Protein Expression and Purification 66 (2009) 131–137

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Expression of HBsAg and preS2-S protein in different yeast based system: A comparative analysis

Nadia Hadiji-Abbes [a], Istabrak Borchani-Chabchoub [a], Henda Triki [b], Radhouane Ellouz [a], Ali Gargouri [a], Raja Mokdad-Gargouri [a,*]

[a] Unité de Génétique du Cancer et Production de Protéines Thérapeutiques, Centre de Biotechnologie de Sfax, Route Sidi Mansour, B.P. 1177 Sfax, Tunisia
[b] Laboratoire de Virologie, Institut Pasteur de Tunis, 13 place Pasteur, B.P. 74, 1002 Tunis Belvédère, Tunisia

ARTICLE INFO

Article history:
Received 6 November 2008
and in revised form 24 February 2009
Available online 20 March 2009

Keywords:
Heterologous expression
HBsAg
PreS2-S protein
Hepatitis B virus
Saccharomyces cerevisiae
Pichia pastoris

ABSTRACT

We have expressed both S and preS2-S genes coding for the hepatitis B small (S) and medium (M) proteins, respectively, in different yeast based expression systems and compared the production level of the recombinant proteins. In *Saccharomyces cerevisiae*, viral genes were expressed under the inducible Gal10/cyc1 and the constitutive PGK promoters using 2 μ replicating vectors. We showed that the yield of S protein was higher than M protein under both inducible (14.27 vs 10.9 mg/l) and constitutive (9.18 vs 6.39 mg/l) conditions, respectively. In the methylotrophic yeast *Pichia pastoris*, the viral genes were expressed in GS115 (Mut$^+$: Methanol Utilizing) and KM71 (Mut^S: Methanol Utilizing Slow) under the control of the alcohol oxidase promoter (AOX1). In Mut^S background, both S and preS2-S genes were expressed at higher levels than in Mut$^+$. In attempt to increase the yield of recombinant viral proteins in *S. cerevisiae*, we have co-expressed both inducible and constitutive vectors harboring the S or preS2-S genes leading to recombinant strains called UTS (containing pDP/S + pYePIT/S) and UTP (containing pDP/preS2-S + pYePIT/preS2-S). We showed that the recombinant S and preS2-S proteins were successfully detected and the production level reached 18.31 mg/l for the S and 13.22 mg/l for the M proteins.

Our comparative study provides evidence that in small scale, *S. cerevisiae* is more suitable for HBsAg and preS2-S proteins production than *P. pastoris* under inducible rather than constitutive condition.

Hepatitis B virus (HBV)[1] is one of the main etiological agents of acute and chronic liver disease that is still a major public health problem as more than 2 billion people worldwide have a history of this infection and there are 350 million chronic carriers [1]. As there is no effective treatment for hepatitis B infection, a preventive vaccine is the only way for its control [2]. The surface antigen of hepatitis B virus (HBsAg) is the primary component of vaccines against hepatitis B virus infection [3].

The envelope of HBV is made of three proteins: the major protein (226 amino acids) encoded by the S gene; the middle protein (281 amino acids) encoded by the preS2-S gene and the large protein (389 or 400 amino acids, according to the subtype) encoded by the preS1–preS2-S region [2,3]. These three proteins are translated from separate initiation codons but share a common reading frame and stop codon. These antigens have been shown to elicit virus-neutralizing and protective antibodies [4]. Production of anti-HBs antibodies after HBV infection provides efficient clearance of the virus and long-term protection. The region of the HBsAg which induces this immune response is designated the "a" determinant. It is exposed on the viral particle surface and is common to all subtypes of the virus [5,6]. The "a" determinant is located within the hydrophilic region of the protein S, between amino acid 110 and 160. There are two pairs of mutually exclusive subtype-specific determinants 'd' or 'y' and 'r' or 'w' (adr, ayr, adw and ayw). It can be classified into nine major subtypes: ayw1, awy2, ayw3, awy4, adw2, adw4, ayr, adrq−, adrq+ reflecting genetic variability [6,7].

To be immunogenic, the HBsAg polypeptide must assemble into particles of 20–22 nm diameters which has been correctly achieved in mammalian cells culture [8], in yeasts *Saccharomyces cerevisiae* [9], *Pichia pastoris* [10] and *Hansenula polymorpha* [11]. Therefore, most of the commercially available HBV vaccines contain the S protein expressed in the yeasts *S. cerevisiae* or *P. pastoris* [9,12] or CHO cells [13]. However, slow or non-responders to the conventional vaccines have been reported. Therefore, it has been suggested that inclusion of the preS2 regions into the vaccines could enhance the antibody response to the S protein and thus circumvent the non-responsiveness to the S protein [14]. Indeed, the preS2 region is known to be highly immunogenic at both B and T cell levels and to give rise to neutralizing antibodies [15]. The

* Corresponding author. Fax: +216 74 440 454.
E-mail address: Raja.Gargouri@cbs.rnrt.tn (R. Mokdad-Gargouri).

[1] *Abbreviations used:* HBV, hepatitis B virus; HBsAg, antigen of hepatitis B virus; PGK, phospho glycerate kinase; GAP, glyceraldehyde phosphate.

1046-5928/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2009.03.006

preS2 region contains a polymerized albumin receptor site, involved in the virus attachment to human hepatocytes and the anti-preS2 antibodies often arise earlier than the anti-S ones [16,17].

The purpose of this study was to express the S and preS2-S genes in different yeast based systems in order to compare their ability to produce these viral proteins in small scale experiments. In *S. cerevisiae*, the viral genes were expressed under galactose inducible (Gal10/cyc1) and constitutive (PGK, Phospho Glycerate Kinase) promoters using 2 μ replicative vectors. We also constructed yeast strains expressing S or preS2-S genes under both inducible and constitutive promoters with the aim to increase the production level. In *P. pastoris*, the S and preS2-S regions were expressed in both Mut^S and Mut^+ strains under the strong and methanol inducible AOX1 promoter. The yield of recombinant proteins were determined by ELISA and a comparison between different expression systems tested will be discussed.

Materials and methods

Strains and media

Saccharomyces cerevisiae strain W303-1B [Matα leu2 ura3 trp1 his3 ade2 canR] was used as host for gene expression and transformed by lithium chloride procedure. The recombinant strains were grown in a shaker flask (200 rpm at 30 °C) in 100 ml of a minimal medium (0.67% yeast nitrogen base) supplemented with 0.5% glucose or 2% ethanol as carbon source. In inducible condition, after growth on 0.5% glucose until OD at 600 nm = 3, 2% galactose was added in the medium and culture was prolonged for 18 h at 30 °C, 200 rpm.

Pichia pastoris strains GS115 (Mut^+, His^-) and KM71 (Mut^S, His^-, Arg^+) purchased from Invitrogen, (San Diego, CA, USA) were used as host for S and preS2-S gene expression.

Escherichia coli strain TOP10 [F-Δlac U169 (U80 lacZ DM15) supE44 hsdR17 recA1 gyrA96 endA1 thi-1 relA1] was used as host for plasmid cloning experiments as described in [18]. Bacteria were grown in Luria–Bertani medium.

Plasmids and DNA manipulation

The HBV S and preS2-S genes were cloned in the YepDP1-8 vector (provided by D. Pompon, CGM, CNRS, Gif/Yvette) under the control of the inducible Gal10/cyc1 promoter and in YepIPT [19] downstream of the constitutive PGK (Phospho Glycerate Kinase) promoter leading to the transformed yeast strains IS, IP (for inducible) and CS, CP (for constitutive) constructed in our previous work [20]. The S and preS2-S genes were isolated from recombinant vectors YepDP/S and YepDP/preS2-S as described by Borchani-Chabchoub et al. [20].

For expression in *P. pastoris*, the BamH1-KpnI DNA fragments containing the S (700 bp) and preS2-S genes (850 pb) were purified and cloned into the pPICZA (Invitrogen) to generate the plasmids pPICZA/S and pPICZA/preS2-S.

P. pastoris transformation and selection

Pichia pastoris strains GS115 (Mut^+) and KM71 (Mut^s) were grown in 500 ml YPD medium at 30 °C until OD at 600 nm = 1.3–1.5. Cells were harvested by centrifugation at 1500*g* for 5 min at 4 °C washed twice in 500 ml ice-cold water. The pellet was then washed twice in 1 ml ice-cold 1 M sorbitol, and centrifuged at 1500*g* for 5 min at 4 °C. The cells were finally resuspended in 1 ml ice-cold 1 M sorbitol and kept on ice until use. The competent cells were transformed with SacI linearized recombinant plasmids DNA (1–5 μg) by pulsed electroporation using a Bio-Rad GenePulser (1500 V, 25 μF, 200 Ω). One milliliter of ice-cold 1 M sorbitol was added to the cells immediately after electroporation. Cells were incubated for 2 h at 30 °C and transformants were selected on YPDS (1% yeast extract, 2% peptone, 2% dextrose, and 1 mol/l sorbitol) plates containing 100 μg/ml zeocin.

Real-time PCR

The ICyler (Bio-Rad) was used for QPCR amplification and detection. QPCR was prepared in duplicates in 25 μl reaction mixture containing 5 μl of template DNA (100 ng), 12.5 μl of SYBR Green PCR Master Mix (Bio-Rad), and 12.5 pmol of forward (5′-ATGG AGAACATCACATC AGGA-3′) and reverse (5′-AAGGTACCTTAAATGT ATACCCAAGAG-3′) primers. The thermal cycling conditions were 10 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C. The fluorescence signal was measured at the end of each extension step at 72 °C. After the amplification, a melting curve analysis with a temperature gradient of 0.1 °C/s from 65 to 95 °C was performed to confirm that only the specific products were amplified.

Gene copy number was determined by the quantification method which presents the target/reference ratio in a sample compared to the calibrator which contains both target and reference genes at a known ratio. The initial concentration of the sample is calculated using the comparative delta Ct method as described by Livak and Schmittgen [21], and the gene copy number is given by the formula: $n = 2^{-\Delta\Delta Ct}$, where ΔΔCt = (Ct target sample−Ct reference sample)−(Ct target calibrator−Ct reference calibrator). Ct is defined as the point at which the fluorescence level rises above the baseline. Beta-actin, used here as reference gene, was amplified with the following primers: forward: 5′-ACACACAGTGTTCCCATC GGT-3′ and reverse: 5′-AAGAACTGGGTGCTC TTCTG-3′.

Southern blot

Genomic DNA was prepared from 1.5-ml cultures of the various *Pichia* transformants expressing HBsAg using the standard protocol. Briefly, cells were washed in SCK buffer (1 M sorbitol, 50 mM citric acid, 150 mM K_2HPO_4) then incubated in buffer A (1 M sorbitol, 50 mM citric acid; 150 mM K_2HPO_4, 10 mM EDTA, pH 8; 0.1% β-mercaptoethanol, 0.3 mg/ml Zymolyase, pH 7) for 2 h at 37 °C to generate spheroplasts. They were then lysed in buffer B (150 mM NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA, pH 8, 1% Sarkosyl). After phenol–chloroform extraction, genomic DNA was recovered by ethanol precipitation. Equal quantities of genomic DNA from different *Pichia* transformants (Mut^S and Mut^+) were digested with HindIII and run on 0.8% agarose gel. The separated fragments were transferred to Hybond N+ nylon membrane (Amersham Pharmacia Biotech) and probed with the radioactive probe (pPICZA/S plasmid radiolabeled with α-P^{32} dCTP using a Rediprime II kit from Amersham Pharmacia Biotech). Following hybridization, the HBsAg containing bands were visualized by autoradiography.

Proteins extraction, Western blot and ELISA

All recombinant strains were cultivated on appropriate media for gene expression. Aliquots of cells corresponding to 20 OD units were harvested by low speed centrifugation (6000 rpm, 5–10 min), resuspended in 0.2 ml lysis buffer (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, pH 8, 150 mM NaCl, 0.1% Nonidet P-40 1 mM PMSF, 2 mg/ml leupeptine and 1 mg/ml pepstatine) containing zirconia glass beads (0.45 mm-Sigma) and lysed by 10 min bursts of vortexing at maximum speed, with chilling on ice for 1–2 min between bursts. After centrifugation at 12,000 rpm for 15 min, the supernatant was collected and protein concentration determined using the

Bio-Rad assay. Total proteins (30 μg) were separated on 12.5% SDS–PAGE and electrotransferred to Hybond P membrane (Amersham Pharmacia Biotech). Immuno-blotting was carried out with anti-HBs antibody coupled to peroxidase (diluted to 1:500) and the complex was visualized using the enhanced chemiluminescence Western blotting detection kit ECL plus (Amersham Pharmacia Biotech). The anti-HBs used is a mixture of three monoclonal antibodies raised against the "ad" and "ay" subtypes (Diasorin). ELISA test was performed using the ETI-Mak 4 kit (Diasorin) to estimate the HBsAg concentration in the soluble cell extracts (10 μg) as described by the manufacture's protocol. A standard curve was prepared using dilution series containing 0–100 ng of HBsAg from the commercially available vaccine Engerix B and included in each assay.

Results

Optimisation of the culture conditions for the expression of preS2-S and S genes in S. cerevisiae

The viral genes preS2-S and S isolated from virus DNA from Tunisian patients with chronic hepatitis B (with accession numbers AF229159 for S and AF214661 for preS2-S in the Genebank database), belonging to the ayw2 subtype (Genotype D) were cloned into the replicating vectors yePDP1-8 and YEpiPT and expressed under the control of the inducible Gal10/cyc1 and the constitutive PGK (Phospho Glycerate Kinase) promoters, respectively [20].

Four recombinant strains were selected: IS, IP (for Inducible S and preS2-S) and CS, CP (for Constitutive S and preS2-S) and cultivated in 100 ml of minimal and rich media supplemented with different carbon sources: glucose, glycerol, galactose or ethanol. Overall, the yield of recombinant proteins is considerably higher on rich than minimal media under inducible as well as constitutive promoters (Fig. 1A and B). Interestingly, we showed that ethanol is the most appropriate substrate for the production of HBsAg and M proteins in both the constitutive and inducible systems. Indeed, in the latter one, we tested all pre-culture media (glucose, glycerol, galactose and ethanol) in combination with either 2% or 0.5% galactose during the induction phase. We showed that a significantly higher level of recombinant preS2-S (Fig. 1A) and S (data not shown) proteins were obtained in presence of 2% rather than 0.5% galactose. In order to define the optimal condition for viral antigens production in the constitutive system, we cultivated the CS and CP strains on glycerol, glucose, galactose or ethanol. Our data showed, once again, that the highest amount of HBsAg was obtained after 48 h culture on 2% ethanol compared to other carbon sources tested (Fig. 1B). Similarly, the ethanol appears as the most appropriate substrate for preS2-S protein production by the CP strain (data not shown). When IS and IP recombinant strains were cultivated in optimal conditions as described above, the yields of HBsAg and preS2-S proteins were estimated by ELISA to 14.24 and 10.9 mg/l under inducible conditions, respectively. However, the amount of viral proteins was lower when gene expression was driven by the constitutive PGK promoter: 9.1 and 6.3 mg/l of S and preS2-S, respectively (Table 1). The Western blot analysis revealed a single 27 kDa protein in both S transformants (CS and IS) whereas a proteolysis product was observed for the M protein in the IP strain, due to the presence of a protease site as already mentioned in several works [22,23]. In the CP strain, no signal corresponding to the preS2-S protein was observed (Fig. 2). This result is in line with our previous report showing that this strain produces the M protein on several carbon sources such as ethanol,

Table 1
Expression of AgHBs by *S. cerevisiae* and *P. pastoris* recombinant strains.

Strains	Conditions	Total proteins (mg/l)	Yield of HBsAg (mg/l)	Specific activity (μg HBs/mg total protein)
S. cerevisiae				
IS	Galactose	1751	14.27	8.16
IP	//	1711	10.9	6.5
CS	Ethanol	1660	9.18	6.86
CP	//	1583	6.39	4.6
UTS	Ethanol/Galactose	2008	18.31	9.7
UTP	//	1928	13.22	8.23
P. pastoris				
GS115 (Mut^+)				
SG9	Methanol	33.33	0.35	10.5
PG1	//	85.36	0.19	2.28
KM71 (Mut^S)				
SK4	Methanol	1587	5.7	3.59
PK6	//	72.66	0.215	2.99

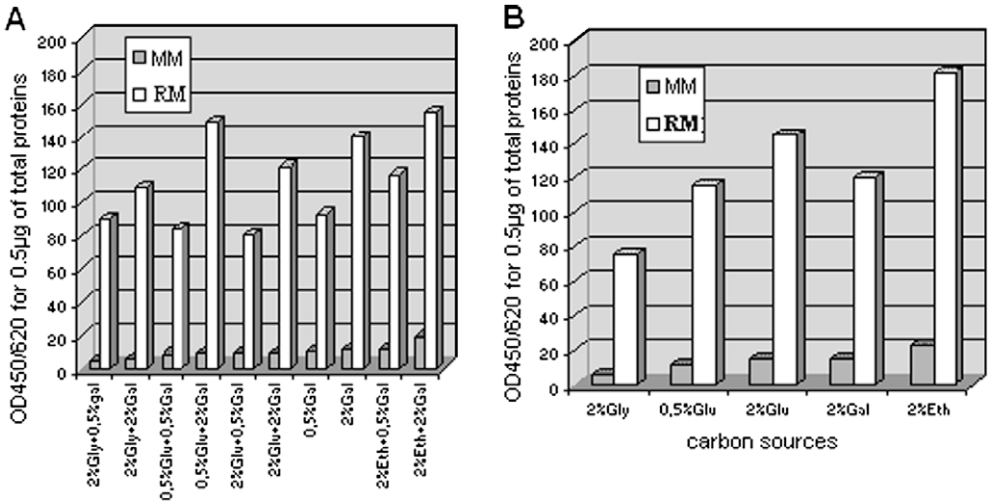

Fig. 1. Comparison of HBV S and preS2-S proteins level by ELISA. Recombinant yeast strains IP (A) and CS (B) were cultivated on minimal (MM) or rich media (RM) with different carbon sources: 2% glucose, 0.5% glucose, 2% glycerol, 2% galactose and 2% ethanol for 24 h. Cultures from IP strain were then supplemented by 0.5% or 2% galactose for Gal10/cyc1 promoter induction. Total proteins were extracted and 10 μg were assayed by ELISA using the ETI-Mak 4 kit (Diasorin). The ratios $OD_{450/630}$ were normalized to the total quantity of proteins.

glycerol, galactose but not on 2% glucose which could be explained by a post-translational negative control [20, see the Discussion].

Cloning of the preS2-S and S genes in P. pastoris and estimation of copies number

The preS2-S and S regions were cloned into the expression vector pPICZA (Invitrogen). The recombinant plasmids (pPICZA/preS2-S) and (pPICZA/S) were integrated in the strains GS115 (Mut$^+$) and KM71 (Mut^S) and the recombinant clones were selected on zeocine supplemented medium. Four clones were retained: SG9, PG1 which have integrated pPICZA/S and pPICZA/preS2-S, respectively, in the GS115 strain and SK4, PK6 which have integrated the pPICZA/S and pPICZA/preS2-S, respectively, in KM71.

Genomic DNA extracted from the selected Mut^S and Mut$^+$ clones were analyzed by Southern blot and Q-PCR to estimate the gene copies number. The Southern blot revealed in the Mut$^+$ clones (SG5, SG9, SG55, PG1, PG13, PG22, and PG47) the same fragment of 4.3 kb in addition to a common band of 2.3 kb corresponding to the endogenous single copy AOX gene also present in the GS115 host strain (Fig. 3A). The intensity of the latter band was taken as reference to estimate the copy number of integrated plasmid. This allowed us to conclude that most of the *Pichia* clones harbored a single copy of the S or preS2-S gene except the PG1 clone which should contain more than 1 copy. In the case of the Mut^S transformants, we showed that SK2, SK3 and SK5 clones have integrated a single copy of the expression cassette while the SK4 appears to harbor more copies since the band of 4.3 kb (corresponding to the expression vector) is about two times more intense than the endogenous AOX reference (Fig. 3B).

Table 2
Determination of the copy numbers in different yeast recombinant strains according to the Livack method: $2^{-\Delta\Delta Ct}$.

Clones	ΔΔCt	Copy number: $2^{-\Delta\Delta Ct}$
SK4	−1.25	2.37
PK2	−0.95	1.92
PK6	−2.25	4.75
PG1	−3.15	8.87

To confirm the Southern blot data and determine precisely the gene copy number in recombinant clones, we performed Q-PCR analysis using the relative quantification based on the β-actin as reference gene. In this experiment, we have chosen the SG9 clone as calibrator since the Ct values of the target (S) and the reference (β-actin) are approximately equal. Using the ΔΔCt method, we have estimated the gene copy number in the recombinant strains to 1, 2.3, 1.93, 4.7 and 8.8 copies for SK3, SK4, PK2, PK6 and PG1, respectively (Table 2). Fig. 4 shows a representative example of Q-PCR data for Mut+ SK4 (2.3 copies) and SK3 (1 copy) strains. Furthermore by ELISA, we compared the yield of recombinant proteins in the 2 Mut$^+$ clones PG1 and SG9. As shown on Fig. 5A, the level of preS2-S protein was lower compared with the AgHBs despite the high copy number in the PG1 clone suggesting that in *Pichia* the

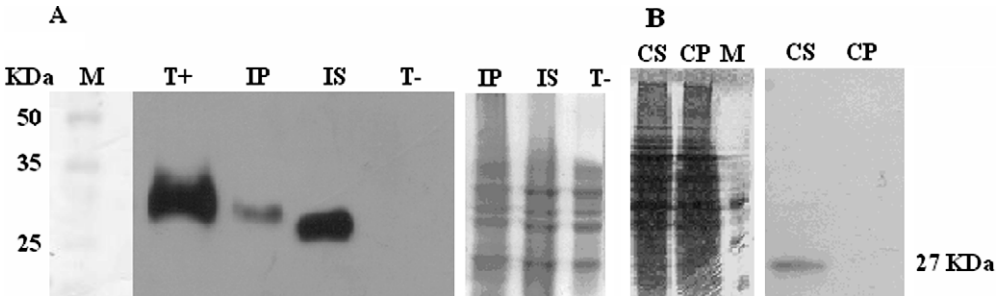

Fig. 2. SDS–PAGE and Western blot analysis of HBV S and preS2-S proteins expressed by the YepDP1-8 inducible vector (panel A; IS and IP strains) and YepIPT constitutive vector (panel B; CS and CP strains) and using an anti-HBs monoclonal antibody coupled to peroxydase (Diasorin). The complex was revealed by ECL plus (Amersham Pharmacia Biotech). IS, IP: recombinant yeast strains producing HBsAg and M protein, respectively, in inducible conditions. CS, CP: recombinant yeast strains producing HBsAg and M protein, respectively, in constitutive conditions. M, Rainbow Molecular Weight (Amersham Pharmacia).

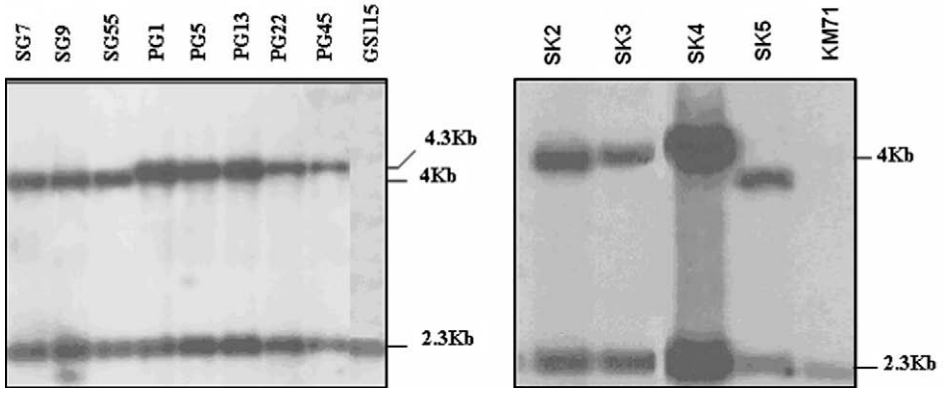

Fig. 3. Southern blot analysis of *Pichia* Mut^s and Mut$^+$ recombinant clones. Genomic DNA (10 μg) prepared from recombinant *Pichia* clones Mut^s (SG5, SG9, SG55, PG1, PG13, PG22, and PG47) and Mut$^+$ (SK2, SK3, SK4 and SK5) or untransformed host strains (GS115 and KM71) was digested with HindIII and subjected to hybridisation with a radiolabelled probe corresponding to pPICZA/S vector. Fragments of 4.3 or 4 kb correspond to the expression vector pPICZA containing the preS2-S or the S gene, respectively. The AOX endogenous gene was visualized as a band of 2.3 kb.

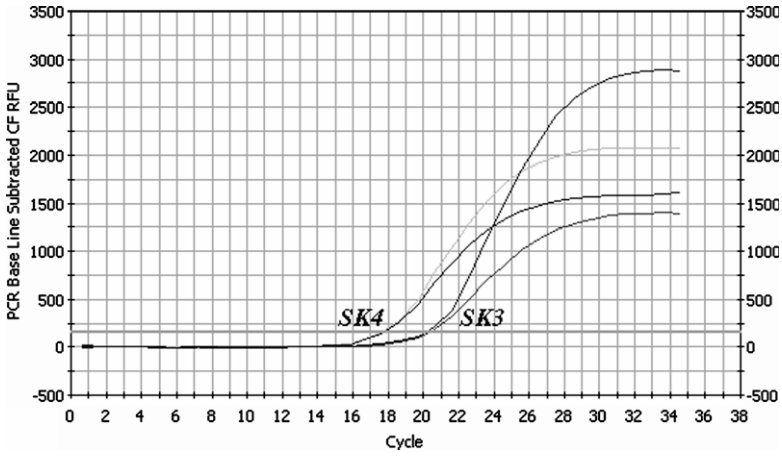

Fig. 4. Representative example of Q-PCR analysis for Mut^S recombinant strains which have integrated 1 copy (SK3) and 2.3 copies (SK4) as determined by the relative quantification using the β-actin as reference gene.

preS2-S protein was not well expressed and probably subject to a proteolysis as already demonstrated in *S. cerevisiae* [22].

Effect of copy number and genetic background on HBsAg expression

In *P. pastoris*, the expression level of many recombinant proteins including HBsAg can be significantly enhanced by utilizing multicopy transformants [24,25]. To study the effect of the gene copy number on HBsAg expression in our system, we cultivated SK4, SK3 strains (in the same genetic background, Mut^S) with different S gene copy number, as described above, in minimal medium supplemented by 0.5% methanol for 24, 48, 72, 96 and 110 h. The yield of HBsAg was estimated by ELISA as described in Materials and methods section. We showed that the production of HBsAg was very low before 96 h and increased at 110 h post-induction (Fig. 5B). Interestingly, we found a direct correlation between the gene dosage and the expression level of HBsAg. The effect of copy number was additive as the 3 copy clone (SK4) resulted in about 3-fold higher yield of HBsAg than in single copy clone (SK3), at both 96 and 110 h post-induction (Fig. 5B). In fact, the copy number influences the HBsAg productivity, since the amount of HBsAg produced by SK4 at 96 h is equal to that produced by SK3 at 110 h. The effect of the Mut$^{S/+}$ genetic background was assessed in two transformants (SK3 and SG9) bearing the same copy number (one copy) of S insert in Mut^S and Mut$^+$ context, respectively. Fig. 4B shows that the level of HBsAg is about 3-fold higher in Mut^S SK3 than in Mut$^+$ SG9 strain. In addition, the optimal amount of recombinant HBsAg was obtained after 110 h post-induction by 0.5% methanol in both contexts Mut$^+$ and Mut^S (Fig. 5C). Once again, the productivity is greatly influenced by the genetic background, as the SK3 produces at 96 h the same amount of HBsAg than SG9 at 110 h post-induction.

Co-expression of the preS2-S and S genes in S. cerevisiae

In an attempt to increase further the antigen production in yeast, we planned to co-transform *S. cerevisiae* by the constitutive and the inducible vector expressing either the S or the preS2 proteins. Therefore, recombinant plasmids YePDP/S + YEpiPT/S and YePDP/preS2-S + YEpiPT/preS2-S were co-transferred in the same yeast cells, leading to two strains named UTS (expressing the S gene under both Gal10/cyc1 and PGK promoters) and UTP (expressing the preS2-S gene under both Gal10/cyc1 and PGK promoters). These strains were selected on minimal media without uracil and tryptophan then cultivated on 2% ethanol for 48 h where only the constitutive promoter was functional, followed by the addition of 2% galactose for 18 h to induce the Gal10/cyc1 promoter. By Western blot, we showed that the recombinant S and preS2-S proteins were successfully detected (Fig. 6). In addition, the production level reached 18.31 mg/l for the S protein and 13.22 mg/l for the M protein (Table 1).

Discussion

Hepatitis B is still a serious problem in human health and much effort was made to counteract this viral infection and to minimize the number of slow or non-responders to the conventional vaccine. Given its important functional role in the HBV life cycle and being a potential target for the development of novel antivirals, the preS region has been expressed with the S gene to generate a more efficient recombinant HBV vaccine [26,27].

In this study, we tested different yeast based expression systems to produce both recombinant HBsAg and M protein of HBV. According to our previous analysis of local virus genotypes, the ayw2 was the most frequent serotype in Tunisia and should be therefore used in order to produce a more appropriate vaccine for our population [28]. The S and preS2-S viral genes were amplified from a Tunisian patient with chronic hepatitis B and inserted into yeast multicopy vectors. The heterologous expression of each gene was driven in *S. cerevisiae* by two promoters: an inducible Gal10/cyc1 and a constitutive one PGK [20]. We have also inserted upstream of the translation initiator codon "ATG" a 10 bp sequence "ACAAAACAAAA" deriving from yeast genes exhibiting high expression level. In fact, it was shown that this sequence improves the translation efficiency. This sequence is usually specific of genes belonging to GAP (glyceraldehyde phosphate) family but it can expand to others, however, the molecular mechanism by which this sequence acts to modulate gene expression is still unknown [29].

We showed that the galactose inducible promoter was more efficient to express either S or preS2-S genes than the constitutive one (Phospho Glycerate Kinase). In fact, the yield of recombinant proteins was 1.5-fold higher under inducible than constitutive conditions. The S gene has been expressed in several yeast, based systems but to our knowledge, this is the first study comparing in the same conditions (the same host strain, replicative origin and transcriptional terminator) the ability of two yeast promoters to express both S and preS2-S genes. We showed that under inducible as well as constitutive conditions, the S region was more efficiently expressed than the preS2-S, which might be due to the degradation

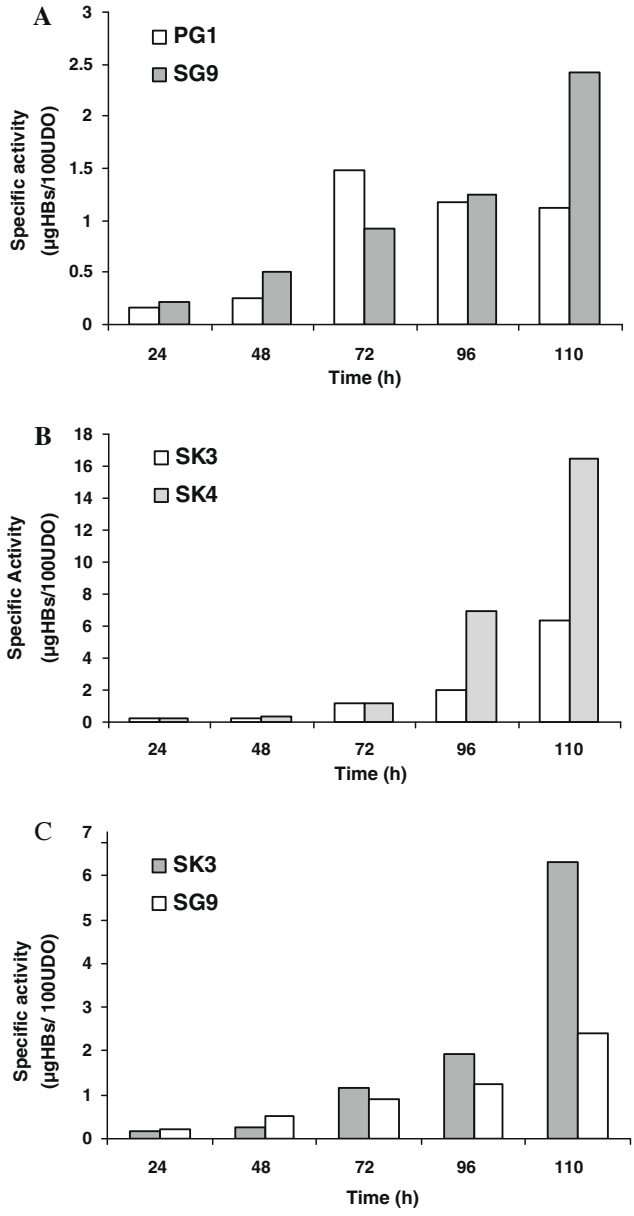

Fig. 5. Kinetics of HBsAg and preS2-S proteins production at different times after methanol induction. (A) Comparison of the yield of AgHBs and preS2-S in Mut$^+$ clones SG9 and PG1. ELISA was performed using the ETI-Mak 4 kit (Diasorin) to estimate the HBsAg and preS2-S yield in 10 µg of total proteins extracted from PG1 (expressing the preS2-S) and SG9 (expressing the HBsAg). A standard curve was constructed using a dilution series (0–1 µg of HBsAg from the commercially available vaccine Engerix B) and was included in the assay. (B) Effect of Mut$^+$/Mut^s context of *Pichia* on the production of recombinant HBsAg. ELISA was performed as described above using 10 µg of total proteins extracted from Mut$^+$ SG9 and Mut^s SK3. Both clones have integrated a single copy of the S gene. Proteins were extracted after 24, 48, 72, 96 and 110 h of methanol induction. (C) Effect of gene copy number on the HBsAg production by recombinant Mut^s strains Aliquots of 10 µg of total proteins extracted from SK4 (3 copies of the S gene) and SK3 (1 copy of the S gene) at different times of methanol induction were analyzed by ELISA as described above.

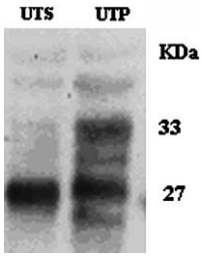

Fig. 6. Western blot analysis of HBsAg and preS2-S protein expression in UTS and UTP strains. Strains were cultivated on 2% ethanol for 48 h where only the constitutive promoter was functional, followed by the addition of 2% galactose for 18 h to induce the Gal10/cyc1 promoter. Thirty micrograms of total proteins were separated on 12.5% SDS–polyacrylamide gel and electrotransferred to Hybond P. The membrane was treated with anti-HBs monoclonal antibody coupled to peroxydase (Diasorin). The complex was revealed by ECL plus (Amersham Pharmacia Biotech).

of the M protein in this heterologous background. This is in accordance with previous works which reported a proteolytic degradation of preS2 sequence in plasma-derived particles [30], in *S. cerevisiae* [31] and in *H. polymorpha* [32]. It was demonstrated that the preS2 region was very sensitive to intracellular protease in *S. cerevisiae* extract and that expression in protease-deficient yeast strain carrying pep4-3 mutation could minimize proteolytic degradation of the preS2 region [22]. The deletion of the site susceptible to trypsin-like protease in the preS2 region reduced the degradation process and enhances the productivity of the M protein [33].

The Western blot analysis revealed, not only the proteolysis of the preS2-S protein, but also a striking feature of the preS2 gene expression in the CP background. Indeed, such expression was absent in presence of 2% glucose. We have already resolved this surprising result in our previous work [20]. We showed that the preS2-S and S genes were well transcribed in both systems and the two corresponding mRNAs had a similar abundance. At the protein level, the HBsAg was produced at a high level with the Gal10/cyc1 and PGK promoter based vectors. However, the preS2-S was well expressed only under the Gal10/cyc1 promoter and was detected at very low level or totally absent during growth on glucose using the PGK promoter based system. Note that on the other carbon sources (glycerol, ethanol, galactose) the preS2-S protein was correctly expressed. The discrepancy between the preS2-S mRNA and protein levels led us to suggest that in yeast, the preS2-S protein synthesis is submitted to a negative translational control which takes place only when glucose was used as carbon source [20].

Our data showed that ethanol is the optimal carbon source for the production of recombinant HBsAg and M proteins in yeast strains IS, IP, CS and CP. This is advantageous since ethanol provides a slight sterilization of the medium and is a cost-effective substrate compared to galactose for example.

In attempt to increase the yield of recombinant viral proteins in *S. cerevisiae*, we co-transformed the same W303-1B host strain by both plasmids pDP1-8/S + YepIPT/S (UTS strain) and pDP1-8/preS2-S + YepIPT/preS2-S (UTP strain). This should enable the recombinant strains to produce the corresponding proteins, firstly during the growth phase through the constitutive PGK promoter, then during the induction phase through Gal10/cyc1 promoter. Indeed, after the culture on 2% ethanol followed by the addition of 2% galactose, both UTS and UTP strains produced higher amount of HBsAg (18.3 vs 14.2 mg/l for IS and 9.1 mg/l for CS) and M protein (13.2 vs 10.9 mg/l for IP and 6.3 mg/l for CP).

Pichia pastoris offers an excellent eukaryotic expression system for large-scale production of functional recombinant proteins. High-level expression and efficient assembly of HBsAg particles has been reported in *P. pastoris* by integrating the S gene under the control of alcohol oxidase (AOX1) promoter. It is well known that to the assembly of the HBsAg polypeptide into particles of 20–22 nm diameter is necessary for the immunogenicity [35]. HBsAg particle assembly does not appear to occur in *E. coli*-based expression systems [36,37]. However, characterization of the recombinant protein produced in *P. pastoris* as well as in *S. cerevisiae* showed that HBsAg exists in a form which is similar to human serum-derived HBsAg 22 nm particles [4,37].

In this work, we have expressed both S and preS2-S of ayw2 serotype in the two genetic background of *Pichia* Mut$^+$ (GS115) and Mut^S (KM71) under the control of the methanol inducible AOX1 promoter. Our data showed that fast growing Mut$^+$ strain (capable of efficient methanol utilization) was less efficient than Mut^S to produce HBsAg and M proteins, which is in agreement with previous reports [34]. Indeed, a single copy Mut^S transformant produced about 2.5-fold more than Mut$^+$ strain harboring a single S gene. Recently, it was shown that Mut$^+$ strains was able to express HBsAg with similar productivity as Mut^S strains when maintained at a limited growth during induction in bioreactor [35].

We also noticed a direct correlation between the gene dosage and the expression level of HBsAg since the 3 copies clone (SK4) resulted in about 3-folds higher yield of HBsAg than the single copy clone (SK3) at both 96 and 110 h post-induction. Similarly, Vassileva et al. have shown that the increase in copy number of the HBsAg expression cassette leads to an increased expression of the HBsAg in Muts strain [25].

Overall, we can conclude that the expression of recombinant S and preS2-S of ayw2 HBV subtype was successful in *S. cerevisiae* and *P. pastoris* but with higher yields using the inducible Gal system in *S. cerevisiae*. More interestingly, we showed that the co-expression of S or preS2-S genes under inducible and constitutive promoters in yeast cells leads to significant increase in the expression levels of the corresponding proteins.

Acknowledgments

We thank Pr Koussay Dellagi as principal instigators of the Hepatitis B project for the helpful discussion and Dr. Khemais Benhadj for improving the quality of the manuscript. This work was supported by a Tunisian grant contract (Contract Programme CBS-SERST 98-02).

References

[1] State of the World's Vaccines and Immunization, WHO/UNICEF Bulletin, Geneva, 1996.
[2] S.A. Rogers, J.L. Dienstag, T.J. Liang, Hepatitis B virus-clinical disease, prevention and therapy, in: R.A. Willson (Ed.), Viral Hepatitis, Dekker, New York, 1997, pp. 119–146.
[3] P. Tiollais, C. Pourcel, A. Dejean, The hepatitis B virus, Nature 317 (1985) 489–495.
[4] R.A. Hitzeman, C.Y. Chen, F.E. Hagie, E.J. Patzer, C.C. Liu, D.A. Estell, J.V. Miller, A. Yaffe, D.G. Kleid, A.D. Levinson, H. Oppermann, Expression of hepatitis B surface antigen gene in yeast, Nucleic Acids Res. 11 (1983) 2745–2763.
[5] L.O. Magnius, H. Norder, Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene, Intervirology 38 (1995) 24–34.
[6] H. Norder, A.M. Couroucé, L.O. Magnius, Molecular basis of hepatitis B virus serotype variation within the four major subtypes, J. Gen. Virol. 73 (1992) 3141–3145.
[7] H. Norder, A.N. Couroucé, L.O. Magnius, Complete genomes phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes, Virology 198 (1994) 489–503.
[8] M.L. Michel, P. Pontisso, E. Sobczak, Y. Malpiece, R.E. Streeck, P. Tiollais, Synthesis in animal cells of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin, Proc. Natl. Acad. Sci. USA 81 (1984) 7708–7712.
[9] P. Valenzuela, A. Medina, W.J. Rutter, G. Ammerer, B.D. Hall, Synthesis and assembly of hepatitis B virus surface antigen particles in yeast, Nature 298 (1982) 347–350.
[10] E. Hardy, E. Martinez, D. Diago, R. Dıiaz, D. Gonzalez, L. Herrera, Large scale production of recombinant hepatitis B surface antigen from *Pichia pastoris*, J. Biotechnol. 77 (2000) 157–167.
[11] S. Schaeffer, M. Piontek, S.J. Ahn, A. Papendieck, Z.A. Janowicz, G. Gellissen, Recombinant hepatitis B vaccines-characterization of the viral disease and vaccine production in the methylotrophic yeast *Hansenula polymorpha*, in: K. Dembrowsky, P. Stadler (Eds.), Novel Therapeutic Proteins, VCH, Weinheim, 2001, pp. 245–273.
[12] J.M. Cregg, J.F. Tschopp, C. Stillman, R. Siegel, M. Akong, W.S. Craig, R.G. Buckholz, K.R. Madden, P.A. Kellaris, G.R. Davis, B.L. Smiley, J. Cruze, R. Torregrossa, G. Velicelibi, G.P. Thill, High level expression and efficient assembly of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*, Bio/Technology 5 (1987) 479–485.
[13] D. Diminsky, N. Moav, M. Gorecki, Y. Barenholz, Physical, chemical and immunological stability of CHO-derived hepatitis B surface antigen (HBsAg) particles, Vaccine 18 (2000) 3–17.
[14] T. Yamada, H. Iwabuki, T. Kanno, H. Tanaka, T. Kawai, H. Fukuda, A. Kondo, M. Seno, K. Tanizawa, S. Kuroda, Physicochemical and immunological characterization of hepatitis B virus envelope particles exclusively consisting of the entire L (pre-S1 + pre-S2 + S) protein, Vaccine 9 (2001) 3154–3163.
[15] W.H. Gerlich, R. Deepen, K.H. Heermann, B. Krone, X.Y. Lu, M. Seifer, R. Thomssen, Protective potential of hepatitis B virus antigens other than the S gene protein, Vaccine 8 (Suppl.) (1990) S63–S68.
[16] D.R. Milich, G.B. Thornton, A.R. Neurath, S.B. Kent, M.L. Michel, P. Tiollais, F.V. Chisari, Enhanced immunogenicity of the pre-S region of hepatitis B surface antigen, Science 228 (1985) 1195–1199.
[17] S. Kuroda, Y. Fujisawa, S. Iino, Y. Akahana, H. Suzuki, Induction of protection level of anti-pres2 antibodies in humans immunized with a novel hepatitis B vaccine consisting of M (preS2 þ S) protein particles (a third generation vaccine), Vaccine 9 (1991) 163–169.
[18] T. Maniatis, E.F. Fritsch, J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor University Press, Cold Spring Harbor, NY, 1982.
[19] I.R. Grahams, A. Chambers, Constitutive expression vector PGK, Methods Mol. Biol. 62 (1997) 159–169.
[20] I. Borchani-Chabchoub, R. Mokdad-Gargouri, A. Gargouri, Glucose dependant negative translational control of the heterologous expression of the preS2 HBV antigen in yeast, Gene 311 (2003) 165–170.
[21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method, Methods 25 (2001) 402–408.
[22] K.E. Langley, K.M. Egan, J.M. Barendt, C.G. Parker, G.A. Bitter, Characterization of purified hepatitis B surface antigen containing pre-S(2) epitopes expressed in *Saccharomyces cerevisiae*, Gene 67 (1988) 229–245.
[23] M. Kobayashi, T. Asano, M. Utsunomiya, Y. Itoh, Y. Fujisawa, O. Nishimura, K. Kato, A. Kakinuma, Recombinant hepatitis B virus surface antigen carrying the pre-S2 region derived from yeast: purification and characterization, J. Biotechnol. 8 (1988) 1–22.
[24] A. Vassileva, D.A. Chugh, S. Swaminathan, N. Khanna, Expression of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris* using the *GAP* promoter, J. Biotechnol. 88 (2001) 21–35.
[25] A. Vassileva, D.A. Chugh, S. Swaminathan, N. Khanna, Effect of copy number on the expression levels of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*, Protein Expr. Purif. 21 (2001) 71–80.
[26] M.W. Pride, C.R. Bailey, E. Muchmore, Y. Thanavala, Evaluation of B and T-cell responses in chimpanzees immunized with Hepagene, a hepatitis B vaccine containing pre-S1, pre-S2 gene products, Vaccine 16 (1998) 543–550.
[27] D.R. Milich, J.E. Jones, A. McLachlan, G. Bitter, A. Moriarty, J.L. Hughes, Importance of subtype in the immune response to the pre-S(2) region of the hepatitis B surface antigen. II. Synthetic Pre-S(2) immunogen, J. Immunol. 144 (1990) 3544–3551.
[28] I. Borchani-Chabchoub, A. Gargouri, R. Mokdad-Gargouri, Genotyping of Tunisian hepatitis B virus isolates based on the sequencing of preS2 and S regions, Microbes Infect. 2 (2000) 607–612.
[29] R.W. Ellis, A. Hagoplan, P.J. Kniskern, D.L. Montgomery, Method for producing hepatitis B proteins in yeast, Eur. Pat. Appli. 88202212.2, 1989.
[30] A.R. Neurath, S.B. Kent, N. Strick, P. Taylor, C.E. Stevens, Hepatitis B virus contains pre-S gene-encoded domains, Nature 315 (1985) 154–156.
[31] T. Imamura, M. Araki, A. Miyanohara, J. Nakao, H. Yonemura, N. Ohtomo, K. Matsubara, Expression of hepatitis B virus middle and large surface antigen genes in *Saccharomyces cerevisiae*, J. Virol. 61 (1987) 3543–3549.
[32] M.R. De Roubin, L. Bastien, S.H. Shen, D. Groleau, Fermentation study for the production of hepatitis B virus pre-S2 antigen by the methylotrophic yeast *Hansenula polymorpha*, J. Ind. Microbiol. 8 (1991) 147–156.
[33] Y. Itoh, Y. Fujisawa, Synthesis in yeast of hepatitis B virus surface antigen modified P31 particles by gene modification, Biochem. Biophys. Res. Commun. 141 (1986) 942–948.
[34] S. Ottone, X. Nguyen, J. Bazin, C. Bérard, S. Jimenez, O. Letourneur, Expression of hepatitis B surface antigen major subtypes in *Pichia pastoris* and purification for in vitro diagnosis, Protein Expres. Purif. 56 (2007) 177–188.
[35] P. Charnay, M. Gervais, A. Louise, F. Galibert, P. Tiollais, Biosynthesis of hepatitis B virus surface antigen in *Escherichia coli*, Nature 286 (1980) 893–895.
[36] J.C. Edman, R.A. Hallewell, P. Valenzuela, H.M. Goodman, W.J. Rutter, Synthesis of hepatitis B surface and coreantigens in *E. coli*, Nature 29 (1981) 503–506.
[37] J.M. Cregg, J.F. Tschopp, C. Stillman, R. Siegel, M. Akong, W.S. Craig, K.R. Buckholz, K.R. Madden, P.A. Kellaris, G.R. Davis, B.L. Smiley, J. Cruze, R. Torregrossa, G. Velicebeti, G.P. Thill, High-level expression and efficient assembly of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*, Bio/Technologie 5 (1987) 479–485.