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Growth rate control in fed-batch cultures of recombinant *Saccharomyces cerevisiae* producing hepatitis B surface antigen (HBsAg)

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Summary. A recombinant *Saccharomyces cerevisiae* producing hepatitis B surface antigen (HBsAg) exhibited growth-associated product formation. By controlling the medium feed rate, based on the calculated amount of medium required for 1 h, a constant specific growth rate was obtained in the range of 0.12–0.18 h^{-1}. In order to prolong the exponential growth phase, the medium feed rate was increased exponentially. A fed-batch cultivation method based on the production kinetics of batch culture enhanced HBsAg production ten times more than in batch culture. The reason for the increase can be explained by the fact that the production of HBsAg is expressed as an exponential function of time when the specific growth rate is controlled to a constant value in growth-associated product formation kinetics. In the scale-up of this culture to 9 l, the specific growth rate could also be maintained constant and the HBsAg production trend was similar to that in a 1-l culture. However, ethanol accumulation occurred at a late stage in fed-bach culture. Ethanol produced was not reutilized and inhibited further cell growth.

Introduction

Hepatitis B surface antigen (HBsAg), used as a vaccine against type B hepatitis caused by the hepatitis B virus, has been purified from human plasma until quite recently. However, this method has the problems of virus contamination and limited supply of plasma from suitable human carriers. The HBsAg protein has been expressed in recombinant yeast with the advent of r-DNA technology (Valenzuela et al. 1982) and is now being produced commercially.

In order to circumvent the conversion of carbon source to ethanol, the fed-batch process has been adopted for yeast growth, by increasing the nutrient feed rate in proportion to cell mass (Fieschko et al. 1987). The control method has been improved with the application of a computer system that monitors the respiratory quotient (RQ) value (the ratio of moles of CO_2 evolved to moles of O_2 consumed) (Wang et al. 1977).

Fed-batch processes are suitable for the cultivation of recombinant cells because of high-cell density and short culture time (generation number). The reason is that the plasmid stability and production activity of recombinant cells are sometimes decreased in proportion to the increase in generation number or culture time (Imanaka and Aiba 1981). Therefore, the expression of a foreign protein using a recombinant yeast has been reported in fed-batch processes (Bitter et al. 1988; Hsieh et al. 1988). In recombinant cell cultivation, the expression efficiency of foreign protein and culture method are dependent on the kinds of promotors and proteins expressed. There are two-stage culture systems and induction systems based on the genetic properties of recombinant cells (Siegel and Ryu 1985).

Recently, Fieschko et al. (1987) developed a fed-batch protocol for high-cell density fermentations of *Saccharomyces cerevisiae* harboring recombinant plasmids. They reported that the recombinant cells were grown under glucose limitation at a specific growth rate below that resulting in ethanol accumulation. In that system, constant specific growth rate was maintained by increasing the flow rate of the nutrient feed pump stepwise according to the culture concentration in the fermentor. They cultivated the recombinant cells to a high density not for growth-associated product formation but for induction.

However, in the case of recombinant yeast exhibiting growth-associated product formation, there are few reports of applied processes based on production kinetics from the point of view of increasing productivity. In a previous paper (Gu et al. 1989), we reported that HBsAg production in fed-batch culture with a controlled constant specific growth rate was ten times higher than that in batch culture. In this case, growth-associated product formation kinetics was noted. For obtaining high productivity of HBsAg, we controlled

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the medium feed rate to maintain the specific growth rate constant based on the production kinetics. In addition, we worked on a scale-up experiment using this method for obtaining high productivity on a production scale. In this report the results from the scale-up experiment of this culture method and the effects of growth rate control on the production of HBsAg are presented and the effect of ethanol produced on growth in the scale-up culture is discussed.

Materials and methods

Equipment. For the cultivation of recombinant yeast, a 2-l (Braun, Melsungen, FRG; Model M) and a 14-l fermentor (New Brunswick Scientific, New Brunswick, N. J., USA) were used with working volumes of 0.9 l and 9 l, respectively. A peristaltic pump (Master Servodyne, Cole Parmer, Chicago, Ill., USA) was used for controlling the medium feed rate between 0.2 and 500 ml/h by adjusting the speed and exchanging the tubes manually. The gas flow rate to the fermentor was adjusted manually to maintain the dissolved oxygen tension (DOT) above 35%. A mixture of air and pure oxygen was used for DOT control. The pH was adjusted to 4.3–4.5 by supplying 2.5 M NaOH and 2.5 M HCl solutions automatically. The temperature was controlled to $30 \pm 0.1°$ C.

Analysis. The analytical methods for measuring dry cell weight (DCW), HBsAg concentration and glucose concentration have been described previously (Gu et al. 1989). The ethanol concentration was measured using a Hewlett Packard (Palo Alto, Calif., USA) 5890 gas chromatograph equipped with a flame ionization detector.

Strain and media. S. cerevisiae strain 20B12 (*alpha, trp1, pep4*) harboring the plasmid encoding HBsAg protein directed by the promotor of yeast alpha factor was used throughout this study (Park et al. 1988).

Details of the media preparation have been given elsewhere (Gu et al. 1989). The feed medium in the reservoir contained 20% glucose, 5% casamino acid, 5.5% YNB (yeast nitrogen base, Difco, Detroit, Mich., USA), 7 ml/l of vitamin solution, 7 ml/l of trace element solution, 70 mg/l of *m*-inositol, 10 mg/l of thiamine-HCl and 1.5 ml/l of antifoaming agent. Vitamin solution consisted of 1.35 g/l of pantothenic acid, 3.05 g/l of niacin, 0.7 g/l of pyridoxin, 0.03 g/l of biotin, and 0.02 g/l of folic acid. Trace element solution consisted of 2 g/l of $ZnCl_2 \cdot 4H_2O$, 2 g/l of $MgMoO_4 \cdot 2H_2O$, 0.5 g/l of H_3BO_3, and 100 ml/l of conc HCl.

Culture methods. The cells streaked on the solid medium plate (agar 2% + batch medium) from a seed-lot system were grown in a Precision (Chicago, Ill., USA) gravity convection incubator at 30° C for 48 h. One isolated colony was inoculated into 15 ml batch medium in a 50-ml conical tube and cultivated at 30° C and 250 rpm in a shaking incubator (Orbit Incubator-shaker, Lab-line, Merlose Park, Ill., USA) for 24 h. The broth of 3–4% pre-seed was inoculated into a 500-ml flask containing 100 ml medium and cultivated for 20 h.

From the seed culture, the cells harvested and resuspended in 20 ml fresh batch medium were inoculated into a fermentor (2 l), then medium feeding was started. The 9-l cultivation procedure was the same as that for 1 l.

Results and discussion

Theoretical backgrounds

Product formation kinetics. In growth-associated product formation, the production rate (*dp/dt*) may be written as

$$\frac{dp}{dt} = k\frac{dx}{dt} \tag{1}$$

$$= k\mu x \tag{2}$$

where p is product concentration, k is production rate constant, x is cell concentration and μ is specific growth rate.

In Eqs. 1 and 2, *dp/dt* is a function of the specific growth rate and the cell concentration. If the specific growth rate is constant in Eq. 2, *dp/dt* exhibits non-growth-associated product formation. Integrating Eq. 2 with time gives

$$p = kx_0 e^{\mu t} \tag{3}$$

where x_0 is initial cell concentration p becomes an exponential function of time.

Control scheme to maintain constant specific growth rate. For the cultivation of recombinant *S. cerevisiae*, where cell growth and metabolism are affected by glucose concentration, we have used the following control scheme to maintain the specific growth rate constant. If $Y_{x/s}$ (cell yield) and μ are constant, the amount of glucose required for 1 h (S) in the exponential growth phase may be written as

$$S = \frac{\Delta X}{Y_{x/s}} \tag{4}$$

$$= \frac{X(e^{\mu} - 1)}{Y_{x/s}} \tag{5}$$

where X is total cell mass at the time of determination, ΔX is cell mass increment for 1 h. In Eq. 4, the amount of glucose used for cell maintenance and product formation during exponential growth was ignored (Aiba et al. 1976; Woehrer and Roehr 1981). Therefore, if we know the values for cell concentration and working volume at a given time, we can calculate the amount of glucose required for 1 h by using Eq. 5. In the experiments, we used constant values for cell yield and specific growth rate, which were obtained from batch culture, and the required amount of glucose was calculated based on cell mass. The calculated value was converted into the feed rate. Using this method, the specific growth rate was maintained constant and the deviation between the anticipated value and real value of cell mass was compensated by hourly determination of cell mass.

Batch cultivation

During the cultivation of recombinant cells, the activity of cells for foreign protein production usually decreases according to the increase in generation number. The reason for the decrease in production activity of cells has been explained by means of several properties of plasmid and gene expression (Kleinman et al. 1986; Parker and DiBiasio 1987). According to our experience, the activity of a recombinant *S. cerevisiae* producing HBsAg decreased below 50% of the maximum yield

at generation numbers 10 to 15 (flask repeat number 3–4). For that reason, we speculated that a fed-batch culture method that supports high productivity with a low generation number is required.

The results from batch cultivation of a recombinant yeast producing HBsAg are shown in Fig. 1. Secondary growth started at the time when sugar was completely consumed and the cells began to take up the ethanol produced. The HBsAg production rate was high in the primary growth phase, but low in the secondary growth phase.

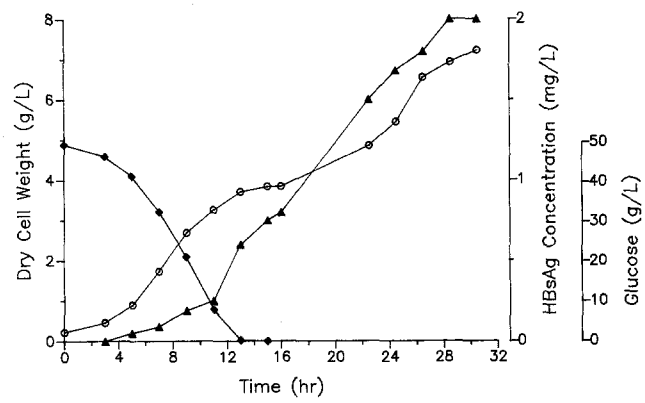

Fig. 1. Batch culture of recombinant *Saccharomyces cerevisiae* producing hepatitis B surface antigen (HBsAg) in a 1-l working volume: O, dry cell weight; ◆, glucose concentration: ▲, HBsAg concentration

Maintenance of constant specific growth rate in fed-batch culture

By analysing the results from the batch experiment that exhibited growth-associated production formation, we thought that higher productivity would be obtained if the exponential growth phase could be extended. Therefore, we carried out a fed-batch culture by maintaining the specific growth rate constant to obtain higher productivity. Because the medium feed rate calculated from Eq. (5) was compensated by the actual cell mass and glucose concentration measured hourly, there was slight modification of the feed rate. The variation in the medium feed rate is shown in Fig. 2.

By using our fed-batch scheme, the cells grew up to 25 g/l and the specific growth rate was maintained constant in 1 l of culture. When the specific growth rate was controlled, HBsAg production was increased ten times compared with that in batch culture and the production yield also increased sharply (Gu et al. 1989). The production rate in the fed-batch culture was represented by an exponential function of time, therefore the HBsAg production profile was similar to that of cell growth.

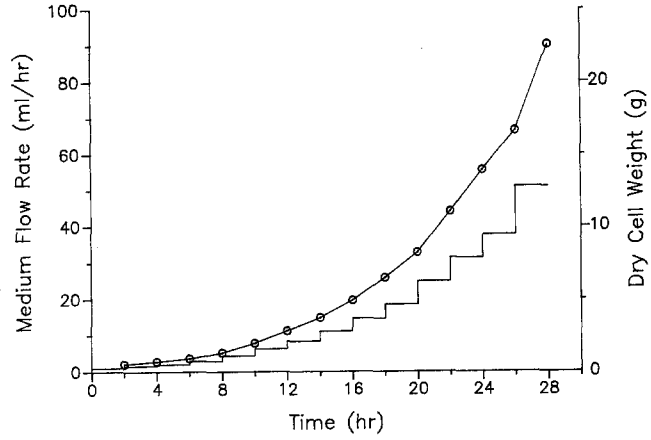

Fig. 2. Variation in the medium feed rate in 1-l fed-batch culture. The glucose concentration in the medium reservoir was 200 g/l: O, dry cell weight; —, medium flow rate

As shown in Fig. 3, the controlled range of growth rate was between 0.12 and 0.18 h^{-1}. When the specific growth rate was above 0.18 h^{-1}, the increment of medium flow rate was so high that glucose assimilation was variable because of the high residual glucose concentration. Accordingly, the trend of cell growth did not show oxidative metabolism. When the specific growth rate was maintained below 0.12 h^{-1}, the cells were not activated easily, so exponential growth decreased. For that reason, the specific growth rate controlled by medium feed should be maintained between 0.12 and 0.18 h^{-1}. The profile of HBsAg production was varied by controlling the specific growth rate.

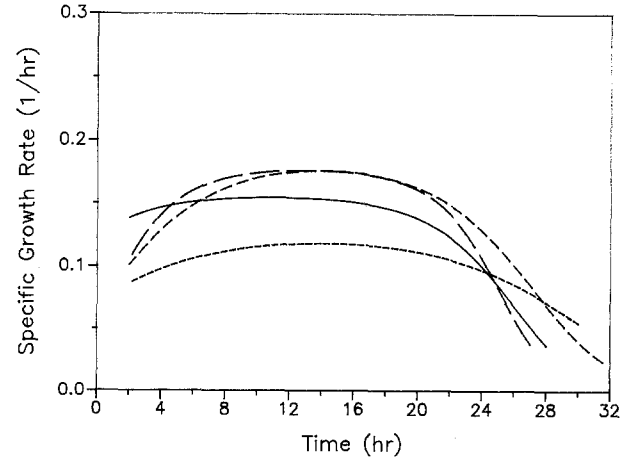

Fig. 3. Profiles of the controlled specific growth rate: ——, 1 l; - - - - -, 9 l; - - -, 9 l; — —, 9 l

Figure 4 shows that the production of HBsAg in a well-controlled system was higher than that in a poorly controlled system. Although the difference in cell mass was small in comparing the well-controlled system with the poorly controlled system, the difference in HBsAg production was large. Because the period mantaining the specific growth rate constant in a well-controlled system was larger than that in a poorly controlled system, the production of HBsAg in a well-controlled system seems to be higher than that in a poorly controlled system.

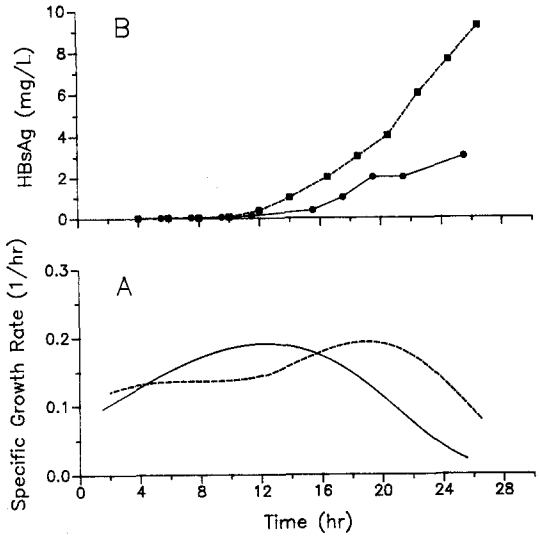

Fig. 4A,B. Comparison of HBsAg production between well-controlled and poorly controlled fed-batch cultures. **A** Profile of the controlled specific growth rate: -----, specific growth rate in a well-controlled culture; ——, specific growth rate in a poorly controlled culture. **B** HBsAg production according to the growth rate control: ■, HBsAg production in a well-controlled culture; ●, HBsAg production in a poorly controlled culture

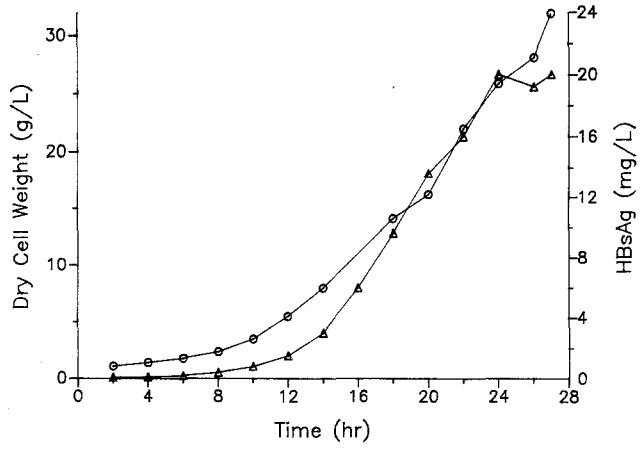

Fig. 5. The time-course profiles of cell growth (○) and HBsAg production (△) in a 9-l fed-batch culture

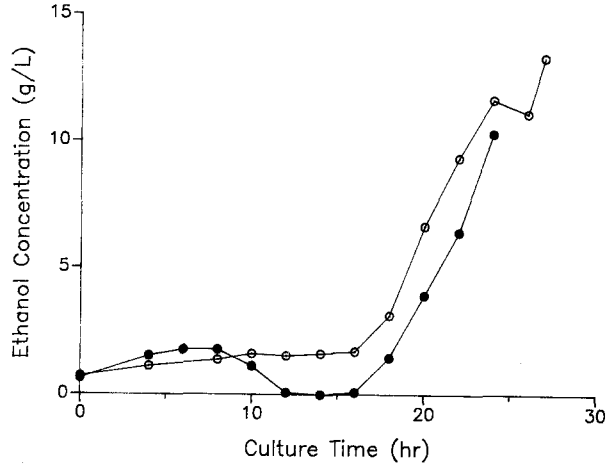

Fig. 6. Trends in ethanol accumulation in two separate 9-l fed-batch culture (○, ●)

Scale-up of the culture

When we carried out the same experiment in 9 l, the specific growth rate was also maintained constant and the time-course profiles are illustrated in Fig. 5. The cell concentration reached up to 31 g/l and the production of HBsAg was ten times higher than that in batch culture. The profiles of cell growth and HBsAg production were similar to those from the 1-l scale.

The production of ethanol increased sharply after 20 h of culture. A mixture of air and pure oxygen was used to avoid oxygen limitation. Wang et al. (1977) reported that the specific growth rate decreased according to the increase in culture time in a fed-batch culture when they used a computer-controlled system. In order to increase the cell mass and lessen the glucose effect, reutilization of ethanol should occur. However, in our case, reutilization of ethanol was not found. Figure 6 shows that ethanol significantly accumulated, especially after 20 h of culture. The accumulation of ethanol seems not to be related to the scale of the culture because ethanol formation always occurred in every fermentative growth phase (Wang et al. 1977; Woehrer and Roehr 1981). The specific growth rate may be decreased by the inhibitory effect of the ethanol produced. Probably due to this growth inhibition by ethanol, the cells finally ceased to grow at 30–35 g/l.

In conclusion, when we controlled the specific growth rate in fed-batch culture with a recombinant yeast exhibiting growth-associated product formation in batch culture, HBsAg production became non-growth-associated and the HBsAg concentration was increased as an exponential function of time. The amount of medium required for 1 h to keep the specific growth rate constant was calculated by an equation based on the cell mass. In addition, production and accumulation of ethanol were related to the feeding rate, which was controlled by the specific growth rate. This culture method was carried out in 9 l and 1 l and the scale-up had little effect not only on the growth rate but also on HBsAg production. We therefore speculate that growth-rate control based on production kinetics may be a very effective technique for high density production of a foreign protein in the cultivation of a recombinant yeast exhibiting growth-associated product formation.

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