Biotechnology and Bioprocess Engineering **2003**, 8: 183-186

Production of Recombinant Hirudin in Galactokinase-deficient *Saccharomyces cerevisiae* by Fed-batch Fermentation with Continuous Glucose Feeding

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Abstract The artificial gene coding for anticoagulant hirudin was placed under the control of the *GAL10* promoter and expressed in the galactokinase-deficient strain (*Δgal1*) of *Saccharomyces cerevisiae*, which uses galactose only as a gratuitous inducer in order to avoid its consumption. For efficient production of recombinant hirudin, a carbon source other than galactose should be provided in the medium to support growth of the *Δgal1* strain. Here we demonstrate the successful use of glucose in the fed-batch fermentation of the *Δgal1* strain to achieve efficient production of recombinant hirudin, with a yield of up to 400 mg hirudin/L.

Keywords: recombinant hirudin, *Saccharomyces cerevisiae*, *GAL* promoter, galactose, fed-batch fermentation

INTRODUCTION

Hirudin is a potent, thrombin-specific inhibitor. It was originally isolated from salivary glands of blood sucking leech *Hirudo medicinalis* [1]. Hirudin is a globular protein with a molecular weight of 7,000 Da and is made up of 65 amino acids. The polypeptide is joined by three intra-molecular S-S bridges and has a sulfated tyrosine residue at position 63 [2].

Hirudin represents a new anticoagulant agent in a field in which heparin has been the only available drug for many years. Hirudin forms equimolar complexes with thrombin, and has other advantages over heparin as well as being twice as strong in its antithrombotic effect [3,4]. For example, it does not require antithrombin III as a cofactor, is not inactivated by antiheparin proteins, has no direct effects on platelets and less likelihood of any hemorrhage, is able to inactivate thrombin bound to clots or the sub-endothelium with a low risk of any allergic reaction, and is a very weak immunogen. Hirudin may also act to stop the greatly increased rate of cell division in cancerous growth. Research is still being undertaken into other advantages of hirudin as a clinical tool of the future [5].

The production of hirudin in much smaller quantities in nature encouraged molecular biologists to express the hirudin gene in different hosts for subsequent large-scale production and purification [6-10]. A variety of vector systems were designed and developed employing appropriate constitutive or inducible promoters for the efficient expression of heterologous proteins in the yeast *Saccharomyces cerevisiae*. Among the strong and inducible promoters used in this yeast, *GAL1, GAL7, GAL10* and their hybrids with *CYC1* or UAS_G have been used more frequently to produce heterologous proteins from *S. cerevisiae*. In these galactose inducible systems, the yeast cells were grown on complex medium containing dextrose to repress the expression of cloned product, and then high concentrations of galactose were added to induce the expression. Therefore the concentrations of glucose, galactose and ethanol play major roles in efficient expression of the system. The recombinant *S. cerevisiae* strains harboring the hirudin gene under the control of the *GAL10* promoter were developed in our earlier studies [9,10]. However, as galactose is an expensive sugar and is needed in higher concentrations (above 30 g/L) for efficient expression, its cost influences the final process cost to a large extent. With this consideration, we developed a mutant strain of *S. cerevisiae*, which can use galactose only as an inducer but not as a carbon source, by disrupting the *GAL1* gene coding for an enzyme galactokinase which catalyses the first step in the galactose utilization [11]. As galactose cannot be used as a carbon source, a carbon source other than galactose should be provided to support the growth of the galactokinase-deficient (*gal1*) null strain. In the present report, we have developed fed-batch fermentation processes with continuous feeding of glucose to support growth without repression of the expression of the hirudin gene from the *GAL10* promoter.

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MATERIALS AND METHODS

Strains and Plasmids

The host strain used in this study was S28G1, a *gal1*-disruptant of *S. cerevisiae* 2805 (*Mat a pep4 :: HIS3 prb1 can1 his3 ura3-52)*, in which most of the open reading frame of the *GAL1* gene had been deleted [11]. YEG*a*-HIR525, in which the synthetic hirudin gene was placed under the control of the *GAL10* promoter and the mating factor (MF *a*1) pre-proleader sequence, was used as a hirudin expression vector [12].

Media and Cultivation

Minimal YNBCAD medium, containing yeast nitrogen base (without casamino acid) 6.7 g/L, casamino acid 5.0 g/L and dextrose 20 g/L, was used for the maintenance and growth of the organism. A single colony of yeast transformant from YNBCAD agar plate was inoculated with 10 mL of YNBCAD medium, incubated overnight at 30°C, transferred to Erlenmeyer flask containing the same medium at 10% (v/v) level, and incubated for 24 h at 30°C. The second stage culture at 10% (v/v) was used to inoculate fed-batch fermentations. The medium used initially for fed-batch consisted of yeast extract 40 g/L, casamino acid 5 g/L, K_2HPO_4 10 g/L, glucose 20 g/L and galactose 16 g/L.

Fermentation

Fermentation was carried out in a 5-L bioreactor with the working volume of 3.5 L under controlled conditions of pH5.4 and 30°C. The agitation speed was varied from 400-800 rpm to keep the dissolved oxygen concentration above 20%. Fed-batch fermentations were carried out using different concentrations of glucose in the feed at the constant flow rate of 25 mL/h using a peristaltic pump placed into the growing yeast culture. At the initial stage, the fermentation was carried out in batch cultivation mode with 2.5 L of YPD medium initially containing 20 g/L glucose and 16 g/L galactose. At 14 h of fermentation, when the pH and dissolved oxygen concentration increased, the glucose feed was started at the constant flow rate, thereby varying the concentration of glucose in the feed.

Analytical Methods

The growth of yeast cells was monitored by the measurement of optical density of culture broth at 600 nm using a spectrophotometer. Hirudin concentrations in the culture broth were determined using the chromogenic method described by Sohn *et al.* [6]. One antithrombin unit (ATU) is defined as the amount of hirudin required to completely inhibit one NIH unit of human thrombin. Recombinant hirudin from Accurate Chemical (USA) was used as a reference.

RESULTS AND DISCUSSION

When glucose is being used as the carbon source for the *gal1* Δ strain, its concentration in the broth is critical. The glucose concentration should be maintained at minimum levels to avoid the repression of hirudin expression and ethanol formation. Thus we have examined the feasibility of a continuous glucose feed as a fed-batch fermentation strategy to achieve high-cell density cultivation of the gal1Δ strain while maintaining high-level hirudin expression from the GAL promoter.

Results of fed-batch using 200 and 300 g/L glucose are shown in Figs. 1(a) and (b), respectively. Initial glucose (20 g/L) was consumed by 10 h of fermentation and additional feeding was started at 14 h to reduce the ethanol concentrations. The maximum 151 mg/L and 225 mg/L of hirudin could be obtained from the fermentations using 200 and 300 g/L glucose, respectively, after 50 h.

Result of fed-batch using 400 g/L glucose is shown in Fig. 1(c). As in the earlier experiments, initial glucose was almost used up by 12 h, but the ethanol formed increased to 13 g/L, which is a level undesirable for efficient expression of hirudin. Thus, the feed containing 400 g/L of glucose was started at 14 h of fermentation to ensure the lower concentrations of glucose and ethanol. After 30 h fermentation, both ethanol and glucose concentrations were almost zero. The concentration of galactose remained unchanged until 30 h of fermentation, after which it started to be converted to an unknown metabolite, which was detected as a novel peak on HPLC analysis (data not shown). Optical density reached the maximum of 160 at 55 h and hirudin was maximally produced at 48 h (290 mg/L); however this was later reduced to 250 mg/L, probably due to the enhanced proteolysis at the late stages of fermentation [13].

Results of 500 and 600 g/L glucose fed-batches are shown in Figs. 1(d) and (e), which have similar profiles. At about 14 h fermentation, the batch mode was switched over to fed-batch mode by continuously supplying the feed at a constant flow rate. Glucose concentration dropped below 1 g/L by 10 h and remained almost zero after 14 h of fermentation. Ethanol concentration was maintained below 11 g/L during the fermentation. Maximum hirudin concentration increased to 390 and 403 mg/L in 500 and 600 g/L glucose fed-batches, respectively, at 48 h. At later stages these concentrations started decreasing. At 660 nm, the maximum optical density of fermentation broth from 500 and 600 g/L glucose fed-batches reached to 160 and 170, respectively, at 52 h of fermentation. The residual glucose concentrations in the culture medium were found to be almost undetectable in this continuous feeding strategy.

In the present study, we demonstrated that the *S. cerevisiae* Δ*gal1* strain could produce high levels of hirudin, up to 403 mg/L, in fed-batch fermentations using glucose as a carbon source and galactose as a gratuitous inducer (Table 1). This production level of hirudin ob-

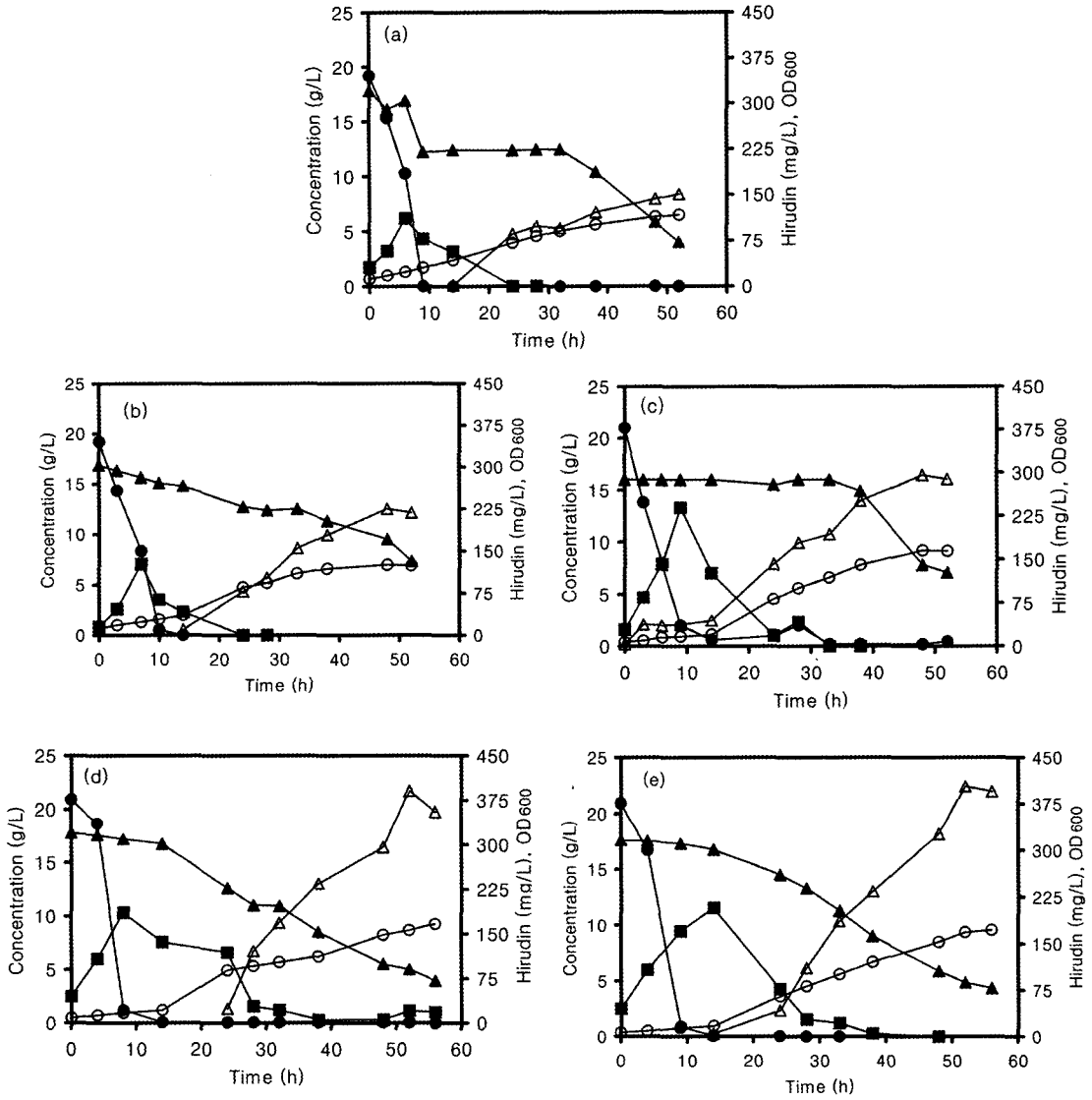

Fig.1 Fermentation profiles of hirudin production using *S. cerevisiae Δgal1* in fed-batches using different concentrations of glucose in the feed. (a) 200 g/L, (b) 300 g/L, (c) 400 g/L, (d) 500 g/L, (e) 600 g/L. ●: glucose, ■: Ethanol, ▲: Galactose, △: Hirudin, ○: OD.

Table 1. Results of fed-batch fermentation of *S. cerevisiae Δgal1* using different concentrations of glucose in the feed

Glucose feed concentration (g/L)	Residual glucose concentration (g/L)	Residual galactose concentration (g/L)	Maximum cell mass (OD_{660})	Maximum hirudin yield (mg/L)
200	ND*	4.0	117	151
300	ND	7.3	125	225
400	ND	5.2	167	295
500	ND	4.0	167	390
600	ND	4.3	172	403

*ND: Not detectable

tained using the Δ*gal1* strain is comparable to the maximum level obtained from the wild type recombinant strain. The cost of the final product could be considerably reduced as this strain uses much less galactose (40 g) than the original recombinant strain which consumes 500 g of galactose [14]. Our results strongly suggest that a continuous glucose feed as a carbon source might be a suitable feeding strategy to avoid glucose inhibition of the *GAL10* product while maintaining support for the growth of the gratuitous strain.

Acknowledgements This work was supported by the Ministry of Science and Technology, Republic of Korea, and by Dong Kook Pharmaceutical Co, Ltd. The authors deeply appreciate their financial assistance.

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[Received April 23, 2003; accepted June 13, 2003]