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Optimization of enterokinase fermentation using a recombinant *Saccharomyces cerevisiae*

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Abstract

The optimization of enterokinase fermentation was performed with a recombinant *Saccharomyces cerevisiae*. The maximum enterokinase yield and specific yield were obtained when the ratio of yeast extract to bacto peptone was 5:5. However, the fraction of plasmid-containing cells was approximately 65% for most nitrogen source ratios. From batch cultures, the minimum DOT level was found to be a key factor. Through DO-stat cultivations, the value was further optimized. As the minimum DOT level increased, the product yield and plasmid-containing cell fraction increased, whereas the ethanol concentration decreased. The optimum agitation speed and aeration rate in a 5 l jar were determined as 700 rpm and 2 vvm, respectively. Under these conditions, a high enterokinase yield of 3.8 mg/l was obtained with the final ethanol concentration of 0.4 g/l. The minimum DOT level was maintained to be 60% or more. Scale-up of enterokinase fermentation from a 5 l jar to a 300 l jar was successfully accomplished based on strategies to maintain a constant impeller tip speed and a minimum DOT level at 60% or more.

Keywords: Enterokinase fermentation; Recombinant *Saccharomyces cerevisiae*; DO-stat; Scale-up

1. Introduction

Enterokinase (enteropeptidase, EC 3.4.21.9) is a membrane-bound serine protease exhibiting a specificity for the sequence of $(Asp)_4$-Lys (D4K). The enzyme motif is found in highly conserved amino termini of trypsinogens from a wide range of species [1]. Enterokinase hydrolyzes trypsinogen to trypsin, and activated trypsin stimulates other pancreatic zymogens [2]. Enterokinase is synthesized as a disulphide-linked hetero-dimer with an amino-terminal heavy chain (120 kDa) and a light chain (47 kDa). The light chain (EK_L) alone exhibits a proteolytic activity while the heavy chain recognizes macromolecular substrates, such as trypsinogen [3,4]. The crystal structure of enterokinase has been defined [5].

In recent years, enterokinase light chains (EK_L) have been widely used to cleave the fusion partner in vitro with a high specificity for $(Asp)_4$-Lys [6]. However, enterokinase can be obtained only in small quantities from bovine duodenal mucosa, which makes industrial scale production too expensive. Recombinant cells of *Escherichia coli* [7] or yeast *Pichia pastoris* [8] encoding bovine EK_L genes were used in an attempt to solve this problem. However, full-scale optimization of recombinant enterokinase (rEK_L) fermentation has not yet been achieved.

Strains of *Saccharomyces cerevisiae* have been used as a host for production of various eukaryotic proteins [9,10]. In fermentations using recombinant cells, control of plasmid stability is particularly important because it directly affects product yield [11–14]. Two stages of continuous cultivation [15–17] and fed-batch processes [18] are used for analysis of plasmid stability.

A recombinant *S. cerevisiae* was constructed to produce C-terminal His-tagged enterokinase for efficient purification and post-cleavage removal [19]. In this study, we investigated key factors for enterokinase fermentations using the recombinant *S. cerevisiae*. Optimum fermentation conditions were determined via batch cultures and DO-stats in 5 l jar vessels. A scale-up of batch fermentation from a 5 l jar to a 300 l pilot was performed.

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2. Materials and methods

2.1. Microorganism and media

The expression host organism used in enterokinase fermentation was *S. cerevisiae* 2805 (pep4::HIS3, pro1-δ, can1, Gal2, his3δ, ura3–52), which was transformed with plasmid pIL20XEK$_L$-Ca-His encoding the C-terminal His-tagged EK$_L$ [19].

The selective medium for seed culture consisted of 20 g/l of glucose, 0.8 g/l of complete supplement medium-Ura (Bio101), and 6.7 g/l of a yeast nitrogen base without amino acids (DIFCO). The fermentation medium for enterokinase production was composed of 20 g/l of glucose, and 10 g/l of yeast extract and bacto peptone. The ratios of yeast extract and bacto peptone were varied. A concentrated galactose solution (200 g/l) was added as an inducer after 24 h of cultivation. For measurement of plasmid-containing cells, YM agar medium (DIFCO) and selective agar medium (selective medium plus 20 g/l agar) were used.

2.2. Cultivations

For optimization of nitrogen sources, seed cultures were performed in 500 ml baffled flasks with 75 ml of sterilized selective medium after inoculation of the recombinant *S. cerevisiae*. Flasks were shaken on a rotary incubator (Jeio Tech, sj-600r) for 24 h at 30 °C and 180 rpm. Cultivated solutions were added at 5% (v/v) as an inoculum to 500 ml baffled flasks with 75 ml of fermentation medium. After 24 h of cultivation, the galactose solution was added at 2%. Then, mixtures were further cultivated for 24 h at 30 °C and 180 rpm.

For fermentations in a 5 l jar, seed cultures were prepared in 500 ml baffled flasks in the same manner as for flask fermentations. Cultivated solutions were added at 5% (v/v) to the 5 l jars (KoBioTech Co., Ltd.) containing 3 l of fermentation medium. Then, the mixtures were cultivated at 30 °C and 500 rpm with an aeration rate of 0.5–2.0 vvm. After 24 h, the concentrated galactose solution was added at 2%, and then further cultivated for 24 h.

DO-stat fermentations in 5 l jars were performed in the same manner as in a 5 l jar. The DOTs in fermenters were maintained at a constant level during cultivation by control of the aeration rate and agitation speed.

For the fermentation scale-up to a 30 l jar (KoBioTech Co., Ltd.), seed cultures were prepared twice. The first culture was prepared in a 500 ml baffled flask in the same manner as described previously. For the second seed culture, the cultivated solution was added at 5% (v/v) to a 5 l jar containing 3 l of selective medium. Then, the mixture was cultivated for 24 h at 30 °C with an agitation speed of 500 rpm and an aeration rate of 2.0 vvm. The cultivated solution was added at 5% (v/v) to the 30 l jar containing 20 l of fermentation medium, followed by cultivation for 24 h at 30 °C with an agitation speed of 460 rpm and an aeration rate of 2 vvm. Then, the galactose solution was added and the mixture was further cultivated.

For the pilot-scale fermentation, the first and second seed cultures were prepared in the same manner as described previously. The third seed culture was made in a 30 l jar containing 20 l of selective medium under the same condition as for a 5 l jar. The pilot fermentation was conducted in a 300 l jar (KoBioTech Co., Ltd.) containing 200 l of fermentation medium at 30 °C with an agitation speed of 150 rpm and an aeration rate of 2 vvm.

2.3. Measurement of cell concentrations and plasmid-containing cells

For measurement of cell concentrations, cell precipitates were isolated by centrifugation of culture broths (Vision Scientific, VS-15000, angle type, 4,000 × *g*, 5 min). After cells were suspended in a 0.9% (w/v) saline solution, the absorbance of the solution was measured at 600 nm on a spectrophotometer (Shimadzu, UV-1201) and values were converted to dry cell weight.

For measurement of plasmid-containing cells, after the cell precipitates were suspended in a saline solution, the solution was spread on plates with either a selective agar medium or a YM agar medium. After 24 h of incubation at 30 °C, the fractions of plasmid-containing cells vs. total cells were estimated from the number ratios of cells grown on the two types of media.

2.4. Measurement of enterokinase activity

Enterokinase activities were measured by a fluorometric assay using the synthetic substrate Gly-(Asp)$_4$-Lys-β-naphthylamide [20]. One milliliter of fluorogenic substrate (0.5 mM GD4K-NA, 25 mM Tris-HCl at pH 8.4, 10 mM $CaCl_2$, and 10% DMSO) was mixed with 0.2 ml of enzyme broth (temporarily stored in a 50% glycerine solution). Then, the solutions were incubated for 5 min in a fluorescence spectrophotometer (Varian, FC-8000) at 37 °C. Enzyme activities were calculated as fluorescence differences versus time with excitation at 337 nm and emission at 420 nm. The enzymic activities were converted into mass per unit volume values of culture broth.

2.5. Measurement of glucose, galactose, and ethanol contents

For measurement of glucose, galactose and ethanol contents, the supernatants were obtained by centrifugation (Vision Centrifuge VS-15000, angle type, 4,000 × *g*) of culture broths. Concentrations were then measured on a Bio-LC (Dionex, DX-500) equipped with an analytical PA-1 column (Dionex, CarboPac, 4 mm × 250 mm), a gradient pump (Dionex, GP 40) and an electrochemical detector (Dionex, ED 40). A mixture (16:84) of a 50 mM NaOH solution and

distilled water at 30 °C was eluted at 5 ml/min as an isocratic mobile phase.

2.6. Measurement of DOT and pH

Dissolved oxygen tensions (DOTs) were measured on a DO-analyzer (KoBioTech Co., Ltd., LA-150). The pH values of culture broths were monitored using a pH-meter (KoBioTech Co., Ltd.).

2.7. Chemicals

All chemicals used were of reagent-grade and were purchased from Duksan, Sigma, and Difco. Complete supplement medium-Ura (CSM-Ura) was a product of Bio101 Co.

3. Results and discussion

Key fermentation factors were sought from medium compositions and cultivation conditions for efficient enterokinase production using a recombinant *S. cerevisiae*. Batch fermentations were carried out at two stages of growth and production in a flask or a fermenter. Since glucose has been reported as the key carbon source in the growth stage and the galactose in production stage by the developers of the microorganism strain used [19], the carbon sources were not further optimized.

3.1. Optimum nitrogen concentrations of fermentation medium

Cultivations were conducted for 24 h (growth stage) in 500 ml baffled flasks containing the fermentation medium. The galactose solution was then added to the flasks at 2% followed by 24 h of further cultivation in the production stage (Fig. 1).

From our preliminary analysis, yeast extract and bacto peptone were found to be the key nitrogen sources for fermentation. Here, therefore, the optimum combination of yeast extract and bacto peptone concentrations in the fermentation medium was determined. The total nitrogen source content in culture medium was fixed at 10 g/l whereas the ratio of yeast extract to bacto peptone was varied from 0:10 to 10:0. Cell growth was enhanced with an increasing ratio of yeast extract to bacto peptone (Fig. 2A), whereas a maximum enterokinase yield of 0.49 mg/l was obtained at a 5:5 ratio. Yeast extract was a good component for cell growth whereas bacto peptone was important for enhancement of the enterokinase yield. There are two general strategies for increasing productivity. One is to use high cell density cultures while the other is to maintain high cell activities for production. The specific enterokinase yields had a pattern similar to the overall yields (Fig. 2B). The maximum specific enzyme yield was 0.045 mg/l of g-cell at a 5:5 ratio, in-

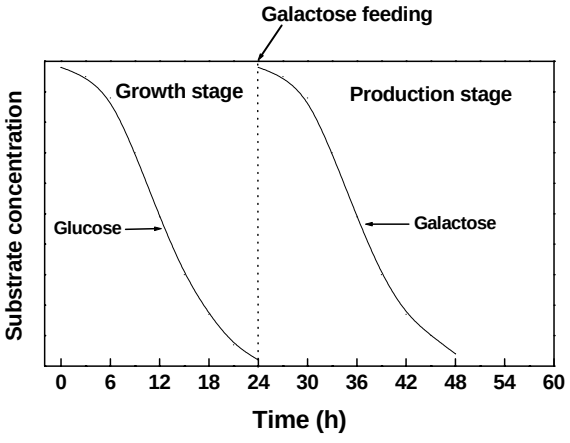

Fig. 1. A schematic diagram of enterokinase fermentations.

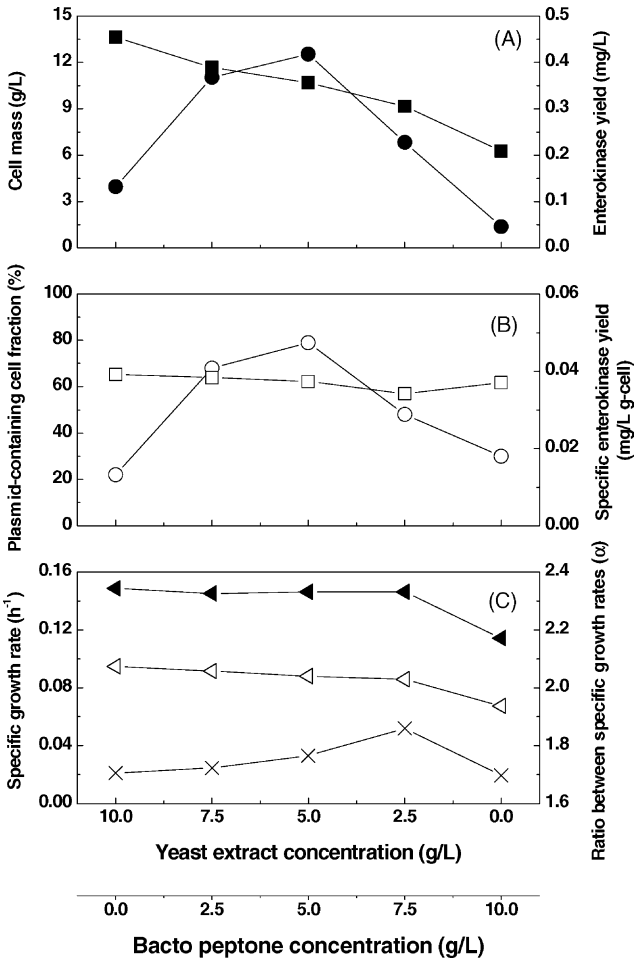

Fig. 2. Cell mass and enterokinase yield at the end of cultivation depending on the combination ratios of yeast extract to bacto peptone in baffled flasks. The total content of yeast extract and bacto peptone was fixed at 10 g/l. Cultivations were performed in a 500 ml baffled flask at 30 °C and 180 rpm for 48 h. (●) Enterokinase yield; (■) cell mass; (○) specific enterokinase yield; (□) plasmid-containing cell fraction; (◀) specific growth rate of plasmid-free cells; (◁) specific growth rate of plasmid-containing cells; (×) ratio of the specific growth rates of plasmid-free and plasmid-containing cells.

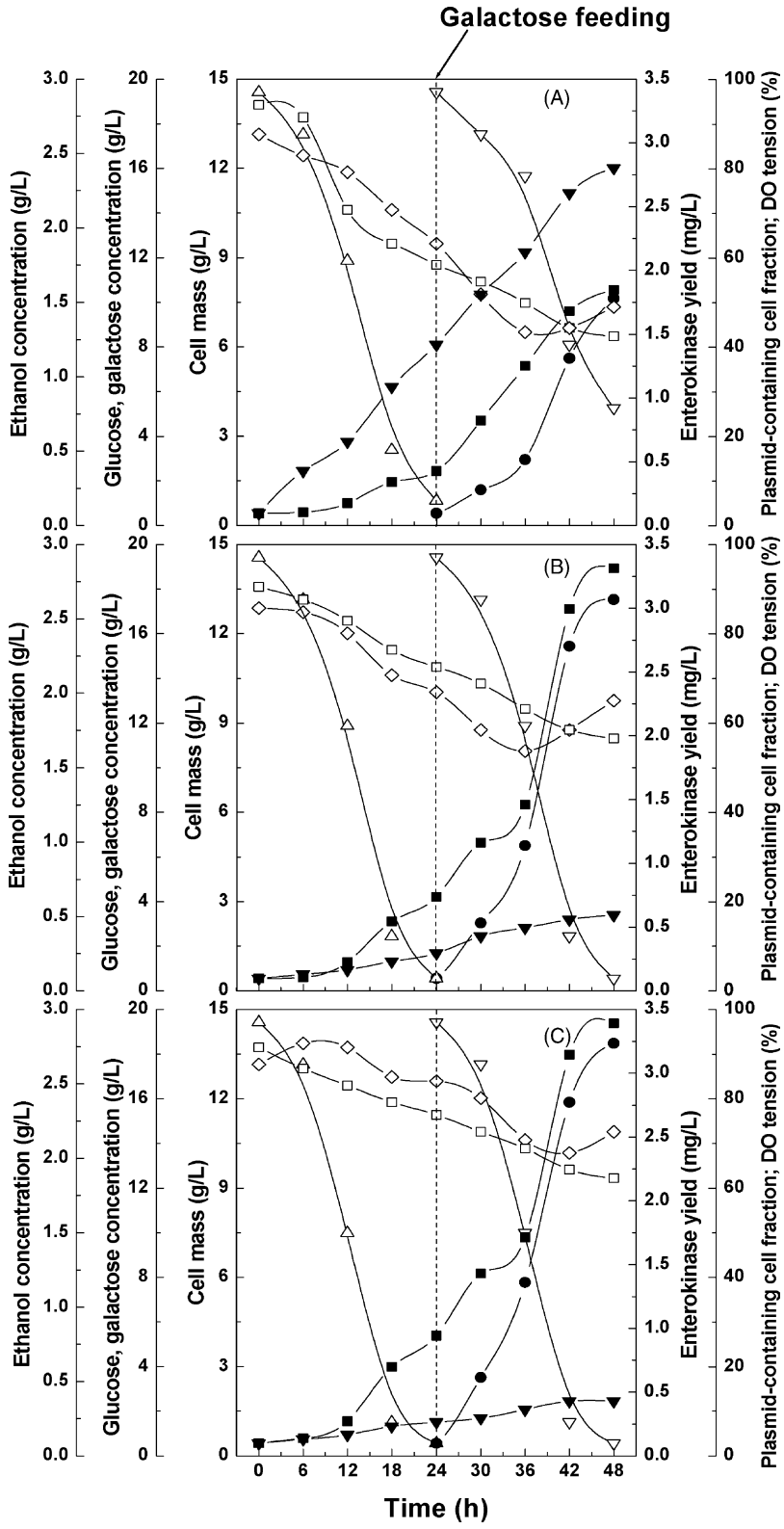

Fig. 3. Patterns of enterokinase fermentation at an aeration rate of: (A) 0.5 vvm, (B) 1.0 vvm or (C) 2.0 vvm in a 5 l jar. Cultivations were performed for 48 h at 30 °C and 500 rpm. (●) Enterokinase yield; (■) cell mass; (▼) ethanol concentration; (△) glucose concentration; (▽) galactose concentration; (◇) DO tension; (□) plasmid-containing cell fraction.

dicating that a good strategy is to obtain a high product yield by enhancement of cell activity rather than by enhanced cell growth.

One of the key factors in fermentations using recombinant cells is to maintain high fractions of plasmid-containing cells. Whether the plasmids do or do not exist inside the host cells can directly affect the overall yield. The product yield in a large scale fermentation can be seriously reduced with an increasing fraction of plasmid-free cells due to repeated transfers. Preliminary analyses showed that the fraction of plasmid-free cells increased significantly with an increasing generation of host cells. The fractions of plasmid-containing cells were estimated (Fig. 2B). The plasmid stabilities of cells were maintained at a constant level regardless of the combination ratios of yeast extract and bacto peptone concentrations. The fraction of plasmid-containing cells was approximately 65% for most nitrogen source ratios. However, enterokinase yields were severely affected by different combinations of the two nitrogen sources. The growth rates of plasmid-containing and plasmid-free cells were estimated. The specific growth rate of plasmid-free cells (μ^-) was higher than the growth rate of plasmid-containing cells (μ^+) (Fig. 2C) probably due to spending of extra metabolic energy for plasmid DNA in non-selection media [21]. μ^- values of approximately 0.157 h^{-1} were obtained at most ratios of yeast extract to bacto peptone, whereas μ^+ values were reduced in a range of 0.084–0.094 h^{-1}. At a ratio of 0:10 the values of μ^+ and μ^- were seriously decreased. The ratio (α) between the specific growth rates of plasmid-free and plasmid-containing cells can be expressed as follows:

$$\alpha = \frac{\mu^-}{\mu^+} \quad (1)$$

As shown in Fig. 2C, the α value increased gradually from 1.69 to 1.87 with varying N-source ratios from 10:0 to 2.5:7.5. However, the value decreased sharply to 1.68 at a ratio of 0:10. The highest α value was obtained at a ratio of 2.5:7.5, while the maximum productivity was achieved at a ratio of 5:5.

3.2. Batch fermentation patterns at various aeration rates

Enterokinase fermentations were performed in 5 l jars containing 5 g/l of yeast extract and 5 g/l of bacto peptone. Galactose was added at 24 h of cultivation. The aeration rate in 5 l batch cultures was varied in a range of 0.5–2.0 vvm and the optimum condition for enzyme production was determined.

At an aeration rate of 0.5 vvm, a low enterokinase yield of 1.8 mg/l was attained at the end of cultivation with a high ethanol level of 2.3 g/l (Fig. 3A). With rapid cell growth, the DOT decreased to 40% and the fraction of plasmid-containing cells was significantly lowered to 40%. Low DOT levels probably cause excessive accumulation of ethanol, resulting in poor growth and low product yields. However, when the fermenter aeration rate was raised to 1.0 vvm, the DOT was maintained at 50% or more, with an enzyme yield of 3.0 mg/l (Fig. 3B). The final ethanol concentration was 0.5 g/l, and the fraction of plasmid-containing cells was maintained at more than 55%. A high enterokinase yield was achieved with a low level of ethanol in the culture broth due to a sufficient air supply. On the other hand, when the aeration rate was increased to 2.0 vvm, the enterokinase yield and the cell concentration at the end of cultivation increased to 3.2 mg/l and 14.5 g/l, respectively (Fig. 3C). The final ethanol concentration was 0.4 g/l, and the fraction of plasmid-containing cells was maintained at 60% or more. The minimum DOT was 65%. Ethanol production can be suppressed directly by maintenance of high DOTs, leading to increases of cell concentrations, plasmid-containing cell fraction, and enzyme yield.

3.3. Determination of the minimum DOT level using DO-stats

DO-stat fermentations were performed in 5 l jars to analyze the effect of DOT on enterokinase production. The DOT in a fermenter was maintained at a constant level during cultivation by control of the aeration rate and the agitation speed. As shown in Fig. 4, with a 20% DOT-stat an enterokinase yield of approximately 1.4 mg/l was obtained. However, as the DO-stat level was raised to 70%, the enzyme yield increased to 3.4 mg/l. The fraction of plasmid-containing cells also increased from 37 to 65% whereas the ethanol yield decreased from 2.5 to 0.4 g/l. Plasmid stability and enzyme yield apparently depend on the ethanol level in the culture broth. Therefore, the DOT level in a fermenter should be maintained at more than 60% for efficient enterokinase production.

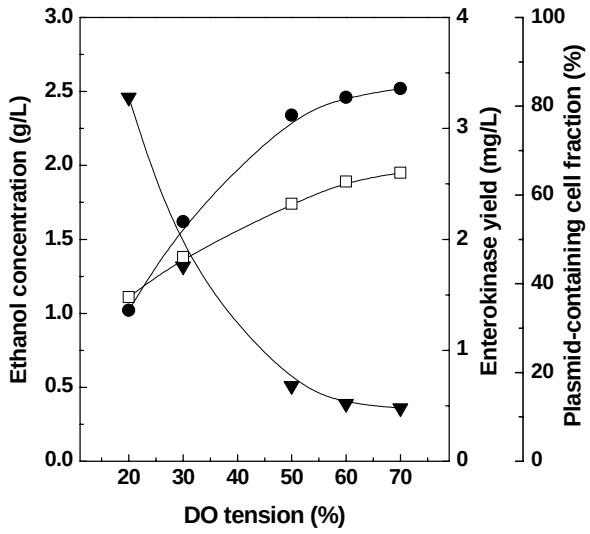

Fig. 4. Patterns of enterokinase fermentation with various DO-stats in 5 l jars. The DOT values were adjusted in a range of 20–70% by control of the aeration rate and the agitation speed. Cultivations were performed for for 48 h at 30 °C. (●) Enterokinase yield; (▼) ethanol concentration; (□) plasmid-containing cell fraction.

Table 1
Optimized enterokinase fermentations from a 5 l to a 300 l jars

	Fermenters		
	5 l	30 l	300 l
Agitation speed (rpm)	700	460	150
Minimum DOT level (%)	69	65	60
Final enterokinase yield (mg/l)	3.8	3.7	3.5

Cultivations were performed for 60 h at 30 °C with an aeration rate of 2 vvm, respectively.

From additional analyses in a 5 l jar fermenter, the optimum agitation speed for maintenance of 60% DOT or more was determined to be 700 rpm with an aeration rate of 2.0 vvm.

3.4. Scale-up from a 5 l to 300 l pilot

A fermentation scale-up of a 5 l jar to a 300 l pilot was performed. A constant impeller tip speed was used as a scale-up factor. Based on this criterion, an impeller rotational speed of 700 rpm in a 5 l fermenter corresponded to 460 rpm in a 30 l jar and 150 rpm in a 300 l pilot. The results obtained from 60 h of cultivations in the fermenters were summarized in Table 1. The DOT levels in a 30 l jar and a pilot were maintained at 60% or more throughout the cultures. The enterokinase yields reached approximately 3.7 mg/l in a 30 l, and 3.5 mg/l in a 300 l jars, respectively, indicating a successful scale-up.

4. Conclusions

Similar plasmid-containing cell fractions were obtained regardless of the ratio of yeast extract to bacto peptone. The specific enterokinase yield depended on this ratio, indicating that the ratio can affect the amount of enzyme formation. The average specific growth rate of plasmid-free cells was higher than the growth rate of plasmid-containing cells, indicating that the number of plasmid-free cells can be increased by repeated transfers, resulting in low product yields.

The DOT level in a fermenter apparently directly affects ethanol production. When an excessive amount of ethanol accumulates in the culture broth, the fraction of plasmid-containing cells is reduced, leading to a low product yield. Therefore, the DOT should be maintained at high levels in order to suppress ethanol accumulation. Based on strategies to maintain a constant impeller tip speed and a minimum DOT, a scale-up of enterokinase fermentation from a 5 l jar to a 300 l pilot was successfully accomplished.

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