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Effects of methanol on expression of an anticoagulant hirudin in recombinant *Hansenula polymorpha*

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A series of batch, fed-batch, and continuous cultures was carried out to analyze the effects of methanol on the fermentation characteristics of recombinant *Hansenula polymorpha* for the production of hirudin, an anticoagulant. Hirudin expression efficiencies were greatly influenced by the methanol concentrations in continuous and fed-batch culture modes. At a steady state of continuous culture, an optimum methanol concentration of 1.7 g l^{-1} was determined at a dilution rate of 0.18 h^{-1} with 1.8 mg l^{-1} h^{-1} hirudin productivity. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 58–61.

Keywords: hirudin; *Hansenula polymorpha*; methanol oxidase promoter; methanol

Introduction

Saccharomyces cerevisiae, used as a host organism for the production of therapeutic proteins and industrial enzymes, has certain limitations such as low product yield and hyperglycosylation of product proteins [1–4,15,17,19,22,24,25]. Due to these limitations, methylotrophic yeasts including *Hansenula polymorpha* and *Pichia pastoris* have been suggested as alternative hosts for heterologous protein production [1,9,10,14]. *H. polymorpha*, a facultative methylotroph, can utilize methanol as a sole source of carbon and energy [5,6,11]. Upon the addition of methanol into a culture grown in a medium containing a low concentration of glycerol, key enzymes involved in methanol metabolism are expressed. The strong inducible promoter elements, such as methanol oxidase (MOX) and formate dehydrogenase, have been used in the cloned gene expression of invertase [23], bean proteins of cocoa [29], phytase [21], aprotinin [30], and spinach glycolate oxidase [8]. Expression studies revealed differences in the regulatory mechanisms of methanol pathway genes between the methylotrophic yeasts [1,8]. In *P. pastoris*, methanol-inducible proteins were observed only after induction with methanol. For *H. polymorpha*, on the other hand, high-level expression of these proteins could also be obtained in glycerol-grown cells according to a repression/derepression mechanism. Many research efforts have been focused mainly on cloning of the genes related to methanol metabolism and peroxisome biogenesis in *H. polymorpha* [5,12,16].

Hirudin, a family of 64–66 amino acid proteins excreted by the blood-sucking leech, *Hirudo medicinalis*, inhibits thrombin, a protein involved in the cascade of events associated with blood coagulation in humans [7]. Hirudin specifically binds to the active site of thrombin, preventing its action on fibrinogen, and thereby interfering with the subsequent blood coagulation. Due to these properties, hirudin has been recognized early as a useful therapeutic agent for cardiovascular diseases. Therefore, an abundant supply of the highly purified and active hirudin was necessary for clinical trials. With limited availability of natural hirudin from leech, the development of recombinant cell fermentation processes for a large-scale production was inevitable [3,19,26–28].

This study was done to analyze the MOX promoter-mediated gene expression pattern in an effort to determine an optimum methanol concentration for hirudin expression in the recombinant *H. polymorpha*. Batch, fed-batch, and continuous culture methods were employed to examine the influences of methanol concentration on the properties of cell growth and hirudin expression in recombinant *H. polymorpha* under various culture conditions.

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Materials and methods

Organism and cultivation conditions

Recombinant *H. polymorpha* DLUV10 (*leu2*, *ura3*) harboring the chromosome-integrating plasmid pUAEM36 was used. *H. polymorpha* DLUV10 was derived from the DL-1 strain by UV mutagenesis to confer the *ura3* mutation. Plasmid pUAEM36 harbors the URA3 selection marker, autonomously replicating sequence (HARS36), and synthetic hirudin gene under the control of the MOX promoter (Figure 1). The MFα1 prepro leader sequence of *S. cerevisiae* was used for the secretion of hirudin. Seed cultures were grown overnight in 5 ml YNB medium (6.7 g yeast nitrogen base, 30 mg leucine, and 20 g glucose) and transferred into a 100-ml flask containing enriched complex medium (30 g yeast extract, 10 g peptone, 15 g KH_2PO_4, and 3.4 g yeast nitrogen base per liter). Flask cultures were carried out in a rotary shaking incubator (Vision Scientific VS1100, Inchon, Korea). All media were heat-sterilized for 10 min at 121°C. Leucine and methanol were filter-sterilized. A bench-top fermenter (KoBiotech KF-3L, Inchon, Korea) was used for fed-batch and continuous cultures. Batch fermentation was switched to a continuous or fed-batch mode at the late exponential growth phase. Medium pH was adjusted to 5.0 with 1 N HCl or 1 N NaOH, and temperature was maintained at 30°C.

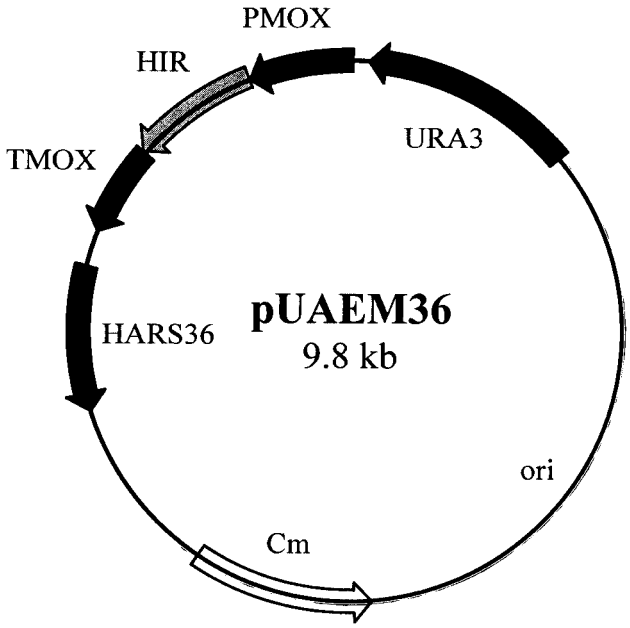

Figure 1 Genetic map of plasmid pUAEM36. PMOX, methanol oxidase promoter; HIR, synthetic hirudin gene in frame with MFα1 prepro leader sequence of *S. cerevisiae*; TMOX, methanol oxidase transcriptional terminator.

Analyses

Dry cell mass was measured at 600 nm. The conversion of optical density to dry cell mass concentration was done using the conversion equation:

$$\text{Dry cell mass } (\text{g L}^{-1}) = 0.14 \times \text{OD}_{600}$$

Culture broth was centrifuged for 10 min at 6000×*g*, and the supernatant fluid was stored at −80°C until further analysis. Methanol concentration was analyzed using a gas chromatograph (YoungLin 600D, Seoul, Korea) equipped with a Carbowax 20M column (Hewlett Packard, Palo Alto, CA). Isopropyl alcohol (2%) was used as an internal standard, and helium gas as a carrier at a flow rate of 30 ml min^{-1}. Oven temperature was linearly elevated from 80°C to 140°C at a rate of 20°C min^{-1}.

Hirudin activity in the culture broth was determined through the antithrombin activity [3,19,26] using a chromogenic substrate, Chromozyme TH (Roche, Mannheim, Germany). The amidolytic cleavage of Chromozyme TH by thrombin (Sigma Chemical, St. Louis, MO) was measured as the rate of increase in absorbance at 405 nm with a microtiter plate reader (Bio-Tek, Winooski, VT). Thrombin (0.6 NIH unit ml^{-1}), diluted culture supernatant fluid, and Chromozyme TH (200 μM) were loaded into the 96-well assay plate, and the reaction was monitored every 30 s for 5 min. One unit of antithrombin activity (ATU) was defined as the amount of hirudin able to completely inhibit one NIH unit of human thrombin at 37°C.

Results and discussion

A batch culture with the methanol medium was carried out to examine the pattern of cell growth and hirudin expression (Figure 2). Maximum dry cell mass was 2.30 g l^{-1} with an estimated cell yield of 0.37 (g dry cell g^{-1} methanol^{-1}). The total amount of hirudin (7.30 mg l^{-1}) produced was almost identical to that

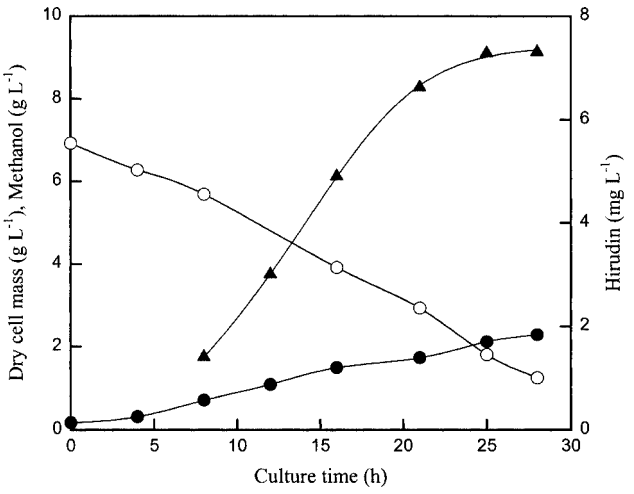

Figure 2 Time course of cell growth, methanol consumption, and hirudin production with recombinant *H. polymorpha* DLUV10 grown at pH 5.0 and 30°C. ●, Dry cell mass; ○, methanol; ▲, hirudin.

obtained by the MOX promoter in the batch culture with glucose (data not shown). Recombinant *H. polymorpha* DLUV10 showed higher specific hirudin expression levels in the methanol medium than in the glucose or glycerol medium. However, since specific

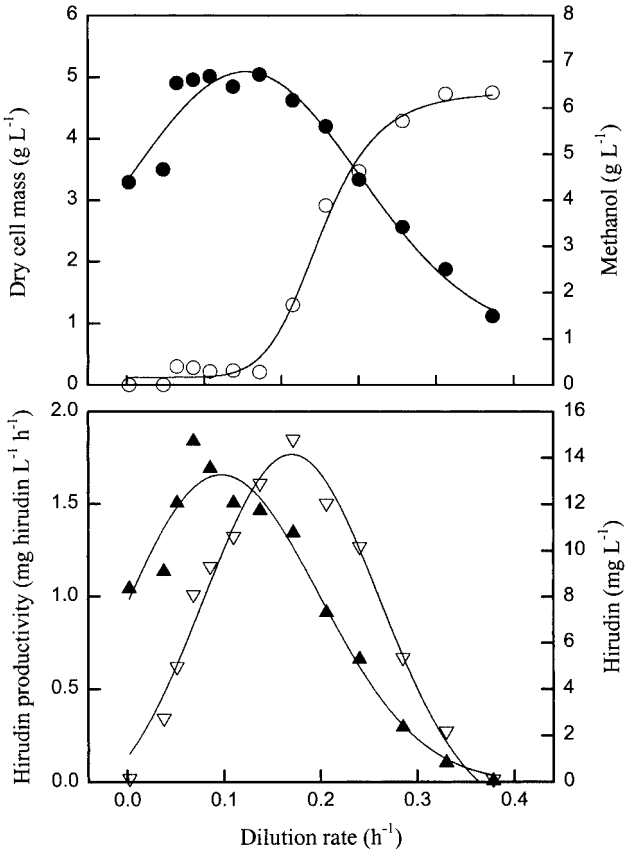

Figure 3 Continuous culture of recombinant *H. polymorpha* DLUV10 grown at pH 5.0 and 30°C. The fermentation mode was switched at the late exponential growth phase. The methanol concentration in the inlet feed was 8 g l^{-1}. ●, Dry cell mass; ○, methanol; ▲, hirudin; ▽, hirudin productivity.

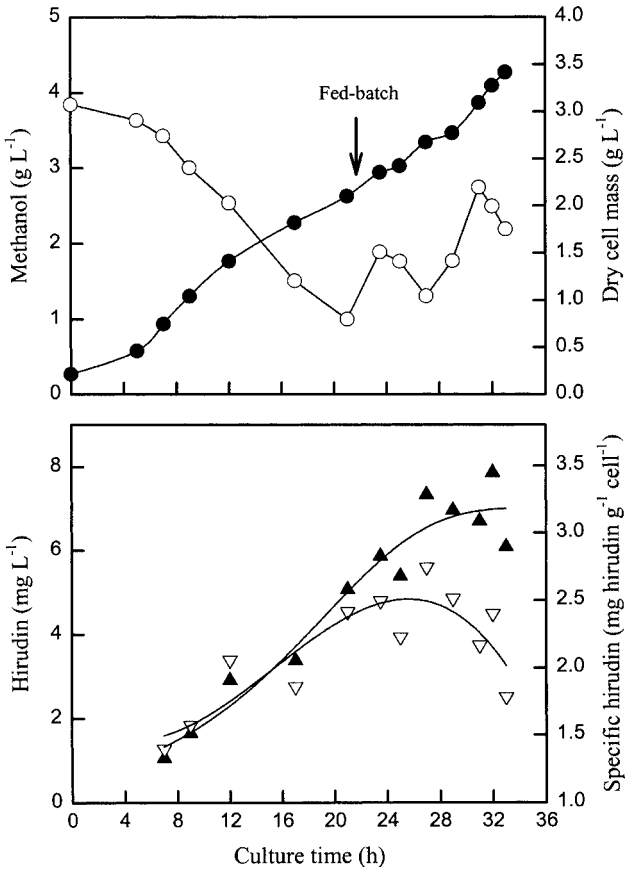

Figure 4 Effects of methanol concentrations on hirudin production in fed-bath cultivation of recombinant *H. polymorpha* DLUV10. ●, Dry cell mass; ○, methanol; ▲, hirudin; ▽, specific hirudin expression level. Methanol (200 g l^{-1}) was intermittently fed to modulate residual methanol concentrations in the fermenter.

growth rates in the methanol medium were lower compared with those obtained from the glucose or glycerol medium, hirudin productivity did not increase as expected.

Characteristics of hirudin production and cell growth were further investigated in continuous and fed-batch cultures. The fermentation mode was switched to the continuous cultivation mode at the late exponential growth phase, and the enriched medium supplemented with 8 g l^{-1} methanol was added. Dilution rates were varied to modulate the residual methanol concentrations at steady states. Maximum hirudin productivity of 1.8 mg l^{-1} h^{-1} was obtained at 0.18 h^{-1} dilution rate and 1.7 g l^{-1} methanol concentration (Figure 3). As the dilution rate increased above 0.20 h^{-1}, hirudin concentration and productivity gradually decreased. Experimental results obtained from the continuous culture clearly suggested that high concentrations of methanol were detrimental to hirudin expression in recombinant *H. polymorpha* DLUV10.

Effects of methanol concentrations were further investigated in the fed-batch culture. A batch culture was grown for 21 h and then switched to the fed-batch mode (Figure 4). Methanol (200 g l^{-1}) was intermittently fed to modulate residual methanol concentrations in the fermenter. Cells continued to grow in the fed-batch mode along with the concomitant expression of hirudin. An increase in the residual methanol concentration caused a gradual reduction in specific hirudin expression. Consequently, hirudin production ceased even with continuous cell growth. Methanol concentrations appeared to exert a greater effect on the hirudin expression than the cell growth. When specific hirudin expression levels were plotted against residual methanol concentrations for different fermentation modes including batch cultures with various initial methanol concentrations, continuous, and fed-batch cultures, recombinant *H. polymorpha* showed a consistent specific hirudin expression pattern for various fermentation modes. Thus, specific hirudin expression levels exponentially decreased with an increase in the residual methanol concentrations (Figure 5).

Methanol is considered a derepressing substrate that enables the highest expression of the enzymes involved in its catabolism [10,20,21]. As mentioned above, use of methanol as the only source of carbon and energy resulted in a very low growth rate and low cloned gene productivity. One of the difficulties associated with methanol feeding in the fed-batch cultivation of methylotrophic yeasts is that the methanol concentration must be maintained within a narrow range to prevent its inhibitory effects on cell growth and cloned gene expression [18]. Guarna *et al* [13] observed that recombinant protein expression in recombinant *P. pastoris* suddenly decreased when the residual methanol concentration exceeded 1.0% (v/v). High concentrations of methanol and by-products, such as formaldehyde and formic acid, formed from methanol metabolism should exert negative effects on the metabolic activity of the cell, and eventually on the productivity of the whole fermentation process.

In this study, effects of methanol concentrations on the expression of an anticoagulant hirudin gene in recombinant *H. polymorpha* DLUV10 were studied carefully to reveal the existence of an optimum methanol concentration in the MOX promoter-mediated expression of hirudin. More research is in progress to establish optimum fermentation strategies by adopting the experimental results obtained in this study.

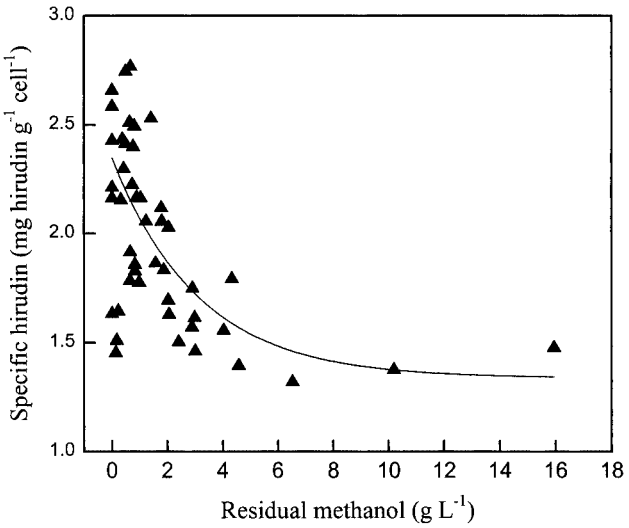

Figure 5 The relationship between specific hirudin expression levels and residual methanol concentrations in various fermentation modes. Data were obtained from shake flasks with various initial methanol concentrations, and continuous and fed-batch cultures. Specific hirudin expression levels for batch cultures were estimated from the exponential growth phases.

Acknowledgements

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