Production of L-lactic Acid from *Escherichia coli* Harboring Recombinant Plasmid with *Rhizopus oryzae ldhA* Gene

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Abstract: Lactic acid is widely used in various industrial applications including the use as a potential precursor for biodegradable plastics. To synthesize the polymers of lactic acid, the production of optically pure monomers is essential. *Rhizopus oryzae* is a potential candidate for applying to L-lactic acid production. Although it produces optically pure L-isomer, *R. oryzae* has some limitations such as its unsuitable morphology when fermented in bioreactors. To solve these problems, genetic modification of *Escherichia coli* has been chosen as a potential alternative to develop as a host for optically pure lactic acid production. In this research, plasmid containing *R. oryzae ldhA* was transformed into *E. coli* cells. The *R. oryzae ldhA* gene on the plasmid could be expressed when chromosomal *ldhA* of *E. coli* was knocked out. The *E. coli* strain with plasmid containing *ldhA* from *R. oryzae* was fermented by using media containing initial 100 g/L glucose. The result suggested that the *R. oryzae ldhA* gene on the plasmid could be expressed and the optimal condition for L-lactic acid production was the use of fermentation broth without Ampicillin in an anaerobic condition. However, the L-lactic acid yield obtained from this strain was still low (5.03±4.149 g/L). This may be resulted from the high amount of residual glucose in the culture that may inhibit L-lactic acid production.
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1. Introduction

Lactic acid has been used in variety of applications including food additives, pharmaceutical and agricultures. Moreover, it can be used to synthesize polymers such as polylactate (PLA) for biodegradable plastic industries (Kricheldorf, 2001; Hester, 2000). With the rapidly increasing demand, the effective process for lactic acid production is necessary to be improved. Currently, lactic acid is preferably biosynthesized by the fermentation of *Lactobacillus* ssp. However, bacteria in this group require complex nutrients and are unable to produce high yield of L-lactic acid at pH value lower than 4 (Skory, 2000). *Rhizopus oryzae*, a filamentous fungus, is a potential choice due to the production of pure optical L-isomer. This organism could produce lactic acid by using starch, cellulose and hemicelluloses as raw material with ammonium salts as nitrogen source (Woiciechowski et al., 1999). However, some limitations still encounter. It was suggested that the L-lactic acid yield obtained from *R. oryzae* was low compared to that of bacteria. Moreover, the problems found when fermented this fungus were the unsuitable morphology for applying in any bioreactors and the contamination of the byproducts, especially ethanol (Narayanan et al., 2004; Skory, 2004). Therefore, genetic engineering is an approach to solve these problems. One of the favorable models is an expression of gene encoding the enzyme lactate dehydrogenase of one species in the other species, for example, *ldhA* from *R. oryzae* expressed in *Saccharomyces cerevisiae* (Skory, 2003) and L-*LDH* from the cow *Bos tauraus* in *Candida utillis* (Ikushima et al., 2009). However, it was reported that the yield of L-lactic acid from these two examples was still low.

For L- lactic acid production, *Escherichia coli* is a potential target to be genetically engineered. The advantages from using this microorganism as an expression system are not only well characterized, but also easy to be manipulated. Plasmids with *ldhA* and *ldhB* from *R. oryzae* could complement the lost of chromosomal *ldhA* in *E. coli* (Skory, 2000). Furthermore, there are many reports on using *E. coli* to express *ldhA* genes from different organisms such as *R. oryzae* (Chang, 1999), *Streptococcus bovis* (Dien et al, 2001) and *Enterococcus facelis* (Mulok et al., 2009). However, to obtain an effective process of L-lactic acid production, there many factors are required to be studied. In this research, plasmid with *ldhA* gene from *R. oryzae* was transformed into *E. coli.* For the expression system, chromosomal *ldhA* and *pta* genes are knocked out to allow the expression of only *ldhA* gene on the plasmid, and reduce some byproducts during fermentation, respectively (Chang et al., 1999). Then, several conditions were tested in a shake flask

scale to find the most effective process for L-lactic acid production which can be useful information for the further production in a larger scale.

2. Material and Methods

2.1 Construction of *E. coli* for L-lactic acid production

All *E. coli* strains used in this work are derivatives of *E. coli* K-12 and are described in Table 1. All strains were cultured in LB media with appropriate antibiotics: 100 µg/mL Ampicillin, 25 µg/mL Chloramphenicol or 100 µg/mL Kanamycin. To construct the strain for L-lactic acid production, the chromosomal *ldhA* gene was knocked out by using cross-over PCR technique (Link et al, 1997). First, a part of *ldhA* gene of *E. coli* strain JC13509, designated as Fragment1, was amplified by PCR with primers pr1 (introducing *Bam*HI site, Table 2) and pr2 (introducing *Bsi*WI site, Table 2). The other part of *E. coli ldhA* gene containing 23 bp homology to Fragment, designated as Fragment2, was amplified by PCR with primers named pr3 (introducing *Bsi*WI site, Table 2) and pr4 (introducing *Eco*RI site, Table 2). Then, these two fragments were annealed by their overlapping regions. The cross-over fragment was amplified by PCR again using pr1 and pr4 primers. The PCR product containing *E. coli ldhA* with *Bsi*WI site in the middle of the fragment was inserted in to pGEM®-T Vector Easy System (Promega, USA), and then transformed into *E. coli* DH5α. Plasmid extracted from Ampicillin resistant transformant, named as pRB73, and *cat* gene (chloramphenicol acetyltransferase gene), amplified by PCR with pr5 and pr6 primers (Table 2) and the pML1 plasmid (obtained from Dr. Steven J. Sandler's Lab) as DNA template, were cut with *Bsi*WI, and then ligated these 2 fragments by using T4 DNA ligase. The ligation reaction was transformed into *E. coli* DH5α and selected on the Chloramphenicol plate. The plasmid containing *ldhA::cat* was named as pRB74 which *ldhA::cat* fragment was further cut from pRB74 by *Bam*HI and *Eco*RI, and then linearly transformed into *E. coli* strain JC13509 containing pKD46 (Datsenko and Wanner, 2000). Chloramphenicol resistant colonies were checked for the integration of *ldhA::cat* onto *E. coli* chromosome by PCR with pr1 and pr4 primers. This *ldhA* knockout strain was named as RB5. Then, P1 transduction (Willetts et al., 1969) was carried out to put *ldhA::cat* from the strain RB5 onto RB6 (containing *pta::kan*) chromosome generating the strain named RB7 with knocked out *ldhA* and *pta* genes.

To construct the plasmid for L-lactic production, *ldhA* gene from *R. oryzae* NRRL395 was cloned into pBlueScript II KS(+). Due to the lack of intron in this *ldhA* gene, the genomic DNA of *R. oryzae* was served as the template for PCR reaction with the following primers: pr33 (introducing *BamH*I site, Table 2) and pr34 (introducing *Hin*dIII site, Table 2). The approximately 1.8 kbp PCR product was cut with *BamH*I and *Hin*dIII, then ligated with pBlueScriptII KS(+) digested with the same enzymes. The ligation reaction was transformed into *E. coli* DH5α. The Ampicillin resistant transformants were selected to extract the plasmids and further cut with *Kpn*I to check the presence of *R. oryzae ldhA* on the plasmid. The pBlueScript II KS(+) with *R. oryzae ldhA* was named as pRB85, and then transformed into *E. coli* RB7 to generate the strain RB24 which is the strain for L-lactic acid production in this work.

Table 1. Strain list

Organism	Strain	Plasmid	Other relevant genotypes	Reference
R. oryzae	NRRL395	-		[a]
E. coli	JC13509[a]	-		[b]
	SS4075	-	*pta::kan*	[b]
	RB1	pRB73		pRB73 in DH5α
	RB2	pRB74		pRB73 in DH5α
	RB5	-	*ldhA::cat*	[c]
	RB6	-	*pta::kan*	SS4075→JC13509
	RB7	-	*ldhA::cat pta::kan*	RB5→RB6
	RB19	pRB85		pRB85 in DH5α
	RB24	pRB85	*ldhA::cat pta::kan*	pRB85 in RB7
	RB29	pBluescriptII KS(+)	*ldhA::cat pta::kan*	pBluescript II KS(+) in RB7
	RB30	pRB85		pRB85 in JC13509
	RB31	pBluescriptII KS(+)		pBluescript II KS(+) in JC13509

[a] Northern Regional Research Laboratry, USA
[b] Sandler's lab, USA
[c] Linear transformation of *ldhA::cat* fragment onto JC13509chromosome

2.2 Production of L-lactic acid in different fermentation conditions

To adjust to the high concentration of glucose and $CaCO_3$ in fermentation broth, each strain was first cultured in 5 ml enrichment broth with pH 6.8 (10 g/L glucose, 5 g/L w/v yeast extract, 5g/L w/v yeast extract, 0.25 g/L KH_2PO_4, 0.25 g/L K_2HPO_4, and 0.5% v/v salt solution composed of 40 g/L $MgSO_4.7H_2O$, 2 g/L $MnSO_4.5H_2O$ and 2 g/L $FeSO_4.7H_2O$). After being incubated at 37° C overnight, each culture was streaked on purification plate (same ingredients as enrichment broth but adding 2.5 g/L $CaCO_3$ and 2% w/v Bacto agar) and incubated at 37° C overnight. Then, culture was transferred to cultivation slants (same ingredients as purification plate except for the addition of 5 g/L $CaCO_3$), incubated at 37° C overnight and kept at 4° C until use (sub-cultured every 2 weeks). To test the production of L- lactic acid, each strain was transferred to another slant and approximately 2 mL pre-culture broth was put into the slant to harvest the

cells, and then transferred to the rest of preculture broth (approximately 48 mL, same ingredients as enrichment broth with the addition of 5 g/L $CaCO_3$). After that, 2 mL of each culture was put into 50 mL of fermentation broth (same ingredients as pre-culture broth except for 100 g/L glucose and 50 g/L $CaCO_3$). For strains harboring plasmids, 100 µg/mL of Ampicillin was added into all media. Each strain was fermented for 4 conditions as the following: (1.) pre-culture at 37^{O}C 24 hr and fermentation at 37^{O}C 48 hr (2.) anaerobic pre-culture at 37^{O}C 24 hr and anaerobic fermentation at 37^{O}C 48 hr (3.) aerobic pre-culture (200 rpm) at 37^{O}C 24 hr and anaerobic fermentation at 37^{O}C 48 hr (4.) aerobic pre-culture (200 rpm) at 37^{O}C 24 hr. and fermentation at 37^{O}C 48 hr with moisture. The concentration of L-lactic acid and residual glucose was analyzed by YSI Select Biochemistry Analyzer model: 2700 (YSI, Inc., USA).

Table 2. Primer list

Primer	Sequence
pr1	5' ACAGGTGGATCCGTCCTTTG 3'
pr2	5' ACCGGTACCGCGTACGCCTGCCG 3'
pr3	5' CGGCAGGCGTACGCGGTACCGGT 3'
pr4	5' GGAATACGGAATTCTGGATCACG 3'
pr5	5' GTAGCGCGTACGATGATTCCGGGGAT CCGTCG 3'
pr6	5' CCATGCCGTACGTGTAGGCTGGAGCTG CTTCG 3'
pr33	5' CTCAGTTTATAGGATCCAAGCAGTC 3'
pr34	5' TGTGTAAGCTTTACAATTCGATTGT 3'

* The underlined regions are enzyme restriction sites

3. Results

The *ldhA* gene from *R. oryzae* NRRL395 was amplified by PCR with pr33 and pr 34 primers. The approximately 1.8 kb PCR product, containing ORF of *ldhA* gene, promoter and ribosome binding site, was extracted, digested with restriction enzymes *Bam*HI and *Hin*dIII, and cloned into plasmid vector pBlueScript II KS(+). Plasmid with *ldhA* gene, giving the bands with the estimated sizes of 800 and 400 bp when it was cut by *Kpn*I, was named as pRB85 (Figure 1).Then, pRB85 was transformed to *E. coli* strain RB7, generated the strain named as RB24, and wild type, generated the strain named as RB30.

When RB24, RB7 and JC13509 were grown on the solid cultivation media with 1%w/v $CaCO_3$, very tiny colonies could be observed for only RB7 in the anaerobic condition (Figure 2) suggesting an importance of *ldhA* for growth under this condition. However, under the same condition, the growth of RB24 indicated that *ldhA* from *R. oryzae* on pRB85 was complemented the loss of *ldhA* on *E. coli* chromosome. Clear zones surrounding colonies of RB24 and JC13509 when grown under both aerobic and anaerobic conditions suggested acid production.

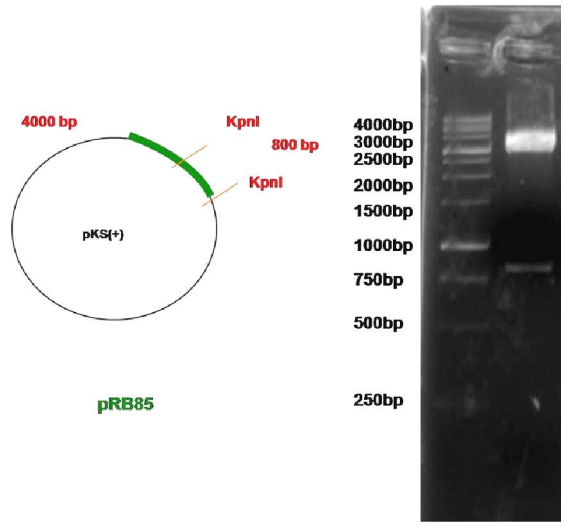

Figure 1. Verification of *R. oryzae ldhA* insertion in pBluescript II KS(+) by *Kpn*I digestion. Approximately 800 and 4000bp bands were generated when pBluescript II KS(+) plasmid containing *ldhA* gene from *R. oryzae* was cut with *Kpn*I.

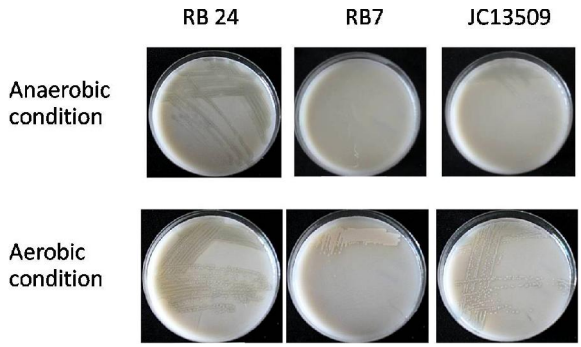

Figure 2. Growth of RB24, the strain harboring plasmid with *ldhA* gene from *R. oryzae* on solid media with $CaCO_3$ under anaerobic and aerobic conditions. All strains were grown on the solid cultivation media with 1% w/v $CaCO_3$ at 37^{o}C for 24 hr under anaerobic condition (top panel)) and under aerobic condition (bottom panel.

To obtain the effective process for lactic acid production of these *E. coli* strains, four fermentation conditions were tested in shake-flask level as the following: (1.) pre-cultured (10 g/L glucose) at 37^{O}C 24 hr and fermentation (100 g/L glucose) at 37^{O}C 48 hr (2.) anaerobic pre-culture (10 g/L glucose) at 37^{O}C 24 hr and anaerobic fermentation (100 g/L glucose) at 37^{O}C 48 hr (3.) aerobic pre-culture (10 g/L glucose) at 37^{O}C 200 rpm 24 hr and anaerobic fermentation (100 g/L glucose) at 37^{O}C 48 hr (4.) aerobic pre-culture (10 g/L glucose) at 37^{O}C 200 rpm 24 hr and fermentation (100 g/L glucose) at 37^{O}C 48 hr with moisture. However, the experiment of condition (2.) did not include the strain RB7 due to the poor growth under

anaerobic condition (Figure 1). Moreover, RB24 was tested if it could produce L-lactic acid when Ampicillin was not added in the media to maintain the plasmid pRB85because the result from Figure 1 suggested the importance of *ldhA* gene under anaerobic growth and the complementation of exogenous *ldhA* gene. It was found that in condition (1.) and (2.) when oxygen is limited or depleted, repectively, RB24 was able to produce L-lactic acid without the addition of Ampicillin (Table 3). This suggested that plasmid with *ldhA* was kept in these conditions. On the other hand, exogenous *ldhA* was not necessary in aerobic condition and then lost when cultured without Ampicillin as seen in condition (4.).

From Table 3, RB24 seemed to be able to produce L-lactic acid in all conditions with the highest amount of 5.03±4.149 g l^{-1} in condition (2.) when Amplicilin was not added to the pre-culture and fermentation media, but the production was relatively low. Moreover, there was high concentration of residual glucose in every culture in every condition that may inhibit the growth and L-lactic production. Therefore, the process for L-lactic acid production from the strain RB24 is required an improvement before going to a larger scale of fermentation.

4. Discussions

Many approaches, including genetic engineering, have been attempted to improve lactic acid production from various microorganisms. Due to the relatively easy manipulation, many efforts have been made to construct recombinant *E. coli* obtained *ldh* genes from other species (Mulok et al., 2009; Chang et al., 1999; Wyckoff et al., 1997; Contag et al., 1990). In this research, *ldhA* gene from *R. oryzae* was cloned into pBlueScriptII KS(+) and then, expressed in *E. coli*. One of the advantages of using *ldhA* from *R. oryzae* is the production of optically pure L-lactic acid. The result suggests that in *E. coli ldhA* knockout strain, it is complemented and also produces L-lactic acid by *ldhA* gene on the plasmid. In an anaerobic condition without the addition of antibiotic, plasmid with exogenous *ldhA* gene can be maintain in the cells suggests the requirement of *ldhA* gene under this condition. Moreover, it seemed that Amplicilin somehow inhibited L-lactic acid production of RB24 strain under an anaerobic condition (Table 3 : condition (2.)).

Although the genetically engineered *E. coli* in this study can produce L-lactic acid, the yield is still low. One of the investigations is the residual glucose after fermentation which may acts as an inhibitor. Therefore, the ingredients such as glucose concentration fermentation broth are needed to be adjusted. Moreover, the genetic instability which is a problem of gene expression on plasmid is observed. To solve this problem, the *ldhA* gene on *E. coli* chromosome is attempted to be replaced by one from *R. oryzae*. However, the *ldhA* genes from these two organisms are less than 40% identity, so there is such a time consuming process needed to do.

Table 3. L- lactic acid and residual glucose concentrations from *E. coli* strains under different fermentation conditions

Condition[a]	Strain[b]	L-lactic acid concentration (g/L)	Residual Glucose concentration (g/L)
(1.)	JC13509	0.42 ± 0.080	81.33 ± 8.031
	RB7	0.43 ± 0.083	98.43 ± 0.7638
	RB24 (No Amp[c])	3.86 ± 4.011	91.93 ± 6.468
	RB24	1.80 ± 2.564	84.70 ± 1.908
	RB29	0.27 ± 0.110	81.67 ± 9.471
	RB30	0.35 ± 0.141	86.77 ± 13.536
	RB31	0.41 ± 0.078	86.30 ± 4.636
(2.)	JC13509	0.34 ± 0.015	78.20 ± 8.455
	RB7	ND [d]	ND [d]
	RB24 (No Amp[c])	5.03 ± 4.149	82.03 ± 6.062
	RB24	0.57 ± 0.238	85.60 ± 10.048
	RB29	0.34 ± 0.053	85.77 ± 11.075
	RB30	0.33 ± 0.015	82.30 ± 5.966
	RB31	0.36 ± 0.031	80.83 ± 9.963
(3.)	JC13509	0.31 ± 0.038	45.00 ± 5.340
	RB7	0.22 ± 0.076	50.03 ± 4.834
	RB24 (No Amp[c])	1.18 ± 1.341	47.27 ± 0.924
	RB24	1.74 ± 1.386	51.40 ± 3.538
	RB29	0.30 ± 0.042	45.53 ± 3.691
	RB30	0.32 ± 0.048	44.17 ± 4.990
	RB31	0.36 ± 0.072	47.80 ± 1.473
(4.)	JC13509	0.27 ± 0.038	33.20 ± 18.654
	RB7	0.22 ± 0.125	46.00 ± 12.601
	RB24 (No Amp[c])	0.25 ± 0.091	48.30 ± 3.560
	RB24	1.95 ± 1.517	53.83 ± 2.196
	RB29	0.20 ± 0.131	40.50 ± 18.357
	RB30	0.28 ± 0.049	46.87 ± 2.290
	RB31	0.34 ± 0.071	40.63 ± 8.165

[a] These conditions are fermentation conditions as mentioned previously in Material and Methods.

[b] All strains, as mentioned in Table 1, are derivatives of JC13509

[c] All strains containing plasmids were cultured with 100 µg/ml Ampicillin. However, RB24 was tested if this strain was able to maintain pRB85 without Ampicillin addition as indicated in the table as RB24 (No Amp)

[e] Not determined due to the poor growth

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