Production of Hepatitis B Core Antigen in a Stirred Tank Bioreactor: The Influence of Temperature and Agitation

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Abstract The influence of temperature and agitation on the growth of *Escherichia coli* expressing hepatitis B core antigen (HBcAg) in stirred tank bioreactor were investigated. The highest specific growth rate for *E. coli* (0.844 h^{-1}) was achieved at a temperature of 37°C and an agitation speed of 250 rpm. The activation energy for the growth of the *E. coli* strain W3110IQ in the stirred tank bioreactor was estimated to be 11 kcal/mol. The highest protein yield was achieved at a temperature of 44°C and an agitation speed of 250 rpm. The relative protein concentration at 44°C is 30 and 6% higher compared to that at 30 and 37°C, respectively.

Keywords: *E. coli*, HBcAg, temperature, agitation speed, bioreactor

Hepatitis B virus (HBV) is the leading cause of hepatitis and other hepatic diseases such as hepatocellular carcinoma and cirrhosis. Despite the availability of an effective vaccine, HBV infections continue to comprise a serious global health problem, with an estimated 400 million chronic carriers worldwide and more than a million deaths from these diseases each year [1]. HBV belongs to *Hepadnaviridae* family of viruses. It is a small enveloped virus with a partially double-stranded circular DNA of about 3.2 kb that replicates by means of reverse transcription [2]. The virion is a 42-nm particle, often called as Dane particle, with an outer layer that consists of host-derived lipid containing 3 related surface antigens (HBsAg). This protein shell encloses an icosahedral nucleocapsid composed of 180 or 240 subunits of core antigen (HBcAg) that contains viral DNA, as well as DNA polymerase and reverse transcriptase [3].

HBcAg is a 22-kDa protein that can be expressed in a variety of host systems, including *Escherichia coli* and yeast cells, where it produces empty and noninfectious core particles that are morphologically and immunologically similar to native capsids that have been isolated from infected hepatocytes [4]. This core particle is a strong immunogen that can function as a T-cell-dependent or T-cell-independent antigen [5,6]. Studies have shown that the core particles can also be used as carriers for the expression and presentation of a variety of heterologous viral epitopes [7], antigens from protozoan parasites [8], and epitopes from malarial parasites [9]. Hence, the hepatitis B core particles have a great potential for being developed into various diagnostic reagents and vaccines, as well as tools for use in gene therapy [10]. Because of its wide range of applications, it is not surprising that the demand for this particular protein will be tremendous in the near future, especially in the biopharmaceutical industry. Thus, there is an urgent need to find an efficient way to produce HBcAg in large amounts in order to meet the high demand of this protein.

Many procedures have been described for the production of HBcAg. A wide range of gene expression systems and methods has been studied to ensure the effective and satisfactory production of HBcAg [11]. However, *E. coli* remains the organism of choice for expressing a recombinant version of this protein, because it is easy to manipulate and its ability to carry multiple copies of plasmids containing the gene for this protein ensures satisfactory production of the protein [12]. Recently, we had reported an optimal production condition for the production of HBcAg in *E. coli* fermentation in shaked flasks [13]. However, no study has been reported about a large-scale production of HBcAg in *E. coli* fermentation in stirred tank bioreactor. In view of this, our goal was to optimize the fermentation process for *E. coli* strain W3110IQ containing the gene that encodes the full length HBcAg protein [14] in a 2-L stirred tank bioreactor.

The *E. coli* W3110IQ cells used in the present study had been transfected previously with a recombinant plasmid (pTacpcore) that contains the coding region of the full

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length HBcAg gene [14]. The *E. coli* stock culture (from glycerol stock at −80°C) was streaked onto a Luria Bertani (LB) agar plate containing 100 μg/mL ampicillin and incubated overnight at 37°C. One well-isolated *E. coli* colony was removed from the agar plate and inoculated into flasks with 5 mL of LB broth containing 100 μg/mL ampicillin. The flasks were incubated overnight at 37°C with vigorous shaking at 250 rpm in a shaker incubator. The culture volume was then increased to 100 mL and used as inoculum in bioreactor operation.

A 2-L bioreactor (Biostat, B. Braun, Germany) was used to grow *E. coli* in the present study. A 50-mL inoculum was inoculated into the bioreactor containing 1 L of LB broth, which had been supplemented with 100 μg/mL ampicillin. The pH, agitator speed, temperature, and airflow rate were set at 7,250 rpm, 37°C, and 0.5 vvm, respectively. When the biomass concentration has reached an optical density of 0.6 at 600 nm (approx 6 h after inoculation), protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG, Promega, USA) to a final concentration of 1 mM. Samples were collected from the bioreactor every hour throughout the fermentation process to determine the biomass and HBcAg concentrations. Fermentation was terminated after 24 h of cultivation, and the cells were harvested by centrifugation (Biofuge Swing-out Rotor, Heraeus, Germany) at 4,000 rpm for 30 min. The resulting pellets were stored at −80°C for further analysis. The effects of temperature (30, 37, and 44°C) and agitational speed (150, 250, 350, and 450 rpm) on the growth of *E. coli* and the HBcAg yield were evaluated. Each experiment was carried out in duplicate; the data show the mean of the results of the duplicate experiments.

The HBcAg concentration was determined using the enzyme-linked immunosorbent assay (ELISA) method. The *E. coli* cells obtained from the fermentation were disrupted enzymatically to release their intracellular HBcAg, according to a method described by Tan *et al.* [15]. The released HBcAg was sandwiched between anti-HBcAg monoclonal antibody (Sigma, UK) and alkaline phosphatase-conjugated anti-mouse antibody (Sigma). The HBcAg concentration was then determined at an absorbance wavelength of 405 nm using *p*-nitrophenyl phosphate (NPP) as a substrate. The relative concentration of HBcAg was calculated by dividing the concentration of HBcAg in each culture by its concentration in the control culture. The dry cell weight was determined after the cell suspension was filtered through a cellulose nitrate membrane filter with a 0.2-μm pore size (Sartorius, Germany) and then dried at 80°C for 1 day.

Fig. 1 shows a typical growth curve for *E. coli* W3110IQ cultivated in the stirred tank bioreactor at 37°C, pH 7, and an agitation speed of 250 rpm. The specific growth rate of *E. coli* varied with agitation speed (Fig. 2A), the highest (0.844 h^{-1}) was achieved at the culture that was agitated at 250 rpm. The specific growth rate in the culture that had been agitated at 150 rpm was approximately 10% lower than in the culture agitated at 250 rpm. Further increased agitation speed from 250 to 350 and 450 rpm has caused a reduction in specific growth rate of *E. coli*. The specific growth rate obtained at the culture agitated at 350 and 450 rpm was 23 and 31% lower than that at 250 rpm, respectively.

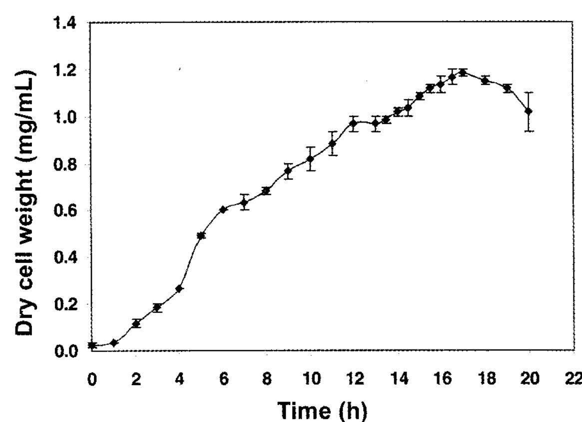

Fig. 1. A typical growth curve for *E. coli* W3110IQ being cultivated in a 2-L stirred tank bioreactor at a temperature of 37°C, a pH of 7, and an agitation rate of 250 rpm.

Agitation speed plays an important role in providing dissolved oxygen into the medium and also homogenizing the fermentation broth. Many bacteria will only grow in the presence of oxygen. However, some bacteria like *E. coli* are facultative; as such, they can grow with or without oxygen. Thus, *E. coli* can grow in the presence of low oxygen levels, albeit at a relatively low rate. When the agitation rate is too low, the *E. coli* growth rate is affected proportionately. High-speed agitation in a bioreactor gives rise to the formation of an undesirable amount of foam. To prevent this, an antifoaming agent is added. The antifoaming agent causes the coalescence of bubbles in the liquid phase of the culture medium, thereby reducing the rate of oxygen transfer [16] and seriously reducing the growth of *E. coli* organisms in that culture [12].

Fig. 2B shows that the highest specific growth rate (0.844 h^{-1}) was achieved at 37°C. The specific growth rate at 30 and 44°C was 33 and 44% lower, respectively than that at 37°C. The study was stopped at 44°C, because the upper limit of the growth temperature for *E. coli* is 45°C; temperatures higher than this may denature intracellular enzymes that are needed for bacterial growth [17]. Environmental temperature affects the growth of bacteria because of the sensitivity of enzyme-catalyzed reactions to temperature. The effect of temperature on the specific growth rate of bacteria can be described quantitatively by the Arrhenius equation [18]:

$$\mu = Ae^{-E/RT} \tag{7}$$

where E is the activation energy of the reaction (cal/mol), R is the universal gas constant (1.98 cal/mol·K), T is the absolute temperature (K), and A is Arrhenius constant (h^{-1}). The value of E for the growth of *E. coli* in a 2-L stirred tank bioreactor calculated using the Arrhenius equation was 11 kcal/mol. This value is very similar to the one we observed in our previous shaked flask fermen-

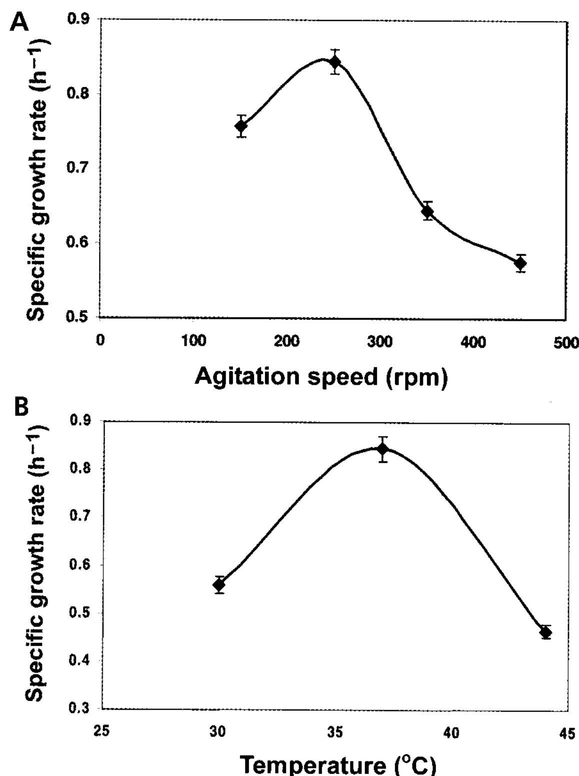

Fig. 2. The effect of (A) agitation speed and (B) temperature on the specific growth rate (μ) of *E. coli* W3110IQ.

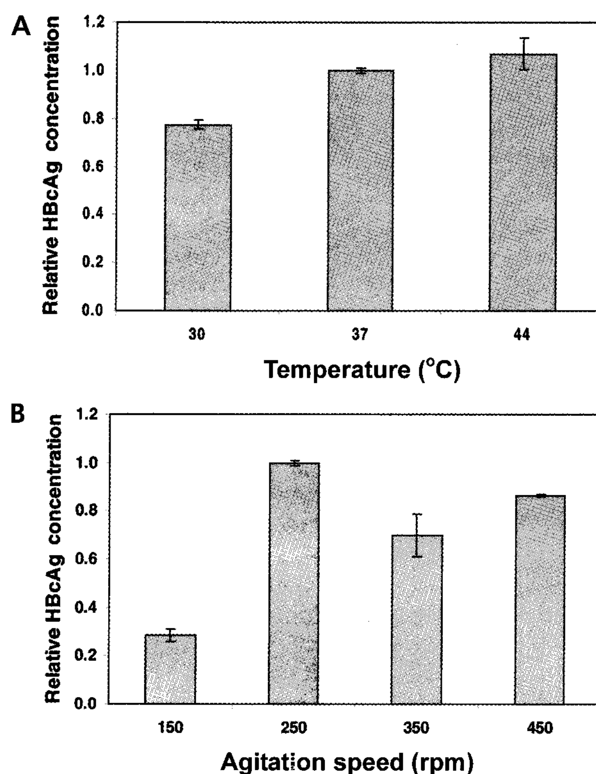

Fig. 3. The effect of (A) agitation speed and (B) temperature on the HBcAg yield of *E. coli* W3110IQ.

tation experiment (10 kcal/mol) [13].

The final HBcAg concentration produced by *E. coli* in the stirred tank bioreactor was quantified using ELISA. The relative HBcAg concentration for each fermentation operation is shown in Fig. 3. The specific growth rate of *E. coli* cultivated at 44°C is the lowest among all the temperatures. However, the relative HBcAg concentration was approximately 6% higher than at 37°C. The relative HBcAg concentration at 30°C was 30% lower than it was at 44°C. The observation that the HBcAg production rate increased with temperature in this study is very similar to the findings in our previous study of flask fermentation [13]. Farewell and Neidhardt [19] reported that the peptide chain elongation rate of *E. coli* continues to increase up to 44°C. Therefore, as long as a protein is stable at the higher temperature, an increase in protein yield should not be surprising. Indeed, Naito *et al.* [20] reported that HBcAg is stable at temperatures as high as 65°C.

Fig. 3B shows that the highest volumetric protein concentration was obtained in cultures that were agitated at a rate of 250 rpm. The lowest HBcAg yield, which was 70% lower than at 250 rpm, was obtained at 150 rpm. The HBcAg yield of cultures agitated at 350 rpm were approximately 30% lower than at 250 rpm, and those obtained at 450 rpm were approximately 14% lower. Coleman *et al.* [21] reported that the greater availability of dissolved oxygen provided by higher rates of agitation results in a higher protein production rate in *E. coli*. Any further increase in agitation speed may cause physical damage to the cells and reduce the protein production rate.

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