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# Production of kefiran from molasses and spent yeast cells by *Lactobacillus kefiranofaciens* JCM 6985

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## Abstract

Kefiran is an exopolysaccharides produced by *Lactobacillus kefiranofaciens* isolated from kefir grains. Kefir grains is a starter of traditional fermented milk that originated in the Caucasian Mountains in Russia. Kefiran contains glucose and galactose at a ratio 1:1. It is widely used as thickeners, stabilizers, emulsifiers and gelling agents. It also has antimicrobial and antitumor activity. The main aim of this work was to produce kefiran from low-cost carbon and nitrogen sources. Firstly, the effect of various sugars including glucose, sucrose, lactose, galactose and lactose on kefiran production were investigated. It was found that lactose gave the highest kefiran production of 283.33±15.3 mg/L followed by glucose (210±20 mg/L) and sucrose (180±5.8 mg/L). When molasses was used as a carbon source, the optimal molasses concentration was 80 g/L which gave kefiran production of 235±5.7 mg/L. The kefiran production using various low-cost nitrogen sources were investigated. Among nitrogen sources tested, spent yeast cells gave the highest kefiran production of 580±10 mg/L. Moreover, when spent yeast cells was hydrolyzed and used as nitrogen source the kefiran production was increased up to 1,286±18 mg/L. These results show that molasses and spent yeast cells can be used as low-cost nutrients for kefiran production.

**Keywords :** *Lactobacillus kefiranofaciens, kefiran, molasses, spent yeast cells*

## Introduction

Exopolysaccharides (EPS) are polysaccha-rides produced from various microorganisms. Factors affecting the production of EPS are composition of the medium such as carbon and nitrogen sources and physical parameters such as pH, temperature and incubation time (1). EPS produced by lactic acid bacterias have received much interesting because lactic acid bacteria are Generally Recognized as Safe (GRAS).The EPS producing lactic acid bacteria can be isolated from dairy products, meat, vegetable and fruit preserve. Kefiran is an exopolysaccharide produced by lactic acid bacteria *Lactobacillus kefiranofaciens* isolated from kefir grains. Kefir grains is

a starter of traditional fermented milk that originated in the Caucasian mountains in Russia. These grains contain a relative stable microbiota immobilized in a matrix of polysaccharides and proteins which have been proven for health beneficial properties. Kefiran contains glucose and galactose at a ratio of 1:1. It is widely used as thickeners, stabilizer, emulsifiers, gelling agents. It also has antimicrobial and antitumor activity (2). However, the production costs of kefiran are still high mainly due to the high cost of carbon source and especially nitrogen sources including tryptone, yeast extract and meat extract (3). Therefore, the low-cost carbon and nitrogen sources should be used for economical production of kefiran. This study aimed to use molasses and industrial byproduct as low-cost carbon and nitrogen sources for kefiran production. The kefiran productions from low-cost carbon and nitrogen sources were optimized and study the optimization of kefrian production by *L. kefiranofaciens* JCM 6985

## 2. Materials and methods

### 2.1 Microorganism strain

*Lactobacillus kefiranofaciens* JCM 6985, the producer of kefiran was obtained from the Japan Collection of microorganisms (JCM) RIKEN, Japan. *L. kefiranofaciens* was precultured in commercial MRS broth (Himedia) at pH 5.5 and incubated without shaking at 25˚C for 48 h.

### 2.2 Media

The production medium used for kefiran production was a modified-MRS consisting of 2% tryptone, 1% yeast extract, 2% meat extract, 0.2% $K_2HPO_4$, 0.4% triammonium citrate, 0.5% sodium acetate, 0.1% Tween 80, 0.028% $MnSO_4.4H_2O$, 0.058% $MgSO_4.7H_2O$, 0.074% $CaCl_2$ and varied carbon sources. Molasses was dissolved in distilled water, pretreated by sulfuric acid, heated at 110˚C for 30 min and filtrated. The clarified molasses were then diluted with distilled water at different concentrations of 20, 40, 60, 80 g/L and used as carbon source for the production of kefiran. Spent yeast cells was hydrolyzed by adjusting pH with 6N sulfuric acid to pH 2.0 and heated at 121°C for 15 min. The hydrolysate was centrifuged and nitrogen-free compounds were removed by ethanol precipitation. The spent yeast cells hydrolysate was also used as nitrogen source in modified-MRS medium.

### 2.3 Culture conditions

#### 2.3.1 Production of kefiran from various sugar.

The seed culture of *L. kefiranofaciens* was inoculated into 100 mL of modified MRS medium with various carbon sources including glucose, sucrose, lactose, galactose and lactose concentration of 20 g/L and incubated at 30˚C for 120 h. The cultivation was performed anaerobically and slowly stirred by a magnetic stirrer.

#### 2.3.2 Production of kefiran from various molasses fermentation

The seed culture of *L. kefiranofaciens* was inoculated into 100 mL of modified MRS medium with molasses concentration of 20, 40, 60 and 80 g/L and incubated at 30˚C for 120 h. The cultivation was performed anaerobically and slowly stirred by a magnetic stirrer.

#### 2.3.3 Production of kefiran from various nitrogen sources

The seed culture of *L. kefiranofaciens* was inoculated into 100 mL of modified MRS medium with replace nitrogen from soybean meal, whey protein, isolated soy protein and spent yeast cells an

equal amount of nitrogen in MRS medium and incubated at 30°C for 120 h. The cultivation was performed anaerobically and slowly stirred by a magnetic stirrer.

**2.3.4 Production of kefiran from spent yeast cells hydrolysate**

The seed culture of *L. kefiranofaciens* was inoculated into 100 mL of modified MRS medium with spent yeast cell hydrolysate as a nitrogen sources and incubated at 30°C for 120 h. The cultivation was performed anaerobically and slowly stirred by a magnetic stirrer.

**2.4 Assays**

Bacterial growth was measured by spectrophotometry at 660 nm. Kefiran in the supernatant named as broth kefiran was precipitated by the addition of the same volume of cold ethanol (-20°C) as that of the sample and then centrifuged at 10,000 rpm, 4°C for 10 min. The precipitate was re-dissolved in distilled water. To remove any remaining undissolved materials, the solution was centrifuged and the clear supernatant was again precipitated. The resulting precipitate was re-dissolved in distilled water. Broth kefiran was then quantified calorimetrically by adding 1 mL of anthrone reagent (0.8 g anthrone, 100 mL $H_2O$ and 300 mL $H_2SO_4$) to 0.1 mL of the broth kefiran solution (4). The mixture was incubated for 10 min at 100°C and cooled to room temperature and the optical density at 620 nm was measured. The concentration of broth kefiran was calculated using the standard curve of glucose. The kefiran surrounding the cells named as capsular kefiran was extracted from the cells by boiling the cells in distilled water at 100°C for 30 min. The mixture was centrifuged and the extracted capsular kefiran was measured. Total kefiran was the sum of broth and capsular kefiran.

## 3. Results and discussion

**3.1 Effect of various carbon sources on cell growth and kefiran production**

Figure 1 shows the effect of carbon sources on growth and kefiran production. It was found that galactose gave the highest dry cell weight of 2.60±0.20 g/L followed by glucose and lactose (2.33±0.28 and 2.36±0.15 g/L, respectively). While lactose gave the highest kefiran production of 283±15 mg/L followed by glucose (210±20 mg/L) and sucrose (177±6 mg/L). The catabolism of lactose begins with the decomposition of disaccharide into glucose and galactose. Both monosaccharide take part in the further syntheses of ATP, biomass, and biosynthesis of EPS (5). Lactose was a suitable carbon source for kefiran production. The highest kefiran production was 712 mg/L (2). The effect of carbon sources on kefiran production by kefir grains was studied. Among the sugars tested (fructose, glucose, sucrose and lactose), the addition of lactose gave the highest biomass of kefir grains of 12 g/L and the highest kefiran production of 4.3 % of kefir grains biomass, followed by sucrose (9 g/L and 3 %, respectively). (6)

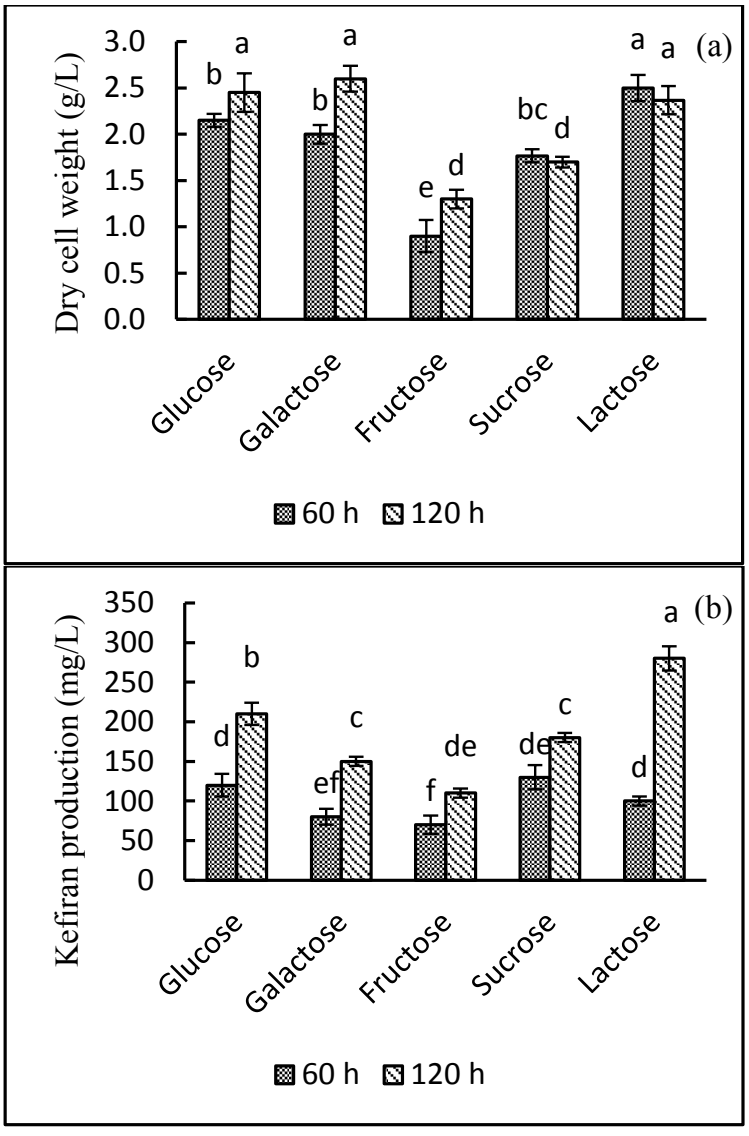


**Figure 1.** Effect of various carbon sources on growth and total kefiran production of L. kefiranofaciens JCM 6985 cultivated using 20 g/L sugar concentration at pH 5.5 and 30°C for 60 h and 120 h. Different letters on the bar indicated significant differences between treatments ($p<0.05$).

### 3.2 The use of molasses for kefiran production

Molasses is the by-product of sugar cane industry. It contains 46 % sugar, 3 % protein, 65% nitrogen-free extract and 8.1% ash (7). Figure 2 shows the effect of molasses concentration on growth and kefiran production. At the highest molasses concentration of 80 g/L, the dry cell weight and kefiran production increased up to 2.43±0.12g/L and 235±6 mg/L, respectively. Several researches showed that molasses can be used as a carbon source for lactic acid bacteria. The production of lactic acid

by *Enterococcus faecalis* using molasses as a carbon source. They found that the use of molasses at a concentration of 333 g/L (170 g sugar/L) gave lactic acid production as high as 134.9 g/L (8). The use of several cheap carbon sources to produce lactic acid by *L. delbrueckii*. They found that the use of sugarcane molasses at 133 g sugar/L, sugar cane at 119 g sugar/L and sugar beet at 105 g sugar/L gave lactic acid production of 120, 107 and 84 g/L, respectively (9).

Molasses could be used for exopolysaccharides production by *Psudomonas fluorescens*. When using cane molasses at a concentration of 50 g/L, 2843 mg/L of exopolysaccharides was produced (1).

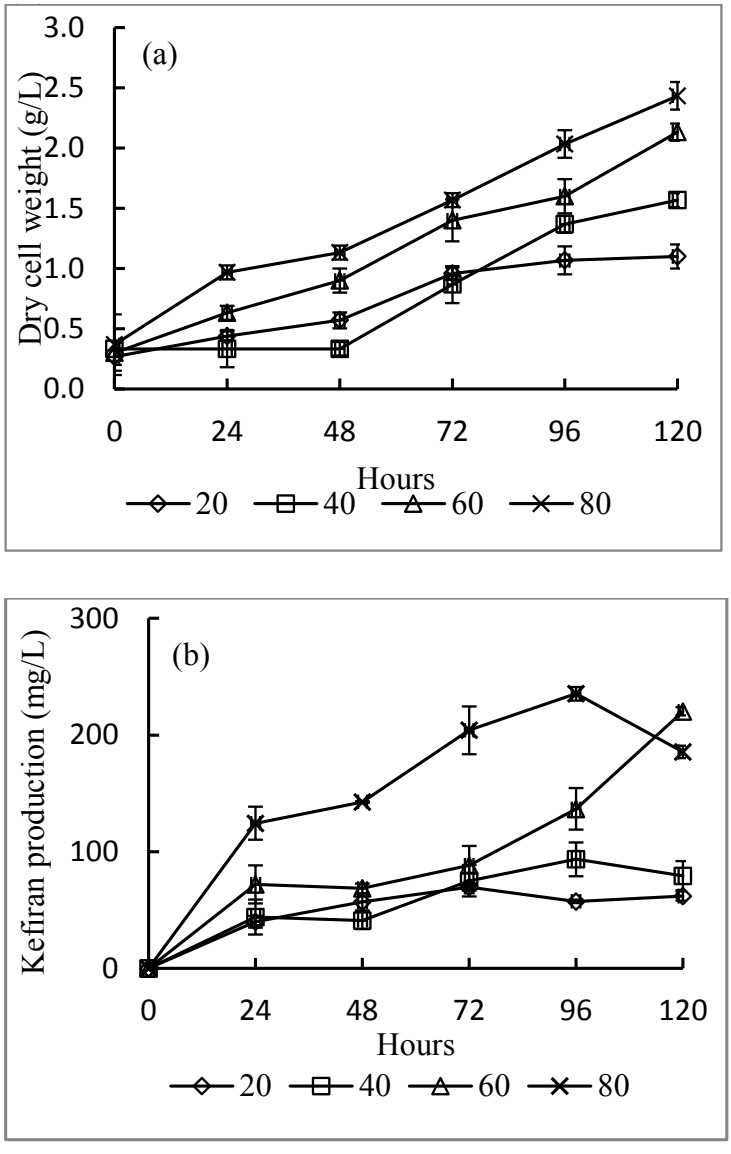


**Figure 2.** Effect of molasses concentration 20, 40, 60 and 80 g/L on growth and kefiran production of *L. kefiranofaciens* JCM 6985 cultivated at pH 5.5 and 30°C for 5 days.

Table.1 shows dry cell weight and kefiran production from various carbon sources. The use of different carbon sources affected the growth and synthesis of EPS. In this study, fructose gave the highest growth yield of 0.15±0.01, while lactose gave the highest production yield of 15.63±0.84 mg/g followed by fructose and glucose. It should be noted that the use of molasses at appropriate sugar concentration of 20 g/L compared with pure sugar was found that molasses gave kefiran at 69±2 but less than pure sugar, however molasses is cheaper than other sugars and could be a better carbon source for economical.

**3.3 Kefiran production by using various nitrogen sources**

Figure 3 shows kefiran production by using various nitrogen sources. It was found that the spent yeast cells gave the highest kefiran production of 580±10 mg/L. This could be because spent yeast cells contains protein at high level of 45-60%, vitamins and minerals. The spent cell yeast is an inexpensive nitrogen source and generally recognized as safe (GRAS) and has good nutrition characteristics (10) Wang and Bi (2008) study the effect of organic and inorganic nitrogen on the production of kefiran by *L. kefiranofaciens* JCM 6985. Various nitrogen sources including peptone, casein, yeast powder, tryptone, yeast extract, urea, ammonium chloride and ammonium sulfate at a concentration of 50 g/L were tested. The carbon source was lactose at a concentration of 100 g/L. They found that the organic nitrogen gave better growth and kefiran production than the inorganic nitrogen. Among the nitrogen sources tested, the yeast powder gave the highest growth while casein gave the highest kefiran production (3).Figure 4 shows kefiran production by *L. kefiranofaciens* JCM 6985 using spent yeast cells hydrolysate. It was found that spent yeast cells hydrolysate gave higher kefiran production of 1286±19 mg/L. This could be because after hydrolysis the protein might be hydrolyzed to lower molecule size that can be easily used by *L. kefiranofaciens* JCM 6985. The effect of baker's and brewer's yeast on growth of *L. acidophilus* EQ57. They found that the autolysis obtained from 100 brewer's yeast gave the highest growth of

**Table 1.** Dry cell weight and kefiran production from various carbon sources

| Carbon sources (sugar concentration) | DCW (g/L) | Kefiran production (mg/L) | ΔS (g/L) | Yx/s | Yp/s(mg/g) |
|---|---|---|---|---|---|
| Glucose (20 g/L) | 2.33±0.38 | 210±20 | 18.92±0.08 | 0.13±0.01 | 11.10±1.05 |
| Galactose (20 g/L) | 2.6±0.20 | 153±6 | 19.14±0.03 | 0.14±0.01 | 8.01±0.3 |
| Fructose (20 g/L) | 1.3±0.10 | 103±6 | 8.54±0.14 | 0.15±0.01 | 12.10±0.68 |
| Sucrose (20 g/L) | 1.76±0.32 | 180±5.8 | 16.21±1.63 | 0.09±0 | 10.90±0.36 |
| Lactose (20 g/L) | 2.37±0.15 | 283±15.3 | 18.13±0.18 | 0.14±0.01 | 15.63±0.84 |
| Molasses (20 g/L) | 1.10±0.10 | 69±2 | 8.70±0.68 | 0.13±0.01 | 7.96±0.61 |
| (40 g/L) | 1.57±0.06 | 93±14 | 9.74±0.25 | 0.16±0.01 | 9.60±1.94 |
| (60 g/L) | 2.13±0.2 | 203±13 | 10.94±0.39 | 0.20±0.02 | 18.56±2.57 |
| (80 g/L) | 2.43±0.11 | 235±5.7 | 26.20.46 | 0.09±0 | 7.79±0.21 |

ΔS: Sugar consumption, Yx/s: Growth yield, Yp/s: Production yield

*L. acidophilus* EQ57 at O.D. 600 nm 1.264 (11). The effect of acid-hydrolysis of spent yeast cells for lactic acid production from *L. rhamnosus* found that the combination of 5 g/L of yeast extract and the spent yeast cells hydrolysate had high performance in lactic acid production with high productivity of 2.63 g/L h and high yield of 98.8 % more excellent than those from the fermentation supplemented with 15 g/L of yeast extract (12).

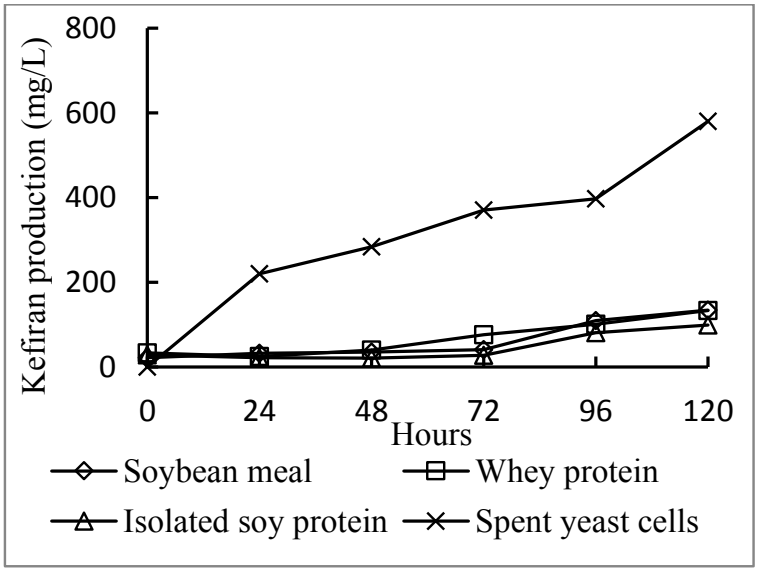


**Figure 3.** Kefiran production by *L. kefiranofaciens* JCM 6985 using various nitrogen sources cultivated at pH 5.5 and 30°C for 5 days.

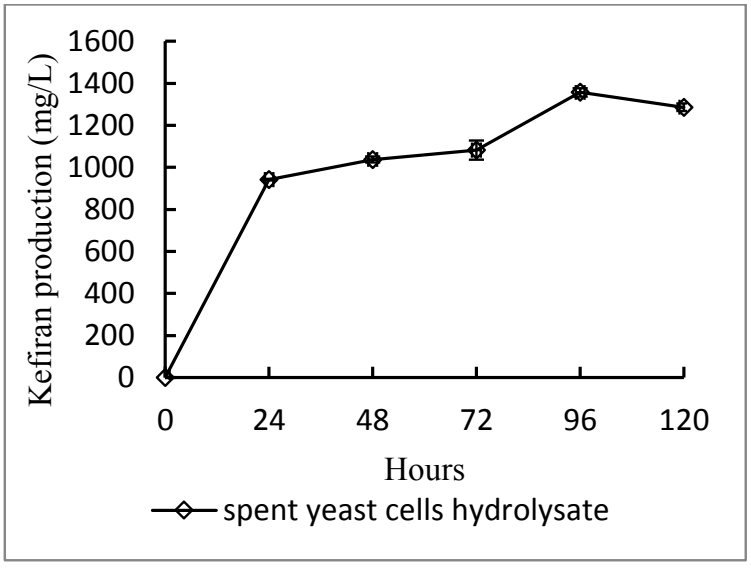


**Figure 4.** Effect of using spent yeast cells hydrolysate on kefiran production of *L. kefiranofaciens* JCM 6985 cultivated at pH 5.5 and 30°C for 5 days.

## 4. Conclusion

In the production of kefiran with different sugar by *L. kefiranofaciens* JCM 6985 lactose gave the highest kefiran production of 283.3±15.3 mg/L followed by glucose and sucrose at 210±20 and 180±5.8 mg/L respectively. Molasses at a concentration of 80 g/L gave the highest kefiran production of 235±5.69 mg/L. When spent yeast cells hydrolysate was used as the nitrogen source, it gave higher kefiran production than the use of spent yeast cells. These results show that it was possible to produce kefiran using molasses as a low-cost carbon source and spent yeast cells as a low-cost nitrogen source.

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