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技术与方法

毕赤酵母发酵生产颗粒性乙肝表面抗原的条件优化

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摘 要: 乙型肝炎病毒的流行对人们的生命健康造成了极大的威胁, 而有效准确的诊断和预防性疫苗是阻止其流行的主要手段, 乙肝表面抗原是诊断试剂和疫苗的主要成分。本试验在构建稳定表达 HBsAg 的毕赤酵母菌株后, 对其发酵条件进行了研究。采用摇瓶分批培养方法, 探讨了不同培养基、溶解氧、诱导物甲醇的浓度以及 pH 值等因素对菌体生长与重组蛋白表达的影响。在 10 L 发酵罐上采用分批补料培养的方法研究了进行扩大培养生产重组 HBsAg。结果表明, FBS 无机盐合成培养基是理想的工业发酵培养基, 溶解氧对菌体的生长与表达有显著的影响, 甲醇诱导最佳终浓度为 1% (*V*/*V*), 发酵的最适 pH 值为 5.4~6.0。发酵罐放大培养后, ELISA 和 SDS-PAGE 分析表明重组 HBsAg 获得了高效表达, 最终菌体生物量达到 310 OD_{600}, 表达量达到 27 mg/L。电子显微镜观察表达重组乙肝抗原可以自组装为 22 nm 类病毒颗粒, 为 HBV 的新一代早期血清学诊断和疫苗的大规模生产提供了一定的参考。

关键词: 发酵参数, 高表达, 优化筛选, 毕赤酵母, 重组乙肝表面抗原

Optimization of Fermentation Conditions for Maximal Recombinant Hepatitis B Surface Antigen Particle Production in *Pichia pastoris*

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Abstract: Hepatitis B virus (HBV) infection can cause the severe threat to the health of the people around the world. It depends upon the development of efficient diagnostic reagent and vaccine to prevent the prevalence of HB. In this study, we constructed the high expression recombinant *Pichia pastoris* and performed the screening tests in shake flasks to obtain the optimal values of several key fermentation parameters. Based on their effects on the growth and expression level of recombinant strains, FBS was the optimal industrial medium. The optimal values for the dissolve oxygen (DO), the final concentrations of methanol and the pH values were 50 mL, 1% (*V*/*V*) and 5.4–6.0, respectively. The optimal values of the parameters simulated in shake flasks were successfully scaled up to 10 L bioreactors to achieve high-throughput production: 310 OD_{600} in biomass and 27 mg/L in recombinant HBsAg. The expressed recombinant HBsAg in *P. Pastoris* was confirmed by SDS-PAGE and Western blotting. Electron microscopy examination

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showed that the purified protein could be self-assembled to 22 nm virus-like particles. The results provided a basis for industrial scale-up production of diagnostic reagent and vaccine of next generation against HB.

Keywords: fermentation parameters, high-throughput, optimization screening, *Pichia pastoris*, recombinant hepatitis B surface antigen (HBsAg)

Introduction

Human HBV can cause chronic infections that frequently lead to the development of cirrhosis and hepatocellular carcinoma [1,2]. Since there has no effective treatment for this infection, preventive vaccine is the widely used and economical way to control HBV infection and its sequel epidemiologically. However, vaccination may fail due to the variety in the age, obesity, renal disease and genetically determined resistance etc [3], therefore, it is a great demand to improve the immunogenicity of the antigen material used for vaccine. The conventional diagnostic antigen for immunoassay of HBsAb is obtained from the plasma of high titer HBV patients. With the popularization of HBV vaccination, it is difficult to find highly viremic individuals to obtain such sera [4]. Variety of cell systems have been used to express recombinant HBsAg, such as mammalian, insect and *S. cerevisiae* cells, which have been successfully used as HBV vaccines for immunization purposes [5], but the glycosylation of the recombinant HBsAg, occurring in 146 Asn within the sequence of 'a' common epitope, affects the immunoreactivity of the product, resulting in the hiding of the epitope, and the industrial production cost is very high. The recombinant HBsAg has also been expressed in *P. pastoris*, but the expression level was not high enough for detecting in SDS-PAGE [6–8]. Up to now, since the current commercial HBV recombinant HBsAg for immunoassay of HBsAb was not satisfactory, especially in their sensitivity [4], the recombinant HBsAg was rarely used for immunoassay instead of blood-origin HBsAg. To scale up production of excellent immunoreactivity and immunogenicity recombinant HBsAg in *P. pasotris*, we constructed the stable recombinant *P. pastoris* for high-level expression of HBsAg, and screened several key fermentation parameters in shake flasks, which greatly affected the growth and expression level of recombinant *P. pastoris*, involving media, DO, the final methanol concentration and pH values, and obtained the optimal parameters for production of HBsAg. The culture process simulated in shake flasks was successfully scaled up to the bioreactor to achieve high-throughput production of HBsAg. The expressed HBsAg could be self-assembled to 22 nm virus-like particles, similar to the natural virus particles observed in the sera of infected carriers of HBV. Our results in this study could provide a basis for industrial scale-up high-throughput production of the recombinant HBsAg in *P. pastoris*, and the HBsAg could be a promising material for the vaccine and diagnostic reagent of next generation.

1 Materials and methods

1.1 Materials

A plasmid containing HBsAg gene (pPIC3.5K-HBsAg, *adr*) was constructed by our laboratory and transformed into *P. pastoris* (Fig.1). Yeast extract, peptone, yeast nitrogen base without amino acid, glycerol and biotin for *P. pichia* cell culture were purchased from Oxoid LTD and Gibco Corporation.

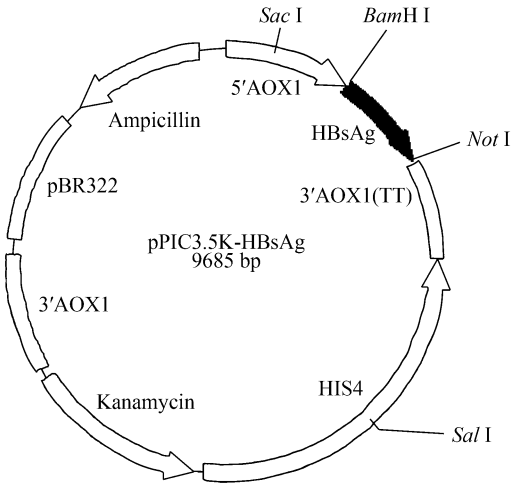

Fig. 1 Construction of the recombinant HBsAg expression vector

The HBsAg gene (680 bp) was inserted into *Bam*H I and *Not* I sites of pPIC3.5K to create the HBsAg expression vector, the HBsAg gene was placed under the control of the methanol-inducible *AOX1* promoter, the *HIS4* marker and Kanamycin selection of transformants on G418 and RDB plates

1.2 Methods

1.2.1 Optimization screening for the parameters affecting the growth and expression level of recombinant *P. pastoris* in shake flasks: Based on their effects on the growth and expression level of recombinant *P. pastoris* in shake flasks, the optimization screening for several parameters, including different media, dissolved oxygen, final

concentrations of methanol, and pH values were performed to obtain the optimal values respectively. At each time point (about 12–24 h) during induction, the samples of highly expressed recombinant strains judged by media were withdrawn to assay the biomass and expression level of the recombinant strains respectively.

1.2.2 Scale-up production of recombinant HBsAg in bioreactor: Fermentation experiments were performed in BIOSTAT-B 10 L bioreactor, which was connected with computer, and controlled by MFCS/win 2.0 (B. Braun Biotech) with an 8 L working volume and control modules for pH, temperature, and dissolved oxygen. The inoculums was grown for 24 h at 30°C in baffled shake flasks (250 r/min) in a complex medium containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glycerol. 500 mL of inoculums was added to 6.0 L of Basal Salts Medium (21.4 mL/L 85% H_3PO_4, 0.74 g/L $CaSO_4$, 14.6 g/L K_2SO_4, 11.9 g/L $MgSO_4{\cdot}7H_2O$, 3.3 g/L KOH, 32 g/L glycerol) and 3.5 mL/L of PTM_1 Trace Salts (6.0 g/L $CuSO_4{\cdot}5H_2O$, 0.08 g/L NaI, 3.0 g/L $MnSO_4{\cdot}H_2O$, 0.2 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.02 g/L H_3Bo_3, 0.5 g/L $CoCl_2$, 20.0 g/L $ZnCl_2$, 65.0 g/L $FeSO_4{\cdot}7H_2O$, 5.0 mL H_2SO_4, and 0.2 g/L biotin). Prior to inoculation, 0.1 g/L of Antifoam 289 (Sigma) was added and the pH was adjusted to 5.0 by adding 30% (*W*/*W*) ammonium hydroxide. The fermentation temperature was 30°C, and the pH was maintained at 5.5 by automatic feeding of 30% (*W*/*W*) ammonium hydroxide. The impeller speed was set from 400 to 800 r/min. Medical grade oxygen was fed to the bioreactor to maintain the dissolved oxygen concentration above 30% of air saturation. The bioreactor was operated in batch mode until all of the glycerol was consumed as indicated by a sharp rise in dissolved oxygen concentration (about 24 h). A glycerol feeding (50% (*V*/*V*)) was then maintained for 3.5 h at a growth-limiting rate of 15 mL/(h·L) of glycerol. After stopping feeding of glycerol for 1 h, a sharp rise in dissolve oxygen would appear, and Methanol feeding was as follows: feeding 3.0 mL/(h·L) of methanol for 1 h, 6.0 mL/(h·L) of methanol for 2.5 h, and 9.1 mL/(h·L) of admixture of methanol and 10 mL PTM_1 /L methanol till 59 h.

1.2.3 Lysis of cells and Quantitative ELISA for recombinant HBsAg expression: Induced yeast cells were harvested and washed with sterile water, and then collected and stored at −80°C. Frozen cells were thawed for 3 h at room temperature, and breaking buffer (25 mmol/L sodium phosphate, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L DTT) was added. The cells (6 mL breaking buffer/g wet cells) were resuspended and then stirred for 15 min, and kept at 4°C for 18 h. The cells were then disrupted by four passes through a sanitized APV Gaulin 30 CD homogenizer at chamber pressures of 12 000 to 14 000 psi, resulting in 95% of disrupted cells. The concentration of soluble HBsAg in yeast cell extracts was determined using a quantitative HBsAg kit (Beijing Wantai Pharmacy Enterprise). Briefly, the clarified extracts were diluted 1000-fold with 20 mmol/L phosphate buffer (pH 7.2). Aliquots ranging from 10 μL to 100 μL were assayed according to the protocol of the manufacturer. To make a reference curve, a series of standard dilution panel containing 0–4 ng of HBsAg working standard was included in each assay.

1.2.4 SDS-PAGE and Western blotting of recombinant HBsAg: 10 μL of clarified extracts from recombinant *P. pastoris* mixed with 10 μL of ddH_20 and 10 μL of SDS-PAGE loading buffer, and 10 μL of sample was applied to SDS-PAGE, using 12% acrylamide separating gel and 3% stacking gel containing 1% SDS. The gel sheets were then stained with 0.025% Coomassie brilliant blue R-250. After equilibration of gels and nitrocellulose membranes in transfer buffer (25 mmol/L Tris·HCl, 192 mmol/L glycine, 3.5 mmol/L SDS, 200 mL/L methanol), the protein was electroblotted onto a NC membrane (Amersham). After the membrane (50 g/L fat free milk/TN buffer (10 mmol/L Tris·HCl, 150 mmol/L NaCl, pH 8.0)) was blocked, it was probed with a mouse-anti-HBsAg-McAb (dilution 1:1000, 50 g/L fat free milk/TN buffer (dilution 1:100 in 5% fat free milk/TN buffer, 20°C–25°C, 1 h), followed by a sheep-anti-mouse coupled to alkaline phosphatase (dilution 1:10 000 in 50 g/L fat-free milk/TN buffer; 20°C–25°C, 1 h). Bound antibodies were detected using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro-blue tetrazolium (NBT) as substrates in 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 100 mmol/L Tris·HCl buffer, pH 9.5.

1.2.5 Purification of the recombinant HBsAg: After ammonium sulfate precipitation, the HBsAg was suspended with 0.1 mmol/L phosphate buffer solution containing 8.5% (*W*/*V*) ammonium sulfate and separated chromatographically through a Phenyl-5PW 0.75 cm × 7.5 cm column (TOSOH, Japan), then the column was washed with 0.1 mmol/L phosphate buffer solution with descendent gradient of ammonium sulfate from 8.5% to 0% (*W*/*V*). Finally, the column was washed by 0.1 mmol/L phosphate buffer solution containing 10% (*V*/*V*) ethanol. After hydrophobic chromatography, 1.0 mL of recombinant HBsAg was

layered on 15 mL of 22% CsCl in 20 mmol/L phosphate buffer (pH 7.2) and centrifuged in a Beckman T70i rotor at 60 000 r/min for 10 hours at 20°C. After spinning, the centrifuge tube was punctured at the bottom and 1.0 mL of fractions of the gradient were collected and analyzed for the presence of HBsAg particle under electron microscope.

1.2.6 Electron microscopy examination: Purified recombinant HBsAg was examined with electron microscope. Briefly, a drop of sample was placed on a 400-mesh copper grid with formvar-carbon film for 2 minutes, then excess sample was blotted off, and the grids were stained with 2% aqueous solution of uranyl acetate and examined in an F30 transmission electron microscope (Philips, Netherlands) operating at 200 kV.

2 Results

2.1 Optimal values for the parameters acquired by the optimization screening

By comparing their effects on the growth and expression level of recombinant *P. pastoris* in shake flasks, we screened for different values of the parameters involving cell and induction process, including media, dissolved oxygen, final concentrations of methanol, and pH values, and acquired the optimal values for the parameters respectively. The results were listed below.

2.1.1 Media: Fig. 2 showed the biomass of yeast and expression level of HBsAg from recombinant *P. pastoris* cultured in different media: FBS, BMMY, BMM and MM at different induction time points. The growth of yeast in different media was consistent with the expression level of HBsAg. Both of them reached the stationary phase after the induction for 60 h. For the growth of yeast and expression level of HBsAg, BMMY displayed best: the differences of 1.8-fold in biomass and 11.5-fold in expression level were observed between BMMY and MM media, but the differences between BMMY and FBS were so small, especially in expression level of HBsAg. Considering the lower price of FBS, we ranked FBS medium as the optimal medium for industrial scale-up production of HBsAg.

2.1.2 Dissolved oxygen (DO): Since dissolved oxygen (DO) was essential to the growth and expression of recombinant strains, it was a key parameter in batch fermentation. Different volume of the medium (50 mL, 100 mL, 200 mL and 300 mL) was loaded into 500 mL flasks, signifying different dissolve oxygen: the more the medium loaded, the less the DO was. As shown in Fig. 3, the DO negatively and obviously affected the growth of yeast and expression level of HBsAg. The biomass of strain and expression level of HBsAg in loaded 300 mL of medium were 50% and 76% lower than in loaded 50 mL of medium respectively. 50 mL of medium loaded into a 500 mL flask was set for the optimal in DO.

2.1.3 Final concentrations of methanol: Different volume of 100% methanol was added to the medium within one day to reach different concentrations of methanol (0.50%, 1.00%, 2.00%, 3.00% and 4.00%). Fig. 4 illustrated the screening for the optimal value. The biomass of yeast and expression level of HBsAg was 55 OD_{600}, 5.6 mg/L respectively at 1.00% final concentration of methanol. Both of them reached the maximum. While the final concentration of methanol was more than 1.0%, it inhibited the growth and expression of recombinant strains: the biomass of yeast and expression level of HBsAg decreased, suggesting that the higher concentrations of methanol was toxic to the strains and sharply depressed the expression of HBsAg. But too low concentration of methanol (0.5%) failed to meet the requirements for the growth and expression of recombinant strains.

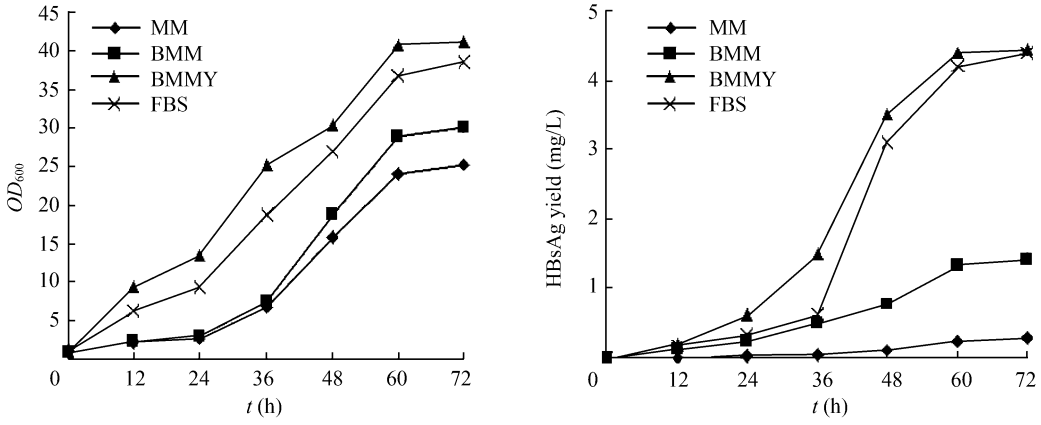

Fig. 2 Screening for different media

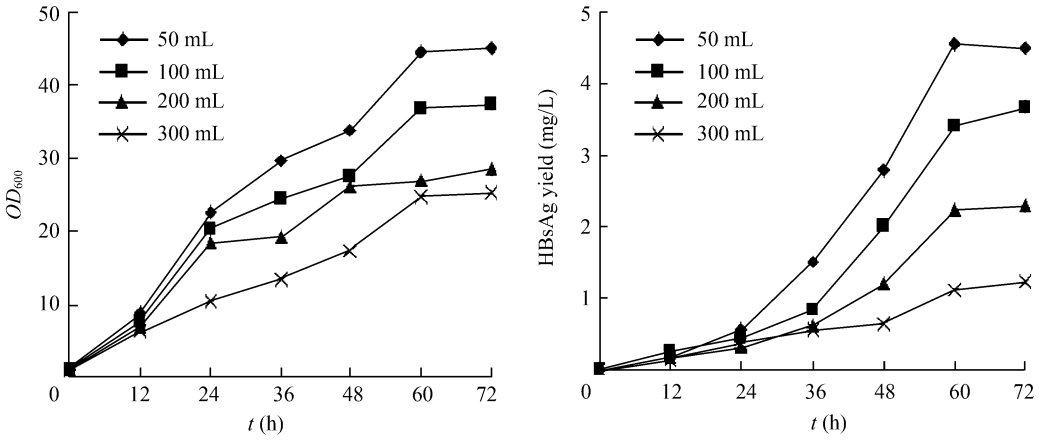

Fig. 3 Screening for different dissolve oxygen

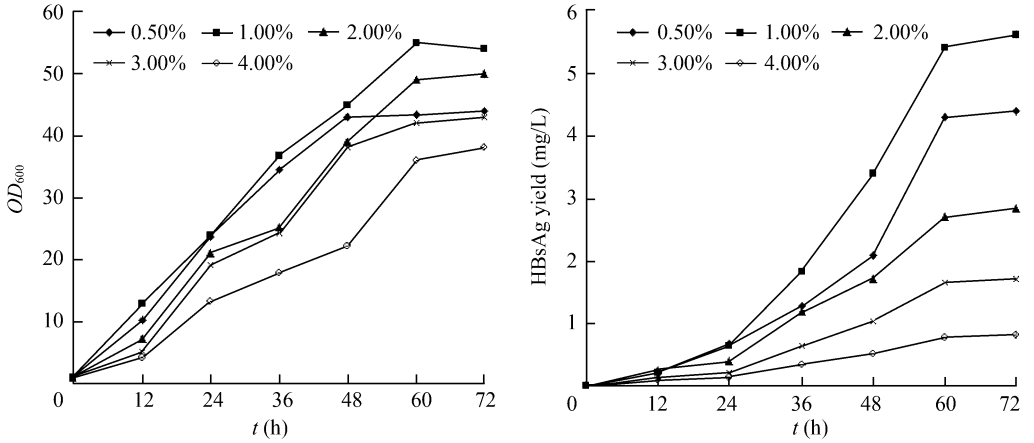

Fig. 4 Screening for different final concentration of methanol

2.1.4 pH value: The screening for different initiative pH values of medium (pH 4.8, pH 5.4, pH 6.0, pH 6.6 and pH 7.2) was illustrated in Fig. 5. It indicated that different pH values differed very slightly in the growth of the strains, but varied greatly in the expression of HBsAg. The optimal initiative pH values ranged from 5.4 to 6.0. Within this pH value of medium, the biomass of the strains and expression level of HBsAg could reach 58 OD_{600} and 5.8 mg/L respectively. The lowest expression level of HBsAg was found at pH value of 7.2, which decreased 84.5% compared to that at pH value of 5.4, suggesting that pH value of medium changed the status of the ions in the medium, thus directly affecting the absorption of the ions and the utilization of the strain.

2.2 Scale-up production of the recombinant HBsAg in bioreactor

The optimal values for the parameters simulated in shake flask were applied to scale-up production of recombinant HBsAg in a 10 L bioreactor. Fig. 6 illustrated the growth and expression of recombinant strains in the bioreactor. After 24 h batch culture, glycerol was limitedly fed to maintain the growth of recombinant strain for 3.5 h, and then the feeding was stopped. When glycerol was consumed as indicated by a sharp rise in DO, methanol was fed at low speed. At this point, the biomass of yeast reached 130 OD_{600}, and then the feeding rate of methanol was increased constantly. Due to the shift from glycerol to methanol, the strains should acclimatize to utilize the methanol, and the growth rate was slow, meanwhile the expression of recombinant HBsAg began. An hour late, the feeding rate of methanol was doubled. Along with the increase of the feeding of methanol, the DO fluctuated greatly, and especially the temperature also fluctuated greatly during 54 h to 66 h. The growth of yeast decreased obviously, while the expression level of HBsAg increased sharply. It indicated that the recombinant strains metabolized actively to mainly synthesize HBsAg. During the fermentation, DO spike was performed constantly to ensure that the concentration of methanol was maintained a relatively

appropriate level in order to meet that methanol was neither devoid of, nor too much to be toxic to the recombinant strains. After 84 h culture, the biomass of yeast reached the maximum, 310 OD_{600}, while the expression level of HBsAg came to the maximum, 27 mg/L at 78 h, therefore 270 mg HBsAg could be obtained from a fed-batch culture in 10 L bioreactor. After that point, the expression level of HBsAg decreased, indicating the optimal time point to harvest cells was 42–48 h after induction. The biomass of the strains and expression level of HBsAg in bioreactor were 5.3-fold and 4.7-fold more than those in shake flask respectively.

2.3 SDS-PAGE and Western blotting of recombinant HBsAg

The silver-stained SDS-PAGE and western blotting of the samples after fermentation were shown in Fig. 7. The sample withdrawn from the control strain (Fig. 7a, lane 2) had a differential band. An approximately ~24 kD band appeared only in the recombinant strain (Fig. 7b, lane 3), but was not in the control strain. Western blotting using anti-HBsAg McAb confirmed that this band was recombinant HBsAg. Although there have been some reports on the expression of recombinant HBsAg in *P. pastoris*, the expression level was not so high that only very weak band or no band of the proteins could be detected in SDS-PAGE, thus, most of these reports were merely descriptive[9,10].

2.4 Purification of the recombinant HBsAg and its electron microscopy examination

The supernatant proteins extracted from crude cell culture were further purified by ammonium sulfate precipitation, hydrophobic chromatography and CsCl density gradient ultracentrifugation (data not shown). The yield of recovery of purified HBsAg was 62.4%, 81.3% and 72.5% respectively, and the total yield of recovery was 36.8%, therefore 100 mg purified HBsAg could be obtained from a fed-batch culture in a 10 L bioreactor.

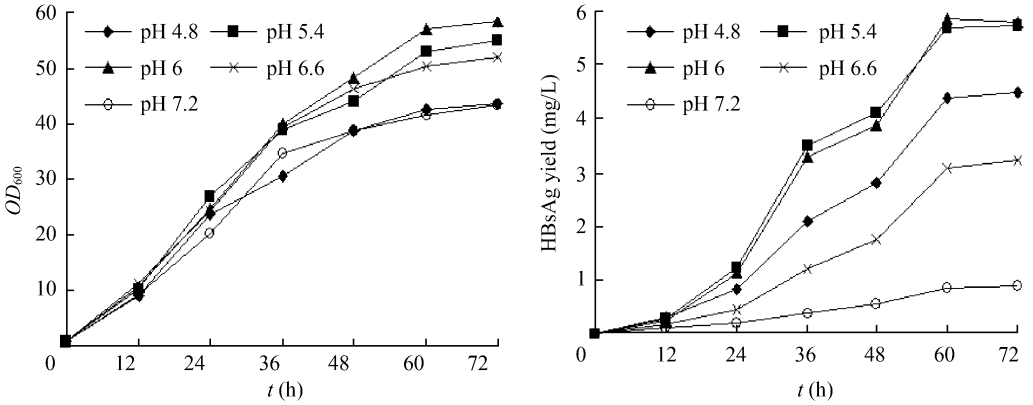

Fig. 5 Screening for different initiative pH values of medium

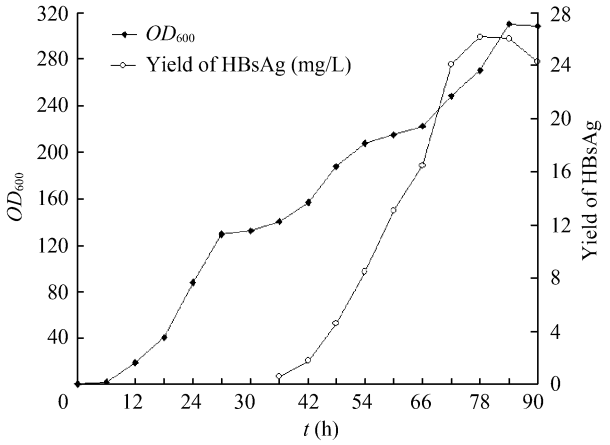

Fig. 6 Curves of the growth of the strains and expression of HBsAg in bioreactor

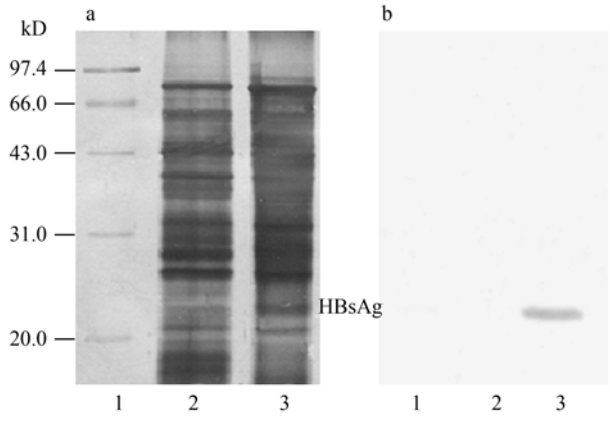

Fig. 7 Identification of the supernatant protein from culture cells SDS-PAGE (a) and Western blotting (b)

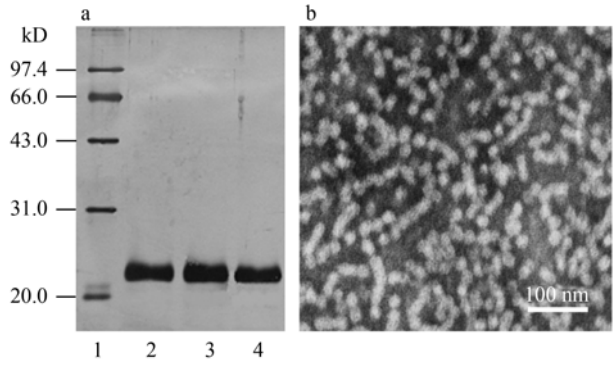

Fig. 8 Purity and electron micrograph of purifed recombinant HBsAg

(a) Purified recombinant HBsAg in silver-stain SDS-PAGE (1: protein marker; 2, 3, 4: purified HBsAg after CsCl density gradient ultracentrifugation). (b) The HBsAg virus-like particles were clearly visible under electron microscope

The purity of recombinant HBsAg was clearly verified by silver-stained SDS-PAGE (Fig. 8a): only the recombinant HBsAg band (about 24 kD) was detected in SDS-PAGE. The electron microscopy examination of the purified HBsAg revealed that large quantities of global structures with an average diameter of 22 nm particles were clearly visible (Fig. 8b). It indicated that recombinant HBsAg expressed in *P. pastoris* could be self-assembled to the virus-like particles, resembling to the natural virus particles observed in the sera of infected carriers of HBV.

3 Discussion

The yeast expression system offers many advantages, but most importantly the recombinant proteins expressed in this system could be synthesized to natural structure. The majority of the recombinant proteins produced in yeast were expressed via *S. cerevisiae* as a host system [11]. The *S. cerevisiae* based expression system used for the expression of HBsAg relied on the use of episomal vectors, which could autonomously replicate plasmid, possessed instability and reduced the overall yield of the desired product. In addition, plasmid maintenance required the use of expensive selective medium, and the yield of product is usually not high with the exception for some products [12,13]. *P. pastoris* offered an excellent eukaryotic expression system for inexpensive, large-scale production of functional recombinant protein because of the strong, tightly regulated promoter elements. *P. pastoris* expression system involving *alcohol oxidase* 1 gene regulatory elements has been characterized as the most efficient one among all varieties of yeast, and the gene of interest is integrated into the genome of the *P. pastoris*. Thus, the gene of the interest acquires better genetic stability than that of the *S. cerevisiae*. It was reported that *S. cerevisiae* derived HBsAg particle vaccine, bound to the cell surface of monocytes through interaction with the lipopolysaccharide binding protein and the lipopolysaccharide receptor (CD14), interfered with the lipopolysaccharide and interleukin-2-induced activation of monocytes, and reduced the capacity to bind monocytes [14]. Although there were some reports on the expression of recombinant HBsAg in mammalian cells, the expression level was not so high that almost no band of proteins could be detected in SDS-PAGE. For the goal of fully exploiting the potential of the *Pichia* system for the production of recombinant HBsAg, we constructed the high expression recombinant *P. pastoris*.

Expression of heterogeneous protein in *P. pastoris* related not only to the gene sequence itself, but also to the genetic characteristics of the recombinant strain and the culture environment [15]. Since the culture and induction process had remarkable effect on the expression of heterogeneous proteins, it was not easy to obtain the satisfactory yield of recombinant proteins. Therefore, optimization screening for the key parameters involving the culture and induction process for expression of heterogeneous proteins is necessary prior to the industrial scale-up production. We screened for different values of the parameters in simulated shake flasks respectively. FBS was optimal for the large scale up production of recombinant HBsAg because of the higher efficiency and lower price. FBS medium could maintain a stable pH value, and benefit the absorption and use of the nutrients. Proper initiative pH value significantly affected the expression level of recombinant *P. pastoris*. The optimal initiative pH values ranged from 5.4 to 6.0. Microorganisms utilized molecular oxygen mainly for respiration. Therefore oxygen availability was an important factor to influence the expression of proteins in both bacterial cells [16] and yeast [17]. Yeast grown on methanol might also require a substantial amount of oxygen for the initial oxidation of methanol to formaldehyde [18]. Methanol taken up by the cells was oxidized to formaldehyde in a coupled reaction involving *alcohol oxidase* (*AOX*) and catalase (*CAT*) in peroxisomes [13]. These reactions utilized molecular oxygen as an ultimate electron acceptor. Foreign protein synthesis needed abundant energy, and it was important to maintain a relatively high DO for expressing foreign protein in *P. pastoris*. For the expression vector under the control of *AOX1* promoter, it was necessary to keep the methanol level within a relatively narrow range, which could be achieved in

bioreactors by different methods [19]. In shake flasks, an apparatus for online monitoring methanol concentrations was introduced, but it was so expensive [20], and any ordinary apparatus for ordinary methanol feeding protocol has not been reported. We established the feeding protocol for our recombinant strains empirically, which worked without any measuring devices. Our data indicated that 1% final concentration of methanol was optimal. Too low or too high concentration of methanol was disadvantageous to the recombinant strains due to the limited carbon supply and toxicity to the strains.

The optimal values of the parameters were acquired in the simulated shake flasks, but whether they could fit the large scale-up production remained to be solved. Our data in this study indicated that the optimal values of the parameters could be successfully scaled-up in bioreactor. Since the pH value, DO, and concentration of methanol were accurately controlled in bioreactor, the yield of HBsAg was greatly increased. The expression level of HBsAg and biomass in bioreactor were 4.7-fold and 5.3-fold than that in shake flasks respectively. The supernatant proteins extracted from crude cell culture had a strong about 24 kD recombinant HBsAg band, and possessed the specific immunoreactivity. The recombinant HBsAg could be self-assembled to 22 nm virus-like particles, similar to the natural virus particles observed in the sera of infected carriers of HBV. The industrial scale-up production of HBsAg provided a promising material for next generation vaccine and diagnostic reagent of HB.

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