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Fermentation study for the production of hepatitis B virus pre-S2 antigen by the methylotrophic yeast *Hansenula polymorpha**

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SUMMARY

Various physico-chemical parameters have been studied in order to improve the production of hepatitis B virus pre-S2 antigen (middle surface antigen) by the methylotrophic yeast *Hansenula polymorpha*. Antigen production was done in two steps: first, production of cells on glycerol (Phase 1), followed by induction of antigen expression with methanol (Phase 2). Dense cultures of *H. polymorpha*, equivalent to 35–40 g/l (dry weight), were readily obtained in small fermenters using minimal medium containing glycerol as carbon source. Antigen expression in this minimal medium, after induction with methanol, was however low and never exceeded 1.6 mg/l of culture. Antigen production was greatly enhanced by adding complex organic nitrogen sources along with methanol at induction time; yeast extract was the best of all the sources tested. In shake flasks, antigen production was proportional to yeast extract concentration up to 7% (w/v) yeast extract. it became clear that the nutritional conditions for good antigen expression were different from those for good biomass production. The effects of yeast extract were reproduced in small fermenters: antigen levels reached 8–9 mg/l in medium containing 6% (w/v) yeast extract during induction with methanol. The mechanisms of yeast extract's effects are still unknown but are probably nutritional. The recombinant *H. polymorpha* strain produced both periplasmic and intracellular antigen. The periplasmic antigen was shown to be present as 20–22-nm particles and was therefore immunogenic. Immunoblotting indicated that part of the pre-S2 antigen was present as a 24-kDa degradation product. These studies have led to a 140-fold increase in volumetric productivity of antigen and to a 4.6-fold increase in specific production.

INTRODUCTION

Yeast systems offer many advantages over bacterial or mammalian systems for the production of eukaryotic proteins. Being eukaryotes, yeasts are able to produce eukaryotic proteins in the right conformation; they can also glycosylate proteins and, in several cases, secrete them [4,18,32]. *Saccharomyces cerevisiae* has been first developed for this purpose [2,3,10,13,26], but attention is also now focusing on some methylotrophic yeasts.

The presence of tightly regulated and highly expressed promoters related to methanol metabolism makes methylotrophic yeasts attractive hosts for efficient heterologous protein expression [4,9,31]. Recent results using *Pichia pastoris* are very promising [4–6,8,11].

The well-known methylotrophic yeast *Hansenula polymorpha* is another potential host for heterologous protein expression [19,22,24]. We have recently used *H. polymorpha* as a host system for the synthesis and secretion of the middle surface antigen [1] of hepatitis B virus (pre-S2 antigen) under control of the methanol oxidase (MOX) promoter [21]. Yields of the antigen in the order of 0.5 mg/l were obtained in shake flask experiments under non-optimized conditions [21]. Since the pre-S2 protein is considered to be a more efficient vaccine [14], it is therefore important to improve the production of this antigen for vaccine and diagnostic purposes. Yields of pre-S2 antigen have been reported to be about one-tenth those of the S antigen using the recombinant yeast *Pichia pastoris* [23].

In this report, we describe the influence of some physiological parameters (growth/induction conditions, medium composition) on the production of *H. polymorpha* biomass and pre-S2 antigen in shake flasks and small fermenters. The aim of these studies was to develop a fermentation process leading to high cell biomass and greatly increased antigen production.

MATERIALS AND METHODS

Strain

A *Hansenula polymorpha* uracil auxotroph was used as a host for the transformation of plasmid pMHUS2; this

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plasmid contains the coding sequence for hepatitis B virus pre-S2 antigen (pre-S2 HBsAg) linked to the methanol oxidase promoter, an origin of replication from *Hansenula polymorpha* (HARS) and the *ura3* gene for selection in yeast cells [21].

Cultures were maintained in 10% glycerol at −60 °C. The strain was first plated on Enriched Yeast Nitrogen Base Agar (see composition below) and incubated for 2 days at 37 °C; these plates were kept at 4 °C for up to 3 weeks.

Media and growth conditions

Shake flask experiments. Shake flask experiments were performed in either Enriched Yeast Nitrogen Base (EYNB) or Medium 1. EYNB consisted of Yeast Nitrogen Base (Difco, Detroit, MI) supplemented with: adenine sulfate, L-trytophan, L-histidine · HCl, L-arginine · HCl, and L-methionine (20 mg/l each); L-tyrosine, L-leucine, L-isoleucine, and L-lysine · HCl (30 mg/l each); L--phenylalanine (50 mg/l); L-glutamic acid and L-aspartic acid (100 mg/l each); L-valine (150 mg/l); L-threonine (200 mg/l) and L-serine (400 mg/l). Medium 1 contained (per litre): KCl, 3 g; $MgSO_4 \cdot 7H_2O$, 4.5 g; $CaCl_2 \cdot H_2O$, 0.6 g; NH_4Cl, 5 g; H_3PO_4 (85% w/v), 6 ml; solution A, 10 ml; solution B, 10 ml; vitamin solution, 4 ml/l. Solution A contained (per litre): $FeCl_3$, 4.08 g; $ZnSO_4 \cdot 7H_2O$, 4 g; $MnSO_4 \cdot H_2O$, 0.6 g; $CuSO_4 \cdot 5H_2O$, 0.66 g; concentrated H_2SO_4, 3 ml. Solution B contained (per litre): H_3BO_3, 0.02 g; $Na_2MoO_4 \cdot 2H_2O$, 0.2 g; KI, 0.08 g; $CoCl_2 \cdot 6H_2O$, 0.45 g. The vitamin solution (50 ml) contained 2 mg of biotin and 200 mg of thiamin hydrochloride. Solutions A and B and the vitamin solution were filter-sterilized (0.2 μm). For preparation of the complete medium, the basal medium containing all of the major elements was first made, its volume adjusted to 975 ml, and its pH adjusted to 4.5 with NaOH prior to sterilization at 121 °C for 30 min; solutions A and B and the vitamin solution were then added. In some experiments, ammonium chloride (BDH Chemicals, Toronto, Canada) was replaced by sodium nitrate (Fischer Scientific, Ottawa, Canada) or various complex organic nitrogen sources obtained from Marcor Development Corp. (Hackensack, NJ), ICN (Cleveland, OH), Sheffield (Norwich, NJ), Deltown (Greenwich, CT), or Difco (Detroit, MI) as specified in Table 3. In all experiments, 1.3% (v/v) methanol was used as a carbon source.

The flasks were inoculated with cultures grown for 2 days in Medium 1 supplemented with 2% (v/v) glycerol. The cells were washed once in saline (0.85% NaCl) before inoculation; the absorbance ($A_{600\,nm}$) of the inoculated media varied from 0.3 to 0.4 at time 0. All shake flask experiments were performed at 37 °C at an orbital agitation rate of 350 rpm (radius of gyration: 2.5 cm).

Fermenter experiments. Fermentations were run in a 3.5-l fermenter (Chemap, Volketswil, Switzerland) having a 2-l working volume. Medium 1 (above) was used initially (Table 2). Medium 1 was later modified, as indicated in the text, in order to avoid limitation of certain elements: the amount of KCl and $MgSO_4 \cdot 7H_2O$ was increased, respectively, to 6 and 6.75 g per litre.

A two-phase fermentation process was developped for antigen production: (Phase 1), production of cell biomass on glycerol in Medium 1 (modified or not as indicated in the text) followed by the addition of methanol (Phase 2) to induce the promoter of methanol oxidase for antigen production. The conditions used in Phase 2 were greatly varied in the course of this study and details are reported where necessary.

The final conditions for antigen production (Phase 2) are described in Table 4 (reference: Fermentation No. 7) and are summarized here: after production of cell biomass on glycerol in modified Medium 1, one volume of induction mixture was added to the culture to start antigen production. The induction mixture consisted of modified Medium 1 (NH_4Cl omitted) containing 12% (w/v) yeast extract and 0.25% (v/v) methanol to give a final concentration in yeast extract of 6% (w/v). The pH of the induction mixture was adjusted to 5.2 prior to autoclaving. Antibiotics (chloramphenicol and ampicillin (sodium salt) at 20 μg/ml each) were also added to the culture to avoid microbial contamination. Both antibiotics were obtained from the Sigma Co. (St. Louis, MO) and filter-sterilized (0.22 μm) before use.

Fermenters were inoculated with 5% (v/v) of 48-h culture of the recombinant *H. polymorpha* in Medium 1 containing 2% (v/v) glycerol. Dissolved oxygen was maintained above 20% air saturation by modulating aeration using a mixture of air (1 l/min) and pure oxygen (0–1.8 l/min). Other conditions were: temperature, 37 °C; agitation, 350 rpm.

Following induction with methanol, further methanol addition, during the antigen production phase, was based on the sudden rise in the dissolved oxygen level (pO_2) resulting from methanol depletion. In most experiments, methanol was added to the culture once the pO_2 level reached 80% air saturation to give a final concentration of 0.12% (v/v). Consequently, the dissolved oxygen level (pO_2) was constantly oscillating between its set value (20%) and its maximal values (100% air saturation). This mode of methanol addition was able to prevent methanol accumulation. In the case of methanol-limited fermentations (example: Table 2, Fermentation No. 4), methanol was pumped into the fermenter to give a final concentration of 0.01% (v/v); under these conditions the pO_2 level always remained high (over 50% air saturation).

Control of pH varied with experimental conditions:

pH was maintained at 4 with 3 M NaOH when minimal media were used but pH was maintained below 6 with 2.5 M HCl during antigen production in rich media.

Plasmid stability. Plasmid stability was measured by plating samples of cultures at the end of the fermentations on YEPD agar (1% yeast extract, 2% peptone, 2% dextrose(glucose), 2% agar) and then replicating individual colonies (minimum: 100 colonies) on Enriched Yeast Nitrogen Base (EYNB) medium without uracil (selective medium). The ratio of colonies growing on the selective medium versus those growing on the YEPD medium gave the proportion of plasmid-bearing cells.

Analytical methods

Absorbance. The absorbance of the cultures was determined at 600 nm using 9 mm diameter glass tubes after appropriate dilutions in PBS buffer pH 7 (per litre: Na_2HPO_4, 1.236 g; NaH_2PO_4, 0.18 g; NaCl, 8.5 g). PBS buffer was used as a reference. Under these conditions, one unit of absorbance corresponded approximately to a dry weight value of 1 g per litre. All readings were adjusted according to the method of Toennies and Gallant [25].

Dry weight. Samples were first filtered through a 0.45-μm membrane (Millipore Corp., Bedford, MA), washed once with 15 ml of PBS buffer, pH 7, and dried at 105 °C to constant weight.

Pre-S2 antigen determination. In general, for each sample, the amount of the periplasmic and of the intracellular pre-S2 antigen was determined. This was carried out using an enzymatic method as follows: cells were first washed in buffer A (1.2 M sorbitol, 50 mM $NaPO_4$ buffer, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and then incubated for 30 min at 37 °C in buffer A containing 10 μg/ml of Zymolase-100T (ICN Immunobiologicals, Lisle, IL) in order to facilitate the breakdown of the yeast cell wall. The samples were centrifuged for 10 min at room temperature in a Centra 4 centrifuge (IEC, Needham, MA) at 15000 × *g* (middle of the 10 cm tube) and supernatant fluids directly tested for antigen concentration (periplasmic antigen). The pellets (cells) were resuspended in 200 μl of buffer B (50 mM $NaPO_4$ buffer, pH 7.5, 5 mM EDTA, 2 mM PMSF), mixed with about 100 mg of 500-μm diameter glass beads (SIGMA, MO) and shaken for 4 min on a Vortex mixer at maximal speed. Following centrifugation for 5 min at room temperature in an Eppendorf centrifuge, the supernatant fluid was collected and saved. The treatment of cells with glass beads was repeated once as described above. The two supernatant fluids derived from this cell treatment were combined to give a fraction containing the intracellular antigen. Quantification of antigen was done using an enzyme-linked immunoassay method (ADI Diagnostics, Willowdale, Canada). For comparison purposes, the kit AUSZYME II (Abbot Laboratories, North Chicago, IL) was also used in some experiments. Human-derived hepatitis B surface antigen particles, supplied with the assay kits, were used as standard antigen. Under our conditions, the AUSZYME II kit gave an antigen response 1.9 times that of the ADI Diagnostics kit for the same samples.

In this report, for each sample, the total amount of antigen was the sum of the periplasmic and the intracellular antigen.

Characterization of the pre-S2 antigen. The antigen obtained from the periplasmic space was partially characterized. Immunoblotting was performed as described by Towbin et al. [27] using anti-hepatitis B rabbit serum (Calbiochem Corp.). Centrifugation of the recombinant antigen on CsCl gradients was performed as reported by Valenzuela et al. [30]. The density of the fractions was determined by refractometry (American Opticals Scientific Instruments, Buffalo, NY). A standard curve was generated using solutions of known CsCl concentrations at 23 °C.

RESULTS

Production of the pre-S2 antigen in minimal and defined medium (shake flasks)

The production of pre-S2 antigen by the recombinant *H. polymorpha* was first studied in various minimal and defined media and at various different pH values (Table 1). For these experiments, the inoculum consisted of glycerol-grown cells washed once in saline. The substrate, in all cases, was methanol at an initial concentration of 1.3% (v/v). The results showed that Medium 1, a minimal medium, was as good as the Enriched Yeast Nitrogen Base medium for the production of the antigen and of cell biomass ($A_{600\,nm}$). In addition, varying the initial pH of Medium 1 or increasing the buffering capacity of the medium by the addition of succinic acid showed some positive effects on biomass production but little effect on antigen production within experimental error. On the basis of these observations, Medium 1 was selected for further optimization of antigen production.

Initial production in fermenter

Experiments were performed in 3.5-l fermenters (working volume: 2.0–2.2 l) in attempts to greatly increase biomass and antigen production. As indicated in Table 2, a two-phase fermentation process was used for this purpose: first, production of cell biomass on glycerol (Phase 1) followed by the addition of methanol (Phase 2) to induce the promoter of methanol oxidase for antigen production. All these experiments were done using Medium 1, a minimal medium rich in minerals (MA-

TABLE 1

Pre-S2 antigen production in minimal and defined medium (shake flasks)[a]

Medium	pH		A_{600nm} (Day 6)	Pre-S2 antigen (mg/l)	
	Initial (Day 0)	Final (Day 6)		Day 3	Day 6
Enriched Yeast Nitrogen Base (EYNB)[b]	7	3.4	1.5	0.022	0.050
Medium 1[b]	3.9	2.6	1.9	0.033	0.056
Medium 1 + succinic acid[c]	4.4	4.4	2.6	0.040	0.062
Medium 1 + succinic acid[c] (experiment 1)	5.4	5.4	2.6	–	0.045
Medium 1 + succinic acid[c] (experiment 2)	5.5	5.5	2.1	–	0.071

[a] Conditions: 250-ml Erlenmeyer flasks containing 50 ml of minimal or defined medium and 1.3% (v/v) methanol; temperature, 37 °C; agitation; 250 rpm.

[b] With these media, 0.2 to 0.5% (v/v) of methanol was still present at day 3; at day 6, methanol was exhaused. In media supplemented with succinic acid, methanol was exhausted at day 3.

[c] Succinic acid: 10 g/l.

Inoculum: cells of *H. polymorpha* grown in minimal medium containing glycerol (2%, v/v) and washed once in a sterile saline solution (0.85% NaCl).

TABLE 2

Two-phase production[a] of pre-S2 antigen in small fermenters: effect of various fermentation conditions

Fermentation No.	Phase	Substrate[b]	A_{600nm} (end of phase)	Aeration conditions[c]	Pre-S2 antigen[d] (mg/l)
1	1	Glycerol (72.5 g/l)[e]	31 (48 h)	Air	<0.01
	2	Methanol (470 ml/l)[f]	36 (300 h)	Air + O_2	1.66 (300 h)
2	1	Glycerol (12.6 g/l)	6 (28 h)	Air	<0.01
	2	Methanol (168 ml/l)	6 (215 h)	Air	0.1 (215 h)
3	1	Glycerol (12.6 g/l)	11 (50 h)	Air	<0.01
	2	Methanol (297 ml/l)	20 (187 h)	Air + O_2	0.73 (187 h)
			47 (360 h)		0.91 (360 h)
4	1	Glycerol (12.6 g/l)	8 (48 h)	Air	<0.01
	2	Methanol-limiting (270 ml/l)[g]	22 (355 h)	Air + O_2	0.93 (355 h)
			44 (409 h)		1.11 (409 h)

All fermentations in Medium 1 (minimal).

[a] Principle: buid-up of cell biomass on glycerol (phase 1) followed by addition of methanol (phase 2) for induction of the production of antigen.

[b] Substrate and amount of substrate (g or ml) used per litre of culture.

[c] Details in MATERIALS AND METHODS.

[d] Antigen concentration measured at least once a day; highest concentration obtained reported.

[e] Glycerol added in two steps: 45 g at the beginning and the rest at 40 h.

[f] Except for fermentation No. 1, in which methanol concentration was maintained below 2% (v/v), all fermentations were carried out as described in MATERIALS AND METHODS.

[g] The volume of methanol brought at each methanol addition gave a final concentration of 0.01% (v/v); under these conditions, pO_2 remained higher than 50% air saturation.

TERIALS AND METHODS). Several observations were made (Table 2):

(a) Antigen production was greatly increased compared to the results in flasks (Table 1) but this increased production was essentially due to increased cell biomass. Antigen yields, however, never exceeded 1.6 mg/l, which was low considering the high cell densities obtained;

(b) Growth of cells and antigen production during Phase 2 on methanol required good provision of oxygen to the culture using a mixture of air and pure oxygen; the results for fermentation No. 2 showed conclusively that aeration with only air gave the poorest results;

(c) Carrying out the fermentation under methanol-limiting conditions (Fermentation No. 4) did not affect antigen or biomass production but led, however, to slower antigen production (Fermentation No. 3 versus Fermentation No. 4). Moreover, antigen production in methanol-limiting conditions was biphasic whereas it was linear under non-limiting methanol conditions (results not shown);

(d) Cell biomass production on methanol (Phase 2) was very limited when high cell biomass levels (Fermentation No. 1) were attained during growth on glycerol, this suggesting that Medium 1 became limiting in one or more nutrient(s).

Effect of various nitrogen sources

Since the different fermentation conditions employed previously, using minimal Medium 1, always resulted in low antigen production, a study of the various medium components, especially nitrogen, was undertaken. This was suggested to us by Dr. A. Demain (MIT; personal communication) and supported by recent observations on the production of a different hepatitis B antigen by *Saccharomyces cerevisiae* [2]. The effect of replacing NH_4Cl with $NaNO_3$ or various complex organic nitrogen sources was first studied in shake flask experiments (Table 3). Greatly improved antigen production was observed only with Hy-Soy (2%) and yeast extract (2%). Antigen production did not necessarily correlate with cell biomass levels: this was particulary evident with yeast extract, at least for the two concentrations of yeast extract tested.

Increasing the concentration of yeast extract from 2 to 7% (w/v) showed a strong positive effect on cell biomass and antigen production (Fig. 1). Cell biomass increased slightly with additional yeast extract but antigen production decreased significantly.

Antigen production in fermenter using rich medium

The results obtained from shake flask experiments prompted us to verify whether the improved antigen production by using very rich medium could be reproduced in small fermenters. Using a 3.5-l fermenter, cell biomass was first produced in 800 ml of modified Medium 1 containing 5 or 15% (v/v) of glycerol (Phase 1); at the end of this phase, glycerol and ammonium ions were exhausted and cell biomass levels reached approximately 50 g (dry weight) per litre. At induction time, one volume (800 ml) induction mixture was added to the culture to induce antigen production. The induction mixture consisted of modified Medium 1 (NH_4Cl omitted) containing

TABLE 3

Effect of various nitrogen sources on cell biomass and pre-S2 antigen productions in shake flasks

Nitrogen source (1% unless otherwise indicated)	Source	Pre-S2 antigen concentration (ng/ml cells)	A_{600nm}
NH_4Cl 5 g/l (control)	BDH Chemicals	42	1.8
$NaNO_3$	Fisher Scientific	30	1.5
NZ amine A	ICN	28	2.7
Soy peptone	Marcor	77	3.1
Edamin	Sheffield	55	3.4
Enzymatic digest of lactalbumin	Deltown	48	2.6
Pancreatic digest of gelatin	Deltown	30	2.4
Acid digest of casein	Deltown	41	3.2
Edamin type S	Sheffield	52	2.7
N-Z-amin type A	Sheffield	31	2.4
N-Z-Case M	Sheffield	33	2.5
Hy Soy 1%	Sheffield	75	3.5
Hy Soy 2%	Sheffield	95	3.5
Bacto-peptone	Difco	31	2.3
Yeast Extract 1%	Difco	16	3.1
Yeast Extract 2%	Difco	177	3.3

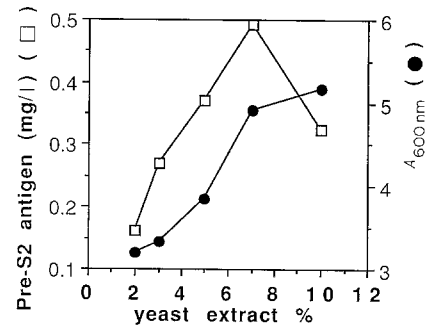

Fig. 1. Influence of yeast extract concentration on antigen and cell biomass production after 6 days (shake flask experiment).

TABLE 4

Two-phase production[a] of pre-S2 antigen in small fermenters using rich medium during induction with methanol: effect of fermentation conditions

Fermentation No.	Conditions		Results	
	Phase 1	Phase 2	Phase 1	Phase 2
5	- Glycerol: 5% (v/v)[b] - Aeration: air[d] - NH_4Cl: 5 g/l	Induction mixture + fed-batch addition of 150 ml of same mixture	A_{600nm} = 28 NH_4^+ = 0 Glycerol = 0	A_{600nm} = 47 Antigen[e] = 7.2 mg/l (164 h)
6	- Glycerol: 15% (v/v)[c] - Aeration: air + O_2[d] - NH_4Cl: 15 g/l	Induction mixture	A_{600nm} = 40 NH_4^+ = 300 mg/l Glycerol = 0	A_{600nm} = 61 Antigen[e] = 8.8 mg/l (96.5 h)
7	- Glycerol: 15% (v/v)[c] - Aeration: air + O_2[d] - NH_4Cl: 15 g/l - KCl (2×), $MgSO_4$ (1.5×)	Induction mixture (2× KCl + 1.5× $MgSO_4$)	A_{600nm} = 70 NH_4^+ = 12 mg/l Glycerol = 0	A_{600nm} = 61 Antigen[e] = 9.6 mg/l (120 h) A_{600nm} = 53 Antigen[e] = 16 mg/l (144 h)

[a] Principle: build-up of cell biomass on glycerol (800 ml of modified Medium 1 (Phase 1)) followed by addition of 800 ml of induction mixture (modified Medium 1 + 12% yeast extract + 0.25% (v/v) methanol) (Phase 2).
[b] Glycerol (63 g/l) added at the beginning of the fermentation.
[c] Glycerol added in three steps: 63 g/l at the beginning of the fermentation, 63 g/l after 1 and 2 days.
[d] Details in MATERIALS AND METHODS.
[e] Antigen concentration measured at least once a day; unless otherwise indicated, only highest concentration reported here.

12% (w/v) yeast extract and 0.25% (v/v) methanol. Methanol served as an inducer and as a carbon source. Since the biomass was already high, growth of the culture after induction was slow and the culture went through only one to two generations over about 150 h; because of the small number of generations, in spite of a very rich non-selective medium, 95–100% of the cells retained at least one copy of the plasmid.

Although different fermentation conditions were used, the level of pre-S2 antigen averaged 8.5 mg/l according to the ADI Diagnostics test. Very little antigen was detected in the culture supernatant (less than 0.1 mg/l). The characteristics of the different fermentations performed and the results obtained are given in Table 4. The profiles for cell biomass and pre-S2 antigen for one of these fermentations (Fermentation No. 7) are shown in Fig. 2. As

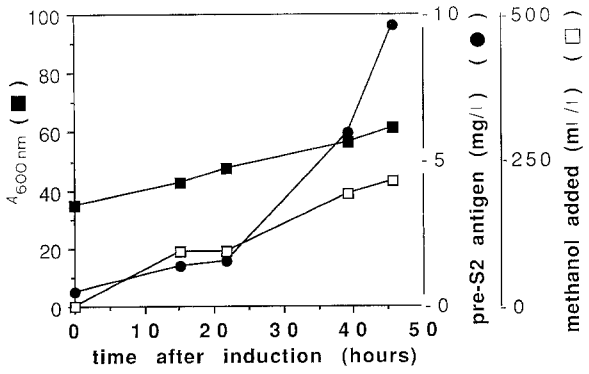

Fig. 2. Production of the pre-S2 antigen in a small fermenter using rich medium. Reference: Table 4, Fermentation n° 7. Time 0 represents the time of induction with methanol (after 2-fold dilution of the culture with the induction mixture).

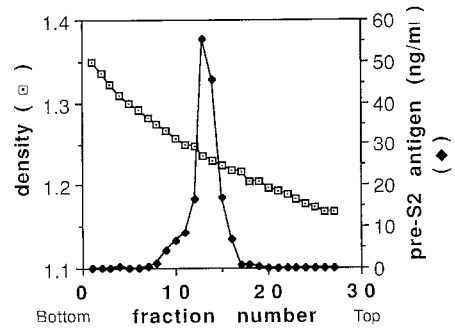

Fig. 3. Cesium chloride gradient centrifugation of the periplasmic pre-S2 antigen produced by *Hansenula polymorpha*.

illustrated in Fig. 2, the kinetics for antigen accumulation in rich medium showed two distinct phases: first, a phase of slow antigen accumulation up to 22 h followed by a phase during which antigen production accelerated; biomass was however produced at the same pace during the whole induction phase.

Characterization of the protein

The pre-S2 antigen produced by our recombinant *H. polymorpha* strain was subjected to equilibrium centrifugation on a cesium chloride gradient in order to verify if the antigen was in the form of 22-nm particles. The periplasmic antigen was used for this purpose. Results showed that the antigen had a density of 1.23–1.24 in a cesium chloride gradient (Fig. 3); the same value of density was obtained for human blood-derived hepatitis B surface antigen (the positive control provided by the ADI Diagnostics assay kit). Recombinant hepatitis B surface antigen produced by *S. cerevisiae* was also shown to have the same density [30].

Periplasmic pre-S2 antigen was further characterized by immunoblotting. Two major forms of the antigen were observed: one form of the antigen showed an apparent molecular mass of 33 kDa, whereas the second form of the antigen had a molecular mass close to 24 kDa. The 33-kDa antigen, as reported previously [21], corresponded probably to a glycosylated form of the pre-S2 antigen; the 24-kDa antigen appeared to be a degradation product. Other minor degradation products were also present.

DISCUSSION

Improvements

The results of our studies show that great improvements in heterologous protein production could be obtained by partial optimization of culture conditions, and, consequently, of microbial growth. The yields of pre-S2 antigen in our studies went from about 0.06 mg/l in minimal medium and shake flasks to an average of 8.5 mg/l in very rich medium and small fermenters, a 140-fold increase in volumetric antigen production. As shown in Table 5, a good part of this increase in antigen production was due to increased cell biomass resulting from the use of rich media. Besides increased biomass, specific antigen production was also improved 4.6-fold (see antigen per absorbance unit, Table 5) between our initial and partially optimized conditions. Several recent reports have shown that rich media may greatly enhance the production of heterologous proteins in several expression systems [2,15–17].

Yields of pre-S2 antigen

As indicated in MATERIALS AND METHODS, antigen levels determined with the ADI Diagnostics kit (this study) were only about half those obtained with the well-known AUSZYME II kit (Abbott Laboratories) used by most other investigators. Consequently, our results should be multiplied by 1.9 (MATERIALS AND METHODS) for comparison with antigen levels determined elsewhere using the AUSZYME II method. Therefore, it can be said that our recombinant *H. polymorpha* strain produced about 16 mg/l (8.5 × 1.9) of pre-S2 antigen according to the AUSZYME II kit. The yields of pre-S2 antigen in this study are about four times inferior to those reported for another recombinant methylotrophic yeast, *Pichia pastoris* strain GS 115/pTB05A [23]. Thill [23] has shown that volumetric antigen yields depend on the strain used.

The yields of pre-S2 antigen cannot directly be compared to those obtained for the more commonly produced hepatitis B *S* antigen, the major component of the viral envelope protein. Yields of recombinant *S* antigen ranging from about 20 to 380 mg/l have been reported in two expression systems [4,10]. It has been observed that the

TABLE 5

Gradual increase in pre-S2 antigen production during optimization

Scale	Medium	A_{600nm}	Pre-S2 antigen (mg/l)	Antigen/A[e]
Shake flask (50 ml)[a]	Minimal	1.9	0.06	1
Shake flask (50 ml)[b]	Very rich	6.0	0.5	2.6
Fermenter (1.6 l)[c]	Minimal	36	1.66	1.5
Fermenter (1.6 l)[d]	Very rich	61	8.8	4.6

[a] See Table 1, Medium 1 alone.
[b] See Figure 1, results at peak for A_{600nm} (cell biomass).
[c] See Table 2, Fermentation No. 2.
[d] See Table 4, Fermentation No. 6.
[e] Relative values.

expression level of the pre-S2 antigen was approximately one-tenth of that of the *S* antigen in the same recombinant system (*P. pastoris*) [23].

Biomass production versus antigen production

The recombinant *H. polymorpha* grew very well in Medium 1, a minimal medium rich in minerals, with glycerol as carbon source. High cell densities, around 35 to 40 g/l (dry weight), could be obtained within 48 h. However, the production of pre-S2 antigen in this medium, after induction with methanol, was relatively low (1.0–1.5 mg/l) and could be increased solely, in our studies, by the addition of very rich medium (6% yeast extract) at induction time in combination with methanol as the inducer. This indicated that the nutritional conditions required for good antigen production or methanol metabolism by our recombinant *H. polymorpha* strain are different from those needed for good cell biomass production. One must therefore differentiate between cell biomass production and antigen production and each step must be optimized separately. Similar observations with other expression systems have been reported [2,13,20,28]. It is now obvious that good cell biomass levels do not necessarily lead to good heterologous protein production.

Possible effects of yeast extract

The beneficial effect of yeast extract on the production of the pre-S2 antigen by our *H. polymorpha* strain is still unexplained. Part of this effect is certainly nutritional as antigen levels correlated well with cell biomass levels ($A_{600\,nm}$) up to about 7% yeast extract in shake flask experiments (Fig. 1). Moreover, as illustrated in Fig. 2, the kinetics for antigen production were different from those for biomass production, which was not true with minimal medium, assuming a same pattern of methanol feeding; biomass was produced at a regular slow pace whereas antigen accumulation was biphasic. The biphasic profile for antigen production suggests that cells may need to accumulate the active ingredient(s) present in yeast extract to a certain level before antigen accumulation becomes stimulated. This also shows that the mechanisms of yeast extract's effect on biomass and antigen production are different. Yeast extract contains not only organic nitrogen, but also vitamins, minerals, sugars, and other organic factors. Yeast extract or other nitrogen sources have also been shown to enhance heterologous protein production in other expression systems [3,15,16,25,28]. Several interesting suggestions have been proposed to explain the beneficial effect of those complex organic nitrogen sources: (a) increased genetic stability of the recombinant microorganism [25]; (b) protection of the heterologous protein from proteolytic degradation [28]; (c) provision of some components necessary for the elongation of proteins [15]; (d) influence on certain regulatory processes for the initiation of transcription, the initiation of translation, or both [15]. It could therefore be an oversimplification to ascribe to yeast extract solely a nutritional effect.

Quality of the pre-S2 antigen

Quality control of the pre-S2 antigen produced by *Hansenula polymorpha* was done using periplasmic antigen as its recovery from cells was simpler than that of intracellular antigen.

It is well known that hepatitis B surface antigens must possess a specific quaternary structure, known as the 20–22-nm particle, to be immunogenic. Equilibrium centrifugation (Fig. 3) of the pre-S2 antigen produced by our *H. polymorpha* strain indicated that the recombinant antigen showed a density very similar to that of human blood-derived hepatitis B surface antigen, strongly indicating that the antigen was in the form of 22-nm particles.

Immunoblotting of the pre-S2 antigen showed the presence of two major fractions: one 33-kDa fraction and one 24-kDa fraction. The 33-kDa antigen was probably a glycosylated form of the pre-S2 antigen as observed previously [21], whereas the 24-kDa antigen was likely a degradation product. In this study, little was done to prevent proteolytic degradation of the pre-S2 antigen and this aspect should be addressed later. We have previously observed serious degradation of the cytoplasmic pre-S2 antigen in *H. polymorpha* [21].

Hansenula polymorpha as expression system

Hansenula polymorpha has been attracting some interest as a host for the expression of heterologous proteins [12,19,22,24]. This yeast has two advantages: (a) a powerful and regulatable methanol oxidase promoter and (b) an ability to use cheap carbon sources such as methanol [22]. The excellent results obtained so far with another methylotrophic yeast, *Pichia pastoris*, act as a stimulant [4,8,18,29]. In addition, methylotropic yeasts may be able to secrete heterologous proteins efficiently [7,8,29], which is of very significant industrial importance.

Using a very rich medium and partially optimized fermentation conditions, we have been able to obtain 16 mg/l of the pre-S2 antigen using one particular recombinant strain of *H. polymorpha*. For comparison, a recombinant *S. cerevisiae* strain yielded about 20 mg/l of the hepatitis B *S* antigen, a less complex form of the viral antigen [10]. Our results show that *H. polymorpha* may be a promising host for heterologous protein production.

Work is in progress to identify which factor(s) in yeast extract is (are) responsible for increased production of the pre-S2 antigen. This identification is essential in order to simplify the culture medium during expression and to

better control the nutritional and physiological conditions required for good heterologous protein production. Further optimization of the fermentation conditions is also in progress.

The value of a recombinant expression system should not be based solely on the results obtained with one protein, as is being recognized more and more [18]. Additional results on several other recombinant proteins are required before making any definite judgement on *H. polymorpha*.

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