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PRODUCTION OF CHEMICALS WITH GENETICALLY MODIFIED *ESCHERICHIA COLI* STRAINS FROM RENEWABLE RESOURCES

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ABSTRACT. The main aim of this study was to create mutant strains with lambda Red recombination system from an *Escherichia coli* strain which was isolated from animal faeces. *E. coli* Ter8/1 strain was genetically modified using lambda Red recombination system and two mutant strains were obtained, TerP01, in which the gene of pyruvate formate lyase was inactivated, and TerPL02, in which the genes of pyruvate formate lyase and lactate dehydrogenase were inactivated. The analysis of product formation in dual-phase fermentation and in minimal salts medium with three carbon sources was performed. The first mutant, TerP01, produced a large amount of lactic acid with small amount of byproduct formation. In the case of the second mutant, TerPL02, lactic acid production has been finished and succinic acid production increased significantly.

Keywords: *Escherichia coli, lambda Red recombination system, fermentation, glycerin, glucose, xylose*

INTRODUCTION

A large part of chemicals are derived from crude oil. Depletion of the earth's fossil energy resources, increasing prices and the adverse environmental effects of oil-based industries have given reasons to develop the production of bio-based chemicals from renewable feedstocks [1, 2]. In many cases, biosynthesis of chemicals is uneconomical, because the product yield and productivity are very low. Metabolic engineering provides a solution to these problems [3].

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An effective and commonly used tool of metabolic engineering is the lambda Red recombination system. This system, developed by Datsenko and Wanner, is an efficient method for preparing insertions, deletions and point mutations in the bacterial chromosomes [4]. The lambda Red system consists of three genes: Gam, Exo and Bet. This three genes encoding the major proteins, which are involved in the process [4, 5]. The first protein is Gam protein which inhibits the RecBCD and SbcCD nuclease activities, protecting linear DNA fragments from degradation [6-8]. The second protein is Exo which has a 5' to 3' double-stranded DNA (dsDNA) exonuclease activity and degrades dsDNA in the 5' to 3' direction generating 3'-ended overhangs [7, 9, 10]. The third protein is Beta which binds stably to the single-stranded DNA (ssDNA) and promotes annealing of two complementary DNA molecules and thereby promotes recombination [6-8]. Ellis et al. [10] demonstrated that Beta is the only protein which is required for recombination with ssDNA.

The recombineering method developed by Datsenko and Wanner has four main steps: (1) creating of a selectable antibiotic resistance gene by polymerase chain reaction (PCR) using primers, which are homologous to the target chromosomal gene, (2) transformation of the strain with a helper plasmid which contains the lambda Red genes, (3) electroporation of the PCR product into the host cell, where the recombination occurs between the target gene and the antibiotic resistance cassette and (4) elimination of the antibiotic resistance cassette [4].

Many researchers have also used this recombination method in order to develop a bacterial strain, which is able to produce chemicals in high yields, mostly in *E. coli* strains [11-13], but has also been used in other microorganisms, such as *Pseudomonas* [14] or in *Salmonella* strains [9].

In this paper, we have been developed a genetically engineered *E. coli* Ter8/1 strain, which was previously isolated from animal faeces by our team [15]. We was successfully used the lambda Red recombination system resulting two mutant strains, *E. coli* TerP01 and TerPL02, in which the pyruvate formate lyase (*pfl*) and pyruvate formate lyase together with lactate dehydrogenase (*ldh*) were knocked out and the fermentation of different carbon sources with these mutant strains have been examined.

RESULTS AND DISCUSSION

The wild-type *E. coli* strain Ter8/1 produced a mixture of acids during the fermentation from different carbon sources (Table 1). As we can see in Table 1, the main product was formic acid for all carbon sources used. In the case of glycerol, no lactate was detected, a small amount of acetate and

succinate were produced. With xylose and glucose, in addition to the acetate and succinate, a small amount of lactate was produced. Therefore, in order to alter the metabolic pathway to another useful product, the *pflB* gene was knocked out at first, thus preventing the production of formic acid (Figure 1). The elimination of *pflB* gene from the chromosomes of *E. coli* Ter8/1 strain was previously published [16]. This *E. coli* Ter8/1 *pflB* mutant is designated TerP01.

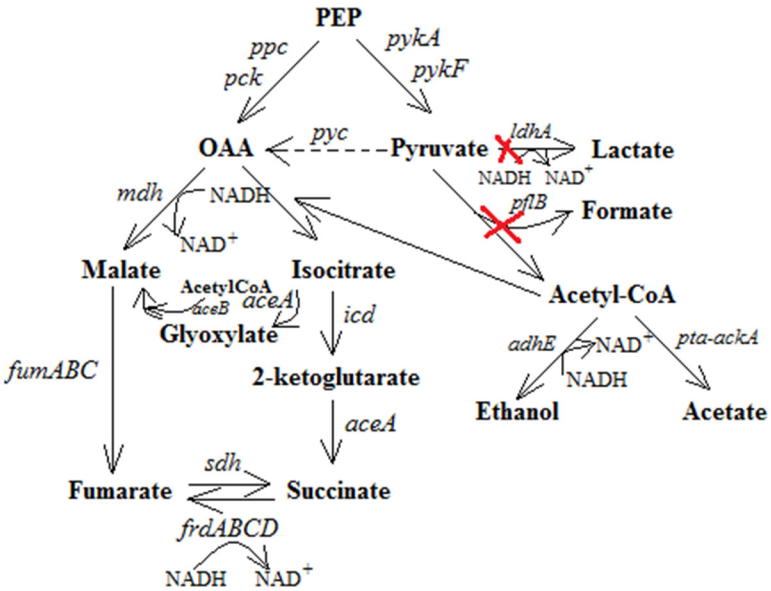

Figure 1. A simplified illustration of metabolic pathways from phosphoenolpyruvate (PEP) to fermentation products in *E. coli*. The genes inactivated in this study are illustrated by red cross bars (pyruvate formate lyase, lactate dehydrogenase). OAA, oxaloacetate; *ppc*, phosphoenolpyruvate carboxylase; *pck*, phosphoenolpyruvate carboxykinase; *pykA* and *pykF*, pyruvate kinases; *pyc*, pyruvate carboxylase; *ldhA*, lactate dehydrogenase; *mdh*, malate dehydrogenase; *pflB*, pyruvate formate lyase; *aceA*, isocitrate lyase; *aceB*, malate synthase; *fumABC*, fumarase isoenzymes; *icd*, isocitrate dehydrogenase; *adhE*, aldehyde-alcohol dehydrogenase; *pta*, phosphate acetyltransferase; *ackA*, acetate kinase; *sdh*, succinate dehydrogenase; *frdABCD*, fumarate reductase; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide

The fermentation products produced in dual-phase fermentation from different carbon sources by *E. coli* TerP01 strain were examined. The substrate utilization and the cell growth decreased with a large reduction in the production of formic acid in all three carbon sources. During glycerol fermentation, TerP01 strain grew slower, than during xylose or glucose

fermentation. The cell yield decreased with 45%, 29% and 6% in glycerol, xylose and glucose fermentation, respectively (Table 1). Production of acetic acid was also declined, succinic acid reduced in the case of glycerol fermentation, but increased slightly in xylose and glucose fermentation. However, the lactic acid formation increased significantly. Lactic acid is a widely used special chemical in the food, pharmaceutical, textile and chemical industries, and it is used as a monomer in the production of polylactate, which is a biodegradable plastic [17, 18]. Several studies deal with lactic acid production with various bacteria and fungi [19-23]. Based on the published studies, the *E. coli* strains correspond well for the purpose of lactic acid production. Good results have been achieved with glucose fermentation, Dien et al. [24] designed an *E. coli* B mutant (FBR11) which produced lactic acid in anaerobic conditions with concentration of 93% of the theoretical maximum. Afterwards, Grabar et al. [25] and Zhu et al. [26] better results have been achieved with the *E. coli* mutants, TG114 and ALS974, respectively.

Table 1. Product synthesis of *E. coli* Ter8/1 and mutant strains after 96 h fermentation in M9 minimal medium with different carbon sources

Strain	Genetic modification	Sub-strate	Cell mass (g/l)	Sub-strate used (mM)	Fermentation product conc (mM)[b]					
					Lac	Lac yield[a]	Suc	Suc yield[a]	For	Ac
Ter8/1	Wild-type	gly	0.4	33	ND	-	14	0.42	62	13
		xyl	0.58	33	7	0.21	10	0.3	52	11
		gluc	0.53	25	13	0.52	4	0.16	57	15
TerP01	Δ*pflB*	gly	0.22	30	29	0.97	6	0.2	ND	4
		xyl	0.41	30	49	1.63	13	0.43	ND	7
		gluc	0.5	27	48	1.77	7	0.26	2	ND
TerPL02	Δ*pflB* Δ*ldhA*	gly	0.21	30	ND	-	27	0.9	ND	5
		xyl	0.4	29	ND	-	35	1.2	ND	2
		gluc	0.42	22	ND	-	28	1.27	ND	ND

ND not detected

[a] Calculated as mol of lactate/succinate produced in the anaerobic phase divided by the mol of substrate metabolized in the anaerobic phase.

[b] Fermentations were carried out in M9 minimal medium supplemented with the appropriate carbon source in concentration of 5 g/l (37°C, 150 rpm).

Abbreviations: gly, glycerol; xyl, xylose; gluc, glucose; Lac, lactic acid; Suc, succinic acid; For, formic acid; conc, concentration. Data represent an average of three experiments.

TG114 mutant produced 98% lactic acid in mineral salts medium and with ALS974 mutant 99% of the theoretical yield in dual-phase fed-batch fermentation was achieved. Zhou et al. [27] and Zhao et al. [28] produced lactic acid with engineered *E. coli* strains from xylose. In complex media, a mutant of *E. coli* B produced 62 g/l lactic acid from xylose, which was 97% of the theoretical maximum [28]. With the *E. coli* W3110 mutant (SZ85) in minimal salts medium, Zhou et. al achieved a yield of 93% [27]. There are some studies which were directed to the production of lactic acid from glycerol [29-31]. A mutant strain of *E. coli* K-12 MG1655 (LA02Δ*dld*) designed by Mazumdar et al. [29] produced lactic acid in minimal medium with a yield of 85%. Tian et al. [30] and Chen et al. [31] published a yield of 78 g lactic acid/100 g glycerol and 75.4 g lactic acid/100 g glycerol with engineered strains of *E. coli*, CICIM B0013-070(pUC-*ldhA*) and B0013-070-pTHldhA, respectively. Our engineered strain, *E. coli* TerP01, produced lactic acid in concentration of 29 mM from glycerol with a molar yield of 0.97 mol/mol (Table 1, Figure 2), which is 97% of the theoretical maximum. From xylose, the mutant strain produced a concentration of 49 mM of lactic acid with a yield of 1.63 mol/mol (Table 1, Figure 2), which is 98% of the theoretical maximum. In the case of glucose, lactic acid concentration was 48 mM and the molar yield was 1.77 mol/mol (Table 1, Figure 2), which is 89% of the theoretical maximum.

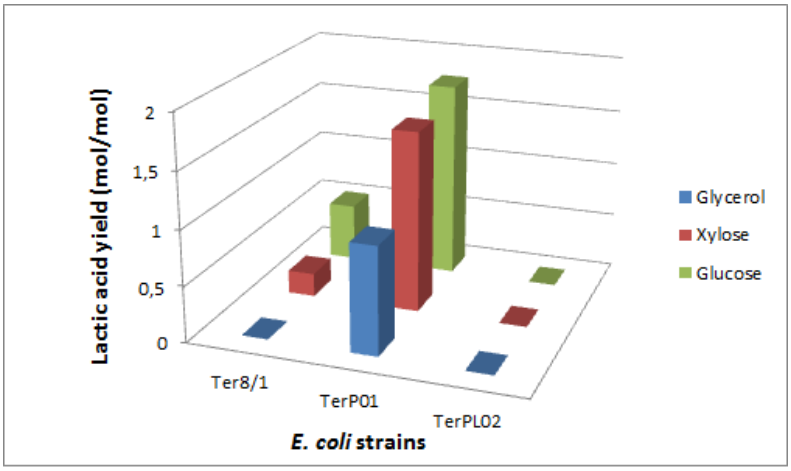

Figure 2. Effect of the genetic modification and substrates on the lactic acid yield

In order to divert the carbon flux to succinic acid, the *ldhA* gene was knocked out (Figure 1). The steps of gene deletion are shown in Figure 3A. The *ldhA*1-FRT-camR-FRT-*ldhA*2 antibiotic resistance gene was first amplified by PCR from pKD3 plasmid using *ldhA*1 and *ldhA*2 primers (Table 3). *E. coli*

TerP01 was second transformed with pKD46 plasmid, which contains the genes required for the recombination [4]. These enzymes catalyzed the recombination between the *ldhA*1-FRT-camR-FRT-*ldhA*2 cassette and the *ldhA* locus in the chromosome. The correct transformants were selected on plates containing chloramphenicol and the pKD46 plasmid have lost by growing at high temperature. For elimination of the antibiotic resistance, the pCP20 plasmid was used. This plasmid encodes the enzyme flippase, which is recognized the FRT sites and removed the section between them, leaving behind a short nucleotide sequence with one FRT site [32]. The correct replacement of the *ldhA* and the later removal of antibiotic resistance were confirmed by PCR analysis (Figure 3B) using *ldhA*3 and *ldhA*4 primers (Table 3). This *E. coli* Ter8/1 *pflB* and *ldhA* double mutant is designated TerPL02.

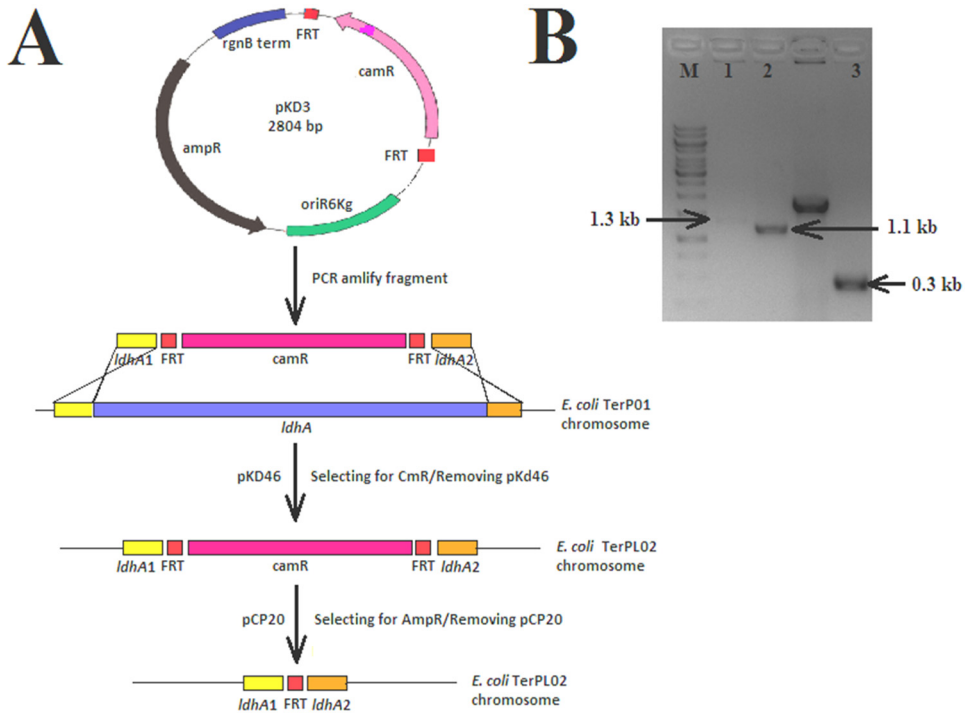

Figure 3. Elimination of *ldhA* gene from the chromosomes of *E. coli* TerP01. **A**. The *ldhA* gene was replaced with the antibiotic resistance gene amplified by PCR using pKD46 plasmid and then, the chloramphenicol resistance was eliminated with the help of pCP20 plasmid. **B**. Verification of deletions by PCR analysis (M: 1 kb DNA molecular weight marker, Fermentas, 1: *ldhA* gene from the chromosomes of *E. coli* TerP01, 2: antibiotic resistance gene from *E. coli* TerPL02, 3: remaining part *of ldhA* gene in chromosomes of *E. coli* TerPL02

During dual-phase fermentation, the cell yield of *E. coli* TerPL02 double mutant strain was a little less, than the cell yield of strain TerP01. Production of acetic acid and formic acid decreased or remained unchanged. After deletion of the second gene, *ldhA*, lactic acid formation stopped and the production of succinic acid increased significantly. Succinic acid is an important chemical in the agricultural, pharmaceutical and food industries. It has a wide range of application and it is a raw material for many special chemicals such as biodegradable polymers and solvents, and fuel additives [3, 33]. There are several studies aimed at the production of succinic acid with genetically modified *E. coli* strains [33-36]. In the production of succinic acid from glucose, researchers have achieved good results, such as Wang et al. [37] obtained a yield of 0.84 g succinic acid/g glucose with an *E. coli* mutant, TUQ19(pQZ26), in dual-phase fermentation. Vemuri et al. [38] achieved a yield of 1.1 g succinic acid/g glucose with *E. coli* strain AFP111(pTrc99A-*pyc*) using complex medium. Andersson et al. [33] produced succinic acid from xylose by *E. coli* strain AFP184 in dual-phase fermentation with a yield of 0.5 g/g. Glycerol was also used as carbon source for the production of succinic acid. Blankschien et al. [36] and Zhang et al. [39] produced succinic acid from glycerol in anaerobic conditions, achieved a yield of 0.69 g succinic acid/g glycerol and 0.49 g succinic acid/g glycerol, respectively. Our results for succinic acid production from glycerol was 0.9 mol/mol (1.15 g/g), which was higher 2.1-fold compared to the wild-type strain and it was 90% of the theoretical maximum (Table 1, Figure 4). The succinic acid yield in xylose was 1.2 mol/mol (0.94 g/g), which was higher 4-fold compared to the Ter8/1 strain and it was 85% of the theoretical maximum (Table 1, Figure 4). In the case of glucose as carbon source, we obtained a yield of 1.27 mol/mol (0.83 g/g), which was higher 7.9-fold compared to the wild-type strain and it was 74% of the theoretical maximum (Table 1, Figure 4).

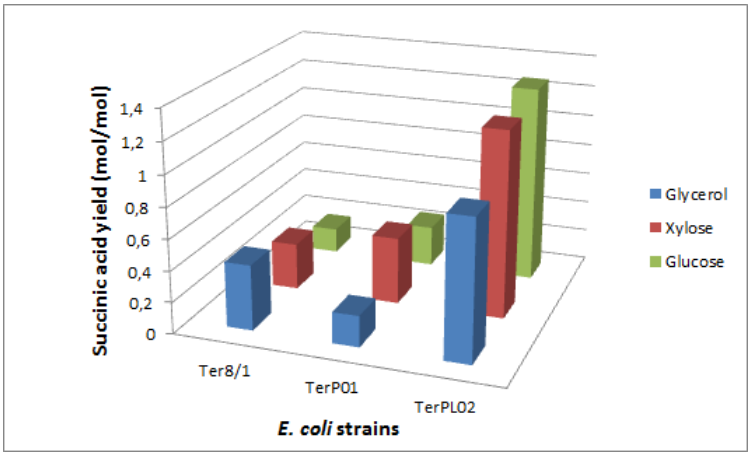

Figure 4. Effect of the genetic modification and substrates on the succinic acid yield

CONCLUSIONS

In this study, an *E. coli* strain Ter8/1, which was isolated from animal faeces, was engineered to produce organic acids by blocking the formic acid and lactic acid formation pathways. The first mutant, TerP01, in which the pyruvate formate lyase was inactivated, was able to convert glucose, xylose and glycerol to lactic acid effectively. Lactate fermentation can theoretically be achieved in *E. coli* with a lactate yield of 2 mol/mol glucose and we obtained a lactic acid yield of 1.77 mol/mol glucose under dual-phase fermentation in minimal medium. From xylose, lactic acid formation can theoretically be reached a lactate yield of 1.67 mol/mol xylose and we achieved a lactic acid yield of 1.63 mol/mol xylose. During the fermentation of glycerol in minimal salts medium with dual-phase fermentation, we obtained a molar yield of 0.97 mol lactic acid/mol glycerol, which was 97% of the theoretical yield. In the case of the second mutant, TerPL02, in which the pyruvate formate lyase and lactate dehydrogenase were inactivated, the succinic acid production increased significantly. The yields obtained from different carbon sources were 0.9 mol/mol glycerol, 1.2 mol/mol xylose and 1.27 mol/mol glucose, which were 90%, 85% and 74% of the theoretical maximum, respectively. Our results confirmed that an isolated strain can be genetically modified and the genetic engineering to reduce the reductive by-product is important in point of view product formation. All these results further suggested that the engineered TerP01 and TerPL02 strains are a promising alternative in the bioconversion of different carbon sources to lactic acid and succinic acid.

In the future, fermentation process will be optimized for lactic acid and succinic acid production in order to improve the productivity and the mutant strains obtained will be further engineered to reduce all of the remaining byproducts.

EXPERIMENTAL SECTION

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2. The *E. coli* strains were cultured in Luria Bertani (LB) broth at 37°C.

To the construction of *E. coli* TerPL02 strain, the lambda Red recombination system was used [4]. The lactate dehydrogenase (*ldhA*) gene was knocked out from the chromosomes of *E. coli* TerP01 (Figure 3). The gene-specific antibiotic resistance cassette was amplified from pKD3 plasmid by PCR using primers *ldhA*1 and *ldhA*2 (Table 3). The PCR program was as follows: 94°C for 5 min,

followed by 34 cycles at 94°C for 15 s, 60°C for 30 s, 68°C for 2 min, plus an additional 7 min at 68°C and a subsequent incubation at 4°C. The PCR product obtained was purified with AccuPrep PCR Purification Kit (Bioneer, South Korea) according to the manufacturer's protocol. The purified PCR product was treated with 1U *Dpn*I in a total volume of 50 µl at 37°C for 2 hours, followed by ethanol precipitation and resuspension in 10 µl dH_2O.

Table 2. Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant characteristics	Source or reference
Strains		
E. coli Ter8/1	Wild-type *E. coli* isolated from animal faeces	[15]
E. coli TerP01	Ter8/1 Δ*pflB*: FRT, sequential deletion of *pflB* in Ter8/1	[16]
E. coli TerPL02	Ter8/1 Δ*pflB*: FRT, Δ*ldhA*: FRT, sequential deletion of *pflB* and *ldhA* in Ter8/1	This study
Plasmids		
pKD46	araBp-gam-bet-exo, bla(ApR), oriR101, repA101(ts)	CGSC Yale University
pKD3	oriR6Kgamma, bla(ApR), rgnB(Ter), FRT-cat-FRT	CGSC Yale University
pCP20	ts-rep, cI857(lambda)(ts), bla(ApR), cat, FLP	CGSC Yale University

Table 3. Primers used in PCR analysis

Primer name	Sequence	Description
*ldhA*1	5'-GAATAGAGGATGAAAGGTCATTGGGGATTAT CTGAATCGGCTGTAGGCTGGAGCTGCTTCG-3'	Used for camR cassette amplification
*ldhA*2	5'-TGTGATTCAACATCACTGGAGAAAGTCTTAT GAAACTCGCATGGGAATTAGCCATGGTCC-3'	
*ldhA*3	5'-GCACAAAGCGATGATGCTGTAG-3'	Used to confirm camR presence in *ldhA* gene and/or *ldhA* disruption
*ldhA*4	5'-CCGTTCAGTTGAAGGTTGCG-3'	

Underlined Nucleotides are homologous to *ldhA* regions

Then, the prepared cassette was electroporated into *E. coli* TerP01 strain, in which the pKD46 plasmid previously has been introduced by electroporation. With the help of L-ara-induced helper plasmid, the recombination takes place between the *ldhA* gene and antibiotic resistance cassette. The transformants were selected on LB agar containing 10 µg/ml chloramphenicol at 37°C. To the

elimination of antibiotic resistance, the pCP20 plasmid was introduced into the mutant cells by electroporation. The cells were selected on LB agar containing 100 µg/ml ampicillin and incubated at 30°C. The successful insertion and deletion of the resistance cassette was confirmed by colony-PCR using *ldhA*3 and *ldhA*4 primers (Table 3). The removal of pKD46 and pCP20 was done by incubation at 42°C for two days. The resulting PCR products were checked on a 1% agarose gel stained with ethidium bromide and visualized using a BioRad transilluminator.

Fermentation condition

Two-phase fermentation was used for analyzing the product formation by mutant *E. coli* strains. In the first step, the cells were grown aerobically in 5 ml LB broth at 37°C and 150 rpm overnight. Cells were harvested by centrifugation at 4500 rpm for 5 min and resuspended in fermentation broth. Fermentation was carried out in M9 minimal medium containing (per liter): 12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, micronutrients final concentration: 1 mM $MgSO_4$, 100 µM $CaCl_2$, $3 \cdot 10^{-9}$ M $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $4 \cdot 10^{-7}$ M H_3BO_3, $3 \cdot 10^{-8}$ M $CoCl_2 \cdot 6H_2O$, $1 \cdot 10^{-8}$ M $CuSO_4 \cdot 5H_2O$, $8 \cdot 10^{-8}$ M $MnSO_4$, $1 \cdot 10^{-8}$ M $ZnCl_2$, $1 \cdot 10^{-6}$ M $FeSO_4 \cdot 7H_2O$. Concentration of the carbon sources, glucose, xylose and glycerol were used in 5 g/l. Cells were placed into a 100 ml flask containing 20 ml fermentation broth and were cultivated aerobically at 37°C and 200 rpm for 16 hours. After 16 hours, cells were harvested by centrifugation at 4500 rpm for 10 min and transferred in sealed 150 ml bottles containing 75 ml fermentation broth with appropriate carbon source and the air above the liquid layer was changed with oxygen free CO_2 gas. The anaerobic production phase was carried out at 37°C for 96 hours.

Analysis

Cell dry-weight was determined by measuring the optical density at 550 nm using a Carry 50 UV-Visible spectrophotometer. We used the 1 OD_{550}=0.34 g dry weight/l simple assumption to estimate the cell mass.

In order to determine the concentration of fermentation end-products, 1 ml of culture sample was centrifuged at 14000 rpm for 10 min and the supernatant was filtered through 0.2 µm pore-size syringe filter. The resulting sample was used for high-performance liquid chromatography (HPLC) analysis. The HPLC system was equipped with UV detector, refractive index detector, and an ion-exchange column (7.8 mm x 300, ICSep Coregel 87H3, Transgenomic). The mobile phase was 0.008 N H_2SO_4 and the flow rate 0.6 ml/min during elution. The column temperature was 50°C. Acids were measured by UV detector at 210 nm and sugars were measured by the RI detector.

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