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Fermentation of recombinant yeast producing hepatitis B surface antigen

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SUMMARY

Fermentations were performed to determine parameters affecting the expression of hepatitis B surface antigen (HBsAg) in the yeast *Saccharomyces cerevisiae* containing the HBsAg gene. These studies emphasized increasing both the relative abundance (HBsAg: cell mass) and total production of HBsAg. Specific activity was increased 70-fold when cells were grown in shake flasks containing nonselective rather than selective medium. The addition of adenine, ammonium sulfate or glucose to the complex medium reduced the production of antigen. Results similar to those achieved in shake flasks were obtained when the growth was performed in fermenters. A nutrient addition system was employed to increase the production of cells and HBsAg. The addition of glucose to the culture medium increased cell mass 6-fold but decreased the production of antigen. This imbalance was corrected by supplementing the glucose with complex nutrients.

INTRODUCTION

Saccharomyces cerevisiae (Baker's yeast) is an excellent host for the production of recombinant-derived proteins, especially those of medical significance, since it is non-pathogenic for man, is free of endotoxin and has been grown at industrial-scale for centuries. A wide variety of products has been expressed in yeast, including hepatitis B surface antigen (HBsAg) [5,7,9]; recombinant-derived HBsAg has been developed by Merck into a vaccine for hepatitis B [8] that represents the first recombinant vaccine licensed for human use as well as the first licensed product derived from recombinant yeast. The primary emphasis of commercial molecular biology has been the enhancement of expression of recombinant proteins. These studies have focused on the choice of promoter, type of expression vector, variation in host cell and the regulation of gene expression [1,6,10]. Less emphasis has been placed on the development of fermentation processes for recombinant microorganisms, despite the importance of this step in the product development.

Growth conditions, such as components of media, affect the physiological properties of *S. cerevisiae*. Enzymes associated with the catabolism of galactose and ethanol are repressed by glucose [2],

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while ammonia inhibits some systems for the uptake of amino acids [3]. The rate at which nutrients are added to the culture also affects the activity and the production of cellular enzymes, as well as the yield of cells. Glucose is an example of such a nutrient [2,4]. When high concentrations of glucose are present initially, which occurs in batch fermentation processes, glucose is fermented to ethanol and the mass of cells produced is relatively low. The conversion of glucose to cell mass can be increased dramatically by controlling the rate at which glucose is added to the fermenter; these systems are referred to as semi-continuous cultures or fed-batch fermentations.

This study focuses on the effects of growth media on the production of recombinant-derived HBsAg. The results of shake flask studies were used to develop a batch fermentation process. Subsequently, a process for fed-batch fermentation was developed to increase the production of HBsAg further.

MATERIALS AND METHODS

Strain

S. cerevisiae, designated as P158 in our culture collection, was received from Chiron Corporation (Emeryville, CA). These cells harbor a plasmid composed of the coding sequence for HBsAg linked to the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter, an origin of replication from the yeast 2μ plasmid, and the *LEU*2 gene for selection in yeast cells [9]. Cultures were maintained as lyophilized cells and stored under inert gas at $-20°C$.

Culture media

Both selective and nonselective media were used. Selective medium (leucine-free; Leu^-) contains the following components per liter: 6.7 g yeast nitrogen base without amino acids (YNB; Difco Laboratories, Detroit, MI), 40 mg uracil, 40 mg adenine, 6 g sodium hydroxide, 10 g succinic acid, 20 mg each of tryptophan, histidine, arginine and methionine, 30 mg each of tyrosine and lysine, and 60 mg threonine. Non-selective medium (YEHD) contains per liter: 20 g yeast extract (Difco Laboratories, Detroit, MI), 10 g soy peptone (HySoy®; Sheffield Products, Lyndhurst, NY) and 16 g glucose. Solid media were prepared by adding 20 g agar.

Growth in shake flasks

Lyophilized stock cultures were reconstituted with distilled water and plated onto Leu^- agar. Colonies were transferred to 50 ml of YEHD or Leu^- medium in a 250 ml Erlenmeyer flask and grown at 28°C and 350 rpm (Model G-26, New Brunswick Scientific Co., Edison, NJ). After 16–18 h of growth, 10 ml inocula were transferred to 500 ml YEHD or Leu^- medium in 2-l Erlenmeyer flasks and the fermentations were continued.

Growth in fermenters

In batch fermentations, 0.5 l of an exponentially growing culture was used to inoculate 9 l of YEHD medium in a 16-l fermenter (New Brunswick Scientific Co., Edison, NJ). The culture was aerated with 5 l/min air and stirred at 500 rpm; temperature was controlled at 28°C. The pH was maintained at pH 5.0 ± 0.1 by the addition of 2 N NaOH and 9 N H_3PO_4. Samples were removed periodically for measurements of glucose, cell growth and production of HBsAg. In fed-batch fermentations, the starting volume in the fermenter was 5 l. After the concentration of glucose had decreased to 1 g/l, a solution of concentrated medium was added to the growing culture; the feed rate was adjusted to maintain the concentration of glucose in the fermenter between 1 and 2 g/l. Concentrated solutions consisted of either glucose (250 g/l) or of a mixture of yeast extract (100 g/l), soy peptone (50 g/l) and glucose (150 g/l).

Harvesting and breakage of cells

Cells were harvested by centrifugation at 9000 $\times$ *g* at 2–8°C. Washed cells were suspended in buffer containing 0.1 M sodium phosphate pH 7.2, 0.5 M sodium chloride and 2 mM phenylmethylsulfonyl fluoride and then broken with glass beads. Cell extracts were clarified by low-speed centrifugation and assayed.

Analytical methods

Growth was monitored by measuring absorbance at 660 nm (A_{660}); cell mass was determined by measurement of dry weight. Levels of glucose were measured enzymatically with glucose oxidase in a Beckman Glucose Analyzer II. Ethanol was measured with an ethanol analyzer (YSI Model 27, Yellow Springs, OH). Levels of HBsAg were determined by radioimmunoassay (AUSRIA II®, Abbott Laboratories, North Chicago, IL) which is specific for particles of HBsAg and by quantitative immunoblot assays which measure the level of HBsAg polypeptides (Dennis-Sykes et al., unpublished data). Relative concentrations of HBsAg were determined by dividing the AUSRIA titer of the test extract by the AUSRIA titer of the Leu$^-$ extract. Plasmid retention was determined by replica-plating colonies from YEHD to Leu$^-$ media; the percentage loss of plasmid is expressed as 100 minus the number of cells growing on Leu$^-$ medium divided by the number of cells growing on YEHD medium.

RESULTS AND DISCUSSION

Effects of culture media

The effects of the composition of the culture medium on the production of cells and HBsAg were examined. When strain P158 was grown in YEHD rather than Leu$^-$ medium, cell mass increased from 2 to 7 g/l (Table 1). More importantly, both the production of HBsAg and its relative abundance were increased; total production increased 70-fold, while the relative abundance (units of HBsAg/dry weight of cells) increased by a factor of 20. Retention of plasmid was found to be quite high; 85–100% of the cells retained plasmids.

Effects of $(NH_4)_2SO_4$

A major difference between Leu$^-$ and YEHD media is the primary source of nitrogen available for cell growth; $(NH_4)_2SO_4$ is the major nitrogen source in Leu$^-$ medium, while both amino acids and peptides provide available nitrogen in YEHD medium. Since sources of nitrogen have been shown to affect the levels and activities of many enzymes of *S. cerevisiae* [3], the effects of $(NH_4)_2SO_4$ on growth of cells and production of HBsAg were examined. As shown in Table 2, neither the addition of $(NH_4)_2SO_4$ to YEHD nor its removal from Leu$^-$ medium had an appreciable effect on cell mass. However, the amount of HBsAg produced when cells were grown in the presence of $(NH_4)_2SO_4$ was decreased 20–70-fold relative to the control flasks.

Table 1

Effect of culture media on cellular growth and production of HBsAg by strain P158 grown in shake flasks

Medium	Final cell growth (A_{660})	Cell mass (g/l)	Relative concentration of HBsAg	Percent loss of plasmid
Leu$^-$	4	2	1	0
YEHD	15	7	70	0–15

Effects of media supplements on cell mass and production of HBsAg

Compounds associated with membrane fluidity (acetate and ergosterol), a major component of HBsAg (leucine), and an amino acid present in potentially limiting amounts (methionine) were added separately to YEHD medium (Table 3). Adenine was added to minimize the possible deleterious effects of the adenine auxotrophy of the host. Most

Table 2

Effect of ammonium sulfate on cellular growth and production of HBsAg by strain P158 grown in shake flasks

Medium	Final cell growth (A_{660})	Cell mass (g/l)	Relative concentration of HBsAg
Leu$^-$	4	2	2
Leu$^-$ without $(NH_4)_2SO_4$	3	3	20
YEHD	15	7	70
YEHD + $(NH_4)_2SO_4$(5 g/l)	14	7	1

of the components tested had no appreciable effects on cell mass or production of HBsAg. However, both cell mass and production of antigen were affected by supplementation of YEHD medium with adenine. When a relatively large amount of adenine (120 mg/l) was added, cell mass was increased from 7 to 12 g/l; however, the production of HBsAg was decreased to 40% of the control. The nature of this phenomenon is not understood.

Batch fermentation studies

The information obtained from these and other shake flask experiments was used to establish a process for the production of recombinant-derived HBsAg in fermenters. Some characteristics of the fermentation are shown in Fig. 1. The initial growth of cells was rapid as glucose was consumed; the growth rate slowed after the glucose had been depleted from the medium. During the inital phase of growth, the level of HBsAg particles was relatively low. The production of HBsAg particles increased greatly after glucose had been depleted from the medium. Since the GAP promoter is constitutive, this change in expression was unexpected; therefore, the levels of HBsAg polypeptides formed during the fermentation were measured. Western blot analyses indicated that the relative abundance of the polypeptides was nearly constant throughout the fermentation (data not shown). This finding suggests that the formation of particles, rather than the expression of polypeptides, is regulated by

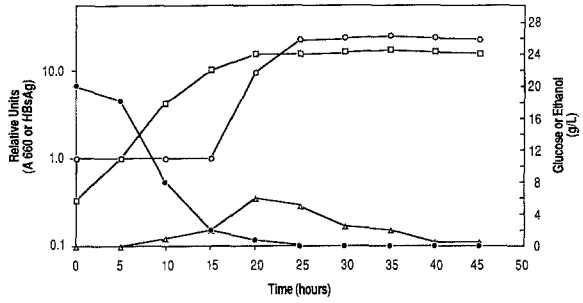

Fig. 1. Fermentation of yeast strain P158. Cells were grown in a 16-liter fermenter and assays were performed as described in the text. □, absorbance (660 nm); △, dissolved ethanol; ○, HBsAg; ●, glucose.

growth conditions. To test the possibility that glucose represses the formation of particles, either galactose or ethanol was used in place of glucose. When test samples were examined the pattern of particle formation was found to be similar to that obtained when cells were grown on glucose. These findings suggest that the assembly of HBsAg polypeptides into particles is not controlled solely by glucose repression.

Fermentation improvements

A more economical process for fermentation required an increase in the production of HBsAg. This was accomplished by three different techniques (Table 4). Firstly, the process was scaled up from shake flasks to fermenters. Relative abundance of HBsAg was constant in both shake flasks (Table 1) and fermenters (Table 4); therefore, proportionally more HBsAg was produced when the fermentation

Table 3

Effect of supplements on cellular growth and production of HBsAg by strain P158 grown in shake flasks

Medium	Cell mass (g/l)	Relative concentration of HBsAg
YEHD	7	70
YEHD + leucine (50 mg/l)	7	84
YEHD + methionine (50 mg/l)	6	70
YEHD + acetate (20 g/l)	7	56
YEHD + adenine (120 mg/l)	12	28
YEHD + ergosterol (20 mg/l)	7	91
Leu$^-$	2	1

Table 4

Production of HBsAg by strain P158 grown in batch and fed-batch fermenters

Mode	Starting medium	Feed solution	Cell mass (g/l)	Relative concentration of HBsAg
Batch	1X YEHD	none	7	70
Batch	2X YEHD	none	12	140
Fed-batch	2X YEHD	glucose	43	4
Fed-batch	2X YEHD	YEHD	48	455

process was scaled up. Secondly, by doubling the concentrations of the components of YEHD medium, the cell mass was increased 2-fold. Since the relative abundance of antigen remained constant, the amount of HBsAg produced also increased 2-fold. Thirdly, a fed-batch fermentation process was developed. During initial studies, glucose was added to the fermenter. The rate of addition was adjusted so that the concentration of glucose in the growth medium was maintained at approximately 1 g/l. This strategy resulted in a dramatic increase in cell mass (from 12 to 43 g/l) over that obtained from a batch process. However, the production of HBsAg decreased 20-fold (from 70 to 3.5 units/l). The cause of this decreased production is not understood; it may be due to a decrease in gene expression or a change in the cellular physiology. This decrease in the production of HBsAg was resolved by using a solution of yeast extract, soy peptone and glucose instead of glucose. Production of cells remained between 40 and 48 g/l, while the production of HBsAg was increased 7-fold over a 1X YEHD batch fermentation. Thus, it appears that there is a nutritional deficiency for production of HBsAg when only glucose is added to the culture; by feeding the more complex solution, this deficiency is overcome.

Clearly, a fed-batch approach has advantages over a batch process. A much larger mass of cells, compared to a batch process, can be obtained in approximately the same period of time. Also, a smaller fermenter can be used, which decreases the capital costs of the process. This approach offers much promise for producing large amounts of recombinant-derived products.

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