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Increased Production of Recombinant Protein by *Escherichia coli* Deficient in Acetic Acid Formation

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Abstract The effect of acetic acid formation deficiency on recombinant *E. coli* fermentation was investigated using a mutant strain deficient in acetic acid formation. A mutant strain which does not grow under anaerobic conditions was isolated. The acetic acid production in this strain was negligible in aerobic batch fermentation. The cloned-gene expression in the mutant strain was higher than the wild-type strain. Fed-batch fermentations with controlled specific growth rates were carried out in order to compare the cloned-gene expression between the wild-type and the mutant strains. The expression decreased along with the specific growth rate in both strains. The cloned-gene expression in the mutant strain was 60% higher than in the wild-type strain at the same specific growth rate.

Key words: *E. coli*, fermentation, acetic acid, cloned-gene expression

Escherichia coli is widely used as a host cell for the production of recombinant proteins [3, 12-14, 16, 21, 24], particularly for the development of effective fermentation methods. In a high cell density culture, one major problem is the formation of undesirable by-products. Among these by-products, acetic acid is the most troublesome, since it inhibits cell growth as well as product syntheses [23]. Acetic acid formation in *E. coli* fermentation is generally thought to be due to a limited oxygen uptake rate [23], an alteration in the carbon flow toward acetic acid as a result of the accumulation of $NADH^+$ [5], or limitations in both the TCA cycle capacity and electron transport system [17]. Han *et al.* [9] suggested that acetic acid formation was the result of limitations in the oxidative metabolism, especially the TCA cycle. Under such limiting conditions, they proposed that *E. coli* reorganizes the oxidative metabolism to ensure that the anabolic requirements are satisfied with respect to the oxidative metabolism, whereas the catabolic demand is met using both the remaining oxidative metabolism capacity and the metabolic pathway leading to acetic acid formation.

Three approaches exist to solving the negative effects on cell growth and cloned-gene product formation: the reduction of the amount of acetic acid formed, removal of acetic acid from the culture broth, and alleviation of the negative effect of the presence of acetic acid. Acetic acid formation can be reduced by controlling the glucose feeding [15, 27], switching carbon sources [10], or lowering the rate of glucose uptake by the use of corn steep liquor [2]. Acetic acid can be removed from the culture broth by dialysis [11, 20]. The negative effect of acetic acid can be alleviated by an efficient supply of oxygen by the use of oxygen-enriched air [6] and by supplementing the culture media with a yeast extract [19] or methionine [8].

Diaz-Ricci *et al.* [4] investigated an interesting alternative for the reduction of acetic acid formation. They used a mutant *E. coli* strain whose acetic acid synthesis is genetically blocked. The mutant strain has a double deletion mutation in the genes corresponding to acetyl phosphotransferase (PTA) and acetate kinase (ACK). However, the mutations in PTA and ACK also resulted in negative effects on the metabolism of *E. coli*. The present study reports on an investigation of the effect of an acetic acid formation deficiency on recombinant *E. coli* fermentation, using two mutants which are deficient in acetic acid formation.

MATERIALS AND METHODS

Bacterial Strains

The *E. coli* strains and plasmid (listed in Table 1) used for this research were kindly provided by Prof. J. H. Lee (Department of Biology, Sung Kyun Kwan University, Suwon, Korea). JIL strains were constructed from MG1655 or TA3516. The wild-type strain JIL1715 was

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Table 1. *E. coli* strains and plasmids used for this research.

		Genotype	Reference
E. coli	MG1655	Wild-type K-12 (λ-, F-)	[7]
	JIL1715	MG1655, *Δ(pro-lac)*	
	JIL1716	MG1655, *Δ(pro-lac), ana*	
	TA3516	F^-, λ^-, *Δ(his-gnd), Δ(pta-ack-dhuA-hisP)*	[1]
	JIL1725	TA3516, *Δ(pro-lac)*	
Plasmid	pMKT2-1	*bla, lacZ*	[18]

used as a basis of comparison. JIL1716 is a mutant strain which does not grow under anaerobic conditions. Its genotype is not identified and is herein referred to as *ana*. Acetic acid production in this strain is negligible in aerobic conditions. JIL1725 is a mutant strain in which acetic acid synthesis is genetically blocked. It has a double deletion mutation in the genes corresponding to acetyl phosphotransferase (PTA) and acetate kinase (ACK).

Fermentation

The batch and fed-batch fermentations were carried out in a fermentor (Korea Fermentor Co., Inchon, Korea) containing 2 l of M56 medium. One liter of the M56 medium contains 2 g glucose, 5.3 g KH_2PO_4, 21.95 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.5 g $(NH_4)_2SO_4$, 0.01 g $Ca(NO_3)_2$, 0.0005 g $FeSO_4 \cdot 7H_2O$, 1 g yeast extract, 100 mg ampicillin, and 100 mg L-prolin. The inocula were prepared using the same medium and grown overnight at 37°C. The cultures were agitated at 500 rpm with an air flow rate of 1 vvm, at 37°C, and a pH of 7.0 was controlled with 5 N NaOH.

In the fed-batch fermentations, the glucose concentration was limited to near zero in order to minimize the acetic acid formation. The specific growth rate was controlled at a constant value with an exponential feeding of a glucose solution. In the early phase of the fermentation, the feed was not added until the glucose in the medium was depleted and the concentration of the dissolved oxygen began to increase. One liter of the feeding medium contained 200 g glucose, 7.8 g $MgSO_4 \cdot 7H_2O$, 100 g $(NH_4)_2SO_4$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.316 g $CaCl_2$, 0.0375 g $MnSO_45H_2O$, 0.0065 g $CoCl_2 \cdot 6H_2O$, 0.0375 g $ZnSO_4 \cdot 7H_2O$, 0.0099 g $CuCl_2 \cdot 2H_2O$, 0.0065 g $Na_2MoO_4 \cdot 2H_2O$, and 5 g L-prolin.

The feeding rate was determined using a mass balance equation of the cell and substrate, represented by $F=(\mu X_0 V_0/Y S_F) e^{\mu t}$, where μ is the set point of the specific growth rate and S_F is the substrate concentration in the feeding medium. The calculated value of the flow rate was converted to analog signals which operated a feeding pump (Cole-Parmer, Ismatec REGLO 100, Vernon Hills, U.S.A.). The dissolved oxygen in the medium was maintained at a concentration above 20% by either regulating the agitation speed or using oxygen-enriched air during the fed-batch operation.

Analytical Procedures

The dry cell weight was obtained by drying washed cells at 90°C for 12 h. The glucose concentration was measured using a Sigma Glucose Kit (Procedure No. 510, St. Louis, U.S.A.). The acetic acid concentration was determined by HPLC. A cation-exchange column (Aminex HPX 87-H, 300×7.8 mm, BioRad, Hercules, U.S.A.) was employed where the flow rate of the mobile phase (0.005 M H_2SO_4) was 0.4 $cm^3 min^{-1}$. The fermentation broth was centrifuged and the supernatant was mixed with 5 mol dm^{-3} H_2SO_4 (1:1). The mixture was centrifuged (15,000 rpm, 15 min) and the supernatant was then filtered through a 0.2 μm membrane. One hundred microliters of the prepared samples and a standard acetic acid solution were used for the analysis of acetic acid by measuring absorbance at 210 nm. The activity of β-lactamase was determined by an iodometric assay [22, 25, 26].

RESULTS AND DISCUSSION

Batch Fermentation

Wild-type strain (JIL1715/pMKT2-1) and mutant strain deficient in acetic acid formation (JIL1716/pMKT2-1)

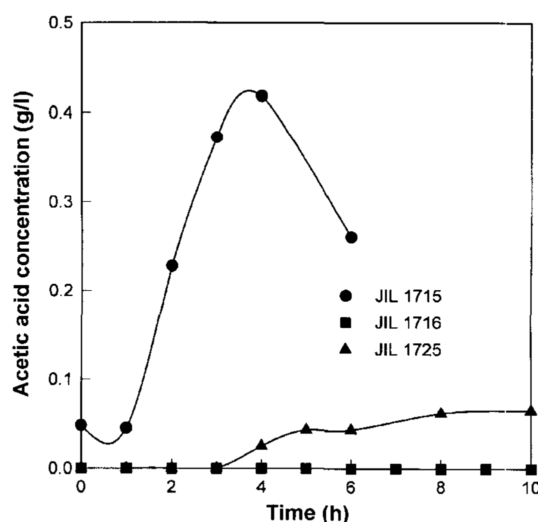

Fig. 1. Acetic acid formation in batch fermentations.

Table 2. Results of batch fermentation.

	JIL1715/pMKT2-1	JIL1716/pMKT2-1
μ_m (h^{-1})	0.88	0.51
Final Cell Concentration (g dm^{-3})	0.83	0.80
$Y_{x/s}$ (g cell per g glucose)	0.43	0.37
Maximum Concentration of Acetic Acid (g dm^{-3})	0.42	0
Maximum Specific Activity of β-Lactamase (units per mg cell)	5.90	6.61

were cultured to investigate the effect of acetic acid formation deficiency on fermentation by recombinant *E. coli*. The specific growth rates of JIL1715/pMKT2-1 and JIL1716/pMKT2-1 during the exponential growth phase were 0.88 h^{-1} and 0.51 h^{-1}, respectively, indicating that the mutant strain grew at a slower rate than the wild-type strain. The final cell concentration of the mutant strain was nearly the same as that of the wild-type. The concentration of acetic acid produced by the wild-type strain reached a maximum value and then decreased as shown in Fig. 1, indicating that the acetic acid produced was used as a carbon source after the consumption of glucose was complete. The glucose in the wild-type strain fermentation was completely consumed after 4.5 h (data not shown), whereas glucose remained in the medium for about 7 h in the mutant strain fermentation. JIL1725 is another mutant strain in which acetic acid synthesis is genetically blocked [1], and exhibits a double deletion mutation in its genes corresponding to acetyl phosphotransferase (PTA) and acetate kinase (ACK). This strain still produced acetic acid as shown in Fig. 1, although the amount produced was much less than in the case of the wild-type strain fermentation. This synthesis of acetic acid has been attributed to the activity of the *ackB* gene product [1]. In the case of JIL1716//pMKT2-1, the acetic acid concentration was zero during the fermentation, indicating that the JIL1716 was more deficient in acetic acid formation than JIL1725.

The JIL1716/pMKT2-1 fermentation resulted in a higher final specific activity of β-lactamase than the wild-type strain. The fermentation results are summarized in Table 2. $Y_{x/s}$ was calculated from the slope of the cell concentration vs. glucose concentration curve during the period of maximum exponential growth. The results of the absence of acetic acid formation and higher expression of a cloned-gene product in JIL1716/pMKT2-1 are of great interest with respect to fed-batch fermentations.

Fed-Batch Fermentation with Controlled Specific Growth Rate

Fed-batch fermentations were carried out at various values of controlled specific growth rate along with an exponential feeding of the glucose solution. Figure 2 shows the cell growth in the fed-batch fermentations of the wild-type strain. The set points of the specific growth rates were 0.1, 0.2, 0.3, and 0.45 h^{-1} in each fermentation. The

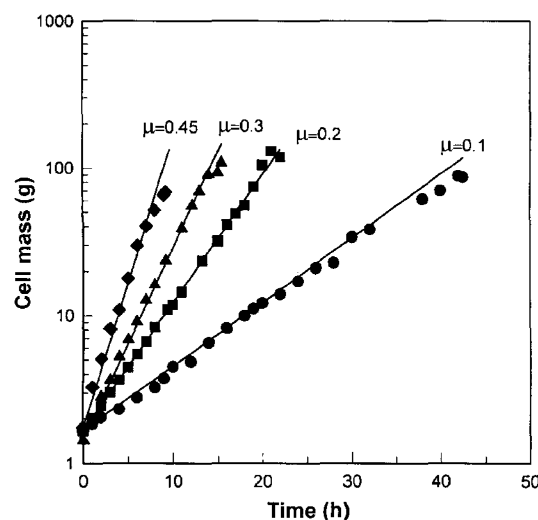

Fig. 2. Cell growth of wild-type strain JIL 1715 in fed-batch fermentations.

specific growth rate in a fed-batch fermentation is not (1/X)(dX/dt) but (1/XV)(d(XV)/dt) (=d[ln(XV)]/dt) since V is not constant. Therefore, the slopes (d[ln(XV)]/dt) of the cell mass curves represent specific growth rates. The cell mass curves of the wild-type strain were linear as shown in Fig. 2, indicating that the specific growth rate was well controlled at the set point in each fermentation. The time indicated by the X-axis in all the subsequent figures is the time after the start of feeding.

Figure 3 shows the cell growth in the fed-batch fermentation of the mutant strain (JIL1716/pMKT2-1). The set points of the specific growth rates were 0.1, 0.17, and 0.3 h^{-1} for each fermentation. These rates were well controlled in the first two cases. However, this was not the case in the third case. The specific growth rate in the third case was maintained at 0.24 h^{-1} rather than 0.3 h^{-1} during the first 5 h. It is thought that this was caused by the limited specific growth rate in the fed-batch fermentation. The specific growth rate in the late exponential phase of the batch fermentation was 0.25 h^{-1}. Unconsumed glucose began to accumulate significantly (data not shown) and the production of acetic acid was observed 6 h after the start of feeding. Thereafter, the specific growth rate decreased even further.

Figures 4 and 5 show profiles of the acetic acid concentration for the wild-type (JIL1715/pMKT2-1) and

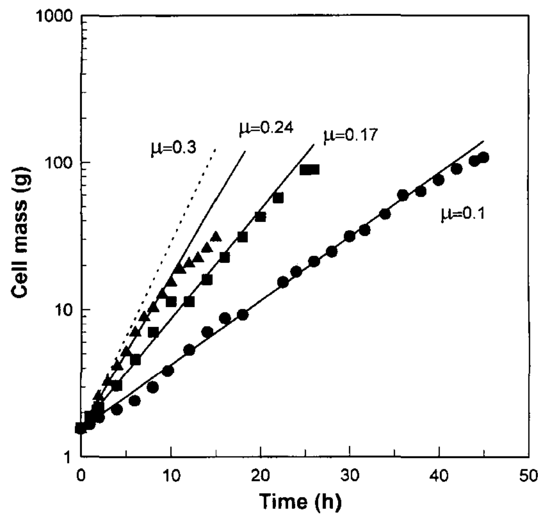

Fig. 3. Cell growth of mutant strain JIL1716 in fed-batch fermentations.

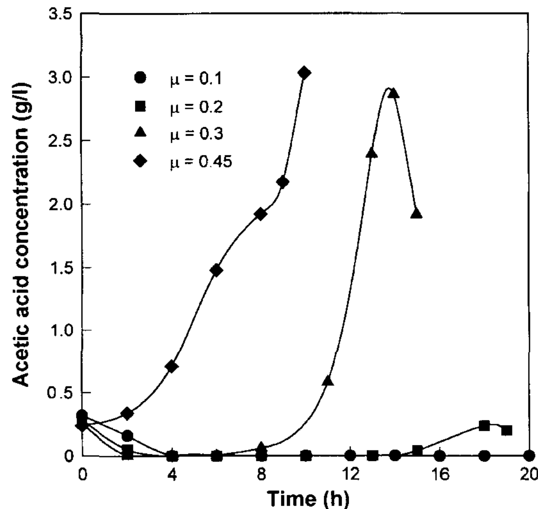

Fig. 4. Acetic acid formation of wild-type strain JIL1715 in fed-batch fermentations.

mutant (JIL1716/pMKT2-1) strains, respectively. The level of acetic acid increased along with the specific growth rate in both strains. Although the mutant strain produced considerably less acetic acid than the wild-type strain, a significant accumulation of acetic acid was observed when the set point was μ=0.3 h^{-1}. As shown earlier in Fig. 1, the acetic acid accumulation was zero in the batch fermentation of JIL1716/pMKT2-1. The acetic acid production in the fed-batch operation of the mutant strain may be caused by a reversion of the mutation. To determine if this, in fact, occurred, we carried out a batch fermentation inoculated with cells producing acetic acid during the fed-batch operation. Acetic acid accumulation was not detected, indicating that such a reversion did not occur. Accordingly, the acetic acid accumulation in the fed-batch operation was

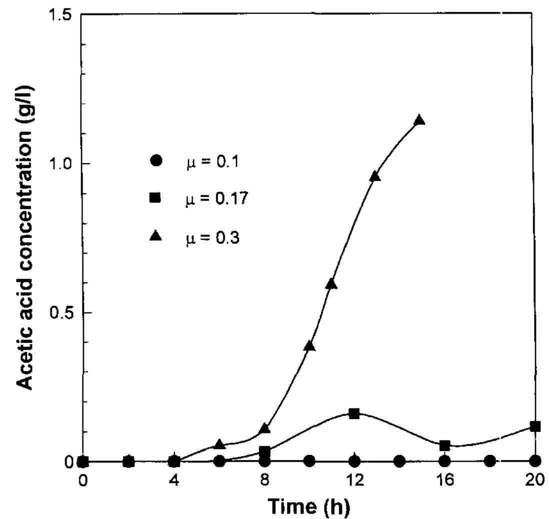

Fig. 5. Acetic acid formation of mutant strain JIL1716 in fed-batch fermentations.

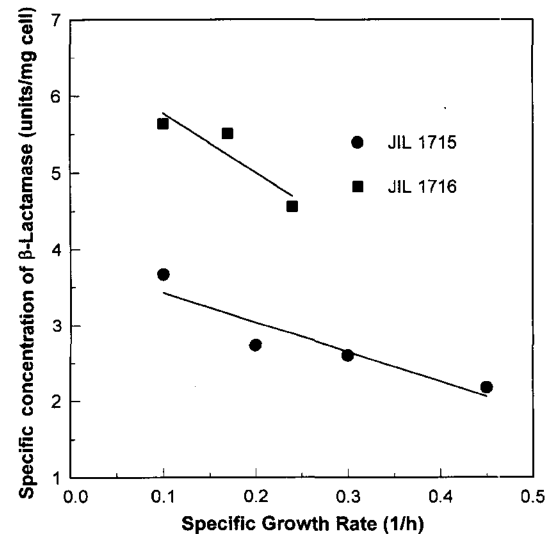

Fig. 6. Expression of β-lactamase in fed-batch fermentations.

most likely the result of the accumulation of glucose in the medium. The glucose level was well controlled at nearly zero in the other cases; however, glucose began to accumulate significantly 6 h after the start of feeding in this case, as described earlier.

The maximum specific β-lactamase concentration depends on the controlled specific growth rate: it decreased linearly as the specific growth rate increased as shown in Fig. 6. The linear relationship between the maximum specific concentration of the cloned-gene product and the controlled specific growth rate was also observed in the case of bovine somatotropin fermentation [28-30]. The β-lactamase expression in the mutant strain was 60% higher than in the wild type strain at the same specific growth rate.

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