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Optimal Conditions for Hepatitis B Core Antigen Production in Shaked Flask Fermentation

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Abstract The effects of various environmental factors such as pH (5, 6, 7, 8 and 9), temperature (30, 37 and 40°C) and rotational speed (150, 200 and 250 rpm) on the growth and the hepatitis B core antigen (HBcAg) production of *Escherichia coli* W3110IQ were examined in the present study. The highest growth rate is achieved at pH 7, 37°C and at a rotational speed of 250 rpm which is 0.927 h^{-1}. The effect of pH on cell growth is more substantial compared to other parameters; it recorded a 123% different between the highest growth rate (0.927 h^{-1}) at pH 7 and lowest growth at pH 5. The highest protein yield is achieved at pH 9, rotational speed of 250 rpm and 40°C. The yield of protein at pH 7 is 154% higher compared to the lowest yield achieved at pH 5. There is about 28% different of the protein yield for the *E. coli* cultivated at 250 rpm compared to that at 150 rpm which has the lowest HBcAg yield. The yield of protein at 40°C is 38% higher compared to the lowest yield achieved at 30°C.

Keywords: *E. coli*, HBcAg, fermentation, pH, temperature, rotational speed

INTRODUCTION

Hepatitis B is a worldwide public health problem. The causative agent of this B-type hepatitis in humans is hepatitis B virus (HBV). The virus causes cirrhosis and liver cancer, and until today there is no effective treatment for this disease [1]. Unfortunately, with all the advances that have been made in medicine all these years, there is still no cure for individuals already infected with HBV. Thus, it is important to prevent hepatitis B by vaccination and diagnosis of HBV infection.

HBV belongs to *Hepadnaviridae* family and contains a nucleocapsid which consists of 180 or 240 subunits of core protein [2]. The virus contains an outer lipoprotein envelope and an inner nucleocapsid which is composed of a double stranded DNA, a reverse transcriptase, and a capsid coat protein. The HBcAg of HBV is a 22 kDa protein that can be expressed in *Escherichia coli (E. coli)* and other host systems where it assembles into capsids [3]. Recombinant hepatitis B capsids have similar morphological and immunological properties to the authentic antigen isolated from infected human hepatocytes [4].

Hepatitis B core (HBc) particle is actually part of a nucleocapsid of the virus. HBc particle is free of DNA and thus it is a non-infectious protein. The particles are widely used as an antigen for the detection of anti-core antigen (HBcAg) antibodies in infected patients. HBc particle has been widely used as a popular choice of virus like particle (VLP) carrier in transformation. HBc particle permits a knowledge-based design of diagnostic reagents, vaccines and gene therapy tools on the basis of the HBc particles [5].

For an effective gene expression and satisfactory production of recombinant protein, the right gene expression system has been chosen. *E. coli* system is the most common used expression system. Just like other bacteria system, *E. coli* system has the advantages like simple physiology, short generation time, consume simple and cheap medium and has been worked successfully with many commercially available expression vectors.

E. coli system was chosen for expressing the recombinant HBcAg because *E. coli* has rapid doubling time, the ability to grow in inexpensive media and produces satisfactory high amount of plasmid; thus more protein can be synthesized. Furthermore, *E. coli* is one of the best understood microorganisms that have been applied in the development of many molecular cloning techniques. *E. coli* reacts differently to different environmental conditions. Thus, environmental factors like pH, temperature and rotation speed can greatly affect the growth rate and protein yield.

Due to the wide application of HBcAg, thus it is an ur-

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gently need to produce this protein in a huge amount for the purpose of diagnosis and vaccine development. Therefore, the main objective of this study was to investigate the effects of various environmental factors on the growth rate and protein yield of *E. coli* W3110IQ hosting the gene encoding HBcAg.

MATERIALS AND METHODS

Cultivation of *E. coli* W3110IQ

E. coli W3110IQ cells harbouring the coding region of the full length HBcAg gene, pTacpcore (from glycerol stock -80°C) was streaked on the Luria Bertani (LB) agar plate containing 100 μg/mL ampicillin. The culture was incubated overnight at 37°C. The culture from the plate was subsequently transferred to LB medium containing 100 μg/mL ampicillin. The inoculated medium was incubated at 37°C with vigorous shaking at 250 rpm. After 5 hours, each bottle was added with 5 μL of Isopropyl-β-thiogalactoside (IPTG) and shaked overnight. The biomass was harvested by centrifugation at 4,000 rpm, 30 min (Biofuge Swing-out rotor, Heraeus, Hanau, Germany). Culture was sampled regularly in an hour period to measure its cell concentration. The effects of various environmental factors such as pH (5~9), temperature (30, 37, 40°C) and rotational speed (150, 200, 250 rpm) on the growth and the HBcAg yield are examined. All the experiments performed in duplicate and the data show the mean of the duplicates.

Specific Growth Rate

During exponential growth phase, the cell concentration doubles at regular intervals. The cell concentration in the culture after time t is given by:

$$X_t = X_0.2^n = X_0.2^{t/t_d} \quad (1)$$

Where t is the time elapsed (h) ,
t_d is the doubling time of the culture (h),
X_0 is the initial cell concentration (mg dcw/L),
X_t is the cell concentration at certain t time (mg dcw/L).

Taking natural logarithms of equation (1):

$$\ln(X_t / X_0) = (t / t_d).\ln 2 \quad (2)$$

Rearranging equation (2) gives:

$$\text{Specific growth rate, } \mu = \frac{\ln 2}{t_d} = \frac{\ln X_t - \ln X_0}{t} \quad (3)$$

Purification and Quantification of HBcAg Particles

The HBcAg particles were purified with sucrose gradient centrifugation as previously described [6]. Purified proteins were analyzed on SDS-PAGE and Western blotting. The relative HBcAg concentrations for various samples were obtained by comparing the intensity of their bands from Western blots. The bands were quantified by using the Sophisticated Volume Tools from the Bio-Rad imaging devices supported by Quantity One [Bio-Rad, Hercules, California, USA]. A volume is the intensity data inside a defined boundary drawn on the images. The intensity data inside the boundary with the data of other objects can be compared using the Volume Analysis Report. The assay was performed in duplicate.

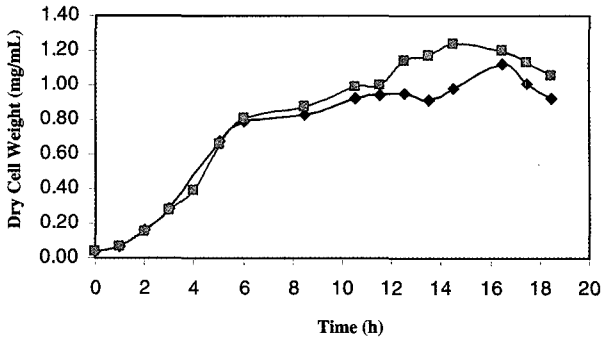

Fig. 1. Growth curve of *E. coli* W3110IQ batch culture. [With IPTG (Diamond), without IPTG (Square)]

RESULTS AND DISCUSSION

The Effect of IPTG on the Growth of *E. coli* W3110IQ

Fig. 1 shows the effect of IPTG on the growth of *E. coli* W3110IQ in batch culture. IPTG is the inducer for the *tac* promoter upstream of the HBcAg gene in the pTac plasmid. Additional of IPTG into the culture medium will switch on transcription of HBcAg gene. IPTG was added to the culture 4 h after the batch culture started. The addition of IPTG to the culture was followed by a reduction in cell biomass compared to the culture without IPTG. The maximum cell concentration achieved in the culture supplemented with IPTG is 11% lower than that without IPTG. The specific growth rate, μ after the addition of IPTG is 0.035 h^{-1} which is 22% lower than the culture without any IPTG supplement. Therefore, the production of recombinant protein by *E. coli* is an additional burden to the cell metabolism and can retard the growth of *E. coli*. Indeed, Brown [7] has suggested that the rapid expression of a cloned gene can result in the energy and molecular drain of the recombinant bacterium, which may affect its growth rate and cell stability.

The Specific Growth Rate of *E. coli* W3110IQ

Temperature, pH and the oxygenation level inside the fermenter are important physical factors that affect the growth and production of microorganisms. Therefore, it is very important to optimize these parameters before the production of HBcAg particles from *E. coli* fermentation can be scaled up to the production scale. Fig. 2(a) shows the specific growth rate of *E. coli* W3110IQ cultivated at

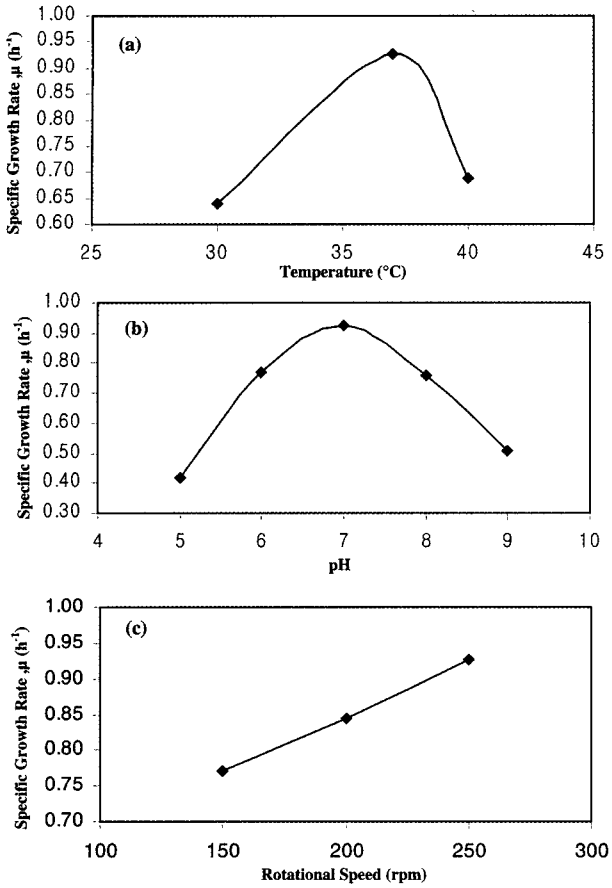

Fig. 2. The effect of environmental parameters on the specific growth rate, μ of *E. coli* W3110IQ. (a) Temperature, (b) pH, and (c) rotational speed of shaking incubator.

different temperatures. The highest specific growth rate is achieved by the culture at 37°C with the value of 0.93 h^{-1}, which is 45% and 30% higher than that at 30°C and 40°C respectively. Temperature affects chemical reaction rates through its actions on cellular enzymes. Generally, the optimum temperature for enzymatic activities in most organisms is in the range of 20 to 40°C. Low temperature inhibits enzyme activity and slows down cell metabolism and consequently cell growth. Temperature which is too high would cause coagulation and irreversible denaturation of cellular enzymes.

The relationship between the specific growth rate, μ, of bacteria and absolute temperature, T can be described by Arrhenius equation [8] as shown below:

$$\mu = Ae^{-\frac{E}{RT}}$$

where E is the activation energy of the reaction,
R is the universal gas constant (1.98 cal mol^{-1} K^{-1}),
A is Arrhenius constant.

Activation energy, E, for the growth of *E. coli* can be obtained from the above equation. The value of E estimated from the current study is 10 kcal/mol. The typical activation energy for growth of bacteria is in the range

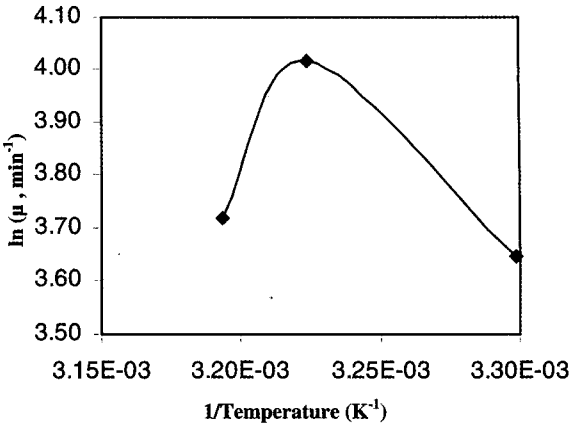

Fig. 3. An Arrhenius plot of the effect of temperature on the specific growth rate, μ of *E. coli* W3110IQ.

of 10 to 20 kcal/mol [8]. The Arrhenius plot (Fig. 3) is frequently used to describe the relationship between μ and temperature. Plotted in this manner, the form of the growth responses of almost all bacteria is similar. The minimum temperature for sustained growth of *E. coli* lies between 7.5 to 7.8°C and the maximum temperature at which growth can be sustained is approximately 49°C [9]

In nature, bacteria can be found at a wide range of pH; however the optimum pH for bacteria growth is between pH 5.0 to 9.0. *E. coli* is a representative of the large neutrophilic class. This narrow range of pH may cause the metabolic problem to *E. coli* because pH is important parameters governing a number of reactions, including ionization and oxidization. Fig. 2(b) shows a comparison of specific growth rate, μ, of *E. coli* W3110IQ cultivated on various pH. It is obvious that the highest specific growth rate was achieved in the culture of pH 7, which is 0.93 h^{-1}. Cultures of pH 6, 8, 9 and 5 have a decreasing trend of μ value. Specific growth rate is lowest for the culture of pH 5, which is 123% lower than that of the culture of pH 7. Our result further confirms the observation of Duffy *et al.* [10] that initial pH of less than 5.5 strongly inhibited the growth of *E. coli* O157:H7. The pH of the extracellular environment has a great influence on enzymatic activities of the cells. The optimum pH for cell metabolism is in neutral range. Duffy *et al.* [10] reported that pH 5.8 is apparently not suitable for the growth of *E. coli*. An increase in the hydrogen ion concentration resulting in a pH drop to a value lower than 7 and a decrease in the hydrogen ion concentration resulting in a pH higher than 7. Both critical changes in pH are often harmful to the cells. This would slowdown the rate of chemical reactions, possibly due to the destruction of cellular enzymes, thereby affecting the rate of growth and ultimately the survival of microorganisms.

The growth of aerobic bacteria in submerged culture is controlled by the availability of nutrients and oxygen. Culture medium is heterogeneous in nature, so the rate of substrate or product transfers at a particular interface always becomes a limiting factor for a particular bioreaction. Materials might transfer between solids and liquid,

gas and liquid, or to the cells. Rotational speeds of a shaker incubator play an important role in providing a good mass transfer to ensure the homogeneity of the culture medium. Also, the cells may be aggregated into flocs, which may limit the mass transfer of nutrients and oxygen into the cells. These flocs have to be disaggregated by the shear stress as a result of vigorous agitation. The growth of many bacteria depends on the presence of oxygen but some bacteria are facultative. Facultative bacteria can grow either in the presence or absence of oxygen. This group includes many *Staphylococci, Streptococci,* and *E. coli.* These microorganisms require a low concentration of oxygen for growth.

Fig. 2(c) clearly shows that the optimum rotational speed is 250 rpm which has the highest specific growth rate of 0.93 h^{-1}. Slowest specific growth rate, 0.77 h^{-1} is achieved at the culture rotated at 150 rpm that is 20% lower than that at 250 rpm. The presence of oxygen is necessary for the process of respiration. Oxygen plays a vital role in ATP formation and the availability of energy in an utilizable form for cell activities. Rotational speed influences the supply of oxygen in the medium. High rotational speed would enhance the mass transfer rate of oxygen. When the dissolved oxygen has decreased in the media, the facultative *E. coli* would grow anaerobically, at a rate of 30% lower than the aerobic growth. The present study clearly suggests that oxygen is indeed very essential in offer an effective growth of *E. coli.* The maximum specific growth rate is achieved at the highest rotational speed used in the present study; therefore further experiment needs to be carried out to determine the optimal rotational speed for cell growth.

The Yield of HBcAg

Fig. 4(a) presents the relative protein concentration of samples cultured at various temperatures. Obviously, the highest protein concentration was obtained from the culture at 40°C. Relative protein concentrations of the culture at 37 and 30°C are 33 and 38% lower than the culture at 40°C, respectively. The result from the present study shows that a higher temperature is favoured for the production of HBcAg protein. This is a very interesting result since the specific growth rate of *E. coli* W3110IQ at 40°C is 30% lower than that of 37°C. This suggests that at higher temperature, the cell machinery for protein synthesis is more effective compared to that of cell growth. Indeed, Farewell and Neidhardt [11] showed that the peptide chain elongation rate continues to increase up to 44°C at the same rate between 25°C and 37°C. Therefore if the particular protein is stable at higher temperature as in the case of HBcAg, increased protein yield at temperature higher than 37°C is not surprising. At low temperature, the cellular enzyme activity is low; consequently cell growth and the protein production rate is also affected. Duffy *et al.* [10] showed that the *E. coli* O157:H7 culture at low temperature (15°C) had undergone a longer lag phase than that at 37°C. A longer lag phase means that the microorganisms need to take a longer time to adapt to the new environment and thus results in a lower growth rate and often a lower protein production rate.

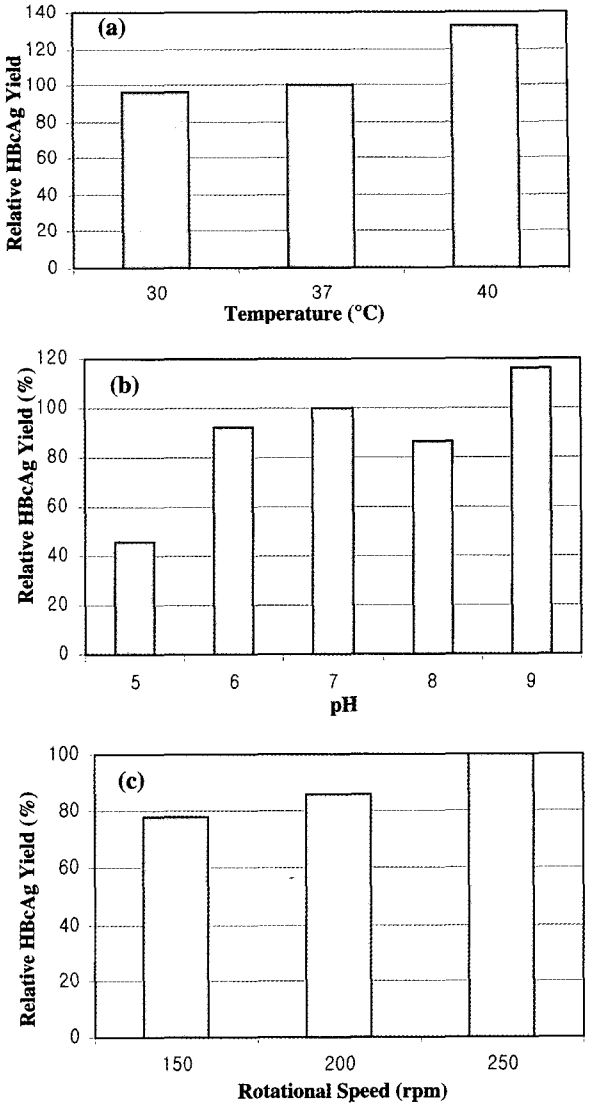

Fig. 4. The effect of environmental parameter on the HBcAg yield of *E. coli* W3110IQ. (a) Temperature, (b) pH, and (c) rotational speed of shaking incubator.

Fig. 4(b) shows that sample at pH 9 gives the highest protein concentration, followed by pH 7, 6, 8 and the lowest protein concentration is that from sample at pH 5. Relative protein concentrations of the samples at pH 7, 6 and 8 are 16, 26, and 34% lower than that of pH 9 respectively. The lowest relative protein concentration is obtained by the sample of pH 5, which is 154% lower than that of pH 9. Generally, the results of the present study show that higher pH favoured the HBcAg production. Indeed, Duffy *et al.* [12] reported that *E. coli* grown at pH 5.6 had lower verotoxin production than cells grown at pH 7.4. It can be deduced that pH 9 is the optimum pH for the maximum HBcAg production by *E. coli* W3110IQ although its specific growth rate is 83%

lower than that of pH 7. A growth condition with pH 5 is certainly to be avoided as it is not favourable for the growth and production of *E. coli* W3110IQ.

From Fig. 4(c), the highest protein concentration is obtained from the sample at 250 rpm. This is followed by the sample of 200 rpm, which is 16% lower than that of 250 rpm. The protein concentration of the 150 rpm is the lowest, which is 28% lower than that of 250 rpm. *E. coli* W3110IQ, like any other *E. coli* is a facultative microorganism. It needs oxygen to grow, reproduce, survive and produce metabolism products. In the absence of oxygen, *E. coli* can switch to anaerobic process, whereby it still manages to survive and produce but at a lower rate. Thus, an effective mass transfer of oxygen in the culture medium would provide an optimum protein production rate for *E. coli* due to the greater availability of dissolved oxygen, which has been proven by Coleman *et al.* [13]. Other than oxygen transfer, vigorous agitation may provide other benefits for the cell growth and protein production. The cells under vigorous shaken conditions are less likely to form clustered in microcolonies than under unshaken or slower shaken conditions. Smaller cell cluster has bigger interfacial areas which enhance the mass transfer rate of nutrient and oxygen form fermentation broth into the cells [13].

Optimum Growth and HBcAg Production Conditions for *E. coli* W3110IQ

From this study, it can be deduced that the optimum growing parameters for *E. coli* W3110IQ is pH 7, 37°C and 250 rpm. However, a higher protein production rate is achieved in the culture at 40°C and pH 9. This means a higher growth rate does not certainly promise a higher protein production rate. Since the growth condition of pH 9 and 40°C show the highest HBcAg protein production, further study is needed for the pH higher than 9 and temperature higher than 40°C. It is highly possible that it might give an even higher HBcAg yield. The same situations occur in studying the effects of rotation speed to the growth and product yield by *E. coli* W3110IQ. The results show that among the three rotational speed (150, 200 and 250 rpm) used in this study; sample with 250 rpm presents the highest growth rate and also the maximum HBcAg yield. Thus, it is possible that a rotation speed higher than 250 rpm can give us a much higher product yield.

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