

Full Length Research Paper

Production of fusion m13 phage bearing the di-sulphide constrained peptide sequence (C-WSFFSNI-C) that interacts with hepatitis B core antigen

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Effects of pH, temperature, and level of mixing on the production of fusion M13 phage bearing the peptide sequence (C-WSFFSNI-C) that interacts with HBcAg were investigated in this study. The optimum pH for the phage production was achieved at pH 7, followed by pH 6 and 8. The highest fusion phage titre was obtained at growth temperature of 37°C, followed by 27 and 42°C. The rotational speed at 250 rpm was the optimal mixing level for the phage production. Further increase of rotational speed to 300 rpm has reduced the phage production to a level lower than that obtained at 200 rpm. The results also showed that the propagation of fusion M13 phage has greatly affected the growth of *Escherichia coli* ER 2738. The viability of the phage produced with the current method was then determined using phage titre and dot-blot assays.

Key words: *Escherichia coli*, filamentous bacteriophage, pH, temperature, mixing.

INTRODUCTION

M13 phage is one of the most studied filamentous phages. This filamentous phage is a flexible long thin rod of almost 0.9 µm length and about 7 nm in diameter. The viral particle is composed of a single-stranded circular DNA (ssDNA) molecule that is encapsulated in a long cylindrical protein coat. The major coat proteins (gpVIII) along the long side are all identical and present in many copies (about 2700 subunits). On either end of the filamentous rod is capped with two different minor coat proteins; gpVII and IX on one side and VI and III on the other side (Stopar et al., 2003).

M13 phage and the closely related filamentous phages

fd and f1, are non-lytic phage that do not kill or prevent the host cells propagation during phage production. M13 phage has several unique and significant characteristics that made it a preferable tool over other phages for many biological applications and studies. One of the applications of M13 phage is phage display. It is achieved by fusing polypeptide libraries to phage coat proteins (gpIII and gpVIII) without disrupting the phage particle assembly (Cesareni, 1992). The resulted fusion phage particles will display the polypeptides on their surfaces and they also contain the encoding DNA. Library members with particular functions can be isolated with simple selections and polypeptide sequences that can be decoded from the encapsulated DNA (Sidhu, 2001). Apart from that, filamentous M13 phage can also be applied to select specific ligands that interact with virus or virus core particle (Ho et al., 2003; Tan et al., 2002) and

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in DNA sequencing (Clark and Russell, 1997).

The most important facet of any study on phage ecology is undoubtedly the influence of environment factors on phage productivity. Environmental factors not only affect phage multiplication but also phage infection mechanism (Burns and Slater, 1982). Viruses including bacteriophages are obligate parasites that necessitate a living host for propagation and it is impossible to discuss their ecology without considering their hosts as well. Temperature, pH, and mass transfer rate (level of mixing) are important environmental parameters that will not only affect the phage infection but also the growth of host cells that are vital for phage multiplication. Therefore, the objective of this study is to optimize the culture condition for optimal production of this fusion M13 phage by manipulating several growth parameters such as pH, temperature, and level of mixing.

MATERIALS AND METHODS

Growth of *Escherichia coli* ER2738 and production of fusion M13 phage

E. coli ER 2738 from glycerol stock was streaked onto a Luria Bertani (LB) agar plate supplemented with tetracycline and incubated for 16 h at 37°C. A well-isolated single colony of *E. coli* cells was inoculated into a 250 ml conical flask containing 20 ml LB broth and incubated at 37°C for 6 – 8 h with vigorous shaking at 250 rpm. A blue plaque (fusion bacteriophage) obtained from plaque assay was inoculated into 5 ml LB medium containing 125 µl log phase *E. coli* cells. The culture was incubated at 37°C for 4 h with vigorous shaking at 250 rpm. The culture was then centrifuged at 12, 100 x g (JA20 rotor, Beckman, USA) for 10 min at 4°C. One millilitre of the supernatant obtained was transferred into a universal bottle containing 0.5 ml *E. coli* cells that have been grown to log phase (OD₆₀₀ = 0.7). The culture was mixed well by gentle shaking at 25°C for 5 min. The mixture was subsequently transferred into a conical flask containing 100 ml LB medium containing 0.5 ml log-phase *E. coli* cells and incubated at 37°C for 4 h with vigorous shaking at 250 rpm. Sampling of culture was carried out regularly in 30 min interval for up to 16 h from cultures containing non-infected and M13 phage-infected host cells to determine the biomass concentrations.

In order to determine the effect of pH (6, 7, 8), temperature (27, 37, 42°C), and rotational speed (200, 250, 300 rpm) on the growth of M13 phage, culture that have been grown for up to 4 h was harvested by centrifugation at 7, 750 x g (JA20 rotor, Beckman, USA) for 15 min at 4°C. The supernatant was separated and precipitated with solution containing PEG/NaCl [20% (w/v) PEG 8000/2.5 M NaCl] overnight at 4°C. The precipitated phage was centrifuged at 7, 750 x g (JA20 rotor, Beckman, USA) for 30 min at 4°C and the supernatant was removed quickly by aspiration. The precipitate obtained was resuspended in Tris buffered saline (TBS) containing 100 mM Tris-HCl, pH 7.5, and 0.9% (w/v) NaCl. Contaminants present in the phage solution were then removed using 0.45 µm and 0.22 µm filters. The amount of phage obtained for each parameter investigated in the current study was determined using phage titration assay.

Specific growth rate

The biomass concentration of *E. coli* culture doubles at regular intervals during exponential growth. Therefore, the biomass con-

centration in the culture at time, t :

$$X_t = X_0 \cdot 2^n = X_0 \cdot 2^{t/t_d}$$

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 the above equation,

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

$$\text{The specific growth rate, } \mu = \frac{\ln 2}{t_d} = \frac{\ln X_t - \ln X_0}{t}$$

Phage titration assay

Ten microlitres of fusion M13 phage bearing the peptide sequence (C-WSFFSNI-C) was diluted to a factor of 10⁻⁹, 10⁻¹⁰ and 10⁻¹¹, respectively, and transferred into three different microcentrifuge tubes containing 200 µl log phase *E. coli* (ER 2738) cells (OD₆₀₀ = 0.7). The mixture was mixed well before adding it into 3 ml top layer agar and poured onto LB agar plate supplemented with IPTG and X-gal. The plates were incubated for 20 min at 25°C until the top agar solidified before further incubation at 37°C for 16 – 18 h. The amount of phage (blue plaque) obtained was calculated as plaque forming unit (pfu).

Dot-blot assay

All the steps performed in this assay were carried out at 25°C. An aliquot of M13 phage was blotted on a nitrocellulose membrane, air-dried and blocked with 10% (v/v) milk diluent (KPL, USA) for 2 h. The membrane was then washed 3 times with TBS supplemented with 0.01% (v/v) Tween 20 (TBST) for 10 min each. The membrane was then incubated with mouse anti-M13 monoclonal antibody [10 µg/ml, GE Healthcare] for 2 h with gentle agitation before washing it again with TBST. After washing 3 times with TBST, secondary antibody, anti-mouse alkaline phosphatase conjugated (1:2500, Chemicon) was added and incubated for another hour. The membrane was washed again with TBST before adding in alkaline phosphatase buffer containing 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) substrates and incubated for 5 to 10 min or until the dot was visible. The reaction was then stopped by washing the membrane with water.

RESULTS AND DISCUSSION

Production of fusion M13 phage retarded the growth of *E. coli* cells

Figure 1 illustrates the effect of fusion M13 phage production on the growth of *E. coli* ER2738. The phage was co-inoculated with *E. coli* at the beginning of the batch culture. The result shows that the presence of fusion phage in the culture has greatly affected the growth of *E. coli*. The specific growth rate of the phage-infected culture was 24% lower than the control culture (containing only the *E. coli* ER2738 cells) in the first 2 h of the culture (lag phase). The growth rate of the phage

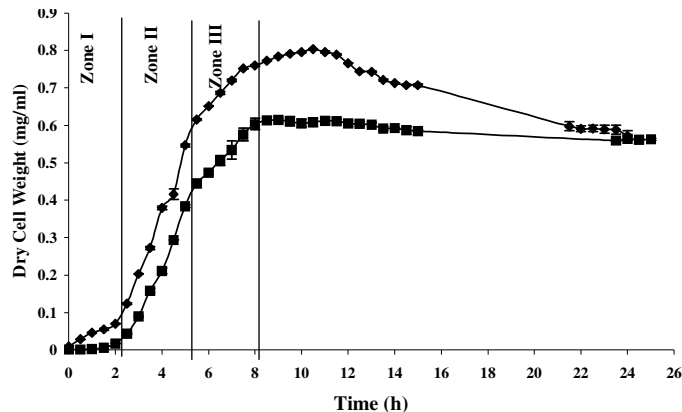


Figure 1. The effect of fusion bacteriophage propagation on the growth of *Escherichia coli* ER2738. (Keys: square; *E. coli* culture infected by fusion bacteriophage, diamond; control *E. coli* culture). The data represent the mean \pm S.D. of triplicate determinations.

infected culture was 8% lower than that of the control culture at the exponential (2-5 h) and deceleration (5-8 h) phases. This slower specific growth rate observed in the phage-infected culture has resulted in a lower biomass concentration compared with the non-infected culture. Therefore, the maximum biomass concentration obtained from the phage-infected culture was 31% lower than that of the control. This could be due to utilization of part of the host cells' biosynthesis machinery in the propagation of the fusion phage that resulted in reduction of biomass production. This observation corresponded with previous findings whereby host cells infected by M13 phage continue to grow and divide but at a decreased rate compared with uninfected cells (Marvin and Hohn, 1969; Smith and Petrenko, 1997).

Effect of pH on the production of fusion bacteriophage

Figure 2a shows the effect of culture pH on the production of fusion M13 phage. The highest specific phage titre (2.99×10^{14} pfu/mg dcw) was achieved at pH 7. The specific phage titre at pH 6 and 8 were 3.55×10^{11} and 2.24×10^{11} pfu/mg dcw, respectively. The propagation of M13 phage is initiated with attachment onto the F-pilus of the *E. coli* cells via the minor coat protein, gp3 at one end of the phage particle (Holliger and Riechmann, 1997). It was reported that the binding of bacteriophage onto the membrane surface of *E. coli* cells has shown to involve electrostatic interaction (Gallusser and Kuhn, 1990). Therefore, the pH of the culture may affect the binding of phage onto the surface of *E. coli*, hence the overall phage propagation rate. In addition, the pH of the extra-cellular environment has a great influence on the enzymatic activities of the host cells, which could affect both phage propagation and growth rate of the host cells. However, in the present study, the growth rate of *E. coli*

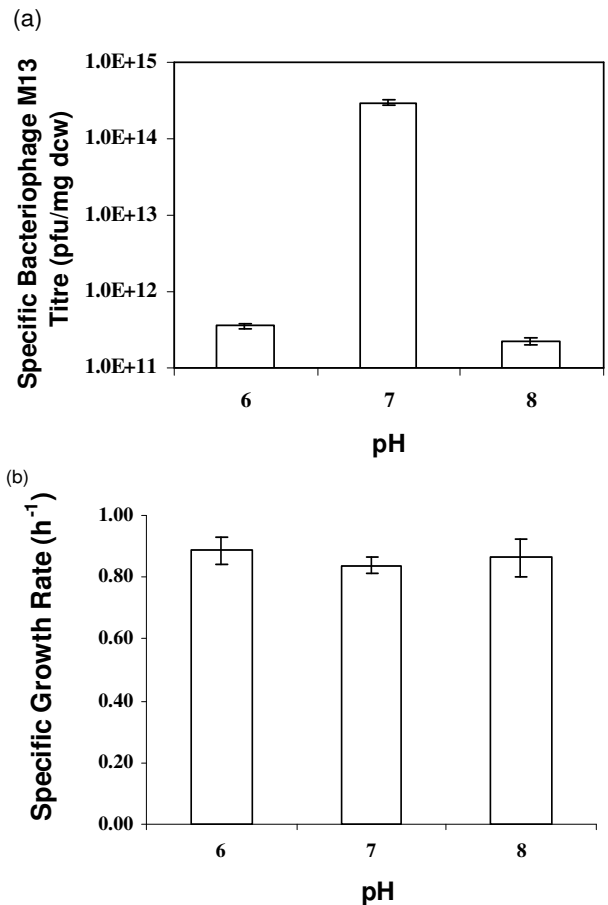


Figure 2. The effect of pH on the (a) fusion bacteriophage production, and (b) specific growth rate of *Escherichia coli* ER2738. The data represent the mean \pm S.D. of triplicate determinations.

cells did not vary much with change of pH as shown in Figure 2b. The highest specific growth rate, μ , (0.888 h^{-1}) achieved at pH 6, which was 6 and 3% higher than that obtained at pH 7 and 8, respectively.

This result was different compared with other *E. coli* strains used in the production of recombinant intracellular product, in which a higher specific growth rate was achieved at pH 7 (Tey et al., 2004). The possible underlying cause for this variation observed in the current study could be due to the greater effect of infection and propagation of M13 phage as shown in Figure 1 than the influence of the culture pH on the growth of *E. coli* host cells.

Effect of temperature on the production fusion bacteriophage

Figure 3a shows the comparison of fusion M13 phage production under different cultivation temperatures. The highest phage titre was achieved at 37°C (2.99×10^{14} pfu/mg dcw), followed by temperatures at 27°C (1.75×10^{12}

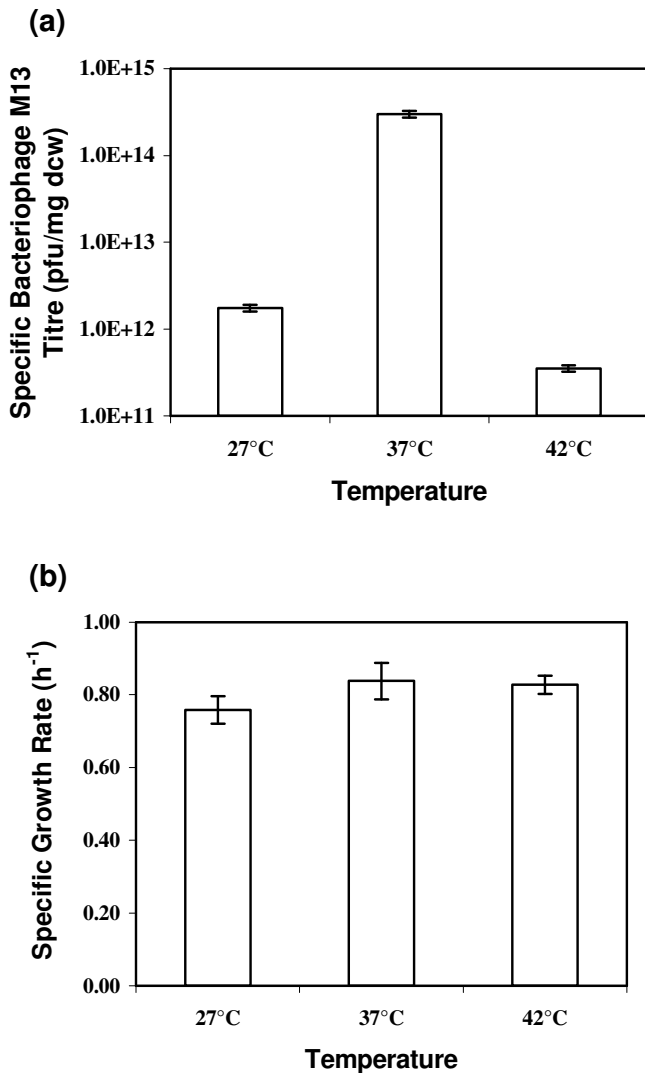


Figure 3. The effect of temperature on the (a) fusion bacteriophage production, and (b) specific growth rate of *Escherichia coli* ER2738. The data represent the mean \pm S.D. of triplicate determinations.

pfu/mg dcw) and 42°C (3.53×10^{11} pfu/mg dcw). Temperature may play an important role in various step of the propagation of bacteriophage such as phage attachment and penetration into the host cells, multiplication and latent period. At low temperature, the movement of phage is slow; hence the attachment of phages onto the surface of host cells will be affected. This means that less phage can be penetrated into host cells; hence lower level of phage will be involved in multiplication stage. On the other hand, the length of latent period will be prolonged at high temperature (Burns and Slater, 1982); therefore longer period will be needed from phage infection stage before moving into phage multiplication stage. In addition, the activities of cellular enzymes are greatly affected by the temperature of the

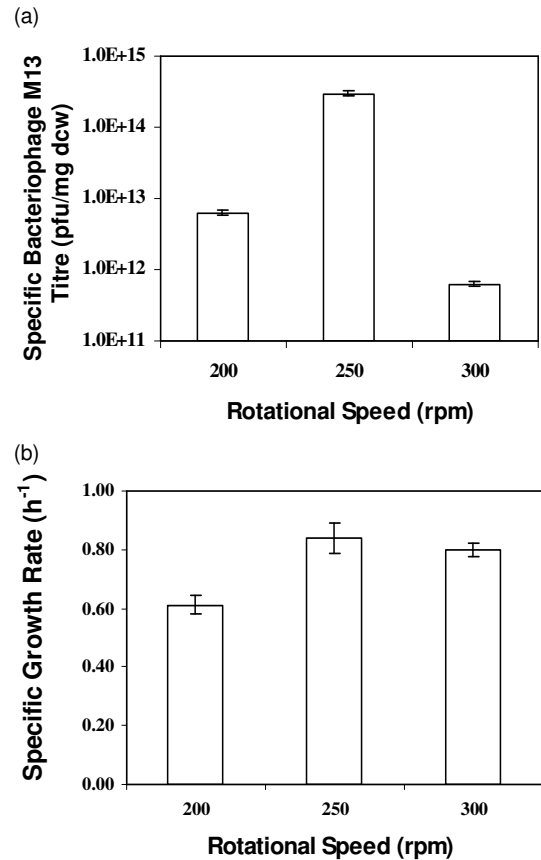


Figure 4. The effect of mixing level on the (a) fusion bacteriophage production, and (b) specific growth rate of *Escherichia coli* ER2738. The data represent the mean \pm S.D. of triplicate determinations.

culture. The optimum temperature for most of the cellular enzymes is in the range of 20 to 40°C. At lower temperature, the enzyme activities will be low and this will slow down the overall cell metabolism, which could lead to lower host cells growth and phage propagation as observed in this study. On the contrary, extremely high temperature will cause irreversible denaturation of cellular enzymes. Therefore, these may explain the reason for lower phage production at 27 and 42°C compared with that obtained at 37°C. In the present study, the specific growth rate of *E. coli* was highest at 37°C ($0.838 h^{-1}$), which was 1 and 11% higher than that at 42 and 27°C, respectively. The specific growth rate at 37°C was only slightly higher than that at 42°C, which could be most probably due to higher phage propagation rate at 37°C that retarded the growth of host cells.

Effect of mixing levels on the production of fusion M13 phage

Figure 4a shows that the production of fusion M13 phage was greatly affected by the levels of mixing. The highest

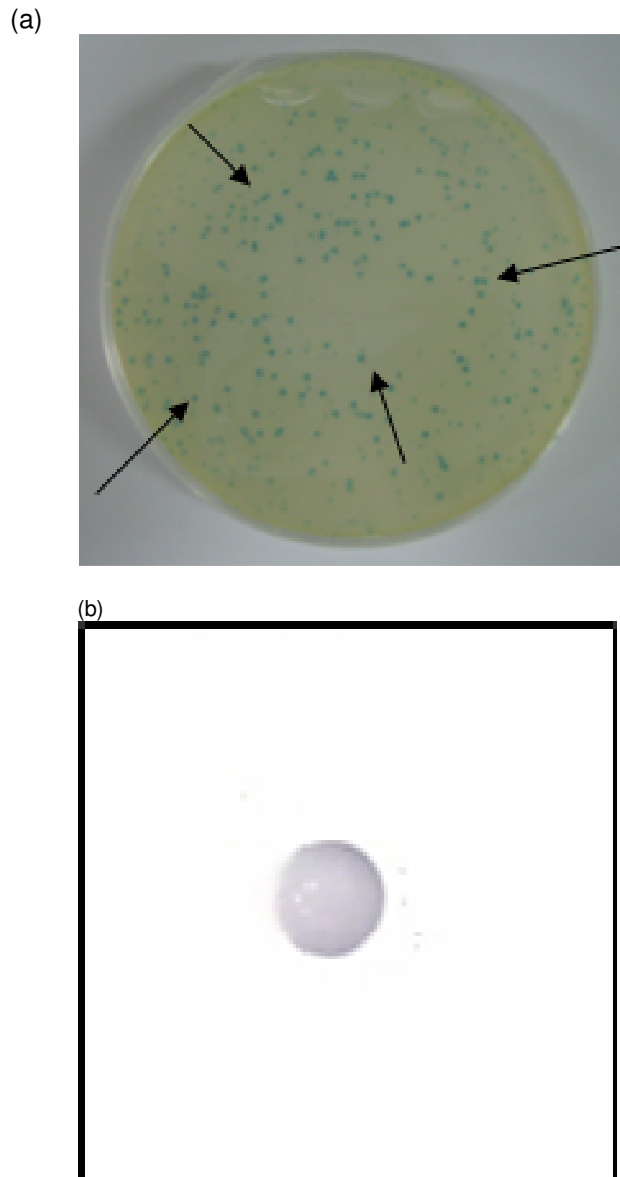


Figure 5. Viability of fusion M13 phage produced. a) The arrows show the presence of blue plaques on the LB agar plate supplemented with IPTG and X-gal, b) Dot-blot test for fusion M13 phage using monoclonal antibody detecting major coat protein (gpVIII) of M13 phage and developed using substrates for alkaline phosphatase-conjugated secondary antibody.

phage production, 2.99×10^{14} pfu/mg dcw was obtained from the culture shaken at 250 rpm. This was followed by 6.33×10^{12} and 6.28×10^{11} pfu/mgdcw from the culture shaken at 200 and 300 rpm, respectively. Figure 4b shows the highest specific growth rate was recorded at the optimal rotational speed of 250 rpm, which was 5 and 37% higher than that of 300 and 200 rpm. Oxygen is an important substance for the vitality of aerobic microbes, hence the growth of host cells and phage propagation are greatly influenced by the presence of oxygen inside the cells. Therefore, the dissolved oxygen level is enhanced

by good mass transfer rate provided by the vigorous shaking of culture medium. Other than enhancing the mass transfer of oxygen, mixing is also required to provide good heat transfer and maintain a uniform environment throughout the medium. The cells under vigorous shaking conditions are also less likely to form clustered microcolonies compared with unshaken or slower shaken ones. As smaller cell cluster has bigger interfacial area, therefore the mass transfer rate of nutrients and oxygen from fermentation broth into the cells will be enhanced (Coleman et al., 2002) and also provide larger surface area for phage attachment. However, higher rotational speed may cause undesirable effect on the binding of phage onto the surface of host cells due to higher shear stress introduced. Therefore, the present study demonstrated that the highest phage production was achieved at the optimum mixing level of 250 rpm.

Viability of fusion M13 phage produced

The viability of the M13 phage bearing di-sulphide constrained peptide (C-WSFFSNI-C) was determined based on formation of blue plaques obtained from phage titration assay as well as the dot-blot test. Figure 5a shows that the M13 phage purified from the current method formed blue plaques, which were positive for fusion M13 phage. This fusion phage carries the *lac* operon, therefore, in the presence of IPTG and X-gal, the infected cells will appear blue colour as clear plaques indicated the presence of environmental wild-type M13, which lacks the *lac* operon. Furthermore, the dot-blot test also showed positive result when these phages were reacted with monoclonal antibody detecting major coat protein (gpVIII) of M13 phage (Figure 5b).

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