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Full Length Research Paper

Engineering of *E. coli* for increased production of L-lactic acid

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An over-expressed L-ldh gene derivative of *Escherichia coli* BAD-ldh was developed. L-ldh gene from *Enterococcus facelis* KK1 consisted of an open reading frame of 954 bp encoding 316 amino acids. L-ldh gene was cloned into pBAD vector and transformed into *E. coli* SZ85 by electroporation. SDS-page and western blotting method confirmed the presence of recombinant L-LDH enzyme with the approximate size of 40 kD. The activity of L-lactate dehydrogenase was achieved at 170 U ml^{-1}. *E. coli* BAD85 was found to produce 0.62 g l^{-1} of lactic acid from 1 g l^{-1} of fructose in 24 h. L-ldh gene from was successfully transformed into *E. coli* SZ85 with the maximum production of L-lactic acid at 0.62 g l^{-1}.

Key words: *Enterobacter,* fermentation processes, genes, lactic acid bacteria, molecular genetics, L-ldh gene.

INTRODUCTION

Lactate is used in various industries such as food, pharmaceutical, medical, and agriculture, where its production is approximately 15 million metric tons per year globally (Blomqvist, 2001; Tsuji, 2002). The demand for lactic acid is rapidly expanding with the introduction of polylactate (PLA), a renewable, biodegradable plastic into the market place (Tsuji, 2002). Lactic acid is the main fermentation product excreted by the gram positive and lactic acid bacteria (LABs) (Franz et al., 1999; Garrity and Holt, 2001; Garvie, 1980). The roles of these LABs have been studied extensively, for better understanding and ability to manipulate their roles especially in fermentation processes. Optically pure isomers can be produced as separate products by microbial fermentation of carbohydrates using chiral-specific L-lactate dehydrogenase or L-lactate dehydrogenase enzymes (Garvie, 1980; Dengler et al., 1997; Grant, 1989; Kochbar et al., 1992). L-isomers are more abundant compared to the D-isomers for most uses of bioplastics. One of the major problems in fermentation using microorganisms is production of a mixture of organic acids (acetic acid, lactic acid, formic, and succinic acid) and ethanol.

The four major models and most widely used microbial platforms for biotechnology, lactic acid production in particular, are *Escherichia coli,* yeasts*, Bacillus subtilis* and *Lactobacillus* strains. There are several advantages offered by the *E. coli* engineered for the L-lactic acid production compared to many other available organisms (Chang et al., 1999; de Graef et al., 1999; Devriese et al., 1995; Snoep et al., 1990). The metabolically engineered *E. coli* has the capability of producing optically pure L-lactic acid with trace amount of other fermentation products (de Graef et al., 1999; Dien et al., 1998, 2001), and are capable of utilizing a wide variety of sugars, including xylose (Devriese et al., 1995; de Graef et al., 1999). In addition to being well characterized (Chang et al., 1999; de Graef et al., 1999; Devriese et al., 1995; Snoep et al., 1990), the *E. coli* strains also have minimal nutritional requirements.

To the best of our knowledge, to date, there is no report on L-ldh gene from *Enterococcus* being expressed in *E.*

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Table 1. Bacterial strains and plasmids

Designation	Genotype/Description	Source or reference
Strains		
E. faecalis KK1	L-ldh D-ldh	Laboratory collection
E. coli W3110	Wild type ATCC 27325	Zhou et al. 2003
E. coli SZ85	W3110 foc A-pfl B:: FRT frdBC adh E:: FRT ack A:: FRT ldh A (L-ldh-frl)	Zhou et al. 2003
Plasmids		
E. coli Top 10	Lac (episome)	Invitrogen
pBAD	pBR 322 ori; P_{BAD} promoter; NcoI, Hind III in multiple cloning site, Amp^{res}	Invitrogen
PCR2.1-TOPO	*Bla kan,* TOPO TA cloning vector	Guzman et al. 1995
E.coli *BAD85*	W3110 foc A-pfl B:: FRT frdBC adh E:: FRT ack A:: FRT d-ldh (L-ldh-frl)	_
pBAD-ldh	Recombinant vector, pBR 322 ori; P_{BAD} promoter; NcoI, Hind III in multiple cloning site, Amp^{res}, *L-ldh* gene from *E. faecalis*	_

coli. In this paper, we describe cloning, sequencing and expression of l-ldh gene from *Enterococcus faecalis* KK1 (Devriese et al., 1993; Scleifer and Kilpper-Balz, 1984) in *E. coli* SZ85.

MATERIALS AND METHODS

Microorganisms, plasmids and growth condition

Strains and plasmids used in this study are listed in Table 1. The stock cultures were maintained at -80ºC in 1.5 ml tubes containing 80% (v/v) glycerol. Luria broth was used to grow *E. coli*. Ampicillin of 50 µg/ml was added and blue white screening method was used for selection of positive transformants. *E. facelis* KK1 was grown in tryptone soya broth (TSB) at 37ºC.

Cloning of the *E. coli* SZ85 L-ldh gene

Genomic DNA of *E. faecalis* KK1 was extracted using alkaline lysis method with modification (Sambrook et al., 1989). The extracted DNA was used as the template for amplification of the L-ldh gene. The PCR amplification was performed with the forward primer F5' AATCTCGAGAATGAAAGTATTTAACAAAACAGTCGC 3' and the reverse primer R 5' CTCGAATTCCTAAGCGTTCGGTTGTAACGATGC 3' under the following conditions: 95ºC for 5 mins, followed by 30 cycles of 1 min at 95ºC ,1 min at 55ºC, and 72ºC for 1 min. An extension step of 5 mins at 72ºC was included after the final cycle. PCR product was gel purified using Gel Band Purification Kit (Qiagen, Germany). The 1 kb purified PCR fragment was ligated into pCR-2.1 TOPO vector (Invitrogen, USA). The transformants were cultured in LB broth with 50 mg ml^{-1} ampicilin at 37ºC. Plasmid was extracted from overnight culture using the alkaline lysis method (Sambrook et al., 1989).

Overexpression of L-ldh gene in *E. coli* SZ85

For expression of L-ldh gene in *E. coli* SZ85, the L-ldh was subcloned into pBAD as follow: pCR2.1 TOPO vector containing the L-ldh gene was digested with XhoI and EcoRI then a 1 kb DNA fragment with L-ldh gene was ligated into pBAD vector, which had been digested with XhoI and EcoRI. The purified pBAD-ldh was electrotransformed into competent *E. coli* SZ85 as described by Sambrook et al. (1989). The electroporation was set up at an electrical pulse of 25 µF capacitance, 2.5 kV, and 200 Ω resistances. The transformants were selected at random on ampicilin selective SOB agar (0.5% NaCl, 1.0% tryptone, 0.5% yeast extract, 1.5% agar, 10 mmol l^{-1} KCl and 20 mmol l^{-1} $MgSO_4$ with 50 µg ml^{-1} ampicilin) after 16-20 h at 37ºC. The recombinant cell harboring the pBAD-ldh was named *E. coli* BAD-ldh. SDS-page was performend based on standard method (Sambrook et al., 1989). Resolved proteins were electro-blotted onto a PVDF membrane using 2X SSC buffer and probed with monoclonal anti-His–alkaline phosphatase conjugated antibody (Invitrogen, USA).

L-lactic acid production by recombinant *E. coli* BAD-ldh

Fermentation was conducted using LB medium with 1% (w/v) fructose as carbon source. The fermentation was carried out in duplicates using 250 ml Erlenmeyer flasks with 10 ml working volume. The initial pH was set at 7.0. The fermentation broth was inoculated with 10% (v/v) inoculum. The flasks were sealed with rubber stoppers and agitated at 150 rpm for 18-20 h at 37ºC. Ampicilin (50 µg/ml) and 0.2% (w/v) L-arabinose were added to the fermentation medium when OD_{550nm} achieved at 0.3-0.5.

Analytical methods

The concentrations of organic acids were determined by HPLC equipped with BioRad Aminex 87H column using 4mM H_2SO_4 as mobile phase at 0.6 ml min^{-1} (Hassan et al., 1996). Sample was centrifuged at 10,000 x g for 5 min to remove cell debris. Supernatant was filtered using 0.2 µm nylon syringe filter. Standard curve was prepared using standard lactic acid. L-LDH activity was determined spectrophotometrically (A_{340}) at 25°C in phosphate buffer (KH_2PO_4, K_2HPO_4, pH 7.4) containing 1 mmol l^{-1} pyruvate and 0.2 mmol l^{-1} NADH. The molar extinction coefficient of NADH at 340 nm (6.22 x 10^3 (mol l^{-1})$^{-1}$$cm^{-1}$) is used for calculating the enzyme units.

RESULTS

Isolation and sequence analysis of L-ldh gene

The L-ldh gene from *E. faecalis* KK1 was amplified with the estimated size of l kb (Figure 1). The same size was also reported for L-ldh gene of Streptococcal strains (Wyckoff et al., 1997), *Streptococcus mutans* (Hillman et al., 1990),

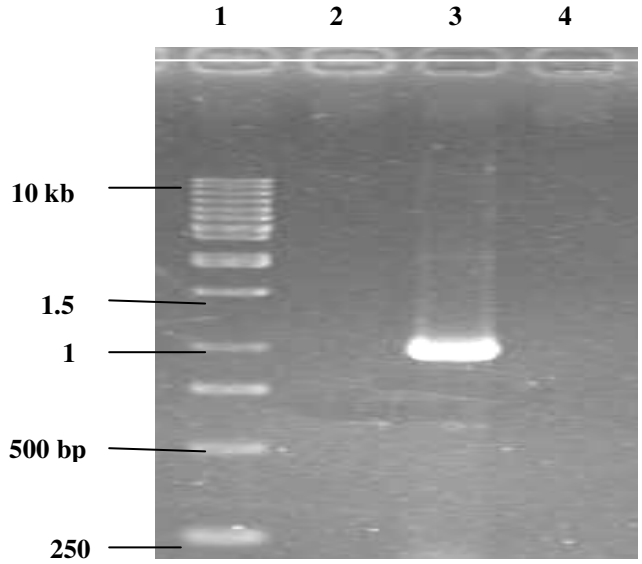

Figure 1. Agarose gel electrophoresis analysis of purified PCR product *L-ldh* gene of *E. faecalis* KK1.

Lactococcus lactis (Llanos et al., 1992) and *Corynebacterium acetobutylicum* (Contaq et al., 1990). Figure 2 shows the alignment of L-ldh gene of different bacteria. Comparison of nucleotide and amino acid sequences with the L-ldh gene of Lactococcal strains revealed a moderate degree of homology (68-70% amino acid). Genera *Lactococcus* and *Enterococcus* were once grouped under the same genus *Streptococcus* and this was reflected in the moderately high homology between the two in L-lactate dehydrogenase amino acid sequences. The only streptococcal strain sharing amino acid homology to L-lactate dehydrogenase amino acid sequence belonging to *E. faecalis* was *Streptococcus pneumoniae* with 45% similarity.

Overexpression of L-ldh gene in *E. coli* SZ85 and analysis of purified protein

The L-ldh gene of *E. faecalis* KK1 was cloned into vector pBAD and was expressed in host strain *E. coli* SZ85. Both PCR amplification and restriction enzyme digestion analyses confirmed the presence of the L-ldh gene in the recombinant pBAD-ldh. SDS-PAGE further confirmed the presence of L-ldh gene products (Figure 3a). A heavily stained protein band with a molecular mass of ~40 kDa was produced by the induced (0.2% arabinose) recombinant *E. coli* BAD-ldh. This value agreed with that of the L-lactate dehydrogenase expressed in other Gram-positive bacterial strains such as *S. mutans* (Hillman et al., 1990), *L. lactis* (Llanos et al., 1992), *Pediococcus acidilactici* (Garmyn et al., 1995) and *C. acetobutylicum* (Contaq et al., 1990). Lane 4 (total protein from *E. coli* SZ85) also obtained a faint band having the same size of ~40 kDa as *E. coli* SZ85 contains L-ldh gene from *P. acidilactici*.

The L-lactate dehydrogenase protein detected by SDS-PAGE analysis was later analyzed by western blotting using anti-His antibody (Figure 3b). The SDS-PAGE analysis demonstrated the predicted size of the recombinant L-lactate dehydrogenase protein of ~40 kDa. The total absence of band in lanes 2, 3 and 4 (Figure 3b) indicated that the presence of faint band of size ~40 kDa in lanes 2, 3 and 4 (Figure 3a) was due to lactate dehydrogenase protein expressed from *L-ldh* gene of *P. acidilactici* and obviously not because of leakage of gene expression. This further confirmed that pBAD vector system offered a tightly regulated expression system. The absence of band of size ~40 kDa in lane 5 (uninduced total protein from *E. coli* BAD-ldh) provided additional evidence that there was no occurrence of leakage of gene expression at all. This result agreed with the findings observed by Siegele and Hu (1997), Huang et al. (2000), Bowers et al. (2004) and Xu et al. (2006) that pBAD expression system offered tight regulation of gene expression. Table 2 shows the L-lactate dehydrogenase activity at induced and uninduced condition where the activity was increased almost 2-fold to 170 U ml^{-1}.

Lactic acid production by recombinant *E. coli* BAD85

During the anaerobic growth of recombinant *E. coli* BAD-ldh in fructose, lactic acid was produced concurrently with cell growth (Figure 4). The concentration of lactic acid produced was achieved at 0.62 g l^{-1} with the productivity achieved at 0.026 g l^{-1} h^{-1}. The cell growth entered death phase after fructose was depleted. At the end of fermentation, the final pH was reduced to pH 5.0 due the acid accumulation. It was apparent that, as pointed out by Russel (1992) and Diez-Gonzalez and Russel (1997) as the medium pH was farther away from the internal pH maintained by the bacteria, inhibition effect increased regardless of whether the lactic acid was in the dissociated or undissociated form. Tang et al. (1989) have shown that both dissociated and undissociated lactic acid had inhibitory effects with the undissociated organic acids being more inhibitory than the dissociated acids. According to Padan et al. (1981), cell maintained the intracellular pH by pumping H^+ out via a cation-dependent proton pumps, which was an energy-intensive process. Meanwhile, the anion concentration increased because the charge on the anion prevented it from transversing the membrane (unless through a transport protein), which led to an osmotic imbalance (Russel, 1992; Roc et al., 1998). Russel (1992) and Roc et al., (1998) further added that increasing the amount of undissociated lactic acid, as occurred when lactic acid concentration was increased or the pH was lowered, led to greater inhibition of cell growth because of pH and osmotic imbalances.

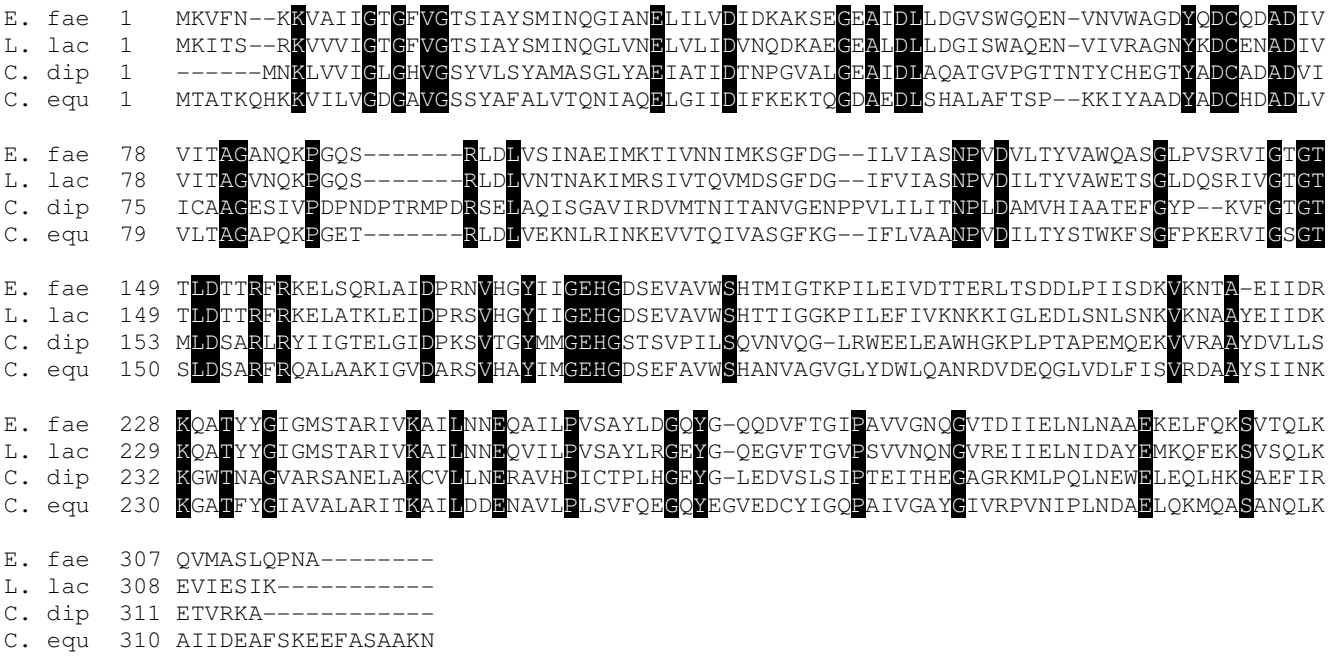

Figure 2. Alignment of L-ldh gene of *E. faecalis* KK1 (E. fae), *L. lactis* (L. lac), *Corynebacterium diphtheriae* NCTC 13129 (C. dip) and *Streptococcus equi* subsp. *zooepidemicus* MGCS10565 (C. equ). Amino acids which are conserved in all sequences are highlighted. -, gap left to improve alignment. Numbers refer to amino acid residues at the start of the respective lines; all sequences are numbered Met-1 of the peptides.

Table 2. L-lactate dehydrogenase activity of uninduced total protein of *E. coli* SZ85, uninduced total protein of *E. coli* SZ85 pBAD, induced total protein of *E. coli* SZ85 pBAD, uninduced total protein of *E. coli* SZ85 pBAD-ldh and induced total protein of *E. coli* SZ85 pBAD-ldh.

Cells	Expression vector	Gene	% L-Arabinose (w/v)	L-LDH activity (U/ml)
E. coli SZ85	-	-	-	85
E. coli SZ85	pBAD	-	-	85
E. coli SZ85	pBAD	-	0.2	86
E. coli SZ85	pBAD	L-ldh	-	89
E. coli SZ85	pBAD	L-ldh	0.2	170

DISCUSSION

Many efforts have been made for the production of L-lactic acid by employing metabolic engineering approach. In 1990, Contaq et al. conducted a study on recombinant *E. coli* PRC 436 whose L-ldh gene from *Clostridium acetobutylicum* B643 was cloned into vectors pPC37 and pPC58 and transformed into *E. coli* FMJ (*ldhA pfl* mutant). The L-lactate dehydrogenase activity was increased by 15-30 folds. Chang et al. (1999) constructed a metabolically engineered *E. coli* RR1 into which L-ldh gene from *Lactobacillus caseii* was introduced. This pta ldhA strain lacked the enzymes phosphotransacetylase and D-lactate dehydrogenase and the L-lactate dehydrogenase activity observed was high. Kyla-Nikkila et al. (2000) did a study on *Lactobacillus helveticus* CNRZ32 and the impact of increasing the L-lactate dehydrogenase activity by additional L-ldh gene. They constructed two stable D-ldh negative *L. helveticus* derivatives by gene replacement. In the first construct, the internal promoter region for *D-ldh* gene was deleted (strain GRL 86). For the second construct, the L-ldh gene replaced the D-ldh structural gene resulting in duplication of the L-ldh gene dosage (strain GRL 89). The maximum L-lactate dehydrogenase activity was found to be 53 and 93% higher in strains GRL 86 and GRL 89 respectively compared to the wild-type strain. This was in contrast to what was observed earlier with L-ldh from *Lactobacillus plantarum.* In this host, inactivation of neither the L-ldh nor the *D*-ldh gene seemed to affect the remaining ldh activity markedly (Ferrain et al., 1994, 1996). The L-LDH activity was increased through gene dosage effect by reintroducing the L-ldh gene in a multicopy plasmid in *L. plantarum.* There was a drastic change in the L-LDH

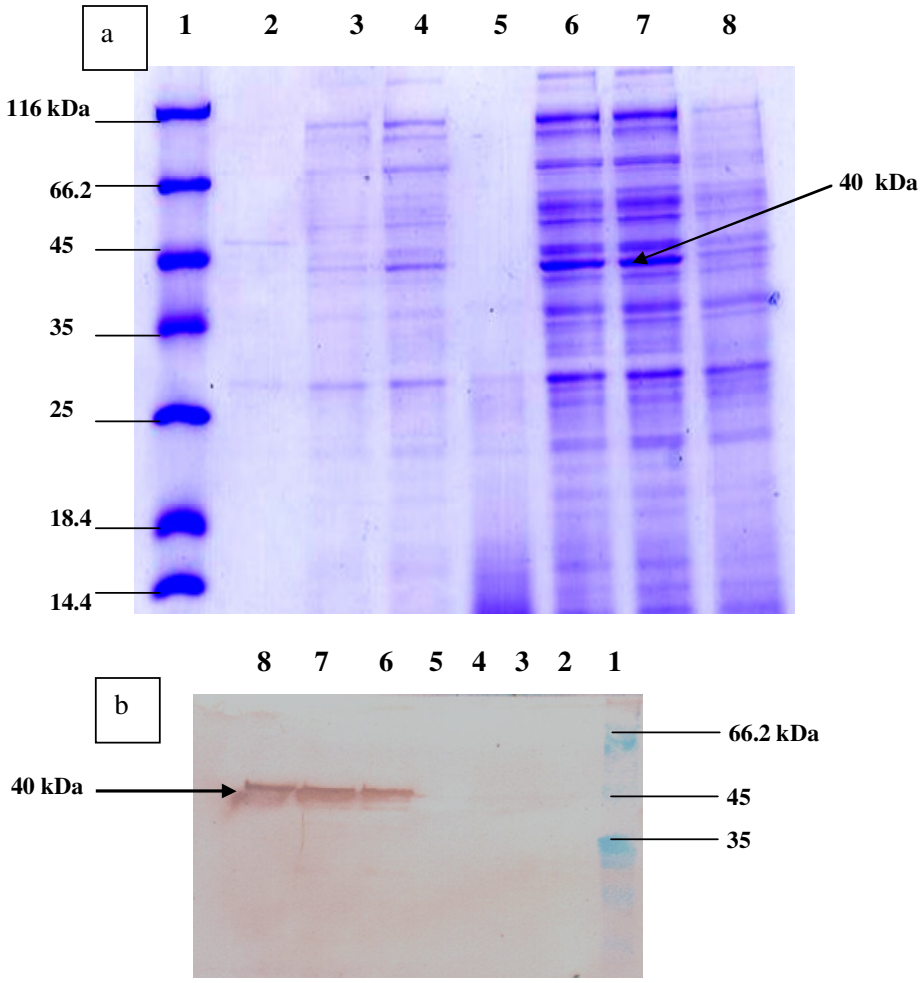

Figure 3 (a) SDS-PAGE and (b) western blot analyses. Lane 1: Protein molecular weight marker (Fermentas Co.), Lane2: Induced total protein of *E. coli* BAD (0.2% arabinose induction), Lane 3: Uninduced total protein of *E. coli* BAD, Lane 4: Total protein of *E. coli* SZ85, Lane 5: Uninduced total protein of *E. coli* BAD-ldh, Lanes 6-8: Induced total protein of *E. coli* BAD-ldh (0.2% arabinose induction).

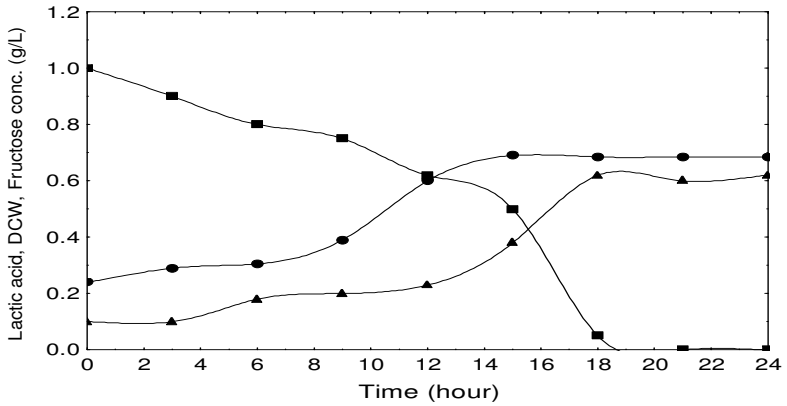

Figure 4. Batch fermentation of recombinant *E. coli* BAD 85 at pH 7.0, 37ºC with 1 g/l of fructose. Closed square: Fructose consumption (g/l), closed circle: Lactic acid (g/l), closed triangle: Dry cell weight (g/k).

activity by 13-fold compared to the wild type. Wyckoff et al. (1997) reported that the L-ldh gene from *Streptococcus bovis* JB1 was highly expressed in *E. coli* FMJ39. Increased LDH activity of about 25% was observed in *S. bovis* JB1 containing the cloned ldh genes on a multicopy plasmid. The specific activity was 13 times greater than that produced when expressed in *E. coli.* In this study, we had constructed a recombinant *E. coli* BAD85, carring L-ldh gene of *E. faecalis*. L-Lactete dehydrogenase activity was found 2-fold higher with 0.2% arabinose induction. The L-lactic acid production was achieved at 0.62 g l^{-1} from 1 g l^{-1} of fructose.

In this study, we used a knockout strain to express the L-ldh gene from *E. faecalis* KK1. The L-ldh gene was amplified and cloned into the expression vector (pBAD) and sequencing results indicated 99% homology with published data. SDS-PAGE and western blotting results showed the expression of L-ldh gene in *E. coli* SZ85. The improved expression system developed can be used for the purpose of enhancing L-lactic acid production. Production of L-lactic acid by recombinant *E. coli* BAD-ldh was studied in batch fermentation using fructose as a carbon source. Cultivation of *E. coli* BAD85 at pH 7.0, 37ºC was able to produce 0.62 g l^{-1} L-lactic acid.

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