**Journal of Industrial Microbiology & Biotechnology (1998) 21**, 1–5

http://www.stockton-press.co.uk/jim

# Production of recombinant hirudin in *Hansenula polymorpha*: variation of gene expression level depends on methanol oxidase and fermentation strategies

CH Kim, HW Seo, HY Kim, JH Sohn, ES Choi and SK Rhee

*Applied Microbiology Research Division, Korea Research Institute of Bioscience and Biotechnology, PO Box 115, Yusong, Taejon 305–600, Korea*

**Various recombinant *Hansenula polymorpha* strains were developed and compared for their level of expression of the anticoagulant hirudin. *H. polymorpha* DL1-57 harboring an autonomously replicating sequence, *HARS36*, efficiently expressed the gene for recombinant hirudin. The effect of methanol oxidase (*MOX*) on the expression of the hirudin gene in *H. polymorpha* DL1-57 was studied, and the fermentation strategies coupled with the *MOX* activity and an antioxidant, tocopherol, were also examined.**

**Keywords:** hirudin; *Hansenula polymorpha*; methylotrophic yeast; methanol oxidase; autonomously replicating sequence

## Introduction

Hirudin from the medicinal leech *Hirudo medicinalis* is a polypeptide of 65 or 66 amino acid residues [1,5]. It acts as a slow, tight-binding inhibitor of thrombin [12]. Hirudin is a trace component of leeches, so many studies to enhance the production of recombinant hirudin have been attempted [2,3,7,8,11]. In this connection, methylotrophic yeasts, including *Hansenula polymorpha*, offering better promoter strength, secretion efficiency and high cell density cultures have attracted considerable attention as new hosts for the production of recombinant proteins [13].

The methylotrophic yeasts are distinguished from other yeasts by the presence of methanol oxidase (*MOX*). As the methylotrophic yeasts have a strong *MOX* promoter, several expression systems using the *MOX* promoter of *H. polymorpha* were developed and applied to the production of recombinant proteins [4,9,13]. Previous studies [4,6,9] confirmed that the expression level of the hirudin gene depends entirely upon the kind of expression cassette and fermentation strategies. In a previous study [6], we asserted that *H. polymorpha* with strong *MOX* activity acts partially like a whole cell enzyme, so that a large portion of the methanol supplied as an inducer is used as a substrate for the enzyme reaction. *H. polymorpha*, therefore, consumes a large amount of methanol to induce the *MOX* promoter. To this end, we developed various kinds of recombinant strains harboring different autonomously replicating sequences of *H. polymorpha* (*HARS*) and some methanol oxidase-deficient mutants (*MOX*$^-$).

As it is also important to understand the roles of the inducer and medium components in the production of hirudin by *H. polymorpha*, the effects of glycerol, yeast extract, fatty acid and tocopherol on cell growth and hirudin production were also investigated.

Correspondence: SK Rhee, Applied Microbiology Research Division, Korea Research Institute of Bioscience and Biotechnology, PO Box 115, Yusong, Taejon 305-600, Korea
Received 4 February 1998; accepted 24 June 1998

## Materials and methods

### Microorganisms and culture conditions

*H. polymorpha* DL1(*leu2*)(ATCC 26021) was used as a host for the integration and expression of the plasmids carrying the hirudin expression cassettes [9]. The recombinant strain with strong methanol oxidase activity used in this study was *H. polymorpha* DL1-057 containing *HARS36*, which shows the characteristic of tandem repeat integration [10]. A methanol oxidase-negative mutant, *H. polymorpha* DL1-005, was derived from strain DL1-057. The medium and culture conditions used were the same as described previously [6]. *H. polymorpha* cells, previously grown on YND agar plates, were inoculated into 500-ml baffled flasks containing 50 ml seed culture liquid medium consisting of 20 g $L^{-1}$ glycerol (Junsei Chemical Co Ltd, Tokyo, Japan) and 6.7 g $L^{-1}$ YNB (yeast nitrogen base without amino acid, Difco, MI, USA). The initial pH was adjusted to 5.5 with 2 N HC1 and 2 N NaOH and then autoclaved for 15 min at 121 °C. After inoculation, flasks were incubated with shaking at 130 rpm and at 30°C for 20 h. To ensure similar cell concentrations at the beginning of the experiments, this seed culture was used as inoculum in all studies. Soybean oil 10 g $L^{-1}$ (Samyang Corp Ltd, Seoul, Korea) and 0.1–2.0% (w/v) d1-$\alpha$-tocopherol (Sigma Chemical Co, MO, USA) were used as a source of fatty acids and as an antioxidant, respectively. They also functioned effectively as an antifoam agent.

### Hirudin fermentation

A 5-L fermentor (NBS BioFlowIII, NJ, USA) was used for the cultivation of *H. polymorpha* DL1-057 (*MOX*$^+$). The fermentor containing 3 L of GYFT consisting of 20–100 g $L^{-1}$ glycerol, 40 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ soybean oil, and 0.1% (w/v) $\alpha$-tocopherol was inoculated with 5% (v/v) of seed culture. Fermentations were examined using two

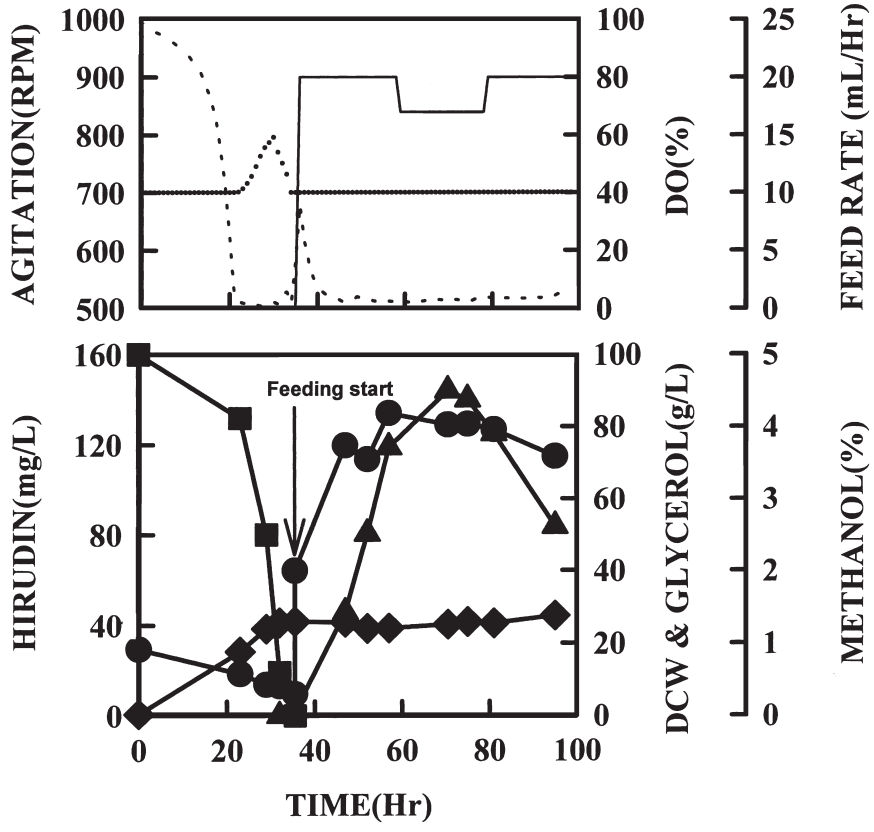


**Figure 1** Fermentation profiles for the production of hirudin by *MOX*$^+$ *H. polymorpha* DL1-057 harboring *HARS*36 using pure methanol as an inducer for *MOX* promoter as well as a carbon and energy source. Feed rate (————); rpm (- - - -); DO (· · · ·). Glycerol (■); methanol (●); hirudin (▲); DCW (◆).

kinds of feeding protocols, methanol fed-batch and mixed-feed fed-batch. For the methanol fed-batch fermentation, GYFT medium containing 100 g $L^{-1}$ glycerol was used for cell growth and the methanol fed-batch was started after depletion of glycerol in the medium. For the mixed-feed fermentation, GYFT medium containing 20 g $L^{-1}$ glycerol was used for the initial cell growth and methanol to glycerol mixtures (3:1, 2:1, 1:1) were used as the feeding solutions for the concomitant growth and induction. Fermentation was carried out at 30°C with an agitation speed of 700 rpm. The pH of the medium was controlled at 5 with 4 N $NH_4OH$ throughout the fermentation.

*H. polymorpha* DL1-005 (*MOX*$^-$) was cultured in a 500-ml baffled flask using 50 ml of GYFT medium containing 50 g $L^{-1}$ glycerol. After depletion of glycerol in the medium, glycerol and methanol were occasionally fed into the medium to maintain their concentrations in the appropriate range.

### *Analytical methods*

Cell growth and the concentrations of methanol, glycerol and hirudin were determined as described previously [6].

## Results and discussion

### *Hirudin production by MOX*$^+$ *H. polymorpha*

Pure methanol or mixtures of methanol and glycerol were used as substrates for the growth of *H. polymorpha*. Methanol acted not only as inducer for the induction of *MOX* promoter but as substrate for cell growth. Figure 1 shows hirudin production by *H. polymorpha* DL1-057 (*MOX*$^+$) in a fed batch fermentation using pure methanol as feeding substrate. Initial concentrations of methanol and glycerol were 1% (v/v) and 100 g $L^{-1}$, respectively. After exhaustion of methanol and glycerol, 45 ml of methanol were added into the fermentor to maintain its concentration above 1.5% and continuous feeding of methanol was initiated at a flow rate of 20 ml $h^{-1}$. The methanol concentration was maintained above 3% during the fermentation. Dissolved oxygen (DO) was controlled above 5% in the DO active mode by automatic control of the agitation rate. The DO level was gradually decreased in proportion to the concentrations of glycerol and methanol from saturation to 5% and then increased suddenly after exhaustion of methanol and glycerol. This signal was used as a starting point for the feeding of methanol. Cell growth almost ceased with the complete consumption of glycerol and the initiation of methanol feeding. Expression of hirudin was initiated after 35 h of cultivation concomitantly with the depletion of glycerol. The maximum concentration of hirudin reached 144 mg $L^{-1}$ with a productivity of 2.1 mg $L^{-1}$ $h^{-1}$ at 70 h of cultivation. Hirudin, which is susceptible to degradation by proteolytic enzymes undesirably produced by the cells, was sharply denatured thereafter [6].

The results of hirudin production by using the 3:1 or 2:1 (w/w) methanol to glycerol mixtures as a feeding solution are presented in Figure 2(a) and (b). The initial concentrations of methanol and glycerol were 0.5% (v/v) and 20 g $L^{-1}$, respectively. After exhaustion of the carbon sources,

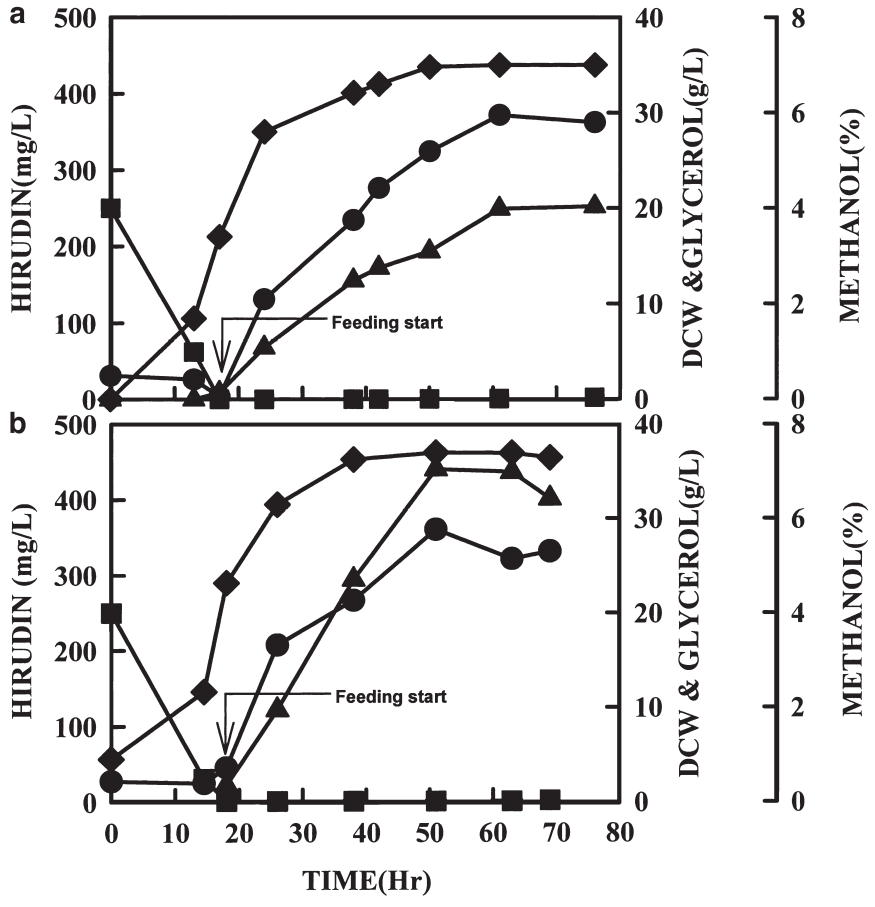


**Figure 2** Production of hirudin by *MOX*$^+$ *H. polymorpha* DL1-057 harboring *HARS*36 using 3:1 (w/w) methanol to glycerol mixture (a) and 2:1 (b) as an inducer for the *MOX* promoter as well as a carbon and energy source. Glycerol (■); methanol (●); hirudin (▲); DCW (◆).

continuous feeding of methanol was initiated at a flow rate of 20 ml $h^{-1}$. Methanol concentrations increased gradually up to 6.0% in the 3:1 solution and 5.8% in the 2:1 solution, whereas glycerol was maintained under 0.2 g $L^{-1}$ after the initiation of feeding. The maximum concentrations and productivities of hirudin were 252 mg $L^{-1}$ and 3.3 mg $L^{-1}$ $h^{-1}$ at 71 h in the 3:1 solution and 440 mg $L^{-1}$ and 8.6 mg $L^{-1}$ $h^{-1}$ at 51 h in the 2:1 solution. The concentration of hirudin was maintained over 12 h at the maximum level and hirudin was denatured slowly thereafter. This was remarkable improvement compared with the previous case (Figure 1), using pure methanol as a feeding solution, with respect to the final concentration of hirudin and productivity. The lower level of hirudin in the 3:1 solution compared with the 2:1 solution might be due to the limitation of glycerol to support growth and maintenance of cells as well as the excess amount of methanol. With excess methanol, *H. polymorpha* cells would act like a whole cell enzyme with strong MOX activity in a short period of cultivation [6]. On the other hand, when glycerol (1:1 solution) was used, the accumulation of glycerol above 1.0 g $L^{-1}$ was concomitant with inhibition of expression of hirudin and the concentration of hirudin barely reached 183 mg $L^{-1}$ (data not shown). As a consequence, the large portion of methanol supplied in excess might be used as a substrate for methanol oxidase, as well as an inducer and a carbon source, and glycerol supplied in excess might inhibit the *MOX* promoter, which resulted in decreased expression of hirudin.

### *Hirudin production by MOX$^-$ H. polymorpha*

As shown in Figure 3, growth of *H. polymorpha* DL1-005 (*MOX*$^-$) in GYFT liquid medium containing 50 g $L^{-1}$ glycerol without methanol was similar to that in the medium containing methanol. However, hirudin was not produced in the medium without methanol, but only in medium containing methanol as an inducer. Thus, hirudin was induced only in the presence of methanol. In addition, the production of hirudin by the *MOX*$^-$ strain was significantly affected by the concentration of methanol at which maximal induction of hirudin was achieved in the range of 0.7–1.0% (v/v) methanol.

Since the *H. polymorpha* DL1-005 does not have methanol oxidase activity, it is not able to utilize methanol as a carbon source. Therefore, an additional carbon source is required to cultivate the *MOX*$^-$ strain. In the present study, glycerol was provided as an additional carbon source for the purpose of cell growth and the concentration of methanol was maintained between 0.7–1.0% (v/v). Production of hirudin by *H. polymorpha* DL1-005 was increased by the additional supply of glycerol (0.1%, w/v). Although cellular growth increased considerably with the increased supply of glycerol above 3 g $L^{-1}$, the production of hirudin decreased

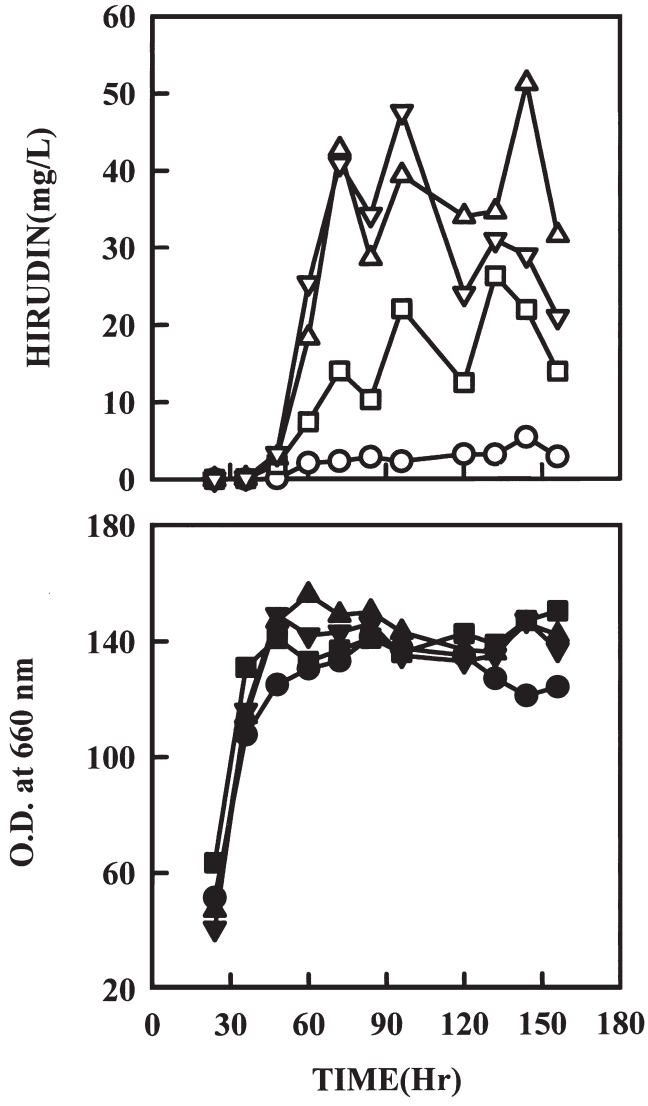


**Figure 3** The effect of methanol on cell growth and hirudin production by *MOX*⁻ *H. polymorpha* DL1-005 harboring *HARS*36. Methanol: 0.0% (○, ●); 0.3% (□, ■); 0.7% (△, ▲); 1.0% (▽, ▼).

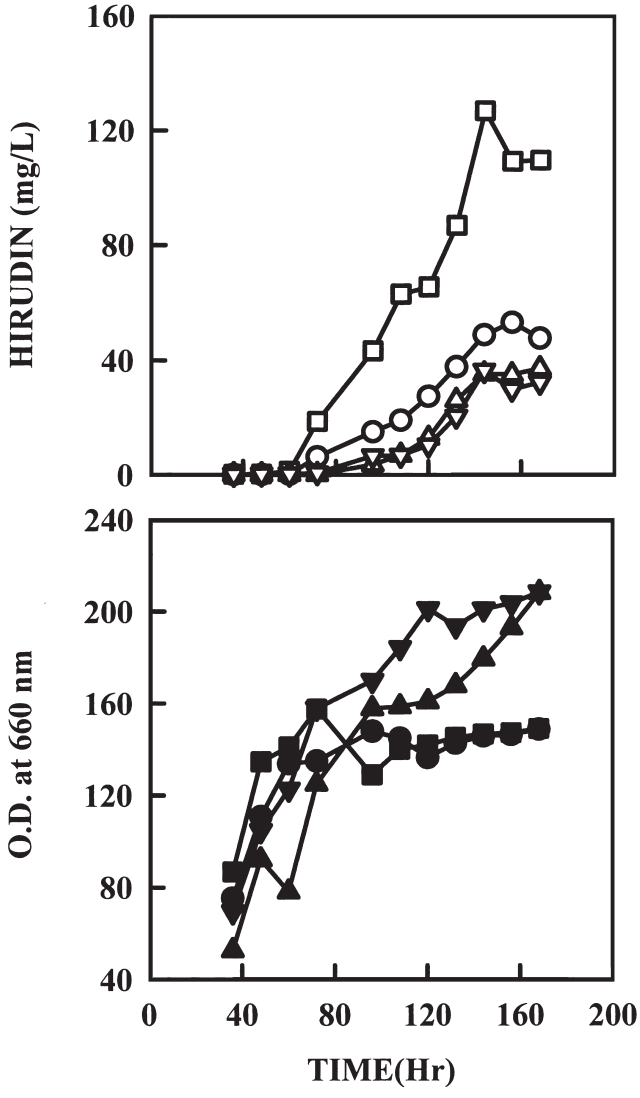


**Figure 4** The effect of glycerol on cell growth and hirudin production by *MOX*⁻ *H. polymorpha* DL1-005 harboring *HARS*36. Glycerol: 0.0% (○, ●); 0.1% (□, ■); 0.3% (△, ▲); 5% (▽, ▼).

when the glycerol was added above 3 g $L^{-1}$ (Figure 4). This suggests that production of hirudin by *H. polymorpha* DL1-005 is repressed by a concentration of glycerol above 3 g $L^{-1}$, indicating that substrate repression occurs. Although growth of *H. polymorpha* DL1-005 increased with an additional supply of yeast extract in a submerged flask culture, the production of hirudin was not significantly affected by the concentration of yeast extract (data not shown).

It was reported in a previous study that the production of hirudin by a *MOX*⁺ *H. polymorpha* DL-1 containing *HARS3* was increased by providing soybean oil as a source of fatty acids and dl-$\alpha$-tocopherol as an antioxidant [6]. In the present study, production of hirudin by *H. polymorpha* DL1-005 was also significantly increased by addition of $\alpha$-tocopherol in the medium (Figure 5). The optimum amount of $\alpha$-tocopherol for the production of hirudin by *H. polymorpha* DL1-005 was between 0.1% and 2.0% (w/v). As shown in Figure 5, however, the production of hirudin by *H. polymorpha* DL1-005 was repressed by addition of $\alpha$-tocopherol above 2.0%. Taking the results described above into account, the production of hirudin by *H. polymorpha* DL1-005 using GYFT medium containing 50 g $L^{-1}$ glycerol, 40 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ soybean oil and 0.1% $\alpha$-tocopherol was examined. After 60 h of cultivation, glycerol and methanol were added to maintain the concentrations in the range of 1–2 g $L^{-1}$ and 0.7–1.0% (v/v), respectively. Maximal production of hirudin by *H. polymorpha* DL1-005 was 215 mg $L^{-1}$ within 160 h of cultivation (Figure 6). Although the consumption of methanol was lower, the concentration and productivity (1.3 mg $L^{-1}$ $h^{-1}$) of hirudin were comparatively lower than those of *MOX*⁺ *H. polymorpha* DL1-005.

In conclusion, the present study shows that the delicate controls of methanol, glycerol, and $\alpha$-tocopherol concentrations in the culture medium are required for maximal production of hirudin by recombinant *H. polymorpha* containing the *MOX* promoter. Further studies will be focused on the development of more productive *MOX*⁻ strains as

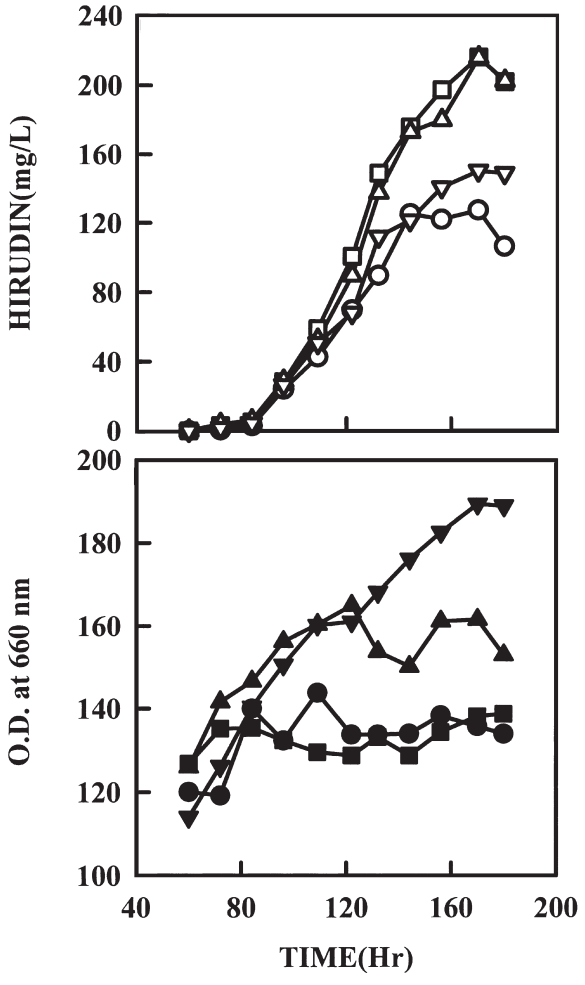


**Figure 5** The effect of tocopherol on cell growth and hirudin production by *MOX*$^{-}$ *H. polymorpha* DL1-005 harboring *HARS*36. $\alpha$-Tocopherol: 0.0% (○, ●); 0.1% (□, ■); 1.0% (△, ▲); 2.0% (▽, ▼).

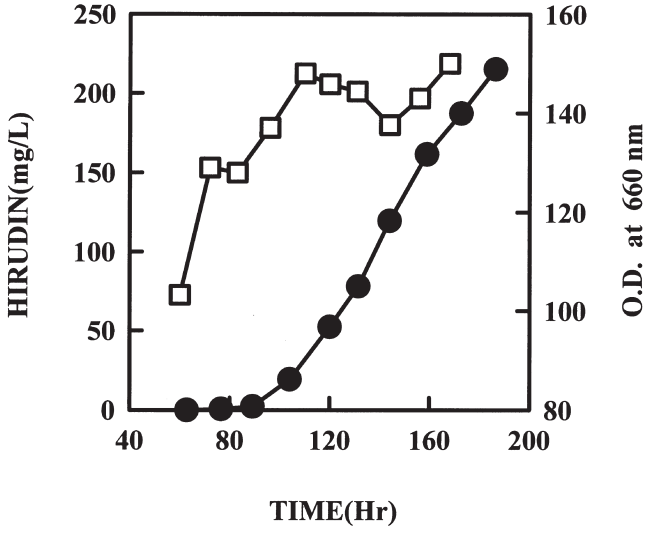


Figure 6 Production of hirudin by *MOX*$^{-}$ *H. polymorpha* DL1-005 harboring *HARS*36. Hirudin (●); cell growth (□).

well as protease-deficient mutants for the enhanced production of recombinant hirudin.

## Acknowledgements

This work was supported by the Ministry of Science and Technology and Dong Kook Pharmaceutical Co, Ltd in Korea.

## References

1 Dodt J, HP Mueller, U Seemuller and JY Chang. 1984. The complete amino acid sequence of hirudin, a thrombin specific inhibitor. FEBS Lett 165: 180–184.
2 Dodt J, T Schmitz, T Schaeter and C Bergman. 1986. Expression, secretion and processing of hirudin in *E. coli* using the alkaline phosphatase signal sequence. FEBS Lett 202: 373–377.
3 Fortkamp E, M Rieger, G Heisterberg-Moutses, S Schweitzer and R Sommer. 1986. Cloning and expression in *Escherichia coli* of a synthetic DNA for hirudin, the blood coagulation inhibitor in the leech. DNA 5: 511–517.
4 Gellissen G, ZA Janowicz, U Weydemann, K Melber, AWM Strasser and CP Hollenberg. 1992. High-level expression of foreign genes in *Hansenula polymorpha*. Biotech Adv 10: 179–189.
5 Harvey RP, E Degryse, L Stefani, F Schamber, JP Cazenave, M Courtney, P Tolstoshev and JP Lecocq. 1986. Cloning and expression of a cDNA coding for the anticoagulant hirudin from the bloodsucking leech *Hirudo medicinalis*. Proc Natl Acad Sci USA 83: 1084–1088.
6 Kim CH, JH Sohn, ES Choi and SK Rhee. 1996. Effect of soybean oil on the enhanced expression of hirudin gene in *Hansenula polymorpha*. Biotechnol Lett 18: 417–422.
7 Loison G, A Findeli, S Bernard, M Nguyen-Juilleret, M Marquet, N Reihl Bellon, D Carvallo, L Guerry-Santos, SW Brown, M Courtney, C Roitsch and Y Lemoine. 1988. Expression and secretion in *Saccharomyces cerevisiae* of biologically active leech hirudin. Bio/technology 6: 72–77.
8 Mendoza-Vega O, C Hebert and SW Brown. 1994. Production of recombinant hirudin by high cell density fed-batch cultivations of a *Saccharomyces cerevisiae* strain: physiological considerations during the bioprocess design. J Biotechnol 32: 249–259.
9 Sohn JH, MY Beburov, ES Choi and SK Rhee. 1993. Heterologous gene expression and secretion of the anticoagulant hirudin in a methylotrophic yeast *Hansenula polymorpha*. J Microbiol Biotechnol 3: 65–72.
10 Sohn JH, ES Choi, CH Kim, MO Agaphonov, MD Ter-Avanesyan, JS Rhee and SK Rhee. 1996. A novel autonomously replicating sequence (*ARS*) for multiple integration in the yeast *Hansenula polymorpha*. J Bacteriol 178: 4420–4428.
11 Sohn JH, SK Lee, ES Choi and SK Rhee. 1991. Gene expression and secretion of the anticoagulant hirudin in *Saccharomyces cerevisiae*. J Microbiol Biotechnol 1: 266–273.
12 Stone SR and J Hofsteenge, 1986. Kinetics of the inhibition of thrombin by hirudin. Biochemistry 25: 4622–4628.
13 Veale RA, MLF Guiseppin, HMJ Van Eijk, PE Sudbery and CT Verrips. 1992. Development of a strain of *Hansenula polymorpha* for the efficient expression of guar $\alpha$-galactosidase. Yeast 8: 361–372.