High Cell Density Culture of Yarrowia lipolytica Using a One-Step Feeding Process

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Yarrowia lipolytica is a potentially useful host for heterologous protein production. To develop an efficient culture method for high cell density cultivation and heterologous gene expression of Y. lipolytica, the effects of medium components and their concentrations on the growth of Y. lipolytica have been investigated. Addition of yeast extract to the culture media was found to significantly reduce the long lag phase encountered when Y. lipolytica was cultivated in synthetic culture media containing high concentrations of glycerol. Therefore, by enriching with 0.3% yeast extract the synthetic culture medium containing 15% glycerol, we could cultivate Y. lipolytica up to 83 g/L dry cell weight in a batch culture. Furthermore, over 100 g/L and 88 units/mL of rice α-amylase activity were obtained in less than 50 h with a one-step feeding process in which a recombinant Y. lipolytica expressing rice α-amylase was cultivated in the 10% glycerol medium enriched with 0.3% yeast extract and fed only once with the concentrated feeding medium (60% glycerol). The easy cultivation of recombinant Y. lipolytica to a high cell density may strengthen its position as a host for heterologous protein production.

Introduction

A variety of biologically active proteins have been produced from yeasts in yields ranging from tens of micrograms to over a gram per liter of culture broth (Herrmann et al., 1995; Rosenfeld et al., 1996; Saliola et al., 1999). However, the expression levels and the quality of the heterologous proteins synthesized often suffer from organism-specific limitations (Faber et al., 1995). Therefore, the attainment of high expression levels of important heterologous proteins will require extensive development in the host organism chosen (Ngsee and Smith, 1990; Schwientek and Ernst, 1994).

Yarrowia lipolytica is currently considered a good host for heterologous gene expression as a result of its ability to secrete large amounts of extracellular proteins such as an alkaline protease, acid proteases, and a ribonuclease (Barth and Gaillardin, 1997; Cheng and Ogrydziak, 1987; Ogrydziak, 1993; Young et al., 1996). Despite its potential for commercially valuable protein production, only a little research on the development of efficient culture techniques for recombinant Y. lipolytica have been performed (Chang et al., 1997; Chang et al., 1998a; 1998b). High cell density culture is a very attractive bioprocess for the production of secreted recombinant proteins in regard to the maximization of volumetric productivity. Therefore, a controlled fed-batch culture technique for recombinant Y. lipolytica was developed so that high cell density culture of a recombinant Y. lipolytica expressing rice α-amylase could be achieved (Chang et al., 1997). However, we thought that we might simplify the fed-batch process and reduce the overall process time.

In this paper, we report a very simple culture method by which recombinant Y. lipolytica was grown to over 100 g/L in less than 50 h while expressing rice α-amylase efficiently.

Materials and Methods

Strains and Medium Composition. The yeast strains used in this study were Y. lipolytica CX161-1B (Mata ade1A) and YLASIn (Mata ade1A xpr2) (Park et al., 1997) in which the rice α-amylase gene was expressed from the XPR2 promoter that was induced at pH 6.8 by protease peptone (Chang et al., 1997). Cultures were maintained on YEPD (yeast extract 1%, bacto-peptone 2%, dextrose 2%) agar plates. The synthetic culture medium used in this study consisted of (per liter) glycerol, 150 g; (NH4)2SO4, 24 g; KH2PO4, 4.5 g; MgSO4·7H2O, 2 g; inositol, 105 mg; thiamine, 2 mg; trace metal solution, 13 mL; vitamin solution, 13 mL; adenine, 1.2 g; and antifoam 289 (Sigma) 0.5 mL. The trace metal solution contained (per liter) FeCl3·6H2O, 2.7 g; ZnCl2, 1.4 g; CaCl2, 2.0 g; Na2MoO4·2H2O, 2.0 g; CuSO4·5H2O, 1.9 g; H3BO3, 0.5 g; MnSO4·5H2O, 20 g; and concentrated HCl, 10 mL. The vitamin solution contained (per liter): pantethenic acid, 5.4 g; pyridoxine, 1.4 g; niacin, 6.1 g; folic acid, 0.04 g; and biotin 0.06 g. In the media with other glycerol concentrations than 15%, the concentrations of all other components were changed proportionally to the glycerol concentration. Yeast extract or casamino acids were added to the synthetic culture medium when needed.

Batch Culture. Precultures were grown for 24 h in baffled shake flasks with GYA medium composed of 1% glycerol, 0.34% yeast nitrogen base without amino acids, and 50 mg/L of adenine. Batch cultures were conducted with 3 L of the synthetic culture medium in a 5-L fermentor (Korea Fermentor, Korea) equipped with pH,
dissolved oxygen, temperature, and foam probes. The temperature was maintained at 28 ± 0.1 °C, and the pH was controlled at 5.5 ± 0.1 with 6 N NaOH. Air flow rate and agitation speed were initially set at 1 vvm and 500 rpm, respectively. Dissolved oxygen concentration was maintained above 20% of air saturation by controlling agitation speed (500–900 rpm) and supplying oxygen-enriched air.

One-Step Feeding Fed-Batch Culture. One-step feeding fed-batch culture experiments were conducted in a 5-L fermentor (BioFlo III, NBS). The operation was initiated as a batch culture with 3 L of the synthetic medium with 0.3% yeast extract. The pH was controlled at 5.5 ± 0.1 with 3 N NH₄OH, and the temperature was maintained at 28 ± 0.1 °C. When glycerol was almost depleted, 1 L of concentrated feeding medium was added to the culture. The culture pH was raised to 6.8 and controlled with 6 N NaOH. The concentrated feeding medium included proteose peptone (0.25% or 2.0%) as an inducer when α-amylase expression was desired.

Analytical Methods. Samples during fermentation were taken at preset intervals using an automatic sampler (Lokas, Korea). Cell concentration was determined by measuring the optical density at 600 nm (Beckman DU650) and converting it to dry cell weight using the predetermined calibration data for the yeast strain. The glycerol concentration in the fermentation broth was measured using a glycerol assay kit (Boehringer Mannheim). Rice α-amylase activity was measured by the 3′,5′-dinitrosalicylic acid method as described in Chang et al. (1997), except that the reaction was started by adding 0.05 mL of enzyme solution to 0.95 mL of substrate solution. Glucose was used as a standard. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose equivalent per min under the given conditions.

Results and Discussion

Effect of Glycerol Concentration on Growth of Y. lipolytica. A recombinant Y. lipolytica strain could be grown to a high cell density by the fed-batch culture process with a controlled feeding strategy (Chang et al., 1997). However, if a simple culture method without sophisticated control strategies for medium feeding is developed for the recombinant Y. lipolytica high cell density culture, the culture method may place Y. lipolytica in an advantageous position over other host organisms for heterologous protein production. As an initial step to develop an easy and convenient fermentation bioprocess we tested whether Y. lipolytica could grow to a high cell density in high concentrations of glycerol. The Y. lipolytica CX161-1B strain was cultivated in the synthetic culture media containing various concentrations of glycerol: 2.5%, 5%, 7.5%, 10%, and 15%. Figure 1 shows that the lag phase increased as glycerol concentrations increased; Y. lipolytica needed longer lag phases before it began to grow in the 10% or 15% glycerol medium. However, once it adapted to the culture media containing high concentrations of glycerol, it grew at similar rates regardless of glycerol concentrations. We thought, therefore, from this result that, if the long lag phase could be reduced, a simple batch culture might be employed to cultivate Y. lipolytica to a high cell density, and then sophisticated controlled fed-batch processes would not be necessary for the recombinant Y. lipolytica high cell density culture.

Effect of Yeast Extract on Growth of Y. lipolytica. It is possible that an unknown nutritional factor(s) present in complex nutrients plays a role in stimulating cell growth under a nonoptimal or stressed growth conditions (Thomas et al., 1994). To investigate how the long lag phase caused by the high concentration of glycerol could be shortened, we studied the effects of yeast extract and casamino acids on the growth of Y. lipolytica in the synthetic culture media containing high concentrations of glycerol. In shake flask cultures, yeast extract significantly reduced the long lag phase encountered in the 15% glycerol medium, but casamino acids did not (data not shown). Then, we grew Y. lipolytica in the 15% glycerol media enriched with 0.1%, 0.2%, 0.3%, and 0.4% yeast extract to see whether yeast extract would have a similar effect on the growth of Y. lipolytica in a jar fermentor in which pH and dissolved oxygen level were tightly controlled. Figure 2 shows that the dry cell weights of Y. lipolytica in the fermentor cultures reached 64–83 g/L in less than 50 h. The highest cell concentration (83 g/L) and cell yield coefficient, Yₓₒᵦ (0.55 g cell/g glycerol) were obtained with the medium containing 0.3% yeast extract. This result clearly indicates that the high cell density culture of Y. lipolytica can be carried out by a simple batch culture if the synthetic culture medium is supplemented with yeast extract, although we do not know what the growth stimulator(s) available in yeast extract is.

High Cell Density Culture Using One-Step Feeding Process. Since the high cell density of Y. lipolytica could be achieved with the batch culture, it was thought that feeding the culture medium only one time would be enough to ensure over 100 g/L dry cell weight. After Y. lipolytica was grown in 3 L of the 15% glycerol medium containing 0.3% yeast extract, 1 L of the concentrated feeding medium (40% glycerol) was added to the culture when glycerol was almost depleted. As shown in Figure 3, Y. lipolytica could be cultivated to over 100 g/L using this one-step feeding process. This experimental result demonstrates that the one-step feeding process enables attaining of Y. lipolytica high cell density culture without using a complicated medium feeding strategy.

Although the long lag phase was significantly reduced by the addition of yeast extract to the 15% glycerol medium, the overall culture time was fairly long (60–75 h) and the length of the lag phase varied from 5 to 15 h depending on the physiological state of the inoculum. To shorten the overall culture time and maintain the culture time relatively constant, we grew Y. lipolytica initially in 3 L of the 10% glycerol medium with 0.3% yeast extract.
and fed the culture with 1 L of the concentrated feeding medium (60% glycerol) when glycerol was almost exhausted. As expected, we could further reduce the lag phase and the overall culture time (about 50 h) using this culture method (data not shown). To test whether this method could be equally applied to recombinant \textit{Y. lipolytica} cultivation, the recombinant YLASIn strain expressing rice $\alpha$-amylase was cultivated in the 10% glycerol medium and fed with the concentrated feeding medium with 0.25% proteose peptone. The dry cell weight reached 105 g/L in 48 h, and 67 units/mL of amylase was produced in the one-step feeding fed-batch fermentation (Figure 4A). The expression level from the XPR2 promoter is affected by the amount of protease peptone and the glycerol to proteose peptone ratio (Chang et al., 1997).

Figure 2. Effect of yeast extract concentration on the growth of \textit{Y. lipolytica} in synthetic culture medium containing 15% glycerol: (A), 0.1%; (B) 0.2%; (C) 0.3%; (D) 0.4%. Open symbol indicates glycerol concentration, and closed symbol indicates dry cell weight of \textit{Y. lipolytica}.

Figure 3. High cell density cultivation of the \textit{Y. lipolytica} CX161-1B strain using a one-step feeding process. \textit{Y. lipolytica} CX161-1B was cultivated in 3 L of 15% glycerol medium with 0.3% yeast extract and fed with 1 L of concentrated feeding medium containing 40% glycerol. Open symbol indicates glycerol concentration, and closed symbol indicates dry cell weight of \textit{Y. lipolytica}. This figure is typical of several independent trials.

Figure 4. High cell density cultivation of the recombinant \textit{Y. lipolytica} YLASIn strain using a one-step feeding process. The recombinant \textit{Y. lipolytica} expressing rice $\alpha$-amylase was cultivated in 3 L of 10% glycerol medium with 0.3% yeast extract and fed with 1 L of concentrated feeding medium containing 60% glycerol. Protease peptone was added to the concentrated feeding medium at a final concentration of 0.25% (A) or 2.0% (B). Arrow indicates the point of feeding. Dry cell weight, $\bullet$; glycerol concentration, $\bigcirc$; ammonium ion concentration, $\triangle$; rice $\alpha$-amylase activity, $\blacktriangleleft$.

To see the effect of the protease peptone concentration on the amylase expression and the growth of the recom-
binant Y. lipolytica in the one-step feeding process, we cultivated the recombinant strain under the same condition except for the concentration of proteose peptone (2% instead of 0.25%). Although the final cell concentrations attained were similar irrespective of the proteose peptone concentration, higher amylase activity (88 units/mL) was attained when measured by comparing with a known amount of amylase. We expect that much higher productivity of amylase can be achieved using this one-step feeding process if the optimal concentration of proteose peptone is determined.

Conclusion

In this study, we showed that a simple one-step feeding process could be successfully employed for the recombinant Y. lipolytica high cell density culture. Complex calculation and expensive control devices are not necessary in the one-step feeding process. Furthermore, the one-step feeding process can be easily applied to a simple two-stage cyclic culture method in which a portion of the culture broth grown in the first fermentor is transferred to the second fermentor that is fed with the induction medium (Chang et al., 1998b; Curless et al., 1991). Therefore, we anticipate that the one-step feeding process may contribute to making Y. lipolytica a favorable host for the high-level expression of commercially important heterologous proteins.

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References and Notes


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