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Optimization of Culture Conditions and Analysis of Plasmid Stability of a Transformant *Bacillus subtilis* for Cytidine Deaminase Production

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The transformant *Bacillus subtilis* ED213 carrying the pSO100 which cloned the *cdd* gene encoding cytidine deaminase (cytidine /2'-deoxycytidine aminohydrolase, EC 3.5.4.5, CDase) originated from wild type *B. subtilis* was cultivated in Spizizen minimal medium (SMM). To overcome poor expression of the *cdd* gene in SMM medium, the medium compositions and growth conditions were optimized. The optimized medium compositions and growth conditions were cytidine concentration of 80 mg/l, glycerol of 25 g/l, and $(NH_4)_2SO_4$ of 10 g/l, along with 37°C and pH 7.0. The intracellular CDase production was increased 3 times from 1,000 unit/ml to 3,200 unit/ml, and extracellular CDase also increased from nearly undetectable amounts to 1,500 unit/ml. The cytidine concentration was signified as the most critical compositional factor for overproduction of CDase by increasing the cell density mainly in culture broth. The plasmids were more stable in cells that were grown in original SMM medium with stability of 90% compared to those grown in optimized SMM medium with stability of 80% after 48 hours cultivation. The most active amplification of plasmid was occurred in the logarithmic phase, which showed a value around four times higher than the initial copy number. In the exponential phase, the CDase production was closely related to the plasmid copy number along with the cell density. However, it was not accorded with cell density at the stationary phase.

Cytidine deaminase (cytidine/2'-deoxycytidine aminohydrolase, CDase) catalyzes the conversion of cytosine nucleosides to the corresponding uracil nucleosides, and is widely distributed in microorganisms (7-9). The function of CDase is to scavange the pyrimidine compounds for nucleotide synthesis and to make the pentose moieties of nucleotides as well as the 4-amino group of cytosine compounds available as sources of carbon and nitrogen (7).

The synthesis of the enzyme is highly inducible in *E. coli* and *S. thyphimurium*, but is not inducible in *B. subtilis*. The CDase derived from *E. coli*, mouse kidney, and yeast (2, 4, 14) has been purified, but it has not yet been purified from *B. subtilis*.

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The *cdd* gene of *B. subtilis* was cloned into *cdd* deficient *B. subtilis* (*Lys*$^-$, *cdd*-1^-, *pyr*-2^- *via E. coli*, and sequenced by Song and Neuhard (10). From the nucleotide sequences, they postulated that CDase from *B. subtilis* is composed of four identical subunits, which have molecular weights of 14 kDa and each subunit is composed of 136 amino acids containing six cysteine residues. Also the nucleotide sequence was revealed to have two promoters, which initiate the transcription of *cdd* gene in the exponential and stationary phases independently.

The plasmid pSO100 (10) harvored in the transformant B. subtilis ED213 was 9.5 kb, and was derived from inserting the 1.2 kb *Eco*RI/*Pvu*I fragment of pSO21 containing *B. subtilis cdd* gene into the corresponding sites of pGB215-110 ΔB (1). This hybrid plasmid showes the temperature-sensitivity, so the cells harboring

this plasmid are killed at temperature above 39℃, and *E. coli-B. subtilis* shuttle vector with two replication origins operated both in *E. coli* and *B. subtilis* (1).

When the transformant *B. subtilis* was cultured in original SMM medium, the *cdd* gene expression occurred with very low efficiency, especially for the extracellular CDase. The detectable amount of extracellular CDase was produced only by adopting a two-stage culture technique, in which the cells were harvested in the late log phase and then recultivated in a 1/10 volume of a fresh medium (11). The purpose of this work was to optimize the culture conditions of transformant *B. subtilis*, to analyze the plasmid stability and copy amplification for overproduction of the CDase, which will facilitate the purification and utilization of CDase in *B. subtilis*.

MATERIALS AND METHODS

Strains

The transforming host was a *Bacillus subtilis* ED40 (*Lys* , *cdd*−*1* , *pyr*−*2*), and the transformant was *Bacillus subtilis* ED213 (*Lys* , *cdd*−*1* , *pyr*−*2*) which harbored pSO100 carrying the *cdd* gene originated from the wild type *B. subtilis* 168 (1).

Media

Medium used for cell cultivation was the Spizizen Minimal Medium (SMM) (13), which contains 5 g of glycerol, 2 g of $(NH_4)_2SO_4$, 14 g of K_2HPO_4, 6 g of KH_2PO_4, 1 g of sodium citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.27 mg of $MnSO_4 \cdot 4H_2O$ per 1 *l* of distilled water. Lysine (50 μg/m*l*) and uridine (20 μg/m*l*), or lysine (50 μg/m*l*) and cytidine (20 μg/m*l*), were supplemented for *B. subtilis* ED40 and ED213 to meet with requirement of its genotype (*Lys* , *cdd*−*1* , *pyr*−*2*). Kanamycin (20 g/m*l*) was added to the minimal medium for cultivation of the transformant *B. subtilis* ED213, in some cases, 0.02% casamino acid (CAA) was added as a growth factor. For medium optimization, the kinds and amounts of various carbon and nitrogen sources were investigated, and the effect of cytidine concentration was also examined along with those of pH and temperature.

Cultivation

2 m*l* of seed culture cultivated at L-test tube for 12 hours was transferred into the 100 m*l* of original or optimal SMM medium adjusted to pH 7.0 in 250 m*l* culture flask. Cultivation was carried out at 37℃ with 150 rpm in a rotatory shaking incubator. The maximum specific growth rate, μ_{max}, was estimated from the slope of the semi-logarithmically plotted cell growth curve.

Preparation of Enzyme

Supernatants were directly used as the sources of extracellular CDase. For intracellular CDase, the harvested cells were suspended in 0.1 M Tris-HCl buffer (pH 7.0), sonicated, and then centrifuged to obtain supernatants.

CDase Assay

CDase activity was determined by the procedure of Hammer-Jesperson *et al.* (3). One unit of CDase was defined as the amount of enzyme which converted one nano mole of cytidine to one nano mole of uridine per minute at 37℃.

Plasmid Stability

Cells were cultivated in original or optimal SMM medium without kanamycin, and then the withdrawn cells were diluted and spreaded on the LB-agar plate not containing kanamycin. After incubation for 12 hours, colonies were transferred onto LB-agar plate containing kanamycin and reincubated at 37℃ for 12 hours. Plasmid stability was defined as the ratio (%) of kanamycin resistant cells containing plasmids to the total cells.

DNA Isolation and Determination of Plasmid Copy Number

DNA isolation was performed by the methods proposed by Koizumi *et al.* (5) and the plasmid copy number (N_p) was determined by the modified method of Moser and Campbell (6). Isolated plasmid and chromosomal DNAs in agarose gel electrophoresis were photographed. Bands densities from negative photograph of gel were measured using a scanning densitometer. The plasmid copy number per equivalent chromosome, N_p, was calculated as $N_p = M_c \times K_p / M_p \times K_c$; where M_c and M_p indicated the molecular weights of the chromosome and plasmid, and K_c and K_p indicated the amounts and chromosomal and plasmid DNA, respectively.

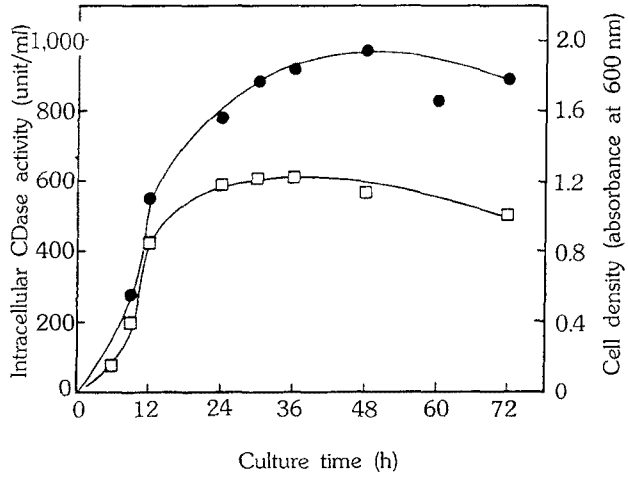

Fig. 1. Growth and CDase prodution of treasformant *B. subtilis* ED213 in SMM medium.
□—□: Cell growth, ●—●: Intracellular CDase.

RESULTS AND DISCUSSION

Growth and CDase Production of a Transformant *B. subtilis* ED213 in SMM Medium

Fig. 1 illustrates the growth and CDase production patterns of the transformant *B. subtilis* ED213 cultivated in original SMM medium at 37℃. After 12 hours of lag phase, cells entered the exponential growth phase, and continued to grow up to 36 hours. Detectable amounts of extracellular CDase was not produced, meanwhile, intracellular CDase with the maximum activity of 1,000 unit/ml was produced proportionally to the cell growth.

Optimization of Culture Conditions

In order to increase the production of extracellular CDase, the medium compositions and growth conditions were investigated. The effect of the amount of cytidine, carbon sources, and nitrogen sources to the production of CDase and to the cell growth of transformant *B. subtilis* ED213 were examined along with pH and temperature. Table 1 shows the effect of the amount of cytidine on CDase production. The cytidine concentration was identified to be the most influential nutritional factor for the CDase production. When cytidine concentration increased from the initial 20 μg/ml in the original SMM medium to 80 μg/ml, the CDase production was drastically increased from 30 to 813 unit/ml for extracellular CDase and from 173 to 2,103 unit/ml for intracellular CDase. The concentration of transformant cells also increased around four times from a cell density of 0.75 to 2.72 (OD at 600 nm).

For the cell growth, the DNA synthesis is a prerequisite. The low production of CDase in unoptimized original SMM medium may be due to the limitation of cell growth caused by the retardation of DNA synthesis. The added cytidine can be converted by CDase to uridine that is consequently used for DNA synthesis, which requires the higher level of CDase synthesis. The excess amounts of cytidine lead to conversion of cytidine into uridine, therefore the cell growth is accelerated.

The effect on the cell growth of various carbon sources, such as glucose, fructose, sucrose, soluble starch, glycerol, mannitol, sorbitol, and lactose were examined as shown in Table 2. The glycerol was selected as the most suitable carbon source and the optimal concentration was 25 g/l (12). Also among the $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_2PO_4$, NH_4HCO_3, $NaNO_3$, and KNO_3, the most suitable nitrogen source selected was 10 g/l of $(NH_4)_2SO_4$. The optimal pH and temperature for CDase production were to be pH 7.0 and 37℃, respectively. CDase production reached a maximum level with 1,066 unit/ml at pH 7.0; however, it decreased at alkaline and acidic pH as shown in Table 3. Especially at pH range of 5.0 to 5.5, the considerable amounts of intracellular CDase were produced to 1,520 and 1,925 unit/ml respectiovely, although, exteacellular CDase was not detected. It seemed that CDase was inactivated severely at acidic

Table 1. Effect of cytidine concentration on CDase production

Cytidine Con.(mg/l)	Cell Density (OD 600 nm)	Extracellular CDase(Unit/ml)	Intracellular CDase(Unit/ml)
20	0.75	30	173
40	1.52	344	527
60	2.24	829	1,764
80	2.72	813	2,103
100	2.79	707	2,352
150	2.48	77	1,301
200	2.80	859	2,441
400	2.56	749	2,744

Transformant *B. subtilis* ED213 was cultivated for 24 h in SMM with different amounts of cytidine at 37℃, pH 7.0, at 150 rpm in a rotary shaker.

Table 2. Effect of carbon sources on the CDase production

Carbon Source	Cell Density (OD 600 nm)	Extracellular CDase(Unit/ml)	Intracellular CDase(Unit/ml)
Glucose	2.59	314	1,352
Fructose	2.52	367	1,362
Sucrose	2.43	197	1,533
Soluble starch	2.01	381	1,497
Glycerol	2.91	635	1,575
Mannitol	3.00	205	542
Sorbitol	1.95	266	1,148
Lactose	0.78	415	7663

Transformant was cultivated for 24 h in SMM medium with cytidine concentration of 80 mg/l at 37℃, pH 7.0, and 150 rpm. The carbon concentrations were 5 g/l.

Table 3. Effect of pH on the production of CDase

pH	Cell density (OD 600 nm)	Extracellular CDase(Unit/ml)	Intracellular CDase(Unit/ml)
5.0	2.84	–	1,520
5.5	3.56	–	1,925
6.0	3.60	137	2,257
6.5	3.12	449	2,281
7.0	3.44	1,066	2,887
7.5	3.44	535	2,637
8.0	2.80	583	2,512
9.0	2.72	219	2,222

Transformant was cultivated in optimal SMM medium for 24 h at 37℃, at 150 rpm on a reciprocal shaker.

Table 4. Comparison of the medium composition of original SMM medium and optimized SMM medium for the CDase production of a transformant *B. subtilis* ED213

Composition	Original SMM (g/l)	Optimized SMM (g/l)
Medium composition		
Glycerol	5	25
$(NH_4)_2SO_4$	2	10
KH_2PO_4	6	6
K_2HPO_4	14	14
Sodium citrate	1	3
$MgSO_4 \cdot 7H_2O$	0.2	0.6
Casamino acid	0.2	0.6
$MnSO_4 \cdot 4H_2O$	0.002	0.001
Cytidine	0.02	0.08
Lysine	0.05	0.05
Kanamycin	0.02	0.02

The cells were cultured in SMM medium (pH 7.0) at 37℃.

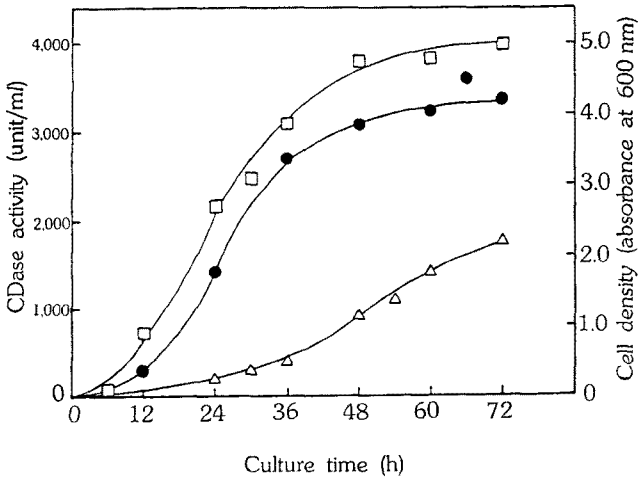

Fig. 2. Growth and CDase production of transformant *B. subtilis* ED213 in optimized SMM medium.
□—□: Cell denstiy ●—●: Intracellular CDase, △—△: Extracellular CDase.

pH and slightly at alkaline pH. Table 4 summarizes the optimal SMM medium composition, and compares it with the original SMM medium composion.

The transformant *B.subtilis* ED213 was cultivated in optimal culture condition as shown in Fig. 2. The CDase production was significantly increased up to 3,200 unit/ml for intracellular CDase and 1,500 unit/ml for extracellular CDase. The increased CDase production in optimal SMM medium may have been mainly caused by increased cell density that was around four times higher than that grown in original SMM medium.

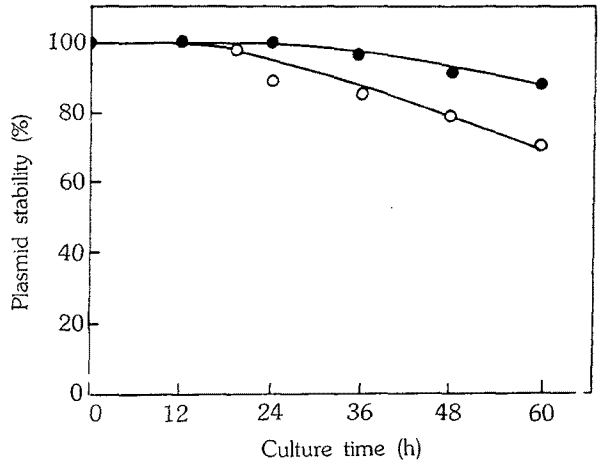

Fig. 3. Comparison of plasmid stability in original and optimized SMM medium.
●—●: Original SMM medium, ○—○: Optimized SMM medium

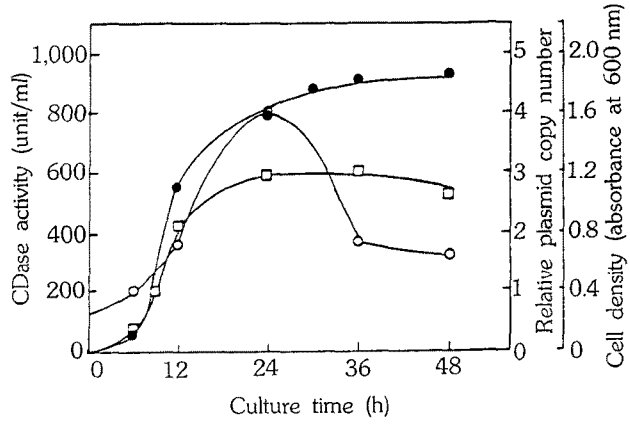

Fig. 4. Changes of plasmid copy number and CDase prodution during the transformant cell growth; plasmid copy number was determined using the scanning densitometer.
●—●: CDase, ○—○: Plasmid copy number, □—□: Cell growth.

Analysis of Plasmid Stability

The comparison of plasmid stability in cells that were grown in original and optimal SMM medium is shown in Fig. 3. In the initial growth stage of 12 hours, plasmids were maintained with 100% of stability in both media. However as cells grew, plasmid-free cells appeared; after 48 hours, over 90% of the cells grown in original SMM medium contained plasmids, on the other hand, 80% of cells grown in optimal SMM medium contained plasmids. This may be due to the competitive instability between plasmid-harvoring cells and plamid-free cells caused by the rapid growth in optimal medium. This kind of plasmid instability can be partially overcome by adding kanamycin into medium during cell cultivation.

Table 5. Comparison of growth parameter in *B. subtilis* ED40 and ED213

Growth parameter / Strains	Maximum specific growth rate μ_m (h^{-1})	Mass doubling time t_d (h)	Cell mass (g/l)
B. subtilis ED40	0.36	1.9	0.8
B. subtilis ED213	0.26	2.67	0.66

B. substilis was cultivated in unoptimaized original SMM medium at 37℃, pH 7.0, and 150 rpm.

Changes in Plasmid Copy Number and CDase Production during the Cell Growth

Changes in plasmid copy amplification and CDase production during the cell growth are depicted in Fig. 4. The CDase production was closely related to plasmid copy number along with the cell density at growth phase. Plasmid copy number was amplified four times at logarithmic phase in which the amplification was closely correlated with active cell growth, however, it decreased rapidly, and then remained constant in the stationary phase. The CDase production also increased until the late log phase together with the increase of plasmid copy number, however, at stationary phase the CDase production was slightly increased even though the copy number was reduced to half. Although reduction of copy number was considered, the ratio of CDase production decreased siginificantly compared to the log phase. This reason can be explained by dual promoters located in front of the *cdd* structural gene and operated in phase dependently; one operated at log phase and the other at stationary phase (10). The promoter which operated at log phase may have been more efficient than that at stationary phase.

Comparison of Growth Parameters in Host and Transformant *B. subtilis*

A growth patterns between *B. subtilis* ED40 and ED 213 were compared to investigate a physiological properties of host and transformant strains as shown in Table 5. As expected, the host cells grew more (Table 5) rapidly and produced more cell mass than transformant cells. It seems that the growth of transformant strain was hindered by sharing common enzymes for DNA replication, transcription, and translation, *etc*, with the plasmid.

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