Co-production of functional exopolysaccharides and lactic acid by *Lactobacillus kefiranofaciens* originated from fermented milk, kefir

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Abstract Kefiran is a functional exopolysaccharide produced by *Lactobacillus kefiranofaciens* originated from kefir, traditional fermented milk in the Caucasian Mountains, Russia. Kefiran is attractive as thickeners, stabilizers, emulsifiers, gelling agents and also has antimicrobial and antitumor activity. However, the production costs of kefiran are still high mainly due to high cost of carbon and nitrogen sources. This study aimed to produce kefiran and its co-product, lactic acid, from low-cost industrial byproducts. Among the sources tested, whey lactose (at 2% sugar concentration) and spent yeast cells hydrolysate (at 6 g-nitrogen/L) gave the highest kefiran of 480 ± 21 mg/L along with lactic acid of 20.1 ± 0.2 g/L. The combination of these two sources and initial pH were optimized through Response Surface Methodology. With the optimized medium, *L. kefiranofaciens* produced more kefiran and lactic acid up to 635 ± 7 mg/L and 32.9 ± 0.7 g/L, respectively. When the pH was controlled to alleviate the inhibition from acidic pH, *L. kefiranofaciens* could consume all sugars and produced kefiran and lactic acid up to 1693 ± 29 mg/L and 87.49 ± 0.23 g/L, respectively. Moreover, the fed-batch fermentation with intermittent

adding of whey lactose improved kefiran and lactic acid productions up to 2514 ± 93 mg/L and 135 ± 1.75 g/L, respectively. These results indicate the promising approach to economically produce kefiran and lactic acid from low-cost nutrient sources.

Keywords Kefiran · Kefir grains · Lactic acid · Spent yeast cells · Whey lactose

Introduction

Exopolysaccharides (EPS) produced by lactic acid bacteria have received much attention because the lactic acid bacteria are Generally Recognized as Safe (GRAS) (Sanalibaba and Çakmak 2016). Lactic acid bacteria that produce EPS can be isolated from dairy products and fermented foods and their produced EPS have been widely used to improve techno-functional properties of food especially yoghurt (Yang et al. 2014; London et al. 2015). Kefiran is a functional exopolysaccharide containing glucose and galactose in about equal amounts. It is widely used as thickeners, stabilizers, emulsifiers, and gelling agents and also has antimicrobial and antitumor activities (Ghasemlou et al. 2012). Kefiran is produced by lactic acid bacteria *Lactobacillus kefiranofaciens* found in kefir grains, a starter of traditional fermented milk that originated in the Caucasian mountains, Russia. These grains contain a relative stable microbiota immobilized in a matrix of polysaccharides and proteins, which have been proven beneficial to human health (Yokoi et al. 1990).

However, the production costs of kefiran are still high, mainly due to the high cost of carbon sources (sugars) and of organic nitrogen sources including tryptone, yeast extract and meat extract (Cheirsilp et al. 2001; Wang and

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Bi 2008). Therefore, utilizing alternative low-cost carbon and nitrogen sources, such as byproducts from agricultural industries, would be economically attractive (Li et al. 2010) and more viable. Whey lactose and mature coconut water are byproducts from cheese and coconut milk production, respectively. They both contain high amount of sugars (mainly lactose in whey; fructose, glucose and sucrose in mature coconut water) that could be used as good carbon sources for microorganisms (Unagul et al. 2007; Cheirsilp and Radchabut 2011; Kantachote et al. 2016). On the other hand, nitrogen-containing byproducts include spent yeast cells from breweries (Suphantharika et al. 2003) and whey proteins from cheese production, and also low-cost nitrogen sources from soybean processing could be considered as alternative nitrogen sources for microorganisms (Loyarkat et al. 2013). These candidate materials for kefiran production are of particular interest to us as inexpensive, abundant and locally available resources.

Lactobacillus kefiranofaciens first accumulates kefiran surrounding the cells, called capsular kefiran, and some of it (called broth kefiran) is then released into the culture broth during cultivation (Cheirsilp et al. 2001). In addition to the production of kefiran, *L. kefiranofaciens* also accumulates a high concentration of lactic acid (Cheirsilp and Radchabut 2011), so it is reasonable to consider the co-production of kefiran with lactic acid. This study aimed to use several industrial byproducts and low-cost nitrogen sources for the combined production of kefiran and lactic acid by *L. kefiranofaciens*.

Materials and methods

Materials

Industrial byproducts such as whey lactose, mature coconut water, whey protein, isolated soy protein, soybean meal and spent yeast cells were obtained from local industries in Thailand. *Lactobacillus kefiranofaciens* JCM 6985, the producer of kefiran, was obtained from the Japan Collection of Microorganisms (JCM) RIKEN, Japan. The kefiran produced by this strain was characterized previously by Maeda et al. (2004). The production medium used for kefiran production was a modified MRS, consisting of 0.2% K_2HPO_4 , 0.4% triammonium citrate, 0.5% sodium acetate, 0.1% Tween 80, 0.028% $MnSO_4 \cdot 4H_2O$, 0.058% $MgSO_4 \cdot 7H_2O$, 0.074% $CaCl_2$ and varied carbon and nitrogen sources. Various sugars including monosaccharides (glucose, galactose, and fructose) and disaccharides (lactose and sucrose) were used as carbon sources at 2% concentration. Spent yeast cells in powder form were pretreated by adjusting pH to 2.0 with 6 N sulfuric acid,

and holding at 121 °C for 15 min. Each alternative nitrogen source was used to replace the original ones in modified MRS medium (2% tryptone, 1% yeast extract and 2% meat extract = 6 g-nitrogen/L). The nitrogen content was 12.2% in whey protein, 6.3% in isolated soy protein, 6.1% in soybean meal, and 5.9% in spent yeast cell hydrolysate. To reduce the costs of nitrogen sources, the most suitable alternative nitrogen source was selected and used as the sole organic nitrogen source in the medium.

Culture conditions and experimental design

Lactobacillus kefiranofaciens was precultured in 10 mL commercial MRS broth (Himedia, India) at pH 5.5, and incubated without shaking at 30 °C for 48 h. The precultured *L. kefiranofaciens* was 10% (v/v) inoculated into 100 mL of modified MRS medium. The cultivation was performed anaerobically in 120-mL screw capped bottles. The medium was stirred at 100 rpm by a magnetic stirrer at 30 °C for 120 h. Optimization of the operating conditions for kefiran production was performed using Response Surface Methodology (RSM) with Box-Behnken experimental design. The three manipulated variables were sugar concentration (x_1), nitrogen source concentration (x_2), and initial pH (x_3), each with three levels in the experimental design, and dry cell weight and kefiran yield were the two observed responses to be modeled and optimized. The design required 17 experimental runs in total, three of which were replications at the central point for estimating experimental variance. The models fit to the data from the 17 runs were second order polynomials. These response surface models show the main, quadratic and interactive effects of the manipulated factors, as follows:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, x_i and x_j represent the manipulated variables or factors, β_0 is the offset term, β_i is a linear effect, β_{ij} is a first order interaction effect, and β_{ii} is a quadratic effect. The goodness of the fit of the model was evaluated from the coefficient of determination (R^2) and from analysis of variance (ANOVA). Response surface plots were made to indicate near optimal conditions, using the fitted quadratic polynomials so that two of the independent manipulated variables were held constant, while the remaining manipulated variable was free to vary.

Batch and fed-batch cultivation

The medium used in this experiment was modified MRS medium with the suitable sugar concentration, nitrogen source concentration and initial pH determined from the previous section. The batch cultivation was carried out in a

1 L bioreactor equipped with pH control system. The agitation speed was maintained at 100 rpm. The air in the medium was removed by introducing carbon dioxide into the medium before inoculation. The temperature was kept at 30 °C. In fed-batch cultivation, the culture with an initial working volume of 0.7 L was first operated in batch mode. The feeding is started in the middle of the exponential phase, and is then repeated every 24 h to maintain the sugar concentration at its near optimal level, which was obtained from RSM. The pH of the culture was controlled at its optimal level according to the RSM using 10 N NaOH solution. The time courses of dry cell weight, broth and capsular kefir, and sugar consumption were determined.

Analytical methods

Bacterial growth was measured by spectrophotometry at 660 nm. The dry cell weight was determined as follows: 10 mL of bacterial suspension was centrifuged at 3500×g for 15 min and the biomass was dried at 60 °C until constant weight. The specific growth rate (μ) was determined for the exponential growth phase as follows:

$$\mu(\text{day}^{-1}) = (\ln X_2 - \ln X_1) / (t_2 - t_1) \quad (2)$$

where X_1 and X_2 are the concentrations of dry cell weight (g/L) at times t_1 and t_2 , respectively. Kefir was recovered and determined according to the method of Maeda et al. (2004). The total procedure took approximately 3 days. The kefir in the culture broth named as broth kefir, was precipitated by adding cold ethanol (− 20 °C) in equal volume to the sample, kept in refrigerator at temperature of 4 °C overnight and then centrifuged at 4500×g and 4 °C for 10 min. The kefir precipitate was re-dissolved in distilled water and centrifuged to remove any remaining undissolved materials. The dissolved kefir was again precipitated and the resulting precipitate was re-dissolved in distilled water. The re-dissolved kefir was quantified colorimetrically by adding one mL of anthrone reagent (0.8 g anthrone, 100 mL H₂O and 300 mL H₂SO₄) to 0.1 mL of the kefir solution. The mixture was incubated for 10 min at 100 °C and cooled to room temperature. The formation of green colored product from the reaction between glucose derivative and anthrone was measured at absorbance of 620 nm. The concentration of broth kefir was calculated using the standard curve generated with glucose. The kefir surrounding the cells named as capsular kefir was extracted from the cells by boiling them in distilled water at 100 °C for 30 min. The mixture was centrifuged. The capsular kefir was quantified similarly as broth kefir. Total kefir is obtained as the sum of capsular and broth kefir. The amount of lactic acid was determined by HPLC equipped with an Aminex HPX-87H

column (Bio-Rad Laboratories, Richmond, CA, USA). The column was eluted with 0.005 M H₂SO₄ at a flow rate of 0.6 mL/min.

All experiments were performed at least in triplicate. Analysis of variance was performed to calculate significant differences in treatment means, and the least significant difference ($P < 0.05$) was used to compare the means, using SPSS software version 16.0.

Results and discussion

Effects of various carbon sources on cell growth and kefir production

Various sugars including monosaccharides (glucose, galactose, and fructose) and disaccharides (lactose and sucrose) were separately used as carbon sources for cell growth and kefir production by *L. kefiranofaciens* (Fig. 1). Among the sugars tested, glucose, galactose and lactose were suitable as carbon sources that gave high cell growth of *L. kefiranofaciens*. The reason that *L. kefiranofaciens* preferentially utilized these sugars might be that they are main sugar components of milk and fermented milk or kefir, from which this strain originated. The most suitable carbon source for kefir production was lactose, which gave the highest kefir yield (283±15 mg/L). Although most lactic acid bacteria use glucose as the primary energy source, some of them prefer lactose. Lactose can be transported via phosphotransferase systems, and the β -galactosidase breaks lactose down to galactose and glucose (de Vos and Vaughan 1994). Ghasemlou et al. (2012) also found that lactose is a suitable carbon source for kefir production. In the research of Zajsek et al. (2013), the effects of added carbon sources on kefir production by kefir grains were studied. Among the sugars added (fructose, glucose, sucrose and lactose), lactose gave the highest biomass of kefir grains and the highest kefir yield of 4.3% based on the kefir grains' biomass, followed by sucrose which gave kefir yield of 3% based on the kefir grains' biomass.

In this study, two industrial byproducts, whey lactose and mature coconut water, were tested as carbon sources for *L. kefiranofaciens*. As the mature coconut water also contained fructose and sucrose, which are not preferable for cell growth and kefir production by *L. kefiranofaciens* (Fig. 1), the dry cell weight and kefir production with mature coconut water were then below those with whey lactose. These results were also confirmed from sugar utilization. *L. kefiranofaciens* consume the favored sugars faster than the less preferred ones. As the *L. kefiranofaciens* grew well and produced the highest amount of kefir with

Fig. 1 Effects of various carbon sources on dry cell weight (DCW), total kefiran production and sugar consumption by *L. kefiranofaciens* cultivated in MRS medium

whey lactose, whey lactose was then chosen for further experiments in the current study.

Effects of various nitrogen sources on kefiran production

To reduce the cost of organic nitrogen sources, all organic nitrogen sources in the original MRS medium were replaced by nitrogen-containing byproducts and cheap nitrogen sources at concentration of 6 g-nitrogen/L. These included spent yeast cell hydrolysate, whey protein, isolated soy protein and soybean meal. Figure 2 shows the production of broth and capsular kefiran by *L. kefiranofaciens* and its sugar consumption. It should be noted that *L. kefiranofaciens* could use all types of nitrogen sources to grow and produce kefiran. The produced broth kefiran were in the range of 173–234 mg/L and the capsular kefiran were in the range of 111–185 mg/L. The total kefiran yield was comparable to that obtained with the original MRS medium (Fig. 1).

Among the low-cost nitrogen sources tested, spent yeast cell hydrolysate gave the highest kefiran production. The use of spent yeast cell hydrolysate as the sole organic nitrogen source in the medium could significantly reduce the costs of kefiran production. Spent yeast cells are

considered as good nitrogen sources because they are enriched with protein, amino acids, minerals, vitamins, and growth factors that could promote the growth of microorganisms (Sombutyanuchit et al. 2001). Yokoi et al. (1990) also pointed out that an essential nutrient for the culture of kefir-producing *Lactobacillus* sp. KPB-167B was yeast extract. Wang and Bi (2008) studied the effects of organic and inorganic nitrogen sources on the production of kefiran by *L. kefiranofaciens* JCM 6985. The various nitrogen sources tested included peptone, casein, yeast powder, tryptone, yeast extract, urea, ammonium chloride and ammonium sulfate at 50 g/L concentration. They found that organic nitrogen sources gave better growth and kefiran production than inorganic nitrogen sources. Among the organic nitrogen sources tested, yeast powder gave the highest cell growth while casein gave the highest kefiran production.

Effects of whey lactose concentration on cell growth and kefiran production

To primarily investigate the effects of sugar concentration in whey lactose based medium, media were prepared with various initial total sugar concentrations ranging within 2–6% (Fig. 3). Spent yeast cell hydrolysate was used as the

Fig. 2 Effects of various nitrogen sources on broth and capsular kefir production and sugar consumption by *L. kefiranofaciens* cultivated in whey lactose-based MRS medium

sole organic nitrogen source at 6 g-nitrogen/L. With increasing initial sugar concentration from 2 to 4%, the cells grew faster and reached the maximal final cell concentration of 2.77 ± 0.15 g/L. The specific growth rate also increased from 0.322 ± 0.007 h⁻¹ to 0.505 ± 0.025 h⁻¹. The growth yield ($Y_{X/S}$) increased from 0.063 ± 0.007 to 0.094 ± 0.006 g-cell/g-substrate, and the product yield of kefiran ($Y_{P/S}$) also increased from 17.10 ± 1.23 to 19.63 ± 0.39 mg-kefiran/g-substrate. However, these yields did not increase further when using higher sugar concentration of 6%. At this sugar concentration, the strain reached slightly lower final cell concentration of 2.13 ± 0.07 g/L and the specific growth rate slightly decreased to 0.493 ± 0.012 h⁻¹ (Supplementary Table 4). This was probably due to the inhibitory effects of high sugar concentration and of high lactic acid concentration produced at this sugar concentration (45.20 ± 0.85 g/L). It should be also noted that at 4% sugar concentration the cells grew slowly after 96 h and the kefiran production stopped at 96 h, even though considerable amount of sugars still remained in the culture. Possible reasons for the cease of kefiran production could be the decrease in pH to unsuitable level (below 4) and/or unsuitable concentration of nitrogen sources for the culture. Several studies have

reported the different optimal sugar concentrations for kefiran production from various carbon sources (Yokoi and Watanabe 1992; Cheirsilp et al. 2001; Harta et al. 2004; Ghasemlou et al. 2012; Dailin et al. 2016). In addition to sugar concentration, the initial pH and nitrogen concentration also significantly affected the cell growth and kefiran production by *L. kefiranofaciens* (Yokoi and Watanabe 1992; Taniguchi et al. 2001; Maeda et al. 2004; Harta et al. 2004; Wang and Bi 2008; Ghasemlou et al. 2012; Dailin et al. 2016). Therefore, to maximize kefiran production three main factors including whey lactose concentration, nitrogen concentration and initial pH, were selected to be optimized. As these three factors may have synergistic effects on cell growth and kefiran production, RSM was used for optimization.

Optimization of culture conditions through RSM

The culture conditions of *L. kefiranofaciens* were optimized through RSM. The effects of three variables, namely whey lactose concentration (WL), spent yeast cell hydrolysate (YH) and initial pH (PH), were investigated. The complete design consisted of 17 trials in total, which contained three replications at the central point for

Fig. 3 Effects of whey lactose concentration on dry cell weight (DCW), broth kefir (open symbols) and capsular kefir (close symbols) production, sugar consumption and pH of *L. kefirifaciens*, when cultivated in whey lactose and spent yeast cell hydrolysate based MRS medium

estimating the experimental uncertainty or variance of replicates. The responses observed were dry cell weight (Y_1) and total kefir (Y_2). The experimental design and the respective experimental results are given in Supplementary Table 1. The regression coefficients (β) and analysis of variance are shown in Supplementary Table 2.

The polynomial fits to dry cell weight (Y_1) and total kefir (Y_2) were as follows:

$$\begin{aligned} \text{Dry cell weight (g/L)} = & -14.20 + 0.043\text{WL} + 3.2125\text{YH} \\ & + 4.215\text{PH} - 0.00065\text{WL}^2 - 0.46\text{YH}^2 - 0.34\text{PH}^2 \\ & - 0.0025\text{WL} \cdot \text{YH} + 0.0025\text{WL} \cdot \text{PH} - 0.15\text{YH} \cdot \text{PH} \end{aligned} \quad (3)$$

$$\begin{aligned} \text{Total kefir (g/L)} = & -6.02114 + 0.00812\text{WL} + 0.50277\text{YH} \\ & + 2.034562\text{PH} - 0.00014\text{WL}^2 - 0.06257\text{YH}^2 \\ & - 0.17837\text{PH}^2 - 0.0073\text{WL} \cdot \text{YH} + 0.001583\text{WL} \\ & \cdot \text{PH} - 0.02861\text{YH} \cdot \text{PH} \end{aligned} \quad (4)$$

Generally, the adequacy of a model is indicated by the coefficient of determination R^2 and the P value. An R^2 close to 1 indicates that the predicted values from models closely follow the experimental results. The models for dry cell weight and total kefir are adequate with R^2 values close to 1, being 0.91 and 0.88, respectively. These indicate that 91 and 88% of the variability in response could be

explained by these fitted models. The P values of the coefficients in these two models indicate their significances ($P < 0.05$). On assessing interactions between the manipulated experimental variables, the P values of cross-terms provide some insights, as they do regarding effects of each single variable by itself. Further statistical analysis of the effects of each variable on dry cell weight and total kefir, in Tables 2 and 3, showed that only the quadratic effects of whey lactose (WL^2) and spent yeast cell hydrolysate (YH^2) were significant ($P < 0.05$) to both responses. In the case of total kefir, the linear term for whey lactose (WL) was also significant ($P < 0.05$). However, the interactions of the three manipulated variables were not significant to dry cell weight or total kefir.

The effects and optimal levels of whey lactose, spent yeast cell hydrolysate and initial pH were visualized by plotting response surfaces and contour maps. Based on the analysis of variance of the second order polynomial models, initial pH had little effect on dry cell weight and total kefir (Supplementary Tables 2 and 3). This is also confirmed by the responses of dry cell weight and total kefir to initial pH, plotted in Figs. 4 and 5. A more steep curvature of dry cell weight responded to spent yeast cell hydrolysate indicates that the dry cell weight changes more due to spent yeast cell hydrolysate than whey lactose (Fig. 4a). Increasing whey lactose from 2% to 4% increased the dry cell weight. Figure 4a, b show that the maximum

Fig. 4 3D surface plot and contour plot of dry cell weight in response to **a** whey lactose concentration versus spent yeast cell hydrolysate while pH was set at 5.5, **b** whey lactose concentration versus pH while spent yeast cell hydrolysate was set at 2.5 g-nitrogen/L and **c** spent yeast cell hydrolysate versus pH while whey lactose concentration was set at 4%

dry cell weight was obtained at moderate whey lactose (4%) and moderate spent yeast cell hydrolysate (2.5 g-nitrogen/L). As the pH decreased during cultivation, a high initial pH was the most favorable for cell growth (Fig. 4b, c). At more acidic initial pH, the inhibitory effects of lactic acid would become more severe than they would at neutral initial pH. Figure 5 shows effects of the three experimental variables

Fig. 5 3D surface plot and contour plot of total kefir production in response to **a** whey lactose concentration versus spent yeast cell hydrolysate while pH was set at 5.5, **b** whey lactose concentration versus pH while spent yeast cell hydrolysate was set at 2.5 g-nitrogen/L and **c** spent yeast cell hydrolysate versus pH while whey lactose concentration was set at 4%

on the production of total kefir. The concentration of whey lactose positively affected kefir production (Fig. 5a). The effects of spent yeast cell hydrolysate on kefir production were smaller than those of whey lactose. As *L. kefirifaciens* produced kefir during both growth phase and non-growth phase, the concentration of carbon source affects the production more than that of the nitrogen

Fig. 6 Effect of pH control and fed-batch cultivation mode on dry cell weight (DCW), broth kefiran (open symbols) and capsular kefiran (close symbols) production and sugar consumption of *L. kefiranofaciens* cultivated in whey lactose and spent yeast cells based MRS medium. In fed-batch, whey lactose (2% sugar concentration) was intermittently added at 24, 48 and 72 h

source. As the model coefficients can be directly compared as a measure of their relative impact, the positive coefficient for interaction term between whey lactose concentration and initial pH ($+0.001583\text{WL} \cdot \text{PH}$) indicates a synergistic effect of two factors. Simultaneously increasing both factors significantly increased kefiran to its maximum level of 640 mg/L (Fig. 5b).

The half radius of curvature shown in Fig. 5b suggested the saturation effect of whey lactose concentration on the kefiran production. There was then very small difference in the kefiran levels achieved at 4–6% sugar concentration. This result also confirmed the results in Fig. 3b. When using the single-factor optimization, the further increase in sugar concentration $> 4\%$ did not significantly increase the kefiran production and its yield. From the economic point of view and for efficient use of whey lactose, the minimum sugar concentration of 4% that gives comparatively high kefiran production and yield was selected as a suitable concentration. The optimal concentration of spent yeast cell hydrolysate and initial pH were found to be 2.5 g-nitrogen/L and 5.5, respectively. Experimental validation of the optimal conditions was performed, giving an acceptable dry cell weight of 2.73 ± 0.15 g/L and a kefiran production of 635 ± 7 mg/L. The predicted results are in good agreement with these experimental results. Under these conditions, *L. kefiranofaciens* also produced lactic acid that reached 32.9 ± 0.7 g/L. Taniguchi et al. (2001)

have reported that the kefiran production by *L. kefiranofaciens* could reach 670 mg/L in the medium containing 5% lactose and 5 g/L yeast extract. Ghasemlou et al. (2012) have reported that the optimal lactose and yeast extract concentrations were 8.84% and 21.3 g/L, or approximately 2.34 g-nitrogen/L. Under these conditions, 712 mg/L kefiran was produced. Nonetheless pure lactose was used in those studies, the production yields were only 8–13 mg-kefiran/g-substrate which was lower than the yields obtained in this current study (16–19 mg-kefiran/g-substrate). Therefore, it could be concluded that whey lactose and spent cell yeast could be used as low-cost nutrient sources for economical production of kefiran.

Effects of pH control on cell growth and kefiran production

The pH of the medium is a key environmental parameter affecting cell growth and product formation by lactic acid bacteria. It has been reported that *L. kefiranofaciens* produced high amount of lactic acid which rapidly lowered the pH of the medium and inhibited the cell growth and kefiran production (Yokoi and Watanabe 1992; Cheirsilp et al. 2001). The pH of the medium was then controlled at 5.5. The results of dry cell weight, kefiran production and sugar consumption under the conditions with and without pH control are shown in Fig. 6. Without control the pH of the

medium declined from 5.5 to 3.9. When the pH was controlled by adding sodium hydroxide, the cells grew faster and gave a higher dry cell weight of 3.16 ± 0.05 g/L (Fig. 6a) and this also improved the specific growth rate by 1.26 folds (Supplementary Table 4). This could be because the inhibitory effect of low pH was alleviated. With the pH control, the cells could consume more sugar (Fig. 6c) and produce more kefiran (Fig. 6b). The final concentration of total kefiran reached 1693 ± 29 mg/L. Regarding lactic acid production, the culture with pH control also gave higher lactic acid (87.49 ± 0.23 g/L) than that without pH control (Table 4). Therefore, it could be concluded that batch cultivation with pH controlled at 5.5 improved dry cell weight, kefiran and lactic acid production by 1.16, 2.67 and 2.66 folds of those without pH control, respectively.

Fed-batch cultivation

Fed-batch cultivation was performed to avoid substrate inhibition and to enhance the production of kefiran by feeding additional substrate. The culture with an initial working volume of 0.7 L was first operated in batch mode. Then 100 mL of whey lactose was added at 24, 48 and 72 h of cultivation time. The pH of the culture was controlled, held at 5.5 throughout the cultivation process (Fig. 6). The *L. kefiranofaciens* grew slightly faster with the addition of whey lactose at 24, 48 and 72 h. The concentration of whey lactose decreased rapidly during first 24 h of cultivation and after each addition. Although the feeding of additional whey lactose did not much affect the final dry cell weight, the total kefiran significantly improved to 2514 ± 93 mg/L (broth kefiran 1827 ± 32 mg/L and capsular kefiran 657 ± 12 mg/L). With the addition of whey lactose every 24 h, the specific growth improved 1.12 fold and lactic acid production was as high as 135.01 ± 1.75 g/L (Supplementary Table 4).

Conclusion

This study demonstrated that whey lactose and spent yeast cells could be used as low-cost nutrients for kefiran production. The culture conditions for kefiran production by *L. kefiranofaciens* were successfully optimized using RSM. A moderate concentration (4%) of whey lactose was suitable for both cell growth and kefiran production. The optimal initial pH was 5.5. Feedback control of the pH alleviated inhibitory effects of low pH and enhanced cell growth, and kefiran and lactic acid production. Moreover, kefiran and lactic acid production were greatly improved by fed-batch cultivation. These results indicate the promising approach to economically produce kefiran and lactic acid from low-cost nutrient sources.

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