Process Biochemistry 51 (2016) 1323–1330

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

A *Klebsiella pneumoniae* bacteriophage and its effect on 1,3-propanediol fermentation

Juntao Shen, Jinjie Zhou, Hongxin Fu, Ying Mu, Yaqin Sun, Yongping Xu, Zhilong Xiu*

School of Life Science and Biotechnology, Dalian University of Technology, Dalian, China

ARTICLE INFO

Article history:
Received 19 March 2016
Received in revised form 22 July 2016
Accepted 27 July 2016
Available online 28 July 2016

Keywords:
Phage infection
Klebsiella pneumoniae
1,3-Propanediol
Sluggish fermentation
Infection time

ABSTRACT

Bacteriophage infection is one of potential threats in bacterial fermentations. In the present study, a phage specially infecting *Klebsiella pneumoniae* was characterized and its effect on 1,3-propanediol fermentation was investigated. It belongs to the *Siphoviridae* family with an icosahedral head of 64 ± 2 nm in diameter and a long tail of 158 ± 3 nm and has a double-stranded DNA of about 45 kbp. The infection kinetics and stability of phage phiKpS2 were also determined. Then, phage infection at different growth phases were carried out in laboratory to evaluate its impact for PDO production. Different sluggish fermentations depending on the infection time at different cell growth phases were observed. Fermentation failed to recover even adding new medium when phage infected at 4 h in fed-batch fermentation. However, the maximum biomass increased 25.8% after about 8 h of lag time when phage infected at 0 h, which led to the increase of PDO production from 69.7 ± 2.1 g/L to 85.2 ± 0.8 g/L. In addition, pH fluctuations were also observed along with cells lysis and growth recovery.

1. Introduction

Microbial conversion plays a key role in the transition from a fossil fuel-based economy to a bio-based one [1,2]. Among the challenges for making bio-based processing more economic, bacteriophage infection is one of constant threats which still needs to be overcome [3,4]. In fact, bacteriophage infection or/and prophage induction, which can happen almost in any bacterial fermentations, contribute to a significant proportion of failed fermentation [5–7]. It can slow or stop fermentation process and cause serious financial losses. Although there are only few reports about phage contaminations, it was indeed observed in many industrial fermentation plants, such as antibiotics, amino acids, dairy and biofuels factories [4,6,8,9]. In the dairy industry, phage infection is the primary cause of slow and incomplete fermentations [9–12]. And in acetone-butanol-ethanol industrial fermentation, phage infections have broken out all around the world and are major problems in the industrial process [8]. Although many efforts have been made, especially in the dairy industry, there were no efficient solutions for preventing phage contamination [12,13].

Klebsiella pneumoniae has the ability to utilize almost all of the major sugars (pentoses, hexoses and cellobiose) and uronic acids derived from hydrolysates of hemicelluloses and celluloses as well as waste glycerol derived from biodiesel production [14–16]. Meanwhile, *K. pneumoniae* is applied for production of many valuable metabolites, such as 1,3-propanediol (PDO), 2,3-butanediol (BDO), hydrogen (H_2), 3-hydroxypropionic acid, and 2-ketogluconic acid [17–24]. It can be concluded that *K. pneumoniae* will be a potential industrial strain to produce bio-based chemicals and fuels. For example, PDO can be used as a monomer for the production of polytrimethyleneterephthalate (PTT), a novel polymer with properties comparable to nylon that can be used in carpets and textile fibers. *K. pneumoniae* was regarded as one of the most efficient one to convert glycerol to PDO [16,23,25]. Especially, PDO fermentation with *K. pneumoniae* has been successfully carried out for pilot-scale experiments by some groups [26–28]. Another important platform chemical, BDO, whose production by *K. pneumoniae* has a history of more than 100 years, has potential applications in the manufacture of pharmaceuticals, antifreeze agent, synthetic rubber, plastics, solvent production, foods and others [29,30]. The industrial-scale fermentation for BDO was first proposed by Fulmer in 1933 [30]. Although it was replaced by fossil fuel-based process soon afterwards, nowadays the bioconversion process is receiving significant interest again [31]. Up to now, *Klebsiella* sp. with the features of broad substrate, high yield and easy cultivation has been the most efficient strain for BDO fermentation [29].

* Corresponding author at: School of Life Science and Biotechnology, Dalian University of Technology, 2 Linggong Road, Dalian 116024, China.
E-mail address: zhlxiu@dlut.edu.cn (Z. Xiu).

http://dx.doi.org/10.1016/j.procbio.2016.07.026
1359-5113/

Phage particles are regarded as the most numerous biological entities on the earth with the number of about 10^{31} [32]. Bacteriophages special for *K. pneumoniae* are also highly abundant in natural environments just as well as its host [18,33]. There exist some studies about *Klebsiella* bacteriophages [34–37]. However, most of these studies aimed at developing preparations for phage therapy.

Hence, considering the abundance of and the risk of contamination by phage especially in industrial-scale fermentations, it is necessary to study the effect of bacteriophage infection on fermentation by *K. pneumoniae*. Bacteriophages were detected from an abnormal PDO fermentation broth as described in our previous study [38]. The present paper focuses on the characterization of phage phiKpS2 which infects *K. pneumoniae* and the impact on the fermentation of PDO.

2. Materials and methods

2.1. Bacterial strains and culture conditions

K. pneumoniae S2 (a mutant from *K. pneumoniae* strain DSM 2026 [27]) was kept in 20% (w/w) glycerol stock solution at −70 °C and used as an indicator strain. Bacteriophage phiKpS2 in this study was isolated from an abnormal PDO fermentation broth following by three consecutive purifications.

Seed and fermentation media had been described by Liu et al. [27] with some modifications. For fermentation medium, KCl and $NaH_2PO_4{\cdot}H_2O$ were substituted by 1.36 g/L KH_2PO_4. Shake-flask experiments for phage infection were carried out in 150 mL with 50 mL seed medium at 37 °C at 200 rpm. Fed-batch fermentation was performed in a 5.0 L bioreactor (Biotech, Shanghai, China) with a working volume of 2.0 L by 10% inoculation. Initial concentration of glycerol is 40 g/L and controlled about at 20 g/L during the fermentation process. Temperature and agitation speed were controlled at 37 °C and 250 rpm, respectively. When the pH was lower than the set-up value (7.0), 5 mol/L NaOH was added automatically. However, it was just kept if the pH was above the set-up value. For bacteriophage infection experiments in shake-flask and fed-batch fermentation, a certain amount of phage suspensions (phage titer 10^{11} PFU/mL) were added at an appointed time. The cell suspension after phage attack was diluted, coated on agar plate, and inculcated at 37 °C for about 16 h. The clones were defined as survivors. At least 10 clones from one time fermentation were isolated after phage attack in fed-batch fermentations following by three consecutive purifications. Then phage resistance was evaluated by observation the formation of phage plaque on plate with high titer of phage suspension. In some situation, phage resistance was also evaluated by coculture of the testing strains with phiKpS2 in 96 well plate and measurement of the optical density at 650 nm (OD_{650}).

2.2. Bacteriophage isolation and purification

Bacteriophages were isolated from an abnormal PDO fermentation broth, as described by Sun et al. [39] with some modifications. Contaminated broth was centrifuged at 10,000 × *g* for 10 min and the supernatant was filtered through a 0.22 μm millipore filter. The phages were then isolated by double-layer plate method [40]. Five milliliter of filtrate and 0.5 mL indicator bacteria were added to 4.5 mL of warm soft agar (0.8%), mixed and poured on Petri dishes to form plaques. After 15 min plates were incubated at 37 °C for about 12 h. Well-isolated individual plaques were picked up, transferred to fresh medium and incubated with the indicator strain for 24 h at 37 °C, following by phage isolation as described above. The procedures were repeated three times. Final purified phages were stored at 4 °C.

2.3. Transmission electron microscopy

Purified phage samples (5 μL, 10^{10} PFU/mL) was adsorbed onto carbon coated copper grids and negatively stained with phosphotungstic acid (2% w/v, pH 4.5) [34]. After drying, the preparation was examined on a JEM-2100 electron microscope (JEOL, Tokyo, Japan). Bacteriophage size was determined from the average of three independent pictures.

2.4. Phage DNA extraction, restriction endonuclease analysis and genome sequencing

DNA was extracted and purified from high titer phage supernatant using a Lambda kit (BLKW Biotechnology Co. Ltd., Beijing, China) following the manufacturer's protocol. Phage DNA was digested with *EcoR* I, *Hind* III and *Sam* I. The DNA fragments were separated by agarose (0.8%) gel electrophoresis in TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA). The bacteriophage phiKpS2 DNA was sequenced in Beijing Genomics Institute, China, and its sequence has been deposited in GenBank under accession number KX587949.

2.5. Optimal multiplicity of infection (MOI)

Infection at different rate of phage particles to host cells was carried out to find the optimal multiplicity of infection. The indicator strain was previous incubated at 37 °C for 5 h and adjusted to 10^8 CFU/mL. And then the phage (adjusted to 10^{10} PFU/mL) and indicator strain were added to seed medium at 7 different MOIs (0.0001, 0.001, 0.01, 0.1, 1, 10, 100). After incubation for 12 h at 37 °C, the phage titer was determined by double-layer plate method [40].

2.6. Adsorption rate

Adsorption experiments were performed at a MOI = 0.1 (phage 10^7 PFU/mL to host cell 10^8 CFU/mL) as described previously [37] with slight modifications. The mixed cultures of phage and its host were incubated at 37 °C. Samples were taken with a time interval of 5 min and immediately centrifuged at 10,000 × *g* and 4 °C for 10 min. The supernatant was used to determine the phage titer.

The adsorption rate constant, which is independent of the cell density and of the time for adsorption, was calculated as Kads [mL/min] = $2.3/(B \times t) \times \log P_0/P_t$, where [B] is the bacterial concentration [mL^{-1}], t is the time of adsorption [min], and P_0 and P_t are the titers of phage at time 0 and t, respectively.

2.7. One-step growth curve

One-step growth curve of phage phiKpS2 was carried out as described before with some modifications [34]. Indicator strain was incubated for 7 h at 37 °C in seed medium. Then cells harvested and added to a fresh seed medium were mixed with phage suspensions to achieve a ratio of MOI = 1. Phages were allowed to adsorb for 5 min at 37 °C. The mixture was then centrifuged (12,000 rpm for 5 min) and resuspended in 25 mL of seed medium. Sample was taken at 15 min time intervals for titration. The latent time, phage burst time and size were calculated from one-step growth curve.

2.8. Sensitivity to temperature, pH and UV

The method to determine phage stability was described in many studies [37,39,41]. Here the stability of phiKpS2 was measured by incubation for 15 min to 60 min at the temperature ranging from 50 to 90 °C, respectively. And phage incubation was carried out for 12 h to assay pH resistance at 37 °C and pH ranging from 3 to 12.