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Enhanced curdlan production with nitrogen feeding during polysaccharide synthesis by *Rhizobium radiobacter*

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ABSTRACT

Curdlan is a secondary metabolite synthesized by *Agrobacterium* sp. and some other bacteria. A newly isolated exopolysaccharide-producing strain was identified to be *Rhizobium radiobacter* CGMCC 12099. The polysaccharide product was confirmed to be curdlan with a molecule weight of 1.4×10^5 Da, and its molecular structure was determined by HPLC and infrared spectrum. Although nitrogen source is necessary for cell reproduction, curdlan production is largely dependent on nitrogen limitation, as well as cell vitality. Here, a nitrogen feeding strategy was investigated to elevate the curdlan production by *R. radiobacter*. The optimal concentration and addition time of $(NH_4)_2HPO_4$ were investigated. The results showed that the enhanced cell density was correlated to the amount of $(NH_4)_2HPO_4$ added. Also, nitrogen addition in earlier fermentation stage was beneficial to the cell growth and curdlan production. Furthermore, continuously feeding strategy was employed by feeding $(NH_4)_2HPO_4$ at a constant rate of 1.24 g/h at 35th h of fermentation for 9 h, achieving a final curdlan production of 65.27 g/L, productivity of 0.544 g/L/h and glucose conversion rate of 38.89%. The curdlan production was improved by 2.1 times compared with that without nitrogen addition. This study provides a feasible and cheap nitrogen feeding strategy to enhance curdlan production.

1. Introduction

Curdlan is an unbranched extracellular polysaccharide composed only of β-(1,3) glucose residue, and is produced by *Alcaligenes faecalis* var. *myxogenes* (Harada, 1965; Phillips et al., 1983) and some other bacteria such as *Rhizobium trifolii* (Ghai, Hisamatsu, Amemura, & Harada, 1981) and *Agrobacterium radiobacter* (Nakanishi et al., 1976). Curdlan was unexpectedly isolated by Harada et al. in 1965, and was named as curdlan for its unique thermo-gelling properties (Harada, 1965; Harada, Masada, Fujimori, & Maeda, 1966). When heated to 55 °C, the curdlan could form a weak gel after cooling down, while when heated to 80–120 °C, a firm gel could be formed irreversibly even upon further heating (Laroche & Michaud, 2007; Zhang, Nishinari, Williams, Foster, & Norton, 2002). Curdlan is insoluble in water, acid solutions, and ethyleneglycol, while easily soluble in alkali, formic acid and dimethyl sulfoxide (Harada, Misaki, & Saito, 1968). In recent studies, the water-soluble curdlan derivatives was obtained by introducing ionic groups on saccharide chain (Suflet, Popescu, Pelin, Nicolescu, & Hitruc, 2015; Zhang & Edgar, 2014). Those properties endow curdlan with wider application in food and pharmaceutical industry (Lehtovaara & Gu, 2011; Nakao et al., 1991; Spicer, Goldenthal, & Ikeda, 1999; Zhang, Liu, & Edgar, 2016).

It has been reported that curdlan could be synthesized by *Agrobacterium* sp. and some other bacteria under nitrogen limited conditions during the late stationary phase of fermentation. A numbers of efforts have been attempted to improve the curdlan production since its discovery. A nitrogen-limited continuous fermentation was conducted by starting with nitrogen sufficient cell growth phase, followed by a nitrogen-free feeding during post-stationary phase (Phillips et al., 1983). A two-step fed-batch fermentation using molasses as cheap substrate was reported for curdlan production, which consisted of cell growth phase and nitrogen-limited curdlan producing phase, and the total production reached 42 g/L after 120 h (Lee et al., 1997). Moreover, many other strategies have been studied to improve curdlan production, such as pH, dissolved oxygen, and addition of uracil and phosphates (Jin & Lee, 2014; Lee & Lee, 2001; Lee, Lee, Kim, & Park, 1999; Yu et al., 2011a; Zhang et al., 2012). It has also been reported that the curdlan production could be enhanced by addition of Tween 80 at a low concentration (Xia, 2013).

Curdlan is a typical secondary metabolite, which occurs in the stationary phase (Phillips et al., 1983), and the biomass is regarded as one of the key factors that affect curdlan production.

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It has been proved that the increase of initial nitrogen concentration could promote cell growth, which resulted in high biomass and curdlan production (Wu, Zhan, Liu, & Zheng, 2008). Furthermore, two fermenters were applied to satisfy different demands for carbon/nitrogen sources ratio during cell growth and curdlan synthesis phases (Zheng et al., 2014). Curdlan could only be produced in nitrogen-limited culture (Phillips et al., 1983), indicating nitrogen depletion is a signal of curdlan synthesis. NtrC is a global regulator (Prival & Magasanik, 1971) of bacterial nitrogen metabolism system. A *ntrC* mutant of *Agrobacterium* sp. was found to be incapable of utilizing NH_4Cl and KNO_3 as nitrogen sources, and therefore resulted in a low curdlan production (Yu, Wu, Zheng, Zhan, & Lin, 2011). Further study confirmed the expression level of both curdlan biosynthesis and nitrogen metabolism related genes were significantly up-regulated under nitrogen limitation (Yu, Wu, Zheng, Lin, & Zhan, 2011).

In this study, we attempted to improve the curdlan production by enhancing the biomass and cell vitality using a newly isolated *Rhizobium radiobacter* CGMCC12099. A novel strategy was presumed to boost the cell density and vitality by feeding nitrogen sources during curdlan synthesis instead of cell growth phase. A diauxie cell growth profile was observed under this nitrogen feeding strategy, as well as a dramatically enhanced curdlan production.

2. Materials and methods

2.1. Microorganism and culture conditions

A curdlan-producing strain was isolated from moist soil in local countryside (Wuxi, China) using an aniline blue dyeing method (Nakanishi et al., 1976). The strain was identified as *Rhizobium radiobacter* and deposited at China General Microbiological Culture Collection Center as CGMCC12099. The seed medium consisted of (in g/L): glucose 40, yeast extract 5, peptone 5, $(NH_4)_2HPO_4$ 2, KH_2PO_4 1.5, $MgSO_4$ 0.75, the pH was adjusted to 7.0. And the agar medium is the same as seed medium except addition of 20 g/L agar. One loop of cells from agar slant was inoculated into a 250-mL Erlenmeyer flask containing 30 mL seed medium. Then, the culture was shaken at 200 rpm and 30 °C as the 1st seed culture. After 18 h of incubation, 6 mL broth was transferred into a 500-mL Erlenmeyer flask containing 60 mL seed medium as the 2nd seed culture which was cultured under the same condition mentioned above. The curdlan fermentation was carried out using fermentation medium consisted of (in g/L): glucose 50, $(NH_4)_2HPO_4$ 2, KH_2PO_4 1.5, $MgSO_4$ 0.75 and 10 mL of trace element solution (5 g $FeSO_4{\cdot}7H_2O$, 2 g $MnSO_4$, 1 g $CoCl_2{\cdot}6H_2O$, 1 g $ZnCl_2$ per liter of distilled water). The medium was sterilized under 121 °C for 15 min. The fermentation was carried out at 30 °C with 10% inoculum of 2nd seed culture.

2.2. Characterization of the polysaccharide product

2.2.1. Aniline blue staining

Aniline blue solution was added into agar medium at 0.05 g/L, and then 1st seed culture cultivated as mentioned above was streaked on agar plate containing aniline dye. The plate was incubated at 30 °C for 3 days, and the stained colonies were compared with earlier reports (Nakanishi et al., 1976).

2.2.2. Thermal gel formation property

Lyophilized polysaccharide powder was homogenized in deionized water to a final concentration of 20 g/L, and then 10 mL curdlan aqueous solution was transferred into a glass tube (18 mm × 180 mm). The tube was boiled in water bath for 10 min, and was cooled down for another 30 min to ensure the gel formation. The gel sample was prepared by cutting a 10 mm-length section from the bottom of the tube for gel strength measurement using texture analyzer (TA-XT2i, Stable Micro System, UK) equipped with a P/0.5 probe.

2.2.3. Chemical composition

Lyophilized polysaccharide powder was hydrolyzed with 1 mol/L H_2SO_4 at 10 g/L. The samples were pre-hydrolyzed under 120 °C for 1 h, and the supernatant was further hydrolyzed for 1 h at 120 °C. After hydrolysis, excessive barium carbonate was added to neutralize H_2SO_4, and the precipitation of $BaSO_4$ was removed by centrifuging at 8000*g* for 10 min. The composition of the hydrolysate was determined by Agilent 1260 Infinity HPLC system (CA, USA) using water as mobile phase at a flow rate of 0.50 mL/min. Temperature of the column was 65 °C, and the injection volume was 10 μL.

2.2.4. Molecular weight

The molecular weight (M_w) of the polysaccharide was determined by high performance gel permeation chromatography (HPGPC) using a HPLC (Waters 501, Miliford, MA) equipped with a differential refractometer (Waters 410), a data module (Waters 746) and a gel permeation column Superose 12 (10 mm × 300 mm). The polysaccharide sample (5 mg/mL) dissolved in 0.5 mol/L NaOH was injected at a volume of 20 μL using 0.5 mol/L sodium hydroxide as mobile phase at a flow rate of 1 mL/min. Dextran (M_w; 15,000–20,000, 60,000–90,000, 143,000, 580,000 and 2,000,000) purchased from Sigma was used as standard samples.

2.2.5. Infrared spectrum

Infrared spectrum was determined on a Fourier transform infrared spectrometer FT/IR- IS10 Nicolet (Thermo, MA, USA) using a potassium bromide (KBr) discs method from 450 to 4000 cm^{-1} at a resolution of 4 cm^{-1}. The values obtained for the polysaccharide sample of this study was compared with commercial curdlan sample purchased from Sigma (CA, USA).

2.3. Curdlan production with different initial biomass

Curdlan fermentation in shake flask was carried out in 500 mL Erlenmeyer flask containing 60 mL fermentation medium. To ensure the reproducibility of each test, certain volume of seed culture was centrifuged at 4000*g* for 5 min, and the cell pellet was inoculated into fermentation culture to meet the requirement for different initial biomass. The flasks were incubated at 30 °C and 200 rpm for 60 h.

2.4. Fed-batch fermentation with ammonium salts addition

The curdlan fermentation was conducted in 3 L stirred tank bio-reactors (Biotech-3BG, Shanghai, China) filled with 2 L of fermentation medium. The 2nd seed culture (200 mL) was inoculated into the curdlan fermentation medium, and the cultivation temperature was 30 °C. Two-stage pH control strategy was conducted as previously described (Lee & Park, 2001). The pH was maintained at 7.0 during the cell growth phase and 5.5 during curdlan producing phase with 2 mol/L NaOH and 2 mol/L H_2SO_4. The dissolved oxygen (DO) was maintained at 30–40% by aeration rate and agitation speed regulation. Concentrated $(NH_4)_2HPO_4$ was added as the nitrogen source during curdlan synthesis stage by peristaltic pump (Longerpump-YZ1515x, Baoding, China). To provide sufficient glucose for curdlan synthesis and cells reproduction, concentrated glucose (500 g/L) was added when residue glucose dropped below 10 g/L.

2.5. Analytical methods

Five milliliters sample was taken from bio-reactor at each time point and centrifuged at 6000*g* for 10 min, and then the supernatant was subjected to analysis of residual glucose and $(NH_4)_2HPO_4$. Ten milliliter of 0.5 mol/L NaOH was added to the precipitate, which was further incubated at 30 °C and 200 rpm for 1 h until curdlan in the precipitate was fully dissolved. Subsequently, the mixture was centrifuged at 6000*g* for 15 min to separate the cell pellets from curdlan alkali solution. The cell pellets was collected and washed with deionized water three times, and then dried to a constant weight at 85 °C to measure the dry cell weight. For the alkali curdlan solution, 3 mol/L HCl was added to precipitate the curdlan, and the final pH of curdlan solution was adjusted to 6.5–7.0. Finally, curdlan was washed with deionized water for three times, and dried to a constant weight at 85 °C. Some fermentation parameters were calculated as follows.

$$\text{Curdlan specific yield (g/g)} = \frac{\text{curdlan production (g/L)}}{\text{drycell weight (g/L)}} \quad (1)$$

$$\text{Curdlan productivity(g/L/h)} = \frac{\text{curdlan production (g/L)}}{\text{fermentation time (h)}} \quad (2)$$

$$\text{Glucoseconversion rate(\%)} = \frac{\text{curdlan prodution (g/L)}}{\text{totalglucose consumption (g/L)}} \times 100\% \quad (3)$$

$$Y_{\text{biomass/NH4}^+}\text{(g/g)} = \frac{w_2 - w_1}{\Delta NH_4^+} \quad (4)$$

In formula (4), w_1 and w_2 are biomass (cell dry weight) measured before and after $(NH_4)_2HPO_4$ addition, and ΔNH_4^+ is the concentration of total $(NH_4)_2HPO_4$ feeding.

The residual glucose was determined by dinitrosalicylic acid method (Miller, 1959), and the $(NH_4)_2HPO_4$ was measured by a modified sodium hypochlorite-salicylic acid spectrophotometry method (Harwood & Huyser, 1970). The pH and DO in stirred tank bio-reactor were detected by in situ pH probe and electrochemical oxygen probe (Mettle Toledo, Switzerland).

3. Results and discussion

3.1. Characterization of the polysaccharide product

To identify the polysaccharide produced by *Rhizobium radiobacter* CGMCC12099, various physical and chemical properties were determined, including aniline blue staining, thermal gel formation, chemical composition, molecular weight and infrared spectrum. Different from other polysaccharides, curdlan could be stained by aniline blue solution and present a clearly blue color. In this study, the polysacchride produced by *R. radiobacter* showed a positive aniline blue stain, indicating the polysacchride was composed largely or entirely of β-1,3-glucosidic bond, a typical characteristic of curdlan and curdlan-type polysaccharide (Harada, Misaki, & Saito, 1968; Nakanishi et al., 1976). Furthermore, when heated at 100 °C for 10 min, aqueous polysacchride suspension formed a firm gel, suggesting the obtained polysacchride was curdlan, and the gel strength was determined to be 589 g/cm^2 which met National Food Safety Standard of China. The monosaccharide composition analysis confirmed that the obtained polysacchride was exclusively composed of glucose as the hydrolysates exhibited a single glucose peak in HPLC analysis. The average molecular weight (M_w) of the polysacchride was determined to be around 1.4×10^5 Da by HPGPC, which was similar to the reported M_w range of curdlan from

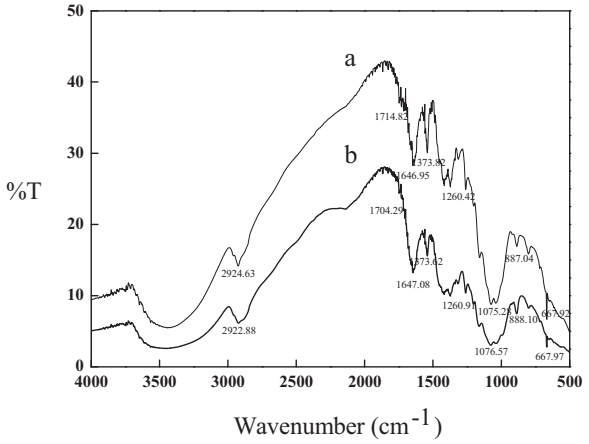

Fig. 1. FT/IR spectra of the polysaccharide produced by *R. radiobacter* CGMCC12099 (a) and the commercial curdlan sample (b).

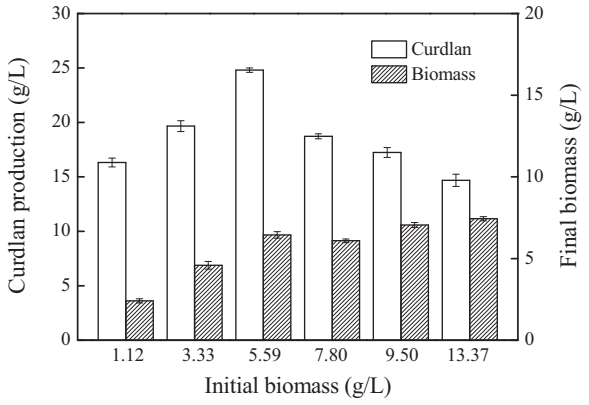

Fig. 2. Curdlan production and final biomass by *R. radiobacter* CGMCC12099 in shake flasks at different initial biomass.

5.3×10^4 Da to 2.0×10^6 Da (Nakata, Kawaguchi, Kodama, & Konno, 1998). As shown in Fig. 1a, a peak at around 890 cm^{-1} was observed in FT/IR spectrum of the polysaccharide, indicating the existence of β-1,3-glucosidic bond, while no characteristic absorption band was found at 840 cm^{-1} which corresponding to α-configuration bond (Kim, Ryu, Choi, Rhee, & Lee, 2003). Band transmittance at 3410 cm^{-1} and 2924 cm^{-1} represent O—H group and C—H stretching, respectively, while absorption band at 1075 cm^{-1} suggests the C—O group, which was consistent with the curdlan spectrum reported previously (Martinez et al., 2015). A similar FT/IR spectrum was obtained for the commercial curdlan sample (Fig. 1b). Thus, the polysaccharide produced by *R. radiobacter* CGMCC12099 was confirmed to be curdlan.

3.2. Effect of initial biomass on curdlan production and cell growth

In our preliminary study, curdlan production started as soon as the ammonium salt was depleted, when cell growth stopped and the biomass remained almost constant during the curdlan synthesis phase. Therefore, the amount of initial cell and its vitality are critical factors for curdlan fermentation. The curdlan production by *R. radiobacter* presented a hump-shaped profile with the increasing initial biomass (Fig. 2), and the maximum was achieved at an initial biomass of 5.59 g/L. Interestingly, cell density exhibited a steady increase when initial biomass was increased from 1.12 to 5.59 g/L, while remained stable at initial biomasses higher than 7.80 g/L accompanied by a reduction in curdlan production. Com-

pared with the maximum curdlan production of 24.8 g/L obtained at 5.59 g/L of initial biomass, curdlan production was reduced by 41% (14.68 g/L) at a high initial biomass of 13.37 g/L, and the curdlan specific yield decreased from 4.28 g/g to 1.97 g/g. Our results indicate that the increased cell density does not necessarily elevate curdlan production, and both cell growth and curdlan synthesis could be inhibited under relatively high biomass. Similar to our observation, no curdlan was detected when the biomass was up to 13.5 g/L, as well as 2-oxoglutarate (Zheng et al., 2014), which is commonly recognized as an indicator of intracellular nitrogen limitation (Leigh & Dodsworth, 2007). Consequently, high cell density may destroy the normal metabolism signal in nitrogen limitation, which causes reduction in both biomass and curdlan production.

3.3. Effect of $(NH_4)_2HPO_4$ feeding time on curdlan production

Curdlan is a typical secondary metabolite produced after cell growth phase. When nitrogen source was depleted, the cells started to produce curdlan while the biomass remained steady. It has been proved that curdlan production could be promoted by enhancing cell density in fermentation (Zheng et al., 2014). Therefore, $(NH_4)_2HPO_4$ was supplemented as the solo nitrogen source to improve the cell growth. According to the unique two-stage curdlan fermentation, three different time points (0 h, 35th h, 75th h) were selected from the cell growth phase, earlier and latter curdlan synthesis phase to investigate the effect of $(NH_4)_2HPO_4$ feeding time on curdlan production. Further study was conducted in a 3-L bio-reactor equipped with pH and DO control system. As shown in Fig. 3, compared with the control without $(NH_4)_2HPO_4$ addition (Fig. 3a), both curdlan production and total biomass were elevated when a final $(NH_4)_2HPO_4$ concentration of 10 g/L was added. Surprisingly, the cell displayed a diauxie growth after $(NH_4)_2HPO_4$ addition (Fig. 3c and d), along with a sharp increase in glucose consumption. The average biomass was about 4 times higher than that of the control, and the highest curdlan production was obtained when $(NH_4)_2HPO_4$ was added at 35th h (Fig. 3c). The cells exhibited a dramatically increased growth upon $(NH_4)_2HPO_4$ addition after nitrogen starvation, which may due to the adapted cellular carbon flux and nitrogen assimilation metabolism that maintains the normal cell growth under nitrogen-limited environment. Our results are consistent with previous reports, in which the expression level of nitrogen metabolism regulating related genes (*ntrB*, *ntrC*, and *nifR*) increased significantly when nitrogen source was exhausted, as well as the carbon metabolism related genes (*exoC*) (Yu, Wu, Zheng, Lin, & Zhan, 2011b). When $(NH_4)_2HPO_4$ was added at 75th h (Fig. 3d), the curdlan production did not show a distinct improvement. Compared with earlier feeding time (0 and 35th h) (Fig. 3b and c), both curdlan production and biomass were lower when ammonium salt was added at 75th h, and the biomass increment per ammonium salts consumption was declined by 23% (Fig. 3d). It is conjectured that the optimal time of $(NH_4)_2HPO_4$ addition should be in the earlier fermentation stage (such as 35th h), when cells could easily transform from curdlan synthesis status to growth status. As a result, the optimal time of $(NH_4)_2HPO_4$ feeding was determined to be 35th h of fermentation.

3.4. Optimization of $(NH_4)_2HPO_4$ feeding concentration

To optimize the amount of $(NH_4)_2HPO_4$ feeding, different concentrations, 2.5 g/L, 5.5 g/L, 11.5 g/L, were investigated (Table 1). The biomass increased with the increasing $(NH_4)_2HPO_4$ concentration, and the highest curdlan production of 60.63 g/L was obtained with 5.5 g/L $(NH_4)_2HPO_4$ at 35th h, as well as a highest productivity of 0.51 g/L/h and glucose conversion rate of 36%. The average level of biomass increment per $(NH_4)_2HPO_4$ consumption was calculated to be 1.12 g/g at 5.5 g/L $(NH_4)_2HPO_4$ addition, slightly higher than the others. Thus, the optimum $(NH_4)_2HPO_4$ concentration was determined to be 5.5 g/L when curdlan production and productivity were improved by 95.3% and 96.2% respectively. This is consistent with our results obtained in shake flasks, in which curdlan production could be enhanced by increasing biomass within a certain range. At 11.5 g/L $(NH_4)_2HPO_4$, cell density grew up to 14 g/L, and the curdlan production was reduced to 37.97 g/L, which was 37.4% lower than the highest level obtained at a relatively lower biomass of 8.52 g/L. Additionally, the glucose conversion rate at cell density of 14 g/L (13.86%) was significantly lower than that of control (31.9%), which may attributed to the vast glucose consumed by fast cell growth. The results demonstrated that the capacity of curdlan synthesis was attenuated under high cell density when 11.5 g/L $(NH_4)_2HPO_4$ was added, whereas a slight improvement in curdlan production (37.97 g/L) was still detected compared with that of control (31.05 g/L).

Table 1
The effect of different concentration of $(NH_4)_2HPO_4$ feeding on curdlan production by *R. radiobacter* CGMCC12099 in 3-L bio-reactor.

Parameter	Control	Concentration of ammonium salts addition (g/L)		
	/	2.50	5.50	11.50
Biomass CDW (g/L)	2.86	5.35	8.52	14.00
Curdlan production (g/L)	31.05	35.7	60.63	37.97
$Y_{biomass/NH4^+}$ (g/g)	/	0.95	1.12	0.97
Curdlan productivity (g/L/h)	0.26	0.30	0.51	0.32
Glucose conversion rate (%)	31.90	33.10	36.00	13.86

3.5. Effect of various $(NH_4)_2HPO_4$ feeding strategies on curdlan fermentation

Our results show that the addition of $(NH_4)_2HPO_4$ could boost not only the cell reproduction but also the curdlan synthesis. Consequently, various $(NH_4)_2HPO_4$ feeding strategy was attempted, including single addition (Fig. 4a), two pulses with different time intervals (Fig. 4b and c), and continuous feeding (Fig. 4d). Pulse-feeding is a widely used strategy in cell fermentation. The pulse-feeding glucose has been applied to improve the production of human-like collagen, and the cell growth was accelerated by the transient glucose starvation (Xu et al., 2012). As shown in Fig. 4b and c, 5.5 g/L $(NH_4)_2HPO_4$ was added at 35th h in two pulses (time intervals of 30 h and 15 h), and there was an obvious increase in biomass upon each $(NH_4)_2HPO_4$ addition, correspondingly, curdlan producing rate was accelerated at each pulse. As shown in Fig. 4b (time internal of 30 h), $(NH_4)_2HPO_4$ was added at 30th h and 60th h, the biomass was doubled after the $(NH_4)_2HPO_4$ addition and reached the maximum cell density of 8.21 g/L. The average cell growth rate showed a little difference between two nitrogen pulses, and the former was higher than the latter, which may be attributed to the relatively lower cell vitality in the mid or late stage of fermentation. This phenomenon was also observed in curdlan fermentation with $(NH_4)_2HPO_4$ addition at 75th h (Fig. 3d). As for curdlan production, the highest titer was 58.12 g/L which was similar to that of single $(NH_4)_2HPO_4$ addition (60.63 g/L) (Fig. 4a). The results indicate that the time point of second pulse (60th h) might be a bit late for the cells to transform from curdlan producing to growth status. Consequently, $(NH_4)_2HPO_4$ pulse-feeding fermentation with shorter time internal of 15 h was preferred. As shown in Fig. 4c, cells grew continuously following two nitrogen pulses, reaching a highest biomass of 8.28 g/L. Furthermore, both curdlan production (61.02 g/L) and productivity (37%) were elevated, compared with single nitrogen addition strategy.

To further shorten the time internal, a continuous $(NH_4)_2HPO_4$ feeding strategy was developed, $(NH_4)_2HPO_4$ solution was fed at

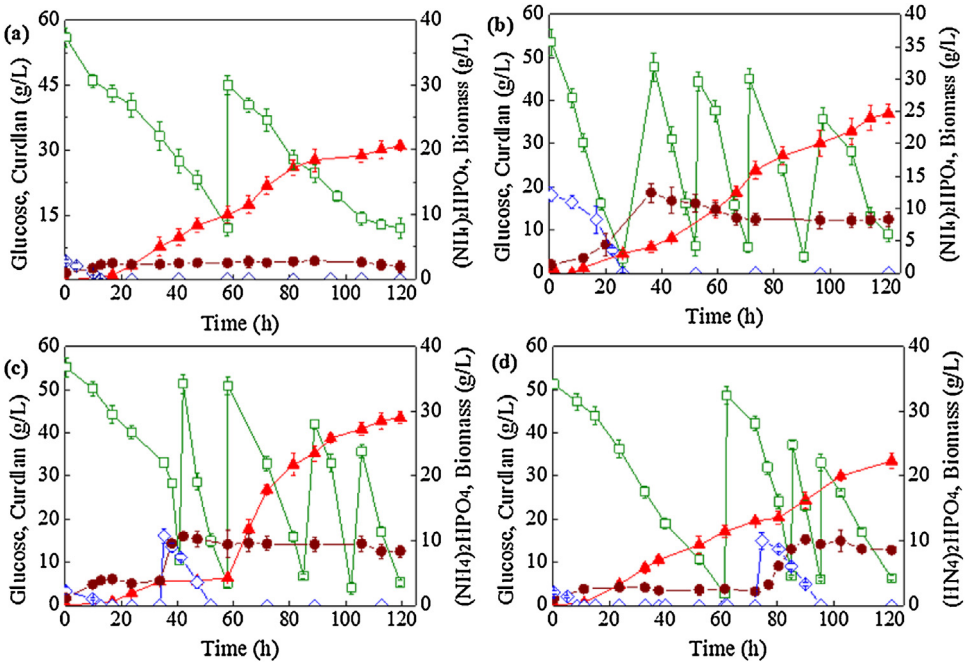

Fig. 3. Time course of curdlan production by *R. radiobacter* CGMCC12099 with different $(NH_4)_2HPO_4$ feeding times in 3-L bio-reactors. (a) Control without addition; (b) addition at 0 h; (c) addition at 35th h; (d) addition at 75th h. Residual glucose (□), $(NH_4)_2HPO_4$ (◇), biomass (●), and curdlan production (▲) are presented.

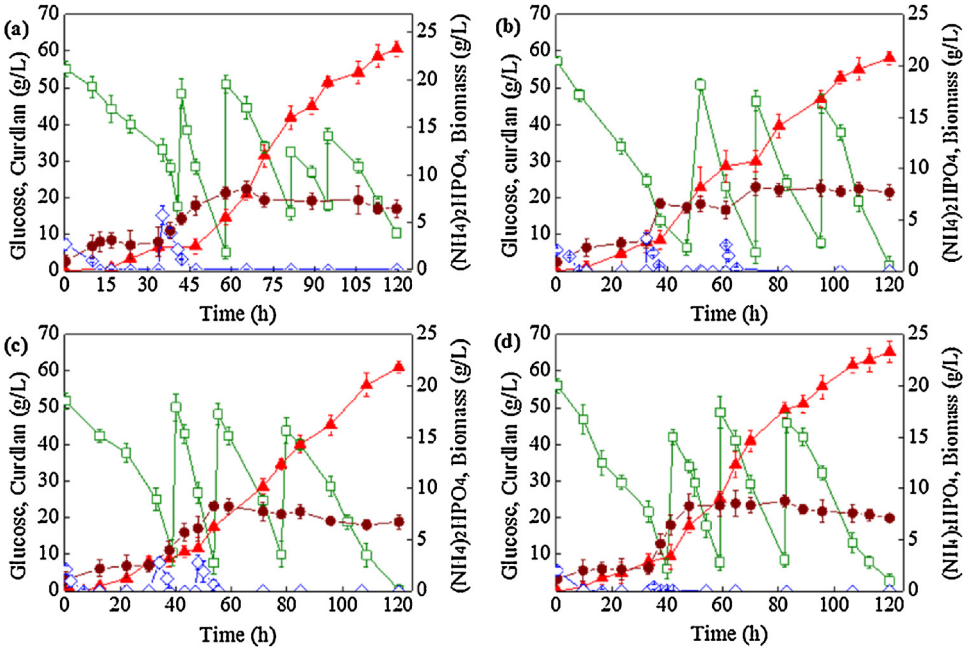

Fig. 4. Time course of curdlan production by *R. radiobacter* CGMCC12099 using different $(NH_4)_2HPO_4$ feeding strategies in 3-L bio-reactors. (a) single addition; (b) two pulses with 30 h time interval; (c) two pulses with 15 h time interval; (d) continuous feeding. Residual glucose (□), $(NH_4)_2HPO_4$ (◇), biomass (●), and curdlan production (▲) are presented.

35th h at a constant rate of 1.24 g/h for about 9 h, amounting to a total concentration of 5.5 g/L. As shown in Fig. 4d, $(NH_4)_2HPO_4$ concentration remained at an extremely low level during the entire feeding process, and the curdlan synthesis was enhanced drastically as soon as the feeding stopped. After 120 h of cultivation, total curdlan production of 65.27 g/L was attained, representing 8% higher than that of the single feeding strategy, and 2.1-fold of that without $(NH_4)_2HPO_4$ addition. The glucose conversion rate and productivity of 40% and 0.54 g/L/h were reached, which were 25% and 109% higher than those of the control. It has also been reported that the ratio of carbon and nitrogen sources (C/N) plays an important role in the curdlan fermentation (Zheng et al., 2014), and the nitrogen feeding strategy may change the ratio of C/N (Wu, Zhan, Liu, & Zheng, 2008). In this study, a continuous feeding strategy favors a relatively stable fermentation environment for better cell growth and curdlan production.

A number of nutrient feeding strategies have been reported to improve the curdlan production (Jin & Lee, 2014; Lee & Lee, 2001; Yu et al., 2011a; Wu, Zhan, Liu, & Zheng, 2008), such as uracil as the precursors of curdlan, polyphosphates as the high

Table 2
Summary of curdlan production using different fermentation strategies.

Fermentation strategy	Titer (g/L)	Productivity (g/L/h)	Specific yield (g/g)	Substrate conversion rate (%)	Reference
Feedback inferential control of optimal pH profile	60	0.5	/	/	Lee and Park (2001)
Optimization of dissolved oxygen combined with pH control	42.8	0.37	13.5	68.9	Zhang et al. (2012)
Selective feeding with ammonia water during cell growth	72	/	6.05	60.6	Wu et al. (2008)
Integration of carbon/nitrogen sources control and sequencing dual fed batch fermentation	67	0.98	6.09	57	Zheng et al. (2014)
Addition of $(NH_4)_2HPO_4$ during curdlan synthesis	65.3	0.54	7.3	40	This study

energy donor for curdlan synthesis, but few of them involved nitrogen feeding strategy. Recent reports on curdlan fermentation were summarized in Table 2. An ammonia water feeding strategy during cell growth phase was reported, resulting a total curdlan production of 72 g/L and a moderate specific yield of 6.05 g/g (Zheng et al., 2014). Compared with ammonia water, $(NH_4)_2HPO_4$ could be added quantitatively without affecting the pH of culture. Additionally, $(NH_4)_2HPO_4$ could provide nitrogen source as well as phosphorus to boost cell growth and curdlan synthesis. In this study, a $(NH_4)_2HPO_4$ feeding strategy was investigated to enhance curdlan synthesis, in which a higher curdlan production of 65.3 g/L and specific yield of 7.3 g/g were achieved.

4. Conclusion

A nitrogen feeding strategy was developed to improve the curdlan production by *R. radiobacter* CGMCC12099 in this study. A diauxie cell growth profile was observed upon $(NH_4)_2HPO_4$ feeding. The time and concentration of $(NH_4)_2HPO_4$ feeding were optimized. Addition of 5.5 g/L $(NH_4)_2HPO_4$ at 35^{th} h resulted in curdlan production of 60.63 g/L with 30% glucose conversion rate. To further elevate the curdlan production, continuous nitrogen feeding strategy was employed by feeding $(NH_4)_2HPO_4$ at a constant rate of 1.24 g/h at 35^{th} h for 9 h. A final curdlan production of 65.27 g/L with a glucose conversion rate of 39.89% were achieved after 120 h of fermentation. Compared with control (without nitrogen feeding), the curdlan production was enhanced by 2.1 times, and the glucose conversion rate was improved by 25%. This nitrogen feeding strategy was proved to be a feasible and cheap method to improve curdlan production, and is therefore highly promising in industrial applications.

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