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Extracellular production of α-amylase during fed-batch cultivation of recombinant *Escherichia coli* using pulsed electric field

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Abstract

In this study, extracellular release of recombinant α-amylase from *Escherichia coli* HB101/pHI301A by high-voltage pulsed electric field (PEF) was demonstrated during fed-batch cultivation. A continuous PEF treatment from the beginning of cultivation caused a decrease in cell growth rate and productivity of α-amylase, although a small quantity of extracellular α-amylase was detected in the culture supernatant. On the other hand, when PEF (12 kV, 3 Hz) was applied intermittently (each for 30 min with an interval of 30 min) from the beginning of stationary phase, the amount of α-amylase released was about 30% of the total amount of α-amylase that was produced in the cells. The release ratio and the total amount of α-amylase released were higher than that of batch cultivation. Suitable PEF treatment can be useful for easy and effective extracellular release of periplasmic proteins by fed-batch cultivation of recombinant *E. coli*.
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Keywords: Pulsed electric field; Extracellular release; Recombinant proteins; Fed-batch cultivation

1. Introduction

Biotechnology has achieved quantum leaps in gene engineering techniques, which could open new fields of opportunity in the production of drugs, medicines, and enzymes by recombinant cells. *Escherichia coli* is one of the most useful bacteria as a host cell for gene engineering due to high levels of expression and synthesis of gene products [1]. To obtain the recombinant proteins produced in *E. coli* cells, cell disruption using ultrasonic treatment or homogenization, which causes the complete disruption of the cell, has been necessary. Total cell disruption results in contamination by intercellular proteins and materials, making it necessary to purify the target recombinant protein.

On the other hand, secretion cultivation, in which recombinant proteins are released extracellularly into the supernatant of the culture, has advantages in the recovery of recombinant proteins. However, secretion of recombinant proteins produced by *E. coli* is complicated by the double membrane structure of Gram-negative bacteria, such as *E. coli*. The recombinant proteins are able to pass through the inner (cytoplasmic) membrane, facilitated by signal peptides, but cannot pass through the outer membrane. This results in the accumulation of recombinant protein in the periplasmic space between the inner and outer membranes. It has been reported that recombinant proteins pass easily into the periplasmic space [2]. However, only a few reports have described the secretion of proteins extracellularly [3–5], and the mechanism of secretion of gene products is little understood [1].

Membrane disruption using high-voltage pulsed electric field (PEF) has been applied to recover intracellular proteins from bacteria, including recombinant *E. coli* cells [8], and in the release of some intracellular proteins from yeast cells [6,7]. In the case of *E. coli* cells, it seems that PEF treatment easily causes outer membrane disruption, allowing the release of the recombinant protein accumulated in periplasmic space [8]. These results suggested that reversible disruption of only the outer membrane during cultivation of recombinant *E. coli* would result in the release of recombinant proteins accumulated in periplasmic

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space into the culture supernatant. In our previous work, it was confirmed that active α-amylase, which had been accumulated in the periplasmic space could be released from recombinant *E. coli* HB101/pHI301A by applying PEF (12 kV, 2 Hz) during batch cultivation. The optimum term of PEF treatment commenced at the beginning of stationary phase, and the optimum PEF application condition is 30 min of application with an interval of 30 min (intermittent PEF treatment) [9]. The amount of α-amylase released when PEF was applied intermittently reached about 30% of the total α-amylase produced intracellularly.

For the production of gene products on an industrial scale, it is necessary to carry out fed-batch cultivation. In batch cultivation, the final cell density of recombinant *E. coli* is low (up to 10^8 cells/mL) due to limitations of the nutrient components such as glucose. Fed-batch cultivation on the other hand allows pH and dissolved oxygen (DO) to be controlled at optimal levels, and additional nutrient components to be added to achieve high cell density (more than 10^{10} cells/mL). Therefore, when fed-batch cultivation is carried out, the amount of gene products will be increased due to a high cell density. It has been reported that high concentrations of gene products could be obtained with fed-batch cultivation [10,11]. If it is possible to release α-amylase extracellularly during fed-batch cultivation using PEF treatment, it seemed that the amount of released α-amylase could be increased.

In this study, we demonstrated the release of recombinant α-amylase extracellularly from *E. coli* HB101/pHI301A by PEF treatment during fed-batch cultivation. The effects of PEF treatment on the production of α-amylase and cell activity were studied experimentally.

2. Experimental

2.1. Microorganism preparation

The recombinant *E. coli* HB101 (Takara Bio Inc., Japan)-bearing pHI301A [12], which produces α-amylase derived from *Bacillus stearothermophilus*, was used in this study. It was reported that 75–82% of α-amylase is accumulated in the periplasmic space [12]. *E. coli* HB101/pHI301A was maintained in L-broth (Bacto-peptone 10 g/L, yeast extract 10 g/L, NaCl 5 g/L, pH 7.2) containing 100 μg/mL of ampicillin, and pre-cultivation was carried out in a shaking flask for 8 h at 37 °C in the 100 mL of Basel medium (composition is shown in Table 1) containing 100 μg/mL of ampicillin. The entire *E. coli*/pHI301A preculture was added to 1000 mL of Basel medium containing 100 μg/mL of ampicillin before PEF treatment. The viability of *E. coli* cells during cultivation was measured by counting colony-forming units (CFUs). The living cell number constant with OD_{660} (4.6×10^8 cells/mL · OD_{660}) was obtained experimentally.

Table 1
Composition of Basel medium

Component	Concentration (mg/L)
KH_2PO_4	4000
K_2HPO_4	4000
Na_2HPO_4	7000
$(NH_4)2SO_4$	1200
NH_4Cl	200
Yeast extract	4000
Glucose	10000
$MgSO_4 \cdot 7H_2O$	2400
$FeSO_4 \cdot 7H_2O$	40
$CaCl_2 \cdot 7H_2O$	40
$MnSO_4 \cdot 2H_2O$	10
$AlCl_3 \cdot 6H_2O$	10
$CoCl_2 \cdot 6H_2O$	4
$ZnSO_4 \cdot 7H_2O$	2
$Na_2MoO_4 \cdot 2H_2O$	2
$CuCl_2 \cdot 2H_2O$	1
H_3BO_3	0.5

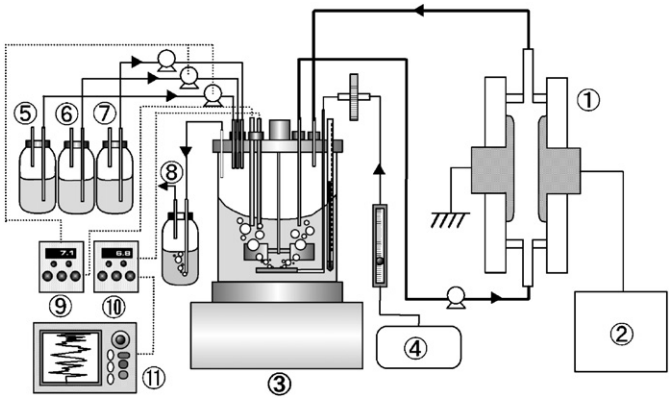

Fig. 1. Schematic diagram of experimental apparatus. 1, treatment chamber, 2, high-voltage pulse generator; 3, jarfermenter; 4, air compressor; 5, 1 N HCl; 6, 10% ammonia water; 7, anti-formed agent; 8, 1 N NaOH; 9, DO controller; 10, pH controller; 11, recorder.

2.2. Fed-batch cultivation

In this experiment, a Jar fermenter M-100 (Tokyo Rikakikai Co., Ltd., Japan) with a working volume of 1 L was used for fed-batch cultivation (Fig. 1). Cultivation temperature was maintained at 37 °C and the pH of the medium maintained at 7.1 using a pH controller PU-1 (Shibata Co., Ltd., Japan) with 1 M HCl and 10% ammonia water. DO was measured using a DO controller DU-1 (Shibata Co., Ltd., Japan), and the value was recoded using a Pen recorder TYPE 3056 (Yokogawa Hokushin Electric Co., Ltd., Japan). DO was observed to decrease with cell growth. When the DO value fell, it was controlled by increasing the agitator rate or air flow rate. When the DO value increased, indicating nutrient depletion, 20 mL of additional medium (Table 2) was added.

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