RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Preparation and application of bacteriophage-loaded chitosan microspheres for controlling of *Lactobacillus plantarum* contamination in bioethanol fermentation

Xianghua Lin, Pei Han, Shijun Dong, and Hao Li*

Beijing Key Laboratory of Bioprocess, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China

***To whom correspondence should be addressed:**

Hao Li

Beijing Key Laboratory of Bioprocess, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, P. R. China;

Phone: +86-010-64447747; Fax: +86-010-64416428;

E-mail address: lihao@mail.buct.edu.cn or lihaoh@163.com

E-mail of all authors:

Xianghua Lin abcflas@163.com

Shijun Dong dsjbuct@163.com

Hao Li lihao@mail.buct.edu.cn

Abbreviation: American type culture collection (ATCC); de Man-Rogosa-Sharpe (MRS); Fourier transform infrared spectroscopy (FTIR); lactic acid bacteria (LAB); *Lactobacillus plantarum* (*L. plantarum*); *Saccharomyces cerevisiae*is (*S. cerevisiae*); scanning electron microscope (SEM);

Shirasu porous glass (SPG); yeast extract peptone dextrose (YPD).

Abstract

Bioethanol fermentation performed by *Saccharomyces cerevisiae* is often inhibited by bacterial contamination, especially lactic acid bacteria (LAB). Bacteriophage with high specificity can efficiently control bacterial contamination. In this study, *Lactobacillus plantarum* bacteriophage was embedded into chitosan by membrane emulsification method and used to control *L. plantarum* contamination in bioethanol fermentation. The prepared bacteriophage-loaded chitosan microspheres were characterized by scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR). The size homogenization of the prepared microspheres was realized through fast membrane emulsification device with Shirasu porous glass (SPG) membrane tube, and the diameter of homogenized microspheres was about 30 μm. The small d value (i.e., 2.015) indicated the narrow range of size distribution. Swelling experiment indicated that bacteriophage was successfully enwrapped into chitosan microspheres. To mimic the bacterial contamination in industrial bioethanol fermentation, *L. plantarum* American type culture collection (ATCC) 8014 was co-cultivated with *S. cerevisiae* CEC B9S-15 in yeast extract peptone dextrose (YPD) broth. Bacteriophage-loaded chitosan microspheres were added into the simulated contaminative fermentation system to evaluate the controlling effect on *L. plantarum* contamination by releasing bacteriophage. Compared with the contamination group, the final ethanol content of bacteriophage-loaded chitosan microspheres treatment group increased obviously (P<0.01). It indicated that *L.* p*lantarum* contamination could be effectively controlled by bacteriophage-loaded chitosan microspheres.

Keyword: Bioethanol fermentation, *Saccharomyces cerevisiae*, *Lactobacillus plantarum*

contamination, bacteriophage, chitosan microspheres

Introduction

Rapidly increased demand for energy results in the gradual depletion of traditional fossil resources and environmental pollution.[1,2] There is thus a need for alternative energy sources.[3,4] As the most promising renewable clean energy, bioethanol has received widely attention due to its security, abundance of raw materials and other particularity,[5-7] and would contribute to the reduction of global warming effect and facilitate alleviating energy crisis.[2,8,9]

Saccharomyces cerevisiae is the most employed microorganism for bioethanol fermentation.[10] However, industrial-scale bioethanol fermentation is often inhibited by bacterial contamination, especially lactic acid bacteria (LAB).[11-15] Contaminative LAB can inhibit *S. cerevisiae* growth and decrease bioethanol yield through secreting lactic acid and competing for nutrients and living space with *S. cerevisiae*.[16] For example, Thomas et al. found that inoculation of *Lactobacillus* (10^7 CFU/ml) before fermentation could lead to about 22% loss of bioethanol yield.[17] Therefore, how to control LAB contamination in bioethanol fermentation is a critical issue.

Traditionally, antibiotics were mainly used to control LAB contamination in bioethanol fermentation.[16] However, overuse of antibiotics caused serious drug residues, bacterial resistance, and even secondary pollution through the proliferation of drug resistance genes.[18,19] Recently, a series of other agents or methods (i.e., sulfuric acid, sulfite, and addition of exogenous lactic acid) have been proposed and performed.[20-22] However, these methods still have their respective limitations. For example, the tolerance of *S. cerevisiae* to lactic acid is limited, and strain screening or genetic modification would be needed to obtain lactic acid-tolerant strains;[22] added

sulfuric acid will corrode fermentation equipment and cannot be easily separated. Therefore, it is necessary to find novel safe and effective methods or agents to control bacterial contamination in bioethanol plants.

Bacteriophage might be an ideal alternative method for controlling bacterial contamination due to its host specificity.[23-27] In fact, bacteriophage has been used to treat bacterial infections and control food borne pathogens.[25,28-30] Recently, it was suggested that the bacteriophage can also be utilized to control bacterial contamination in bioethanol fermentation.[31-33] Solomon and his colleagues have revealed that adding an appropriate amount of bacteriophage into fermentation system can substantially prevent growth of LAB, and keep the concentrations of acetic acid and lactic acid no more than 0.30% (w/v) and 0.80% (w/v), respectively.[31] However, real-time monitoring of fermentation conditions was needed for delivery of bacteriophage timely at different stages. Such operations are time-consuming and uneconomical. If sustained release of bacteriophage can be realized, it will greatly reduce monitoring and other costs. Biocompatible material can be used to wrap bacteriophage through chemical cross-linking and realize the sustained release of bacteriophage for persistent controlling of LAB contamination in bioethanol fermentation.

Due to their good biocompatibility, chitosan, alginate, carrageenan, pectin, polyamino acids, bacterial cellulose, and other polymer materials can be used to encapsulate active substances.[34-37] Owning excellent biocompatibility, biodegradability, bio-adhesion, as well as non-toxic, antibacterial characteristic, etc., chitosan has been widely used to wrap active substances or drugs in different industries, especially the pharmaceutical industry.[38-41]

In this study, chitosan was used to wrap bacteriophage that is specific for *Lactobacillus plantarum* American type culture collection (ATCC) 8014 to realize the sustained release of bacteriophage and persistent controlling of *L. plantarum* contamination in bioethanol fermentation. Bacteriophage-loaded chitosan microspheres were prepared by membrane emulsification method, and subsequently characterized by Fourier transform infrared spectroscopy (FTIR), scanning electron microscope (SEM), and particle size analysis. Moreover, swelling of the prepared microspheres under different pH values (i.e., pH 4 or 6) was performed to verify the enwrapping of bacteriophage. Finally, effect of bacteriophage-loaded microspheres on the *L. plantarum* contamination in bioethanol fermentation was evaluated by measuring the ethanol content.

Materials and methods

Strains, media and culture conditions

S. cerevisiae CEC B9S-15 used in this study was cultured in yeast extract peptone dextrose (YPD) broth (2% glucose, 1% yeast extract, 2% peptone) at 30°C and shaken at 170 rpm in 250 ml cotton-plugged flasks. The *L. plantarum* ATCC 8014 strain used in this study was purchased from Institute of Microbiology, Chinese Academy of Sciences and grown at 30°C in de Man-Rogosa-Sharpe (MRS) broth (1% peptone, 1% beef extract, 1% yeast extract, 2% glucose, 0.5% sodium acetate, 0.2% diammonium hydrogen citrate, 0.2% dipotassium hydrogen phosphate, 0.058% magnesium sulfate, 0.025% manganese sulfate, and 0.1% (v/v) tween 80, pH 6.8).

The bacteriophage targeting *L. plantarum* 8014 was isolated and purified from duck feces samples through the double agar layered method.[42] Single bacteriophage suspension was

propagated on *L. plantarum* 8014 and the bacteriophage titer was determined by the double agar layered method.[42] The bacteriophage lysate was suspended in SM buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, and 0.01% gelatin, pH 7.5), and stored at 4°C.

Reagents

Liquid paraffin was purchased from Tianjin Yongda Chemical Reagent Co., Ltd (Tianjin, China). Chitosan (MW=720000, degree of deacetylation=80.0-95.0%) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Span80 and 25% glutaraldehyde were purchased from Tianjin Fu Chen Chemical Reagents Factory (Tianjin, China). Petroleum ether, ethyl acetate, ethanol, peptone, beef extract, yeast extract, diammonium hydrogen citrate, glucose, sodium acetate trihydrate, dibasic potassium phosphate, magnesium sulfate, manganese sulfate, Tween 80, NaCl, KBr and glacial acetic acid were acquired from Beijing Chemical Works (Beijing, China). SPG membrane tube was purchased from SPG Technology Co. (Miyazaki, Japan). Water used in this study was purified by a Mill-Q system (Millipore, Bedford, MA, USA). All other chemicals and organic solvents used in this study were of analytical grade.

Preparation of bacteriophage-loaded chitosan microspheres by membrane emulsion cross-linking method

Chitosan microspheres were prepared by emulsion cross-linking method according to what reported by Jose et al.[43] The chitosan (4% w/v) was dissolved in dilute acetic acid solution as aqueous phase in prior to complete dissolution at 4°C. The prepared chitosan-dilute acetic acid solution (aqueous phase) was mixed with liquid paraffin (oil phase) in a volume ratio of 1:2, and

appropriate activated bacteriophage lysate (3.6×10^3 PFU/ml) in a volume ratio of 1:8 with chitosan solution and emulsifier Span80 were simultaneously added to generate coarse emulsion by magnetic stirring at 800 rpm at 40°C for 15 min.

The size homogenization was realized through FMEM-500M fast membrane emulsification device (National Engineering Research Center For Biotechnology, Beijing, China）with SPG membrane tube (SPG Technology Co., Miyazaki, Japan) (Fig. 1). As prepared chitosan microspheres were W/O type, hydrophobic SPG membrane tube was selected with the outside diameter of 1 cm and length of 12.5 cm (Fig. 1b). SPG membrane tube was immersed in liquid paraffin and sealed with paraffin before keeping at room temperature overnight till SPG membrane tube was thoroughly wetted. Nitrogen pressure was set as 0.72 MPa to decrease the effect of instability of nitrogen pressure on the homogenization. SPG membrane tube was mounted onto the fast membrane emulsification device prior to using ethyl acetate to rinse the residual material in the apparatus and subsequent rinsing with liquid paraffin three times. After being added to pre-mixed reservoir, coarse emulsion was pressed into the inner side of SPG membrane tube to form homogenized size droplets from the outer periphery under nitrogen pressure (0.72 MPa). Emulsifier molecules that adsorbed on the droplet interface could not only reduce the interfacial tension to promote departing of droplet from the surface of the film, but also prevent aggregation of droplets. The outflow from the spout of the membrane tube center was collected for repeated operation.

Fig. 1

After pressing and flowing through the membrane three times repeatedly, the fine emulsion

was obtained prior to addition of 25 wt% glutaraldehyde solution for subsequent cross-linking for 1 h (the molar ratio of amino and aldehyde groups was 1:0.7). Then the sample was washed with petroleum ether before centrifugation (4000 rpm, 5 min) for acquiring chitosan microspheres. The obtained chitosan microspheres were dried in a vacuum freeze drier.

Moreover, pure chitosan microspheres without bacteriophage were also prepared using the same method mentioned above.

Characterization of bacteriophage-loaded chitosan microspheres

FTIR spectra of the pure chitosan and bacteriophage-loaded chitosan were obtained on a Varian 3100 Fourier transform infrared spectrometer (Varian Inc., America). The samples were thoroughly mixed with KBr, an IR-transparent matrix, and compressed into disks. Scanning was performed from 4000 cm^{-1} to 400 cm^{-1} at a resolution of 4 cm^{-1}. The morphologies of pure chitosan microspheres without bacteriophage and bacteriophage-loaded chitosan microspheres were observed and photographed by SU-1510 SEM (Hitachi High-Technologies, Tokyo, Japan).

After being dispersed in deionized water for ultrasonic dispersing uniformly, microspheres particle size distribution was measured by a Mastersizer 2000 laser particle size analyzer (Malvern Instruments Ltd, UK). Moreover, the distribution width of particles was calculated according to the following equation:

$$d = (D(0.9)-D(0.1))/D(0.5)$$

d refers to the size distribution of particles.

All the measurements were performed at ambient temperature.

Determination of swelling properties of bacteriophage-loaded chitosan microspheres

Bacteriophage-loaded chitosan microspheres were soaked into different pH value solutions (i.e., pH 4, pH 6) for respectively 0 h and 3 h before centrifugation (8000 rpm, 3 min). After diluted 100-fold, the supernatant was employed to evaluate the release of bacteriophage from the microspheres by the double agar layered method.[42]

Inhibition of *L. plantarum* contamination using bacteriophage-loaded chitosan microspheres

To mimic the bacterial contamination in industrial bioethanol fermentation, *L. plantarum* was co-cultivated with *S. cerevisiae* in YPD broth at the beginning of the culture. *S. cerevisiae* and *L. plantarum* were inoculated into YPD broth at cell densities of 4×10^5 and $1.6\text{-}2.3\times10^6$ cells/ml, respectively. To assess the effect of bacteriophage-loaded chitosan microspheres on preventing *L. plantarum* contamination, prepared microspheres were added into the *S. cerevisiae* and *L. plantarum* co-cultivation system. Moreover, free bacteriophage particles and pure chitosan microspheres without bacteriophage were also respectively considered as positive control and negative control and added into the co-cultivation system. So, five groups, that is control group A (only *S. cerevisiae* in YPD broth), contamination group B (*S. cerevisiae* and *L. plantarum* were co-cultured in YPD broth), negative control group C (*S. cerevisiae*, *L. plantarum* and pure chitosan microspheres without bacteriophage were co-cultured in YPD broth), bacteriophage-loaded chitosan microspheres treatment group D (*S. cerevisiae*, *L. plantarum* and bacteriophage-loaded chitosan microsphere were co-cultured in YPD broth), and positive control

group E (*S. cerevisiae*, *L. plantarum* and bacteriophage were co-cultured in YPD broth), were cultivated at 30°C and shaken at 170 rpm. Amount of ethanol in the culture broth was measured using gas chromatography (GC) (Shimadzu Corporation, Kyoto, Japan). The experiment was conducted in triplicate.

Statistical analysis

Data were analyzed by SPSS13.0 for Windows. Differences showing *P*-values less than 0.05 were considered statistically significant.

Results and discussion

FTIR spectroscopic analysis

FTIR spectroscopic analysis was performed to identify the possible functional groups of chitosan involved in bacteriophage-loaded chitosan microspheres. The FTIR spectra of pure chitosan and bacteriophage-loaded chitosan microspheres were shown in Fig. 2. It could be seen that bacteriophage-loaded chitosan microspheres demonstrated a similar FTIR spectrum to that of pure chitosan. The characteristic absorption bands at 3434.71 cm^{-1} and 3413 cm^{-1} could be referred to the stretching vibration of -OH group present in chitosan. The bands observed at 2922.98 cm^{-1}, 2863.47 cm^{-1}, 2922.98 cm^{-1}, 2860.50 cm^{-1} were due to the $-CH_2$ stretching vibration of chitosan. And the absorption bands at 1652.56 cm^{-1} and 1649.59 cm^{-1} were attributed to the C=N stretching vibration of chitosan (Fig. 2).

Compared to pure chitosan microspheres, bacteriophage-loaded chitosan microspheres had higher absorption intensity at 2922.98 cm^{-1}, 2860.50 cm^{-1}, and 1649.59 cm^{-1} (Fig. 2). The higher

absorption intensity of the -CH_2 group and C=N double bond was related to the glutaraldehyde cross-linking reaction. For bacteriophage-loaded chitosan microspheres, the FTIR spectrum confirmed considerable changes for the glutaraldehyde based on the shape and frequencies of the bands, indicating chitosan microspheres were successfully prepared by Schiff base reaction. The following swelling experiment was performed to further verify the success of bacteriophage package.

Fig. 2

SEM observation

Both pure chitosan microspheres without bacteriophage and bacteriophage-loaded chitosan microspheres were spherical with a smooth surface (Fig. 3a and b). Bacteriophage-loaded chitosan microspheres were slightly smaller than pure chitosan microspheres without bacteriophage. Diameters of most microspheres without bacteriophage ranged from 50 μm to 150 μm (Fig. 3a). While diameters of most bacteriophage-loaded chitosan microspheres were about 25 μm, and there were still some small microspheres with diameter less than 5 μm adhered to each other (Fig. 3b).

Fig. 3

Size distribution of SPG membrane-homogenized chitosan microspheres

Size distribution of SPG membrane-homogenized chitosan microspheres and average particle diameter were measured by particle size analyzer. The D (0.1), D (0.5) and D (0.9) value of pure chitosan microspheres without bacteriophage was 23.454 μm, 34.784 μm, and 60.923 μm, respectively (Fig. 4a). The small d value (i.e., 1.077) indicated the narrow range of size

distribution of pure chitosan microspheres without bacteriophage. Moreover, surface area average particle diameter D [3, 2] was 32.715 μm with a little specific surface area (i.e., 264 m^2/Kg). The uniformity value (i.e., 0.569) was good and consistent with the normal distribution (Fig. 4a), which indicated that the uniformity of the prepared pure chitosan microspheres without bacteriophage can be optimized by SPG membrane emulsification cross-linking approach. Homogenized chitosan microspheres have more advantages in the application in comparison to the non-homogenized microspheres. For example, homogenized microspheres can not only improve the utilization of wrapped substances, but also facilitate the study of the relationship between particle size and effect of the wrapped substances.

The result of bacteriophage-loaded chitosan microspheres showed that the D (0.1), D (0.5) and D (0.9) was 10.536 μm, 29.482 μm, and 69.952 μm, respectively (Fig. 4b). The small d value (i.e., 2.015) indicated the narrow range of the size distribution of bacteriophage-loaded chitosan microspheres. Therefore, the homogenized bacteriophage-loaded microspheres might be more effective for controlling of bacterial contamination in bioethanol fermentation. Moreover, surface area average particle diameter D [3, 2] was 19.812 μm with a little specific surface area (i.e., 303 m^2/Kg). The uniformity value (i.e., 0.688) was not very good, but also was in accord with the normal distribution (Fig. 4b), which indicated that the uniformity of the prepared bacteriophage-loaded chitosan microspheres could also be optimized by SPG membrane emulsification cross-linking approach.

Fig. 4

Effect of pH on the release of bacteriophage from the prepared microspheres

Lactic acid secreted by contaminative LAB in bioethanol fermentation can result in the decrease of pH value.[16,44] Moreover, the pH value varied within the range of pH 4 and 6 in the presence of LAB contamination during the bioethanol fermentation. Therefore, pH values of 4 and 6 were selected for swelling of chitosan microspheres to evaluate the release of bacteriophage from the prepared microspheres.

Before swelling, no plaques were detected on the plate (Fig. 5a and b). This indicated that the structure of the prepared microspheres were structurally-complete and would not be broken to release bacteriophage without being exposed to soaking in acidic solutions (i.e., pH 4, pH 6). After swelling in different pH value solutions (i.e., pH 4, pH 6) for 3 h, plaques could be detected on the double layer agar plate (arrow in red circle in Fig. 5c and d). Due to the low degree of cross-linking, a large number of amino residues existed in the microsphere network structure. Under acidic conditions (i.e., pH 4 or 6), amino groups were protonated, and hydrogen bonds and electrostatic attraction could be damaged. Under such situation, the increased osmotic pressure between the inside gel and the outside solution would lead to the microspheres network structure swollen and broken with the release of enwrapped bacteriophage. Such result further confirmed the successful wrap of bacteriophage into the prepared chitosan microspheres. Sustained breakdown of chitosan microspheres also provided the basis for sustained release of bacteriophage.

Moreover, more plaque number was detected after being soaked in pH 4 solution in comparison to that being soaked in pH 6 solution. Based on the number of plaque formed by released bacteriophage from prepared microspheres after being soaked in different pH value

solutions (i.e., pH 4, pH 6), the specific amount of bacteriophage loaded onto chitosans was estimated. After being soaked in pH 4 or 6 solution for 3 h, the encapsulation rate of bacteriophage was respectively estimated as about 16.7% and 5.6%. Therefore, no less than 16% of the added bacteriophage could be successfully enwrapped into the prepared microspheres. As the pH value decreases gradually from pH 6 to 4 in the batch bioethanol fermentation process, above result also indicated that the prepared microspheres would be consistently broken and provide the opportunity for persistent release of bacteriophage and controlling of bacterial contamination. Moreover, the result indicated that the bacteriophage could be released from the prepared bacteriophage-loaded chitosan microspheres in response to changed pH value. More released bacteriophage particles in response to pH 4 than that to pH 6 also provided an opportunity for the preparation of pH responsive microspheres to realize the intelligent controlling of bacterial contamination in the future.

Fig. 5

Effect of bacteriophage-loaded chitosan microspheres treatment on *L. plantarum* contamination during the bioethanol fermentation

To mimic the bacterial contamination at the laboratory scale, *L. plantarum* was co-cultivated with *S. cerevisiae* in YPD broth, and bacteriophage-loaded chitosan microspheres were added to evaluate the treatment effect. In the contamination group (mixed co-culture of *S. cerevisiae* and *L. plantarum*), the presence of *L. plantarum* inhibited the ethanol production ($P<0.01$) (Fig. 6). Moreover, the addition of pure chitosan microspheres without bacteriophage did not restore the ethanol content to the level in the control group. However, the addition of bacteriophage or

bacteriophage-loaded chitosan microspheres reversed the inhibition of *L. plantarum* on the ethanol production ($P<0.01$) (Fig. 6). Moreover, compared with the control group with only *S. cerevisiae* cultured in YPD broth, the final ethanol production in the bacteriophage or bacteriophage-loaded chitosan microspheres treatment group did not decrease ($P>0.05$) (Fig. 6). These results demonstrated that bacteriophage-loaded chitosan microspheres could be efficient for controlling of *L. plantarum* contamination in the fermentation of *S. cerevisiae*.

Indifference of ethanol content between bacteriophage and bacteriophage-loaded chitosan microspheres treatment groups ($P>0.05$) also demonstrated that chitosan itself had no side effect on bioethanol fermentation performed by *S. cerevisiae*. The excellent biodegradability and biocompatibility of chitosan would also contribute to the biosafety of bacteriophage-loaded chitosan microspheres for controlling the bacterial contamination occurred during fermentation and would facilitate the potential application of bacteriophage in the future.[45-48] Moreover, compared to the direct addition of bacteriophage, sustained release of bacteriophage from bacteriophage-loaded microspheres would enhance the effect of bacteriophage through prolonging the action time; and potential pH responsive release of bacteriophage from the microspheres would decrease the necessity of fermentation process real-time monitoring and facilitate realizing of intelligent controlling of bacterial contamination in the future.

Conclusions

In summary, bacteriophage that is specific for *L.* p*lantarum* ATCC 8014 was successfully enwrapped into chitosan microspheres. The prepared bacteriophage-loaded chitosan microspheres

could effectively control the *L. plantarum* contamination in the bioethanol fermentation of *S. cerevisiae*. Moreover, sustained release of bacteriophage from microspheres would enhance the effect of bacteriophage through prolonging the action time; and potential pH responsive release of bacteriophage would facilitate realizing of intelligent controlling of bacterial contamination in the future.

Acknowledgments

This work was supported by National Nature Science Foundation of China (No. 31201413) and the Fundamental Research Funds for the Central Universities (No. YS1407).

Notes and references

1. T. Prior, D. Giurco and G. Mudd, *Global Environ. Chang*., 2012, **22**, 577-587.
2. M. Höök and X. Tang, *Energy Policy*, 2013, **52**, 797-809.
3. I. Dincer, *Renew. Sust. Energy Rev*., 2000, **4**, 157-175.
4. N. L. Panwar, S. C. Kaushik and S. Kothari, *Renew. Sust. Energy Rev*., 2011, **15**, 1513-1524.
5. K. A. Gray, L. Zhao and M. Emptage, *Curr. Opin. Chem. Biol.*, 2006, **10**, 141-146.
6. N. Sarkar, S. K. Ghosh, S. Bannerjee and K. Aikat, *Renew. Energ.*, 2012, **37**, 19-27.
7. Li. K, Liu. S and X. Liu, *Int. J. Energy. Res.*, 2014, **38**, 965-977.
8. M. Hoel and S. Kverndokk, *Resource and energy economics*, 1996, **18**, 115-136.
9. G. P. Peters, R. M. Andrew, T. Boden, J. G. Canadell, P. Ciais, C. L. Quéré, G. Marland, M. R. Raupach and C. Wilson, *Nat. Clim. Change.*, 2013, **3**, 4-6.

10. H. Widiastuti, J. Y. Kim, S. Selvarasu, I. A. Karimi, H. Kim, J. S. Seo and D. Y. Lee, *Biotechnol. Bioeng.*, 2011, **108**, 655-65.

11. M. Beckner, M. L. Ivey and T. G. Phister, *Lett. Appl. Microbiol.*, 2011, **53**, 387-394.

12. N. V. Narendranath, S. H. Hynes, K. C. Thomas and W. M. Ingledew, *Appl. Environ. Microbiol.*, 1997, **63**, 4158-4163.

13. K. A. Skinner and T. D. Leathers, *J. Ind. Microbiol. Biotechnol.*, 2004, **31**, 401-408.

14. M. Balat, *Energy Convers. Manage.* 2011, **52**, 858-875.

15. Y. Katakura, C. Moukamnerd, S. Harashima, and M. Kino-oka, *J. Biosci. Bioeng.*, 2011, **111**, 343-5.

16. N. V. Narendranath and R. Power, *Appl. Environ. Microbiol.*, 2005, **71**, 2239-2243.

17. K. C. Thomas, S. H. Hynes and W. M. Ingledew, *J. Appl. Microbiolo.*, 2001, **90**, 819-828.

18. N. V. Narendranath, K. C. Thomas and W. M. Ingledew, *Appl. Environ. Microbiol.*, 2000, **66**, 4187-4192.

19. Y. G. Zhu, T. A. Johnson, J. Q. Su, M. Qiao, G. X. Guo, R. D. Stedtfeld, S. A. Hashsham and J. M. Tiedje, *P. Natl. Acad. Sci. USA*, 2013, **110**, 3435-3440.

20. W. H. Day, W. C. Serjak, J. R. Stratton and L. Stone, *J. Agr. Food Chem.*, 1954, **2**, 252-258.

21. A. Muthaiyan, A. Limayem and S. C. Ricke, *Prog. Energy Combust. Sci.*, 2011, **37**, 351-370.

22. I. Watanabe, T. Nakamura and J. Shima, *J. Ind. Microbiol. Biotechnol.*, 2008, **35**, 1117-1122.

23. C. A. Spencer, 2014.

24. J. B. Silva and D. Sauvageau, Doctoral dissertation, University of Alberta, 2013.

25. S. Hagens and M. J. Loessner, *Appl. Microbiol. Biotechnol.*, 2007, **76**, 513-519

26. P. A. Khatibi, D. R. Roach, D. M. Donovan, S. R. Hughes and K. M. Bischoff, *Biotechnol. Biofuels*, 2014, **7**, 104.

27. M. D. S. Franzin and M. R. Prioli, U.S. Patent 8,859,743., 2014-10-14.

28. S. Adhya and C. Merril, *Nature*, 2006, **443**, 754-755.

29. J. N. Housby and N. H. Mann, *c*, 2009, **14**, 536-540.

30. E. Kutter, D. De Vos, G. Gvasalia, Z. Alavidze, L. Gogokhia, S. Kuhl and S. T. Abedon, *Curr. Pharm. Biotechno.*, 2010, **11**, 69-86.

31. E, B, Solomon and D. Okull, U.S. Patent Application 12/245,272. 2008-10-3.

32. E. J. Summer and M. Liu, U.S. Patent Application 13/465,700. 2012-5-7.

33. D. R. Roach, P. A. Khatibi, K. M. Bischoff, S. R. Hughes and D. M. Donovan, *Biotechnol. Biofuels*, 2013, **6**, 20.

34. Y. Qiu and K. Park, *Adv. Drug Deliver. Rev.*, 2012, **64**, 49-60.

35. P. Mukhopadhyay, K. Sarkar, S. Bhattacharya, A. Bhattacharyya, R. Mishra and P. P. Kundu, *Carbohyd. Polym.*, 2014, **112**, 627-637.

36. A. Chang, *Irab. Polym. J.*, 2015, **24**, 161-169.

37. L. Li, R. Ni, Y. Shao, S. Mao, *Carbohyd. Polym.*, 2014, **103**, 1-11.

38. F.N. Allouche, E. Guibal and N. Mameri, *Colloids Surf., A*, 2014, **446**, 224–232

39. J. L. Wang and C. Chen, *Bioresource Technol.*, 2014, **160**, 129-141.

40. I. Salcedo, C. Aguzzi, G. Sandri, M. C. Bonferoni, M. Mori, P. Cerezo, R. Sánchez, C. Viseras and C. Caramella, *Appl. Clay Sci.*, 2012, **55**, 131–137.

41. W. Wei, G. H. Ma, L. Y. Wang, J. Wu and Z. G. Su, *Acta biomater.*, 2010, **6**, 205-209.

42. M. H. Adams, *Bacteriophages. Interscience Publishers, NY*, 1959: 27-30.

43. S. Jose, J. F. Fangueiro, J. Smitha, T. A. Cinu, A. J. Chacko, K. Premaletha and E. B. Souto, *Colloids Surf., B*, 2012, **92**, 175-179.

44. X.Wu, S. Staggenborg, J. L. Propheter, W. L. Rooney, J. Yu and D. Wang, *Ind. Crop. Prod.*, 2010, **31**, 164-170.

45. P. Baldrick, *Regul. Toxicol. Pharm.*, 2010, **56**, 290-299.

46. M. Dash, F. Chiellini, R. M. Ottenbrite and E. Chiellini, *Prog. Polym. Sci.*, 2011, 36, 981-1014.

47. S. Rodrigues, M. Dionísio, C. R. López and A. Grenha, *J. Funct. Biomater.* 2012, **3**, 615-641.

48. J. H. Park, G. Saravanakumar, K. Kim and I. C. Kwon, *Adv. Drug Deliver. Rev.*, 2010, **62**, 28-41.

Figure Captions

Fig. 1. Preparation procedure of bacteriophage-loaded chitosan microsphere. (a) Membrane emulsification device schematic diagram; (b) membrane emulsification membrane tube schematic diagram.

Fig. 2. FTIR spectra of (a) pure chitosan and (b) bacteriophage-loaded chitosan microspheres.

Fig. 3. SEM of (a) pure chitosan microspheres without bacteriophage, scale bar = 50 μm, and (b) bacteriophage-loaded chitosan microspheres, scale bar = 5 μm.

Fig. 4. Microspheres particle size distribution (SPG membrane emulsification) of (a) pure chitosan microspheres without bacteriophage, and (b) bacteriophage-loaded chitosan microspheres.

Fig. 5. Effect of pH on the release of bacteriophage from microspheres. (a and b) Before swelling; (c and d) after swelling; (a and c) pH 4; (b and d) pH 6.

Fig. 6. Effect of bacteriophage-loaded chitosan microspheres treatment on *Lactobacillus plantarum* contamination during the culture of *Saccharomyces cerevisiae*.

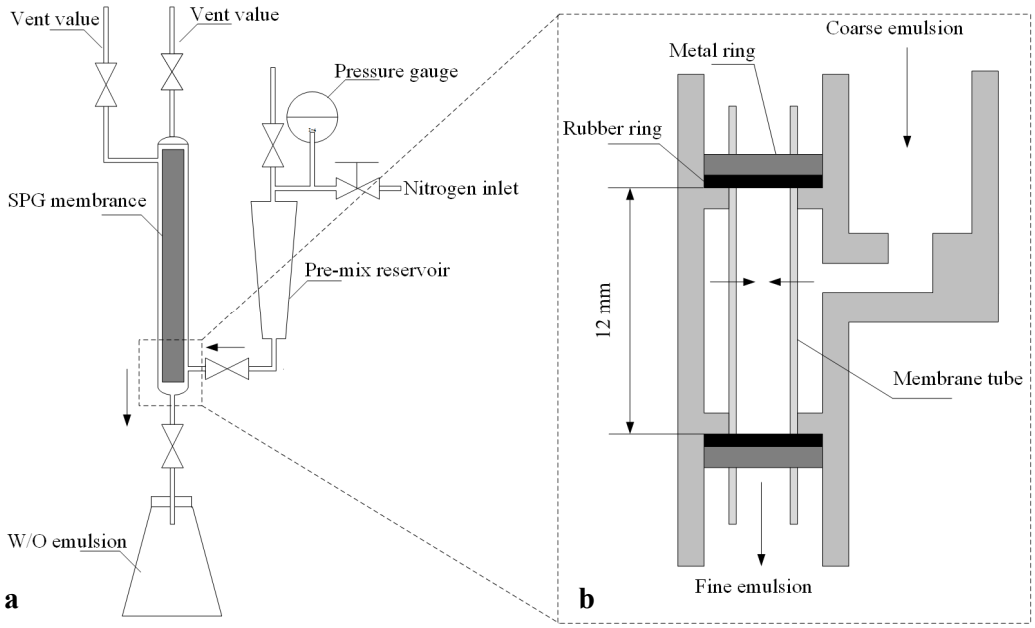

Fig. 1

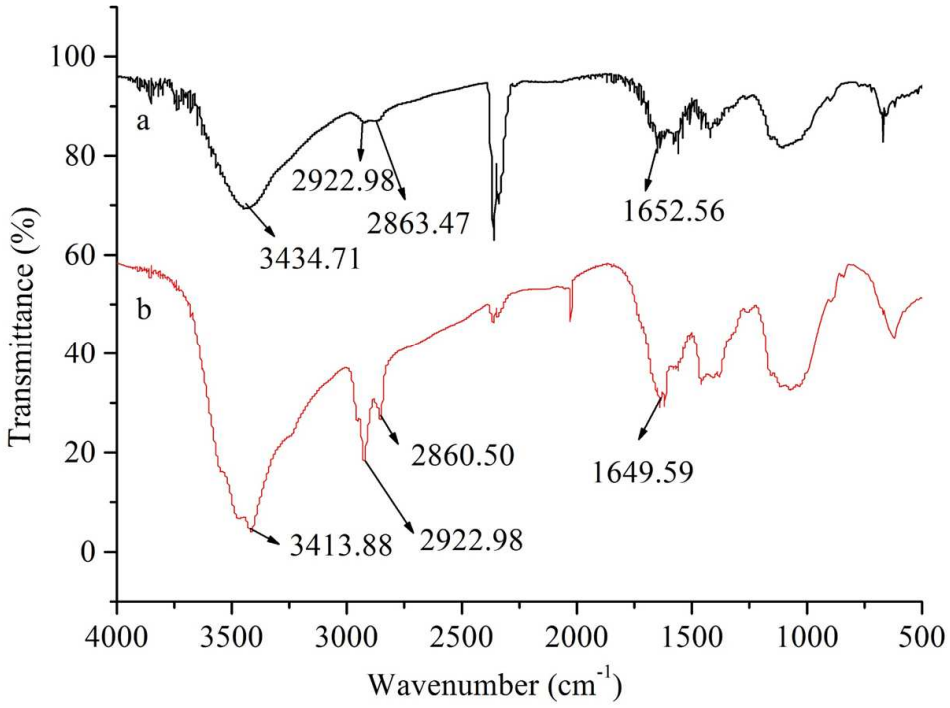

Fig. 2

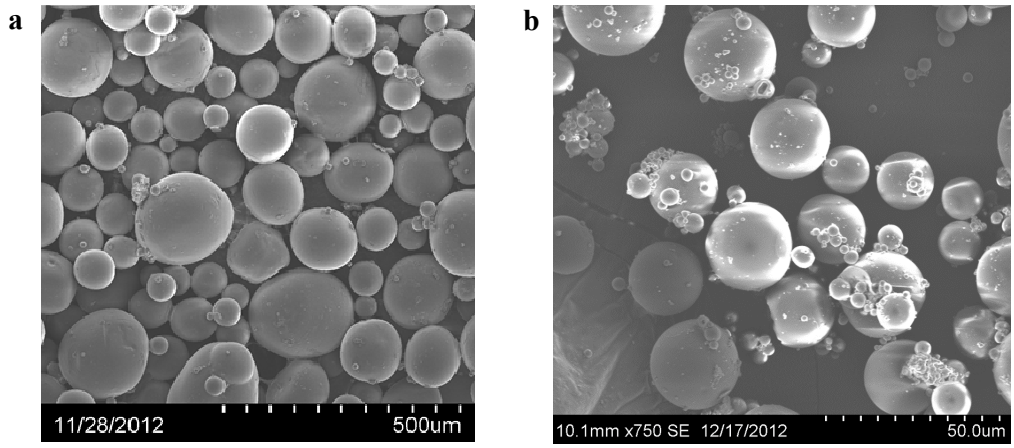

Fig. 3

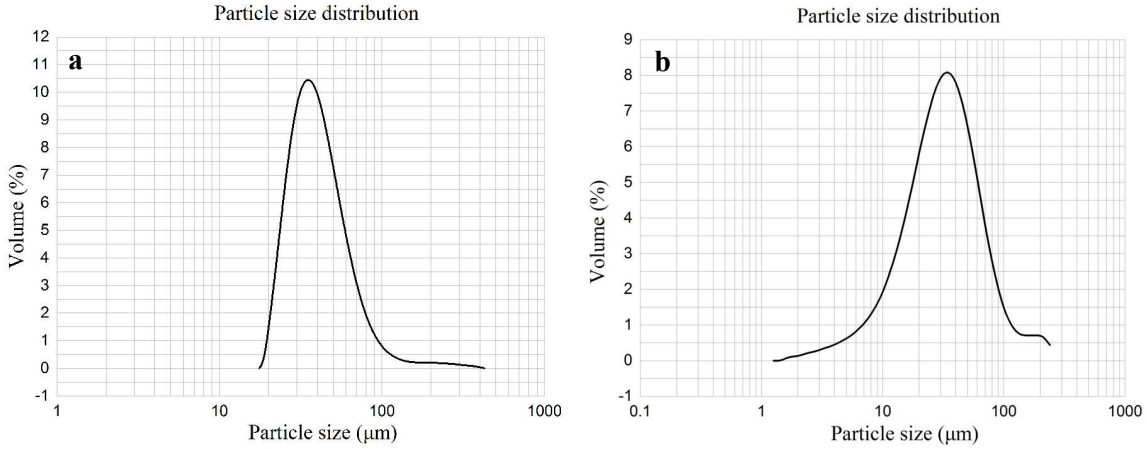

Fig. 4

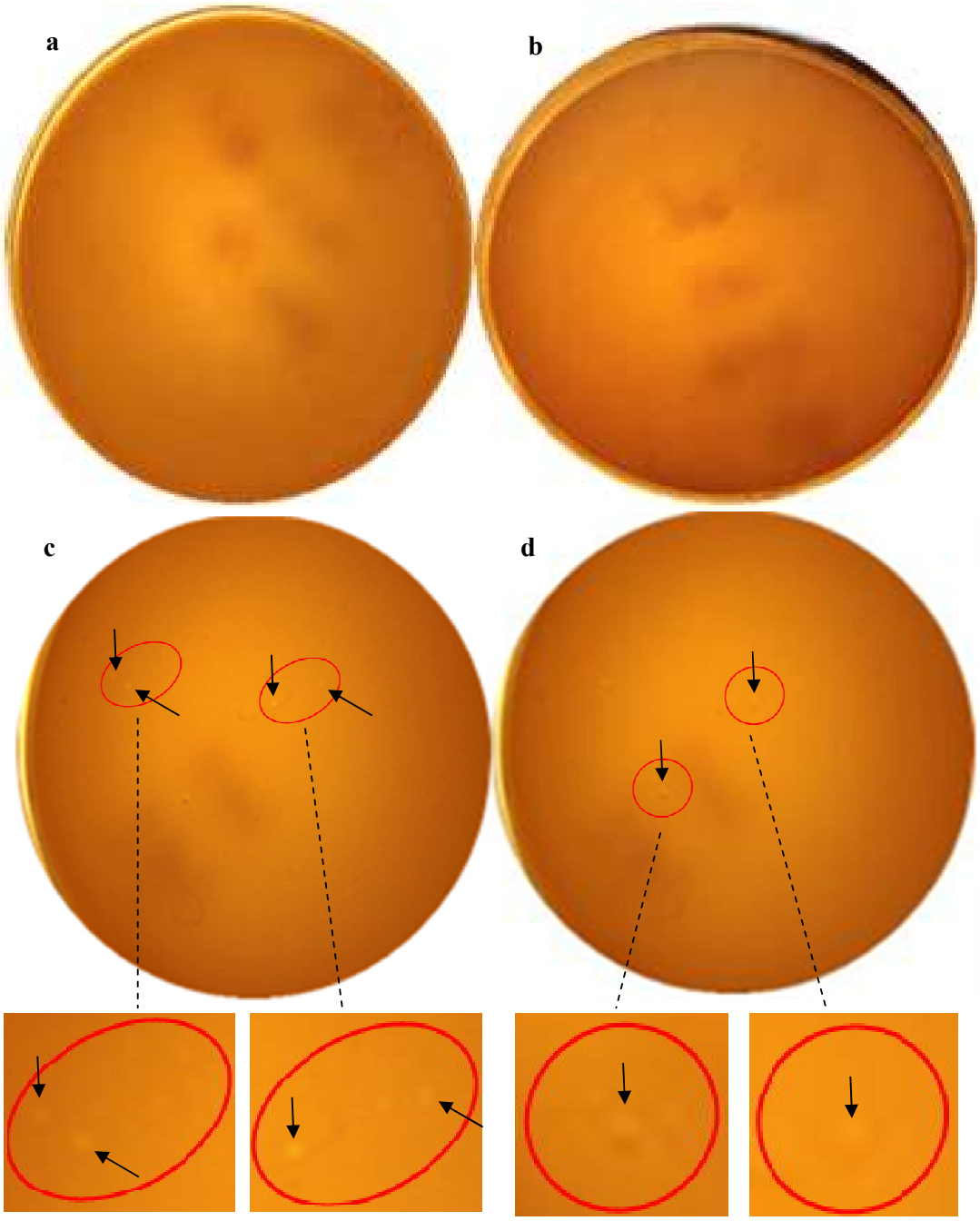

Fig. 5

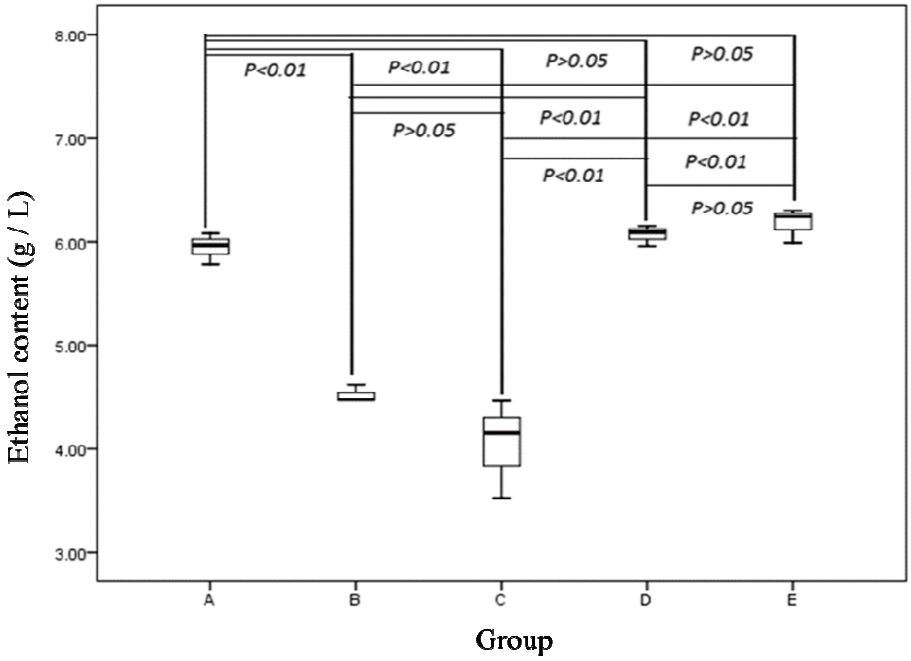

Fig. 6

Graphical Abstract

Bacteriophage that is specific for *Lactobacillus plantarum* ATCC 8014 was successfully enwrapped into chitosan microspheres. The prepared bacteriophage-loaded chitosan microspheres can effectively control *L. plantarum* contamination in bioethanol fermentation. Moreover, sustained release of bacteriophage from microspheres would enhance the effect of bacteriophage through prolonging the action time; and potential pH responsive release of bacteriophage would facilitate realizing of intelligent controlling of bacterial contamination in the future.