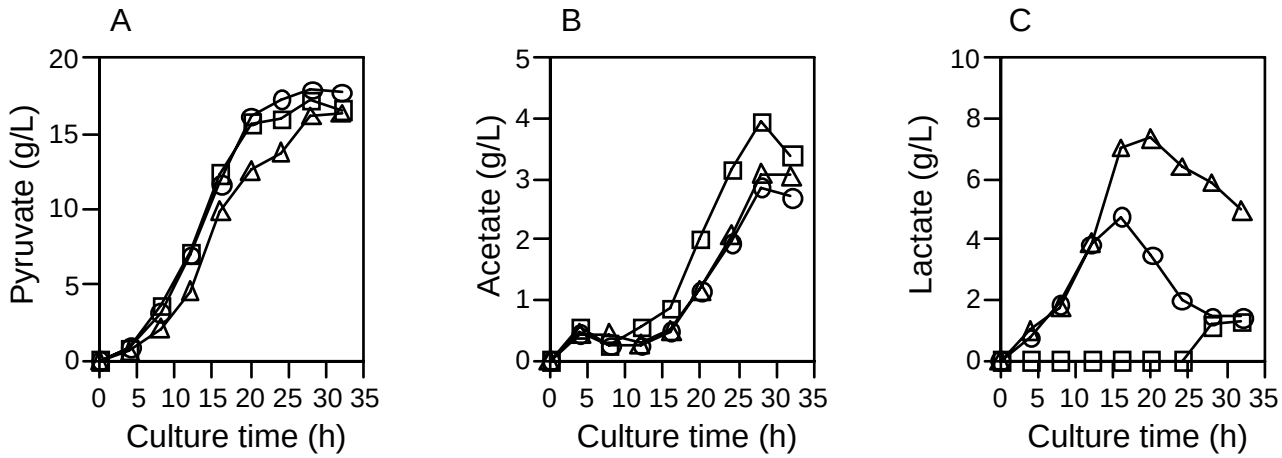


Fig. 3. Wada et al.