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ORIGINAL PAPER

Maximizing filamentous phage yield during computer-controlled fermentation

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Abstract Filamentous phage such as M13 and fd consist of a circular, single-stranded DNA molecule surrounded by several different coat proteins. These phages have been used extensively as vectors in phage display where one of the phage coat proteins is genetically engineered to contain a unique peptide surface loop. Through these peptide sequences, a phage collection can be screened for individual phage that binds to different macromolecules or small organic and inorganic molecules. Here, we use computer-controlled bioreactors to produce large quantities of filamentous phage in the bacterial host *Escherichia coli*. By measuring phage yield and bacterial growth while changing the growth medium, pH and dissolved oxygen concentration, we found that the optimal conditions for phage yield were NZY medium with pH maintained at 7.4, the dO_2 held at 100% and agitation at 800 rpm. These computer-controlled fermentations result in a minimum of a tenfold higher filamentous phage production compared to standard shake flask conditions.

Keywords Fermentation optimization · Filamentous phage production · Phage display

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Introduction

Filamentous bacteriophages (phages) are viruses that infect bacterial cells. Filamentous phage belongs to a class of phage known as Ff, comprised of strains M13, fl, fd, and ft. They have a circular, single-stranded DNA genome that encodes ten viral proteins, five of which make up the coat proteins that envelope the DNA. To infect bacteria, the phage must bind to the F-pilus on the host bacterium via the pIII protein. The phage DNA enters the bacterium and is converted to a double-stranded replicative form (RF). This RF DNA is used as template to make more single-stranded DNA copies for new phage particles, and is transcribed and translated to synthesize the viral proteins. [1]. New phage particles are assembled as they are being secreted from the membrane of the host bacterium. In this way, filamentous phages are different from lytic phage—filamentous phages do not kill their bacterial host in the process of secretion, nor do they prevent cell division. However, their intracellular presence slows the growth of the host bacterium resulting in the appearance of turbid plaques on a bacterial lawn.

Phage display is a technique that allows identification of peptide ligands that bind to any given target molecule [2]. A phage-display library is created by inserting random oligonucleotides into the gene of a surface-exposed loop of a protein encoded by a filamentous phage gene; libraries are usually constructed by manipulating either the minor capsid protein pIII gene (Type 3 vectors) or the major capsid protein pVIII gene (Type 8 vectors). When the recombinant gene is expressed, the foreign peptide is 'displayed' as an extension at the N-terminus of one of the coat proteins on the surface of the phage particle. Several of these random peptide phage libraries are commercially available and have a complexity in the order of 10^9–10^{11};

within this library, each phage has a unique surface peptide that has the potential to bind to another molecule. Through a process of affinity selection (bio-panning) and standard microbiology cloning methods, the tiny fraction of peptide sequences that bind to the target molecule can be separated from the complex mixture of non-binding peptide sequences.

Phage display is a very powerful technique that has been applied to an ever-expanding array of biotechnology applications. The technology was pioneered in 1985 by Smith [2], and was rapidly expanded to include both in vitro and in vivo applications such as epitope mapping of monoclonal antibodies [3, 4], protein purification, alternatives to antibodies of high specificity and nanomolar affinity [5], identification of protease substrates [6], application to drug delivery systems, and gene delivery systems to mammalian cells [7] as well as many others. Recently, these biological applications of phage display have been extended to identifying peptides that bind to inorganic substrates as shown in the following examples. The technology has contributed to the understanding of protein interactions with metallic surfaces [8–11]. This knowledge is being applied to the improvement of medical implants [12]. The list of metals to which phage-displayed peptides bind continues to grow [12–21]. Some of these semiconductor binding phages are being utilized in novel nanotechnology applications; for example, phages are being engineered to assist in the synthesis of nanowires [14, 15]. In these applications, the phages act as nucleation sites for the formation of zinc sulfide and cadmium sulfide nanocrystals [15]. Other metal-binding phages are being considered for use as biofilms to decrease corrosion of metals [17]. In a novel application, we recently reported the use of filamentous phage in the recognition of naturally-occurring minerals for potential use in a biomining setting [21].

As these diverse applications progress toward industrial practice, there will be a need for the large-scale production of phage-displayed peptides. Currently, studies requiring large-scale production have been limited to 1-L shaker flasks. With the advent of modern fermentation technology, we set out to optimize the phage yields in computer-controlled fermenters relative to standard shaker flasks. In the current study, we chose to optimize fermentation protocols for an fd-tet-derived phage vector. This phage contains a tetracycline resistance gene and displays an eight amino acid random peptide on all copies of the pVIII major coat protein. Peptides displayed over the entire surface of the bacteriophage (as a fusion with pVIII coat protein) are believed to have a much greater affinity for a target compared with peptides displayed as a fusion with the five copies of pIII [5]. We demonstrate that the important determinants for maximal phage yield are pH, dO_2, and the composition of the culture medium.

Materials and methods

Bacteriophage and bacterial strain

The *E. coli* strain K91 was the host strain used and is described in Smith and Scott [22]. The bacteriophage 'landscape' phage library was constructed and kindly provided by Dr. Valery Petrenko [5] of Auburn University. We previously screened this random peptide P8 library against chalcopyrite ($CuFeS_2$), and identified a peptide sequence of interest [23]. The phage displaying the sequence DSQKTNPS was used in all of the fermentation experiments in the current study.

Media and inoculum

NZY broth was prepared as follows: 10 g NZ-Amine (Sigma-Aldrich, Oakville, ON, Canada), 5 g yeast extract (BD Biosciences, Mississauga, ON, Canada), 5 g NaCl in 1 L distilled H_2O, and the pH was adjusted to 7.4. NZY plates contained NZY broth with 11 g/L select agar (Invitrogen, Burlington, ON, Canada) and 20 μg/ml tetracycline (Sigma-Aldrich). Super broth (SB) was prepared as follows: 10 g MOPS (3(N-Morpholino) propanesulfonic acid; Sigma-Aldrich), 30 g tryptone (BD Biosciences), 20 g yeast extract (BD Biosciences) in 1 L distilled H_2O, and the pH adjusted to 7.4. A bacteriophage-infected colony was picked from an agar plate and added to 40 mL NZY broth (for two 1-L fermentation runs) or 60 mL of NZY broth (for three 1-L fermentation runs) containing 20 μg/ml tetracycline. The inoculum was shaken at 37 °C and 250 rpm overnight. With the exception of one rich medium condition which was performed in SB, all fermentation runs were performed in 1-L NZY media without tetracycline. An inoculum of 20 mL of was used in each fermentation (representing 2% v/v).

Fermentations

Fermentation equipment was provided by the Single Cell Fermenter Suite at the Centre for Blood Research, University of British Columbia. Bioreactors, biocontrollers, pH and dissolved oxygen (dO_2) sensors were from Applikon Biotech Inc. (Foster City, CA, USA). Two or three bioreactors were set up at one time; the total number of fermentations for this study was 18 that were used to test seven different conditions. All the fermentations were performed in 1 L of medium using 3-L Applikon bioreactors which each had a glass dished-bottom, a Rushton

impeller in the stirrer shaft, three baffles, and an L sparger. One Rushton impeller was installed at the bottom of the stirrer shaft while the standard second impeller was removed to avoid foaming. An ADI 1010 bio-controller (Applikon Biotech Inc.) controlled and monitored pH, temperature, and dO_2 levels in the P.I.D. control system (proportional gain, integral time, and derivative time). ADI 1032/1030 stirrer controllers were used to set agitation at the remote/local mode.

One liter of medium was poured into each bioreactor for batch fermentation, autoclaved, and cooled to room temperature. The fermentation temperature was set to 37 °C and controlled with a solenoid valve that controlled the balance between cooling water and heating jacket (P-gain 250, I-time 0, and D-time 0). Agitation was set to 800 rpm throughout the fermentations, except those tested for the effect of slower agitation (200 and 400 rpm). pH sensors were calibrated with pH 4.0 and pH 7.0 buffer solutions (Fisher Scientific) before autoclaving. During fermentations, pH levels were either uncontrolled or were maintained with two pneumatic pumps connected to 0.2 N HCl and 0.1 M NaOH bottles (P-gain 100, I-time 200, and I-time 0). The dO_2 sensors were calibrated by purging pure O_2 gas into the autoclaved bioreactors at 0.5 vvm for 5 min at 800 rpm. For low oxygen experiments (20% dO_2), aeration was set to 20% and controlled with two solenoid valves connected to O_2 (0.5 vvm) and N_2 (0.5 vvm) rotameters (P-gain 5, I-time 3,000, and D-time 0). In the low agitation experiments (200 and 400 rpm), aeration was set to 100% and controlled with a solenoid valve connected to O_2 (1 vvm). For other experiments, dO_2 levels were automatically monitored. The record time interval for the on-line parameters (Temp, pH, rpm, dO_2) was 1 min. Significant fermentation conditions were repeated multiple times to get the reported values.

Measurement of cell growth

Cell growth was measured in two different ways. Off-line samples (at 5, 6.75, 8.75, and 10.75 h) were collected, and the OD_{595} was measured with a spectrometer (Perkin-Elmer Lambda 3B). Cell growth curves were measured by using light scattering sensors from DASGIP (Jülich, Germany). OD sensors were installed in the bioreactor head plates, autoclaved, and calibrated before inoculation. These sensors collect and record light scattering signals from the sensors every minute. The correlation curve between the DASGIP OD4 module reading and the off-line OD_{595} values showed a good linear relation with corresponding correlation coefficients of R^2 close to 1 (see Figs. 1, 2a). The DASGIP output (Fig. 1, purple line) agreed well with manual readings (Fig. 1, closed diamond symbols) taken approximately every 2 h during the second half of the fermentation.

Preparation of high density cells of non-infected K91

A colony of non-infected K91 cells was transferred to 2 mL of NZY medium, and incubated overnight at 37 °C with shaking at 250 rpm. The overnight culture was diluted 50-fold into 4 ml of SB medium, and incubated at 37 °C with shaking at 250 rpm until an OD_{595} of 1.0–1.5 was reached. At this point, the speed of the shaker was reduced to 100 rpm for 10 min to allow the bacteria to regenerate any potentially sheared F pili. The bacterial culture was then placed on ice and used within 2 h.

Titration of bacteriophage

The off-line samples were collected at each time point and centrifuged at 3,000 rpm for 5 min at room temperature to remove bacteria. The supernatant (containing bacteriophage) was transferred to a new tube. Dilutions of the bacteriophage were made in NZY medium, then 10 μL of each of the diluted bacteriophage samples were added to 30 μL of freshly prepared high density *E. coli* K91 host cells. Samples were incubated for 10 min at room temperature to allow for infection. Tetracycline resistance genes were induced by the addition of 160 μL of NZY medium containing 0.2 μg/mL tetracycline. Samples were then incubated at 37 °C for 30 min with shaking at 100 rpm. Spot titers were performed by adding 30 μL of the phage infected bacteria to an NZY agar plate containing 20 μg/ml tetracycline. After the drops were absorbed by the agar, the plates were inverted and incubated overnight at 30 °C. Tetracycline-resistant transducing units (TU) were counted, and their concentration was calculated.

Results and discussion

Fermentation profiles of bacteriophage-infected K91 cells

The fermentation profiles of phage-producing bacteria were first investigated in flask-mimicking conditions (Fig. 1). The temperature (37 °C) and agitation (800 rpm) were fixed, and multiple parameters were monitored. The initial dO_2 level was 100% but dropped suddenly after 7 h; by 8 h, the dO_2 level was 60% and reached a minimum of 40% by 9 h. The pH of the NZY started at 7.11 and quickly decreased as soon as fermentation started. The pH reached its lowest point of 6.31 at 4.75 h, but by 7 h, the pH had increased to 6.63 and stayed at this value for the duration of the fermentation run. In this control experiment, the bacteriophage-infected cells grew exponentially and appeared to be approaching a plateau at the end of the fermentation period of 10.5 h. During this period, the phage production

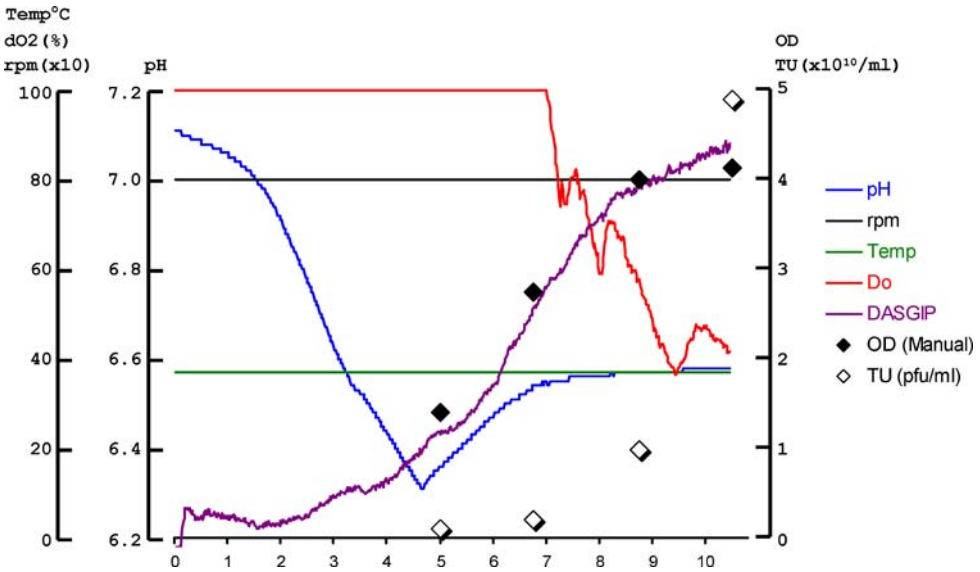

Fig. 1 The control fermentation profile of filamentous bacteriophage-infected *E. coli* K91 cells

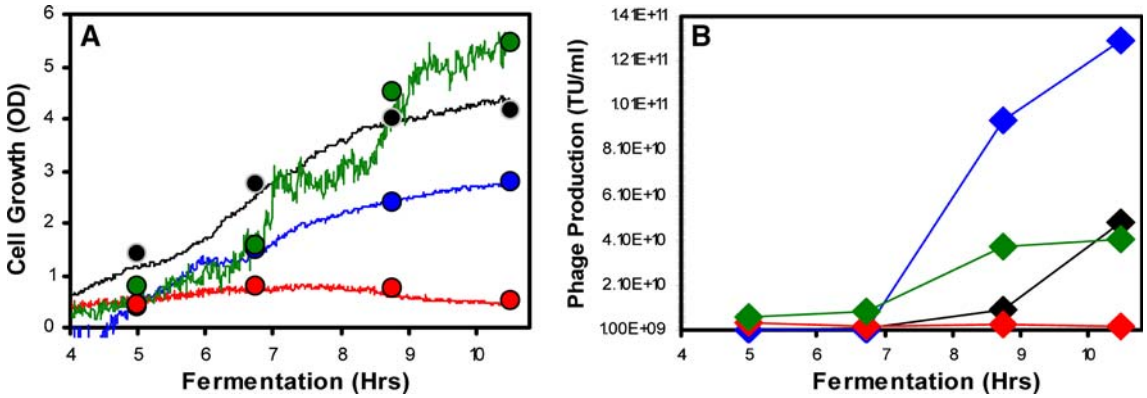

Fig. 2 Cell growth (**a**) and phage production (**b**) from fermentations with NZY medium (*black*), NZY medium with the pH maintained at pH 7.4 (*blue*), NZY medium with the pH maintained at 7.4 and dO_2 levels of 20% (*red*), or SB medium with the pH maintained at 7.4 (*green*)

lagged behind bacterial growth until the bacteria reached mid-log phase at which point there was a dramatic increase in phage production. The bacteriophage yield at the end of this fermentation was 4.9×10^{10} TU/mL. Phage production in this NZY control experiment was similar to reported values of fd-tet bacteriophage production, which are approximately 5×10^{11}–1×10^{12} phage particles/mL; this would be equivalent to $\sim$2.5–5×10^{10} TU/mL (1, 22). It should be noted that fd-tet phage have a lower infectivity ($\sim$20 viral particles to produce one tetracycline resistant transducing unit) and lower production than most other phage vectors [1].

Fermentation optimization for bacteriophage production

To find the optimal fermentation conditions for the production of filamentous bacteriophage, four different fermentation conditions were assembled (Fig. 2a, b): (1) NZY medium as control, (2) NZY medium with pH maintained at 7.4, (3) NZY medium with pH maintained at 7.4 and decreased dO_2 level to 20%, and (4) SB medium with pH controlled at 7.4. Representative fermentation profiles for each condition are shown in Fig. 2.

Effect of pH control

Compared to the NZY control fermentation with uncontrolled pH, the fermentation performed with the pH maintained at 7.4 produced only 67% of the final cell mass (OD 4.14 vs. OD 2.78 at 10.75 h) but produced a 2.65-fold higher phage yield [4.90×10^{10} vs. 1.30×10^{11} TU/mL, Fig. 2a (blue) and Fig. 2b (blue)]. This yield represents a 165% increase in phage production above the level achieved without any pH control. Controlling pH during fermentation not only increased the total amount of bacteriophage but also increased bacteriophage production (i.e., the amount of phage particles produced by a single

bacterium). The bacteriophage production in the NZY control fermentation (Fig. 2b, black) increased significantly only after 7 h (20% of total at 8.75 h). In contrast, the pH-controlled fermentation produced 73% of total phage at the same time point (Fig. 2b, blue). Phage production had not reached a plateau by the end of the 10.75 h fermentation, and would have probably reached a higher level with a longer fermentation time. We conclude that maintaining the pH at 7.4 (or near neutral) is critical for maximal phage production from the fd-tet phage.

Effect of dO_2 level

Fermentation with reduced dO_2 levels produced very little cell mass (12%) and much less bacteriophage (6%) compared to even the NZY control fermentation (Fig. 2a, b, red). Consequently, the dO_2 was held at 100% for the rest of the fermentation runs.

Effect of bacterial growth medium

It could be hypothesized that as host cell density increases, the phage could infect the increased number of host cells more rapidly, resulting in higher phage production. However, the opposite was observed. When compared with NZY with the pH maintained at 7.4, fermentation in rich medium (SB) with the pH maintained at 7.4 produced almost twice the cell mass (197%), but produced only a fraction of the bacteriophage (32%) (Fig. 2a, b, green). The phage production in the SB was comparable to the phage production of the control (i.e., no pH control) fermentation. It has been reported that the most efficient fd-tet phage infection occurs when starved cells are used [1]. Our data support this observation in that high nutrient media is counter-productive to a higher phage yield.

Effect of agitation

To evaluate the potential effects of agitation on phage yield, three identical fermenters were prepared that contained NZY medium (pH 7.4); the fermenters were agitated at 200, 400 or 800 rpm. Unfortunately, aeration in the 200 and 400 rpm fermenters was not sufficient to allow bacterial growth. As a result, three more fermenters were prepared, and dO_2 was set to 100% by delivering oxygen into the media when necessary. No oxygen purge was necessary for 800 rpm, but the O_2 valve was periodically open after 3 and 6 h at 200 rpm and 400 rpm, respectively (data not shown). After 10.5 h of fermentation, cell growth was 76% (200 rpm) and 80% (400 rpm) of the control fermenter (agitated at 800 rpm); similarly, phage production was 93% (200 rpm) and 81% (400 rpm) of the control levels. The reduction in both cell growth and phage production at the end of the fermentation in the 200 and 400 rpm fermenters was probably caused by foaming resulting from the purging with oxygen, which was required to maintain a dO_2 level of 100%. We conclude that higher agitation speeds and aeration were not detrimental to the integrity of the filamentous phage in the liquid medium, and phage were still able to infect bacteria at the highest agitation level used.

Influence of the interaction between bacteriophage and host cells

Unlike lytic phage such as the well-studied phage λ, filamentous bacteriophages do not lyse their host cells during the infectious cycle. As a result, phage yield is probably very dependent on the growth status of the bacterial host. To minimize the risk of the appearance of wild-type-like phage, a lower nutrient medium (such as NZY) is commonly used for preparing recombinant bacteriophage cultures. To test whether fermentation conditions that allow less cell growth result in higher yields of filamentous bacteriophage, we performed multiple fermentations and quantified the bacterial growth (in OD_{595}) and phage production at the end of the fermentation (Fig. 3). Except for the case with low oxygen fermentation (NZY + pH 7.4 + dO_2 20% which had low bacterial growth and a corresponding low phage production), the greater the bacterial cell mass (Fig. 3, black bars), the lower the bacteriophage production (Fig. 3, gray bars).

The ratio of bacteriophage production normalized by cell mass was used as a representation of phage yield (Figs. 4, 5). The highest yield was obtained by using NZY

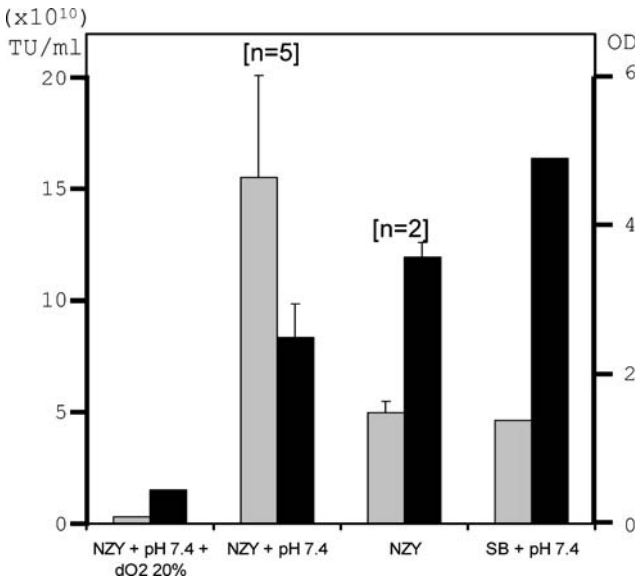

Fig. 3 A comparison between bacteriophage production (*gray bars*) and cell growth (*black bars*) in different fermentation conditions

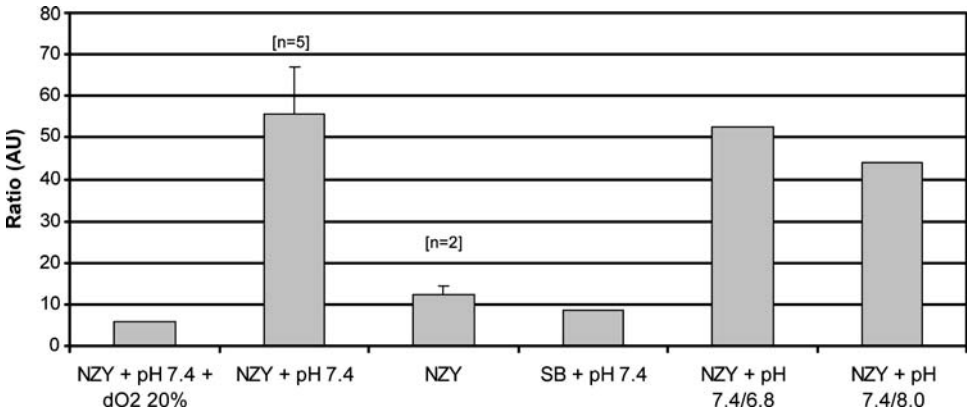

Fig. 4 The ratio of bacteriophage production to cell growth in fermentations from different conditions (*AU* arbitrary units derived from TU/mL per OD_{595})

medium with pH maintained at 7.4 resulting in a phage to bacterial ratio (P/B) of 55.7 ± 12.3 ($N = 5$, Fig. 4). The lowest yield was NZY medium with pH maintained at 7.4 with dO_2 controlled at 20%, resulting in a P/B ratio of 5.9 (Fig. 4). These results represent a tenfold difference in absolute phage yield. As shown in Fig. 4, the P/B ratios were 12.6 ± 2.1 ($N = 3$) in the control fermentation (i.e., no pH control) and 8.4 in the SB (pH 7.4) fermentation which represent 22.6 and 15%, respectively, of the maximum phage production. Since controlling pH significantly increased bacteriophage production, two other experiments were performed. Fermentation was initiated at pH 7.4 until the second sampling (6.75 h), and then the pH was shifted to 6.8 (slightly acidic) or to pH 8.0 (slightly basic) for the rest of fermentation. Changing the pH by ±0.6 pH units did not show significant changes in bacteriophage production by the end of fermentation (Fig. 5). However, the fermentation shifted to pH 6.8 showed a more rapid increase in bacteriophage production than the fermentation shifted to pH 8.0.

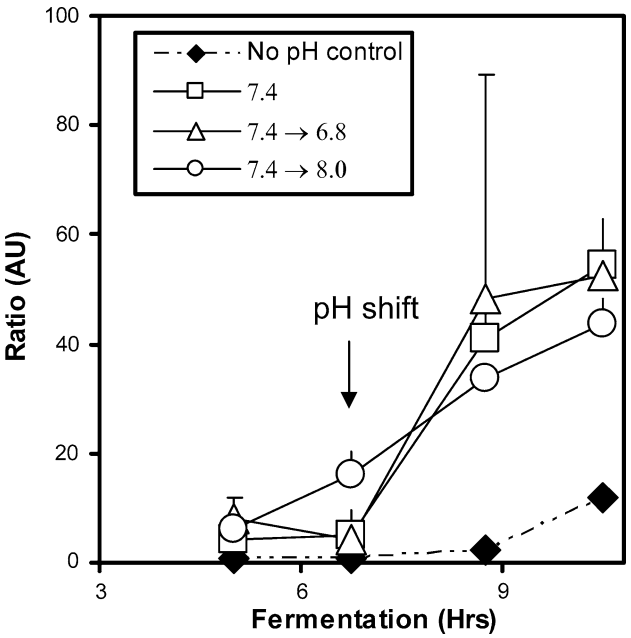

Fig. 5 The effect of changing the pH ± 0.6 pH units on the ratio of bacteriophage production to cell growth

Factors influencing the effect of pH during controlled fermentation on bacteriophage yield

A pH drop generally occurs toward the end of a fermentation due to the accumulation of cytotoxic metabolites such as CO_2 or acetic acid as a byproduct of TCA cycle. However, in this study, it was found that the pH starts to drop as soon as bacterial inoculums were added to the bioreactors (Fig. 1). It is not clear what caused this early acidification. On-line measurement of cell growth gave continuous cell growth curves during the fermentation, and provided important information about the interaction between cell growth and bacteriophage production. In pH-controlled fermentations (four repeated experiments shown in Fig. 6, blue lines), there was a slowdown of bacterial cell growth at 6 h (blue arrows) that did not occur in the other fermentation conditions used. Phage production was still insignificant (2% of total phage production, Fig. 6, gold diamond) at 6 h. It has been reported previously that in bacteriophage-infected cells, the generation time is slowed by 50% compared to uninfected bacteria [1]. Fermentation with non-infected cells was not tested in this study, but cell growth of pH-controlled fermentation was about 67% of the NZY control fermentation (Fig. 2a). Since phage production in the pH controlled cells was much higher than in pH uncontrolled cells, this may help to explain the slower growth of the cells in controlled pH conditions. Interestingly, the SB medium profile showed a similar growth slowdown that occurred much later (Fig. 6, green). Bacteriophage production in SB medium was much lower than NZY medium when both are pH controlled (Fig. 2b, blue vs. green). These results indicate that the pH control of fermentation and the status of the host bacterial cell growth are essential for maximal filamentous phage yield.

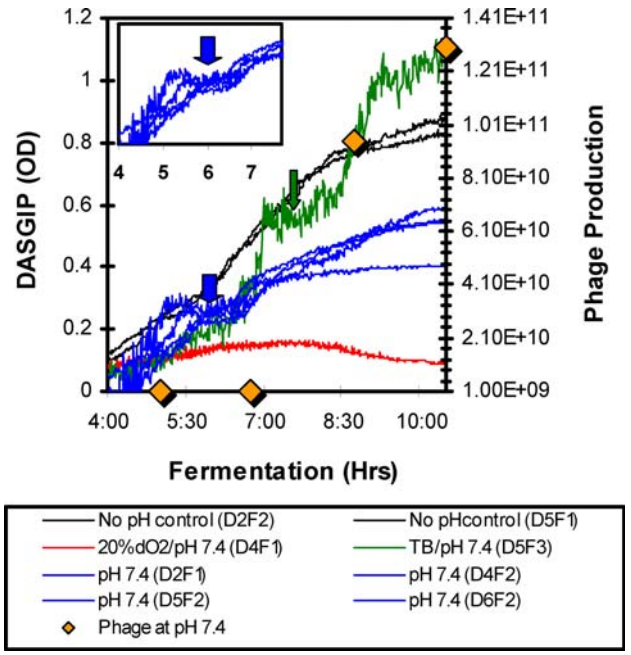

Fig. 6 The interaction of host cell growth and bacteriophage production in different fermentation conditions

Conclusion

Optimization of the fermentation conditions to produce maximal yields of filamentous bacteriophage in *E. coli* was studied. In our studies, we introduce a ratio concept to reflect the overall phage yield compared to the host bacterial cell growth. The optimal conditions for phage yield were NZY medium, with agitation at 800 rpm, pH maintained at 7.4 and 100% dO_2. Under these conditions, yields of filamentous phage in the order of 10^{14} TU/L (equivalent to approximately 2×10^{15} phage particles/L) were obtained during the 10.75 h fermentations. Higher yields of phage may be obtainable by extending the fermentation period. Using these conditions, we conclude that compared to shake flasks, computer-controlled fermentation increases the phage yield by a minimum of tenfold. We are currently testing these conditions with other filamentous phage such as M13, and with phage that displays different peptides.

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