



King Saud University

Saudi Journal of Biological Sciences

www.ksu.edu.sa
www.sciencedirect.com



ORIGINAL ARTICLE

Bioprocess development for kefiran production by *Lactobacillus kefiranofaciens* in semi industrial scale bioreactor



Daniel Joe Dailin ^a, Elsayed Ahmed Elsayed ^{b,c}, Nor Zalina Othman ^a,
Roslinda Malek ^a, Hiew Siaw Phin ^a, Ramlan Aziz ^a, Mohamad Wadaan ^b,
Hesham Ali El Enshasy ^{a,d,*}

^a Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), 81130 UTM, Skudai, Malaysia

^b Bioproducts Research Chair, Zoology Department, Faculty of Science, King Saud University, 11451 Riyadh, Saudi Arabia

^c Natural and Microbial Products Department, National Research Centre, Dokki, Cairo, Egypt

^d City of Scientific Research and Technology Application, New Burg Al Arab, Alexandria, Egypt

Received 23 December 2014; revised 26 May 2015; accepted 3 June 2015

Available online 10 June 2015

KEYWORDS

Lactobacillus kefiranofaciens;
Kefiran;
Polysaccharide production;
Medium optimization;
Submerged culture;
Stirred tank bioreactor

Abstract *Lactobacillus kefiranofaciens* is non-pathogenic gram positive bacteria isolated from kefir grains and able to produce extracellular exopolysaccharides named kefiran. This polysaccharide contains approximately equal amounts of glucose and galactose. Kefiran has wide applications in pharmaceutical industries. Therefore, an approach has been extensively studied to increase kefiran production for pharmaceutical application in industrial scale. The present work aims to maximize kefiran production through the optimization of medium composition and production in semi industrial scale bioreactor. The composition of the optimal medium for kefiran production contained sucrose, yeast extract and K_2HPO_4 at 20.0, 6.0, 0.25 g L⁻¹, respectively. The optimized medium significantly increased both cell growth and kefiran production by about 170.56% and 58.02%, respectively, in comparison with the unoptimized medium. Furthermore, the kinetics of cell growth and kefiran production in batch culture of *L. kefiranofaciens* was investigated under un-controlled pH conditions in 16-L scale bioreactor. The maximal cell mass in bioreactor culture reached 2.76 g L⁻¹ concomitant with kefiran production of 1.91 g L⁻¹.

© 2015 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author at: Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Johor Bahru, Johor, Malaysia. Tel.: +60 07 5531573.

E-mail address: henshasy@ibd.utm.my (H.A. El Enshasy).

Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

1. Introduction

The exopolysaccharides present in the kefir grains were first discovered and named as kefiran by La Rivière et al. (1967). Kefiran is a type of water soluble polysaccharide that is produced by lactic acid bacteria, i.e. *Lactobacillus kefiranofaciens* (Kandler and Kunath, 1983). Kefiran contains equal amounts

<http://dx.doi.org/10.1016/j.sjbs.2015.06.003>

1319-562X © 2015 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

of glucose and galactose (Kooiman, 1968). Kefiran is used widely in the pharmaceutical industrial sectors. Polysaccharides synthesized from microorganisms, especially bacteria, remain free from market monopoly (Sutherland, 2001), and therefore, there are needs to acquire adequate knowledge about process optimization to produce high yields from these valuable products. However, they are present only in a small fraction of today's biopolymer market due to different economic factors (De Vuyst et al., 2001). Kefiran is regarded as safe, since kefir grain has long been consumed. It has recently been reported to have antibacterial, anti-fungal, anti-inflammatory activities and cicatrizing agents for use against a variety of infections (Cevikbas et al., 1994; Schneedorf et al., 2005; Furuno and Nakanishi, 2012). In addition, an enhancing effect on the production of interferon β cortisol and noradrenaline in human cell lines has been reported, and a possible use as a stress reducing food component has been hypothesized (Kabayama et al., 1997). The oral administration of kefir into mice shows that kefir possesses the antitumor activities and delayed type hypertension induced by picryl chloride (Murofushi et al., 1983; Shioimi et al., 1982). Besides, kefir also protects Caco-2 cells from cytopathic effects induced by *Bacillus cereus* infection (Medrano et al., 2009). Another study by Maeda et al. (2004) reported that kefir lowered the serum cholesterol level in spontaneous stroke-prone rats. Kefiran does not inhibit the absorption of cholesterol in food; however, it traps enterohepatics-circulating cholesterol in the intestine. It contributes to the prevention of hepatic disorders caused by cholesterol and orotic acid. Furthermore, it has various prevention functions where it also acts as a preventive for liver disorder and decreased intestinal histamine concentration (Maeda et al., 2005). Furthermore, the oral administration of kefir has been reported to induce changes in the balance of immune cells in a murine model (Pérez et al., 2011). Moreover, kefir reduces atherosclerosis in rabbits fed with high cholesterol diet (Ishii et al., 2010).

The optimization of kefir production generally proceeds via the determination of the best carbon source suitable for cell growth and kefir production. Suitable carbon sources can be either complex or simple such as glucose, sucrose, fructose, xylose and lactose. According to Yokoi and Watanabe (1992), lactose was found to be the best chemically defined carbon source for the production of kefir by *Lactobacillus* sp. KPB-167B, compared to glucose and sucrose. Moreover, El Enshasy et al. (2011) found that sucrose is the best carbon source for xanthan production, and that increasing the sucrose concentration above a certain limit did not have any significant influence on the cell specific production rate. Moreover, the effect of nitrogen sources on the production of kefir and cell growth has been also investigated. According to Cheirsilp et al. (2003), the higher the C/N ratio is, the lower the total kefir production. Phosphate salts are inorganic compounds that are generally used in the production medium to enhance bacterial growth. To the best of our knowledge, there are no available data on the effect of phosphate on the growth of *L. kefiranofaciens* and kefir production. However, previous reports documented the effect of phosphate on the growth of bacterial strains producing other types of polysaccharides. Gunter and Ovodov (2005) showed that cell growth and production of polysaccharides were limited by the absence of phosphate. Moreover, Duguid and Wilkinson (1953) proved that the maximal cell growth of *Aerobacter aerogenes* and polysaccharide

production could be obtained at phosphate concentration of 0.12 g L^{-1} . Moreover, xanthan production was highly effected by the concentration of phosphate (Souw and Demain, 1979).

The present work focuses on maximizing the production of kefir in semi-industrial scale. Firstly, the bioprocess was optimized in small scale where different cultivation media have been selected for preliminary experimentation to investigate their potential for the production of higher yields of kefir. Furthermore, the composition of the best suitable medium was optimized in shake flask level. Finally, the process was scaled up to a pilot scale 16-L stirred tank bioreactor to investigate the kinetics of cell growth and kefir production under uncontrolled pH conditions.

2. Methods and materials

2.1. Abbreviations

- X_{max} : maximal cell dry weight [g L^{-1}]
- dx/dt : growth rate [$\text{g L}^{-1} \text{ h}^{-1}$]
- μ : specific growth rate [h^{-1}]
- Kp_{max} : maximal production of kefir [g L^{-1}]

2.2. Microorganism

The strain used in the present study for kefir production is *L. kefiranofaciens* ATCC 8007. The bacterium was activated in Man-Rogosa-Sharpe (MRS) broth medium (Difco, BD Diagnostic Systems, Maryland, USA) consisting of (g L^{-1}): peptone casein, 30; meat extract, 10; yeast extract, 6.0; sodium acetate, 5.0; ammonium citrate, 2.0; glucose, 0.2; magnesium sulfate, 0.2; manganese sulfate, 0.05; dipotassium phosphate, 2.0. The pH of the medium was adjusted to 5.5. The master cell bank culture was inoculated into agar plates and incubated for 48 h to produce the working cell culture. Stock cultures were preserved in 50% glycerol (v.v⁻¹) at $-78 \text{ }^\circ\text{C}$.

2.3. Inoculum preparation

The cells were activated on an agar plate prior to cell propagation in the broth medium. The inoculated plates were then incubated at $30 \text{ }^\circ\text{C}$ for 24 h. Inoculum was prepared by inoculation of 250 mL Erlenmeyer flasks containing 50 mL of MRS liquid medium with 1 mL of working cell bank cultures. The inoculated flasks were then incubated at 200 rpm and $30 \text{ }^\circ\text{C}$ for 24 h in a rotary shaker (Innova 4080, New Brunswick Scientific, NJ, USA). The production medium either in the Erlenmeyer flasks or the stirred tank bioreactor was then inoculated with 5% (v.v⁻¹) of the inoculum.

2.4. Culture media for cell growth and kefir production

L. kefiranofaciens ATCC 8007 cells were cultivated on five different cultivation media available from the literature to determine the most suitable medium for cell growth and kefir production. The composition of different investigated cultivation media is summarized in Table 1. The selected cultivation media contained different carbon sources, including glucose, lactose, starch and sucrose. Additionally, the most suitable

Table 1 Different industrial media used for *L. kefiranofaciens* growth and kefiran production.

Medium No	1	2	3	4	5
Glucose	20				
Lactose		100			
Sago starch			100		
Soluble starch				100	
Sucrose					100
Meat Extract	10	20	20		
Yeast Extract	5	10	10	10	10
Casein peptone	10				
K ₂ HPO ₄	2	2	2	2	2
Sodium Acetate	5	5	5	5	5
Triammonium citrate	2	4	4	2	2
MgSO ₄ ·7H ₂ O	0.2	0.58	0.58	0.2	0.2
MnSO ₄ ·H ₂ O	0.05	0.28	0.28	0.05	0.05
Tween 80	1	1	1	1	1
Tryptone		20	20		
CaCl ₂ ·2H ₂ O		0.74	0.74		
Initial pH	5.5	5.5	5.5	5.5	5.5
References	Kandler and Kunath (1983)	Cheirsilp et al. (2003)	Yeesang et al. (2008)	Maeda et al. (2004)	Maeda et al. (2004)

cultivation medium was further optimized, where different concentrations of sucrose (0.0–100 g L⁻¹), yeast extract (0.0–14 g L⁻¹) and K₂HPO₄ (0.0–2 g L⁻¹) were evaluated for their effect on the production of kefiran. The weight of carbon sources corresponding to different cultivation media was calculated per flask, dissolved in 10 mL of water and was then sterilized separately in test tubes. Afterward the content of the test tubes was then added to the fermentation medium before inoculation under aseptic conditions. The inoculated flasks were incubated on the rotary shaker (Innova 4080, New Brunswick, NJ, USA) at 200 rpm and 30 °C.

2.5. Bioreactor cultivation

Batch cultivations in stirred tank bioreactor were carried out using 16-L pilot scale stirred tank bioreactor (BioEngineering, Wald, Switzerland) with a working volume of 8-L. The reactor was sterilized *in situ* at 121 °C for 20 min, cooled and then inoculated with inoculum. The optimized medium was used with the same cultivation conditions as in shake flasks in terms of inoculum size, temperature and pH. The agitation speed was maintained at 400 rpm throughout the cultivation, while aeration was performed using filtered sterile air and supplied continuously to the bioreactor at a rate of 1.0 v.v⁻¹ min⁻¹. The stirrer is equipped with two 6-bladed rushton turbine impellers ($d_{i(\text{impeller diameter})} = 85 \text{ mm}$; $d_{i(\text{tank diameter})} = 214 \text{ mm}$, $d_i/d_{i-1} = 0.397$). Foam was suppressed by the addition of silicon-based antifoam grade A (Sigma-Aldrich Inc., MO, USA). During the cultivation process, pH and dissolved oxygen concentration were determined using liquid filled pH electrode and DO polarographic electrodes (Ingold, Mettler-Toledo, Switzerland), respectively. The initial medium pH was adjusted to 5.5 by cascading the pH controller with acid/base feeding peristaltic pumps connected with 2.0 M HCl and 2.0 M NaOH, respectively. During bioreactor cultivation, the pH was not controlled.

2.6. Sample preparation and cell dry weight determination

Samples in the form of two flasks containing 50 mL each or 50 mL broth in the case of bioreactor, were withdrawn at different time intervals during the cultivation in a centrifugation tube. Cell dry weight was determined by gravimetric methods in duplicate. The culture broth was centrifuged in 50 mL falcon tubes at 9000 rpm for 15 min to precipitate the cells. The supernatant was collected in another falcon tube for kefiran analysis. The cells were then centrifuged again under the same condition. After the second centrifugation, the supernatant was discarded and the cells were dried at 65 °C in an oven for 48 h.

2.7. Kefiran determination

Extracellular kefiran was recovered from the culture supernatant and determined according to Piermaria et al. (2009). Kefiran was precipitated overnight by the addition of an equal volume of cold absolute ethanol at 4 °C. The resulting precipitate was collected by centrifugation at 9000 rpm for 15 min, dissolved in hot distilled water and precipitated with ethanol. This step was repeated for three times to obtain pure kefiran. The supernatant of the centrifugation was discarded and the kefiran was dried at 65 °C in an oven for 48 h.

2.8. Statistical analysis

Data were analyzed with the help of SPSS 9.0, and the results were given as mean ± SD of three separate experiment replicates. The mean comparison between different evaluated parameters was performed using ANOVA one-way analysis of variance. Statistical significance was defined when $p < 0.05$.

3. Results and discussion

3.1. Medium screening for cell growth and kefiran production

The cell growth and kefiran production of *L. kefiranofaciens* ATCC 8007 were determined in five different media as described in materials and method section. The inoculated flasks for each medium were incubated at 30 °C on a rotary shaker at 200 rpm for 72 h. Samples were taken at the end of the fermentation for the analysis of pH, cell growth and kefiran production. The most suitable medium for kefiran fermentation was selected based on kefiran production. Fig. 1 shows the kefiran produced for five different media. From this figure, it can be observed that medium 5 gave the highest kefiran production of about 0.72 g L⁻¹. This was followed by media 3, 4, 2, and 1, where the produced kefiran was 0.62, 0.31, 0.20, and 0.09 g L⁻¹, respectively. After 72 h incubation, the maximal cell dry weight obtained in medium 4 was 4.49 g L⁻¹ followed by media 3, 1, 2, and 5, where the cell biomass reached 1.70, 1.53, 1.06, and 1.01 g L⁻¹, respectively.

Media 4 and 5 differ only in the carbon source used, i.e. soluble starch and sucrose, respectively. From the data presented in Fig. 1, although sucrose as the carbon source gave the lowest cell dry weight in medium 5, it gave the highest production of kefiran compared to soluble starch used in medium 4. This indicates that the production of kefiran is not highly dependent on the amount of biomass achieved by the end of the fermentation, but rather on the type and concentration of nutrients added. These results are in good agreement with those obtained by [Elsayed et al. \(2013\)](#), who investigated the effect of medium supplementation with various short-chain carboxylic acids on the kinetics of cell growth and natamycin production by *Streptomyces natalensis*. Their results showed that the natamycin production increased due to medium supplementation with acetate or propionate and not due to the change in biomass. While their butyrate supplemented medium resulted in a decreased specific natamycin production, with higher increase in cell biomass. They concluded that the production of the secondary metabolite is largely affected by the type of nutrients added to the cultivation medium rather than the concentration of the produced cell biomass. Based on these data, medium 5 composed of (g L⁻¹): sucrose, 100; yeast extract, 10; Tween 80, 1; K₂HPO₄, 2; sodium acetate, 5;

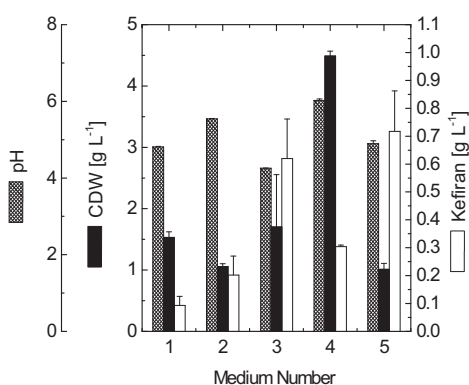


Figure 1 Cell growth, kefiran production and pH profile during the growth of *L. kefiranofaciens* in 5 different cultivation media in shake flask level.

triammonium citrate, 2; MgSO₄·7H₂O, 0.2; MnSO₄·5H₂O, 0.05 was chosen for further optimization.

3.2. Kinetics of cell growth and kefiran production in un-optimized medium

Cultivations were carried out in shake flasks for 72 h to investigate the kinetics of cell growth and kefiran production by *L. kefiranofaciens* using the selected production medium. During cultivations, samples were taken every 6 h for the determination of pH, cell dry weight and kefiran. Fig. 2 shows the profile of pH, cell growth and kefiran production for the un-optimized medium. It can be clearly observed that kefiran was not produced during the first 6 h of cultivation, which can be considered as the lag phase where the cells were adapted to the new environment. After that, the cells grew exponentially with a growth rate of 0.021 g L⁻¹ h⁻¹ and reached their maximal cell mass (1.02 g L⁻¹) after 60 h. On the other hand, kefiran production increased proportionally to the cell mass with a production rate of 0.015 g L⁻¹ h⁻¹, where it reached a maximum of 0.81 g L⁻¹ after 72 h. The pH increased up to 36 h of the start of cultivation and then started to drop reaching a final pH of 4.70.

3.3. Effect of sucrose concentration on cell growth and kefiran production

Carbon source is considered to be a very important component of the cultivation medium, since it is generally used as a source for energy and biosynthesis of kefiran. Previously, lactose was conventionally used as a good source for the high production of kefiran ([Yokoi and Watanabe, 1992](#)). However, our results showed that medium 5 containing sucrose exhibited significant effect on cell growth and kefiran production, which were in accordance with the results of [Wang and Bi \(2008\)](#), who found that sucrose was able to support kefiran production, where 1.48 g L⁻¹ were produced after 72 h. Furthermore, [Harta et al. \(2004\)](#) investigated the influence of the addition of different monosaccharides, disaccharides and their mixtures into kefir grain production. They found that sucrose (at 19.75 g L⁻¹), after fructose, was the second best carbon source suitable for maximal kefiran production. Accordingly, the effect of different sucrose concentrations on cell growth and

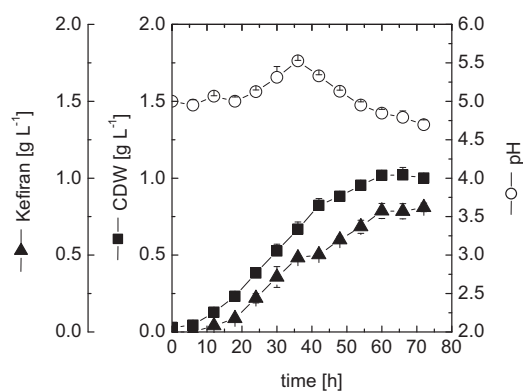


Figure 2 Change in biomass, kefiran production and pH in medium number 5 during shake flask cultivation at 30 °C for 72 h.

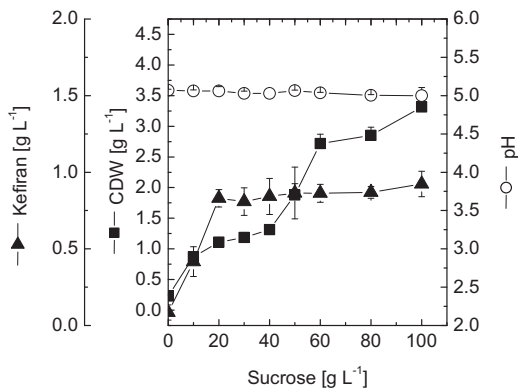


Figure 3 Cell growth, kefiran production and pH profile as affected by different sucrose concentrations. Cultivations were conducted for 72 h in shake flasks culture at 200 rpm and 30 °C.

kefiran production was evaluated by cultivating the cells in different sucrose concentrations ranging from 0.0 to 100 g L⁻¹. The results obtained in Fig. 3 show that increasing the sucrose concentration from 0.0 to 100 g L⁻¹ increased the cell growth from 0.23 to 3.3 g L⁻¹, respectively. Moreover, cultivating the cells at the highest sucrose concentration had no inhibitory effect on their growth. This may be attributed to the low accumulation of reducing sugars, which is not high enough to inhibit the cell growth. On the other hand, the production of kefiran was significantly affected by the sucrose concentration. The maximal kefiran production (0.83 g L⁻¹) was obtained at sucrose concentration of 20 g L⁻¹ after 72 h. Further increase in the concentration of sucrose did not highly increase the production of kefiran. These results were in accordance with those obtained by Yeesang et al. (2008), where they reported that further increase in reducing sugar concentration above the optimal concentration does not significantly increase kefiran production. This can be explained on the basis that the concentrations of sucrose higher than 20 g L⁻¹ may restrict the ability of cells to uptake sucrose, thus inhibiting the biosynthesis of kefiran. No significant changes in the pH were detected during the fermentation as the concentration of sucrose increased.

3.4. Effect of yeast extract concentration on cell growth and kefiran production

Yeast extract is generally known as a source required for nitrogen and growth factors. In order to determine the effect of yeast extract on kefiran production, *L. kefiranofaciens* cells were cultivated on medium containing 20 g L⁻¹ sucrose and different concentrations of yeast extract ranging from 0.0 to 14 g L⁻¹. As shown in Fig. 4, yeast extract exhibited a significant effect on cell growth and kefiran production. Less growth and kefiran production was observed when none yeast extract added to the production medium. Increasing the concentration of yeast extract increased the production of kefiran, where it increased from 0.377 to 0.948 g L⁻¹ as the concentration increased from 2.0 to 6.0 g L⁻¹, respectively. This corresponds to about 151.5% improvement in the produced kefiran. These results were in agreement with those reported by Taniguchi et al. (2001), who found that the addition of 5 g L⁻¹ yeast extract is essential for kefiran production and resulted in the production of 650 mg L⁻¹ of kefiran in the culture medium.

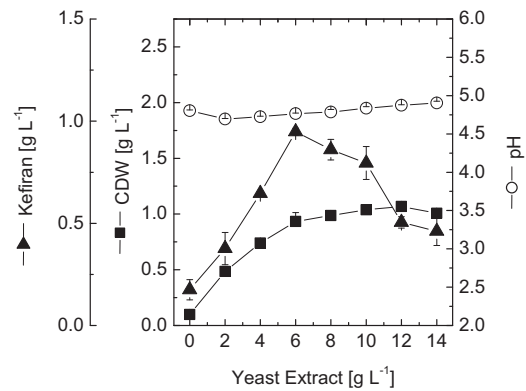


Figure 4 Cell growth, kefiran production and pH profile as affected by different yeast extract concentrations. Cultivations were conducted for 72 h in shake flasks culture at 200 rpm and 30 °C.

Further increase in the concentration of yeast extract adversely affected the production of kefiran. The concentration of cell biomass reached 0.933 g L⁻¹ at 6 g L⁻¹ of yeast extract, where it remained more or less constant at higher yeast extract concentrations. Taniguchi et al. (2001) showed that cultivating the cells without yeast extract resulted in poor growth and kefiran was hardly produced. According to Hsieh et al. (2006), the production of polysaccharides increased with lower concentration of nitrogen sources. Accordingly 6 g L⁻¹ yeast extract was chosen as the optimal concentration for further optimization steps.

3.5. Effect of phosphate concentration on cell growth and kefiran production

Phosphate ions are important for cell growth and kefiran production. Different concentrations of K₂HPO₄ were tested ranging between 0.0 and 2.0 g L⁻¹. The addition of phosphate showed a significant effect on kefiran production, cell growth and pH. In this study, the absence of phosphate resulted in a very poor growth and kefiran production. Increasing the phosphate concentration to 0.25 g L⁻¹, resulted in a significant increase in kefiran production (1.187 g L⁻¹), corresponding to a 25.2% increase. However, further increase in the concentration of phosphate up to 0.5 g L⁻¹ of phosphate; significantly decreased the production of kefiran concentration to 0.867 g L⁻¹ (Fig. 5). The addition of higher phosphate concentrations, i.e. above 0.5 g L⁻¹, showed no effect on both cell growth and the concentration of kefiran. These results were in agreement with those obtained by Hsieh et al. (2006), who investigated the production of polysaccharides under different limitations. They found that phosphate concentration greatly affects the production of the polysaccharide as well as cell biomass. Their results revealed that the absence of phosphate adversely affected both polysaccharide production and cell biomass. Moreover, they also found that increasing the phosphate concentration above the optimal concentration favors cell growth rather than polysaccharide production, which they attributed to the decrease in the activity of enzymes catalyzing the biosynthesis of the polysaccharide. They concluded that lower molecular weight polysaccharides (56–152 KDa) require phosphate concentrations lower than 1 g/L⁻¹, while higher

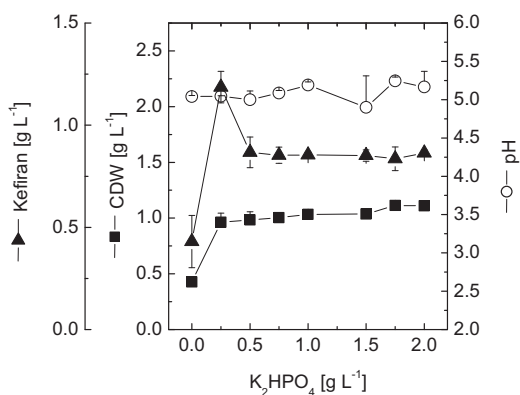


Figure 5 Cell growth, kefiran production and pH profile as affected by different initial phosphate concentrations. Cultivations were conducted for 72 h in shake flasks culture at 200 rpm and 30 °C.

molecular weight polysaccharides (92–277 KDa) are mostly produced at phosphate concentrations above 2 g/L^{-1} .

3.6. Kinetics of cell growth and kefiran production in optimized growth medium

The kinetics of cell growth and kefiran production were evaluated for the cells cultivated in the finally optimized medium in comparison with the un-optimized medium. The optimized production medium contained ($g L^{-1}$): sucrose, 20; yeast extract, 6; phosphate, 0.25; sodium acetate, 5. Fig. 6 shows the kefiran production, cell growth and changes of pH value, where the obtained data showed a sigmoid representation during the fermentation. The maximal concentration of kefiran obtained reached 1.28 $g L^{-1}$, which corresponds to an increase of 58.02% in comparison with the production obtained in the un-optimized medium (0.81 $g L^{-1}$). Furthermore, the optimized production medium gave higher cell biomass, where the maximal cell biomass reached 1.57 $g L^{-1}$ (1.0 $g L^{-1}$ in the un-optimized medium, Fig. 5). These obtained results are in good agreement with the results obtained by Dailin et al. (2014), who investigated medium optimization of kefiran production using *L. kefiranofaciens* in shake-flask level. Their

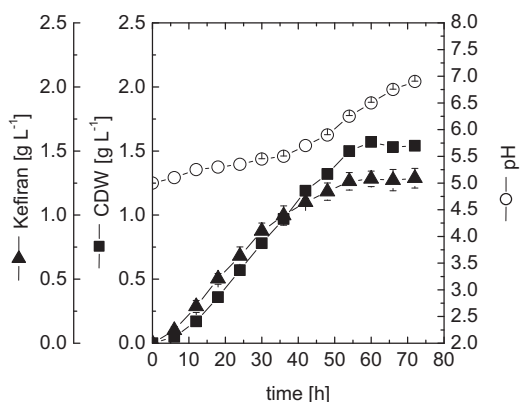


Figure 6 Cell dry weight, kefiran production and pH profile in optimized medium during fermentation in shake flasks at 30 °C for 72 h.

optimized medium increased the maximal cell biomass by about 90% to reach 1.92 $g L^{-1}$, concomitant with 5-fold increase in kefiran production (1.29 and 0.26 $g L^{-1}$ for optimized and non-optimized medium, respectively).

3.7. Kinetics of cell growth and kefiran production during batch cultivation in 16-L stirred tank bioreactor under uncontrolled pH condition

In order to evaluate the scalability of the cultivation process, the cell growth and kefiran production were studied in a 16-L pilot scale stirred tank bioreactor using the optimized medium under un-controlled pH conditions. The results obtained in Fig. 7 show that cells grew exponentially with a growth rate of 0.051 $g L^{-1} h^{-1}$, reaching a maximal cell mass of 2.76 $g L^{-1}$ after 72 h. As reported by Elmarzugi et al. (2010) for lactic acid bacteria, the maximal cell growth was obtained when *Lactococcus lactis* cells were cultivated under uncontrolled pH conditions. Kefiran was produced along during the exponential growth phase which shows that kefiran production is associated with the cell growth. The rate of kefiran production was 0.053 $g L^{-1} h^{-1}$, which is almost likely the same as the growth rate, where the maximal kefiran produced reached about 1.9 $g L^{-1}$ at 40 h. pH increased along the cultivation process, reaching 8.45 by the end of the cultivation.

3.8. Comparison between different optimization steps

The results presented in Table 2 show clearly the effect of different optimization steps on the process development of kefiran production by *L. kefiranofaciens*. It can be seen that the kinetic parameters for cell growth and kefiran production were greatly enhanced by optimizing the production medium in shake flask as well as bioreactor levels. The optimization of different components of the production medium increased the maximal cell biomass by about 57% (from 1.0 to 1.57 $g L^{-1}$), which was also reflected on the cell growth rate that increased from 0.021 to 0.033 $g L^{-1} h^{-1}$, and the maximal specific cell growth increased from 0.0011 to 0.0053 h^{-1} . The increased cell mass production resulted in a concomitant increase in the maximal production of kefiran, which increased from 0.81 to 1.29 $g L^{-1}$, which corresponds to 48% increase.

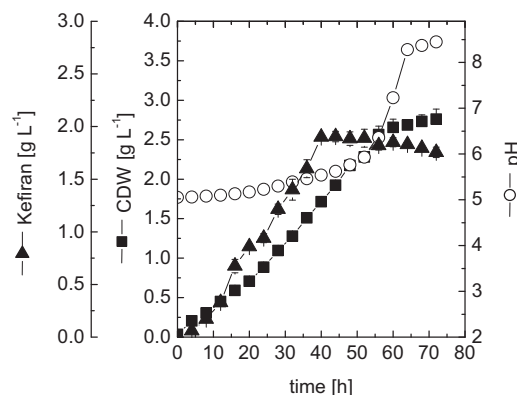


Figure 7 Kinetics of cell growth and kefiran production during batch cultivation of *L. kefiranofaciens* in 16-L stirred tank bioreactor under uncontrolled pH condition.

Table 2 Kinetics of cell growth and kefiran production by *L. kefiranofaciens* in shake flask and in bioreactor using different media and different cultivation conditions.

Parameters	Shake Flasks (medium optimization)		Bioreactor
	Initial medium	Optimized medium	Optimized medium
X_{\max} [g L ⁻¹]	1.0000	1.5700	2.760
dx/dt [g L ⁻¹ h ⁻¹]	0.0210	0.0330	0.053
μ [h ⁻¹]	0.0011	0.0053	0.015
Kp_{\max} [g L ⁻¹]	0.8100	1.2900	1.910

Abbreviations: X_{\max} : maximal cell dry weight; dx/dt : growth rate; μ : specific growth rate; Kp_{\max} : maximal production of kefiran.

This can be attributed to the fact that providing the cells with the most suitable concentrations of nutrients enables the cell machinery to perform at their optimal conditions, thus producing maximal concentrations of cell biomass and kefiran. On the other hand, cultivating *L. kefiranofaciens* at the optimal medium compositions in 16-L stirred tank semi-industrial scale bioreactor resulted in a more pronounced increase in cell growth and kefiran production. The maximal cell biomass obtained bioreactor cultivation reached 2.76 g L⁻¹, where the cells grew with a growth rate of 0.053 g L⁻¹ h⁻¹, corresponding to an increase by about 75.8% and 60.6% from the values obtained in shake flask cultivation, respectively. For kefiran production, the bioreactor cultivation under uncontrolled pH conditions resulted in an increase of about 48.1% from the shake flask cultivation (increased from 1.29 to 1.91 g L⁻¹). The increased cell mass and kefiran production in bioreactor cultivation can be attributed to better cultivation conditions in terms of better oxygenation and mixing in bioreactor. Such increased cellular growth and productivities in bioreactor cultivation when transferred from shake flask level have been previously investigated for the production of different primary as well as secondary metabolites (El Enshasy et al., 2011; Then et al., 2012; Elmarzugi et al., 2013).

4. Conclusions

The aforementioned results clearly revealed that the optimization of the cultivation medium resulted in a significant increase in both cell growth and kefiran production by about 170.56% and 58.02%, respectively. Continuous improvement was achieved by scaling up the process from shake flask level to 16-L bioreactor. Maximal cell mass of 2.76 g L⁻¹ concomitant with high kefiran production of 1.91 g L⁻¹, corresponding to a 49.22% increase was achieved under the uncontrolled pH cultivation in 16-L bioreactor.

Acknowledgments

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding this Research group No. RG-1435-047. The Authors are also thankful for Research Management Centre, Universiti Teknologi Malaysia for funding the project entitled: Bioprocess optimization for efficient kefiran production by *Lactobacillus kefiranofaciens* in semi-industrial scale. Vote No. QJ130000.2609.06J04

References

- Cevikbas, A., Yemni, E., Ezzedenn, F.W., Yardimici, T., Cevikbas, U., Stohs, S.J., 1994. Antitumoral, antibacterial and antifungal activities of kefir and kefir grain. *Phytother. Res.* 8, 78–82.
- Cheirsilp, B., Shoji, H., Shimizu, H., Shioya, S., 2003. Interaction between *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae* in mixed culture for kefiran production. *J. Biosci. Bioeng.* 96, 279–284.
- Dailin, D.J., Elsayed, E.A., Othman, N.Z., Malek, R.A., Ramli, S., Sarmidi, M.R., Aziz, R., Wadaan, M.A., El Enshasy, H.A., 2014. Development of cultivation medium for high yield kefiran production by *Lactobacillus kefiranofaciens*. *Int. J. Pharm. Pharm. Sci.* 7, 159–163.
- De Vuyst, L., De Vin, F., Vaningelgem, F., Degeest, B., 2001. Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *Int. Dairy J.* 11, 687–707.
- Duguid, J.P., Wilkinson, J.F., 1953. The influence of cultural conditions on polysaccharide production by *Aerobacter aerogenes*. *J. Gen. Microbiol.* 9, 174–189.
- Elmarzugi, N., El Enshasy, H., Abd Malek, R., Othman, N., Sarmidi, M.R., Aziz, R., 2010. Optimization of cell mass production of the probiotic strain *Lactococcus lactis* in batch and fed-batch culture in pilot scale levels. In: Vilas, A.M. (Ed.), *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. Formatex Research Centre, Badajoz, Spain, pp. 873–879.
- Elmarzugi, N., El Enshasy, H.A., Elsayed, E.A., Taleb, A., Keleb, E., Naji, S., Elrmithi, O., Othman, N.Z., 2013. Production of α -amylase using new strain of *Bacillus polymyxa* isolated from sweet potato. *IOSR J. Pharm. Biol. Sci.* 5, 73–79.
- El Enshasy, H., Then, C., Othman, N.Z., Homosany, H.A., Sabry, M., Sarmidi, M.R., Aziz, R., 2011. Enhanced xanthan production process in shake flasks and pilot scale bioreactors using industrial semi-defined medium. *Afr. J. Biotechnol.* 10, 1029–1038.
- Elsayed, E.A., Farid, M.A., El Enshasy, H.A., 2013. Improvement in natamycin production by *Streptomyces natalensis* with the addition of short-chain carboxylic acids. *Process Biochem.* 48, 1831–1838.
- Furuno, T., Nakanishi, M., 2012. Kefiran suppresses antigen-induced mast cell activation. *Biol. Pharm. Bull.* 35, 178–183.
- Gunter, E.A., Ovodov, Y.S., 2005. Effect of calcium, phosphate and nitrogen on cell growth and biosynthesis of cell wall polysaccharides by *Silene vulgaris* cell culture. *J. Biotechnol.* 117, 385–393.
- Harta, O., Iconnmpoulou, M., Bekatorou, A., Nigam, P., Kontominas, M., Koutinas, A.A., 2004. Effect of various carbohydrates on the production of kefir grains for use as a novel baking starter. *Food Chem.* 88, 237–242.
- Hsieh, C., Tseng, M.H., Liu, C.J., 2006. Production of polysaccharides from *Ganoderma lucidum* (CCRC 36041) under limitations of nutrients. *Enzyme Microb. Technol.* 38, 109–117.

- Ishii, I., Uchida, M., Inoue, C., Akisato, Y., Watanabe, K., Hosoyama, S., Toida, T., Ariyoshi, N., Kitada, M., 2010. Kefiran reduces atherosclerosis in rabbits fed a high cholesterol diet. *J. Atheroscler. Thromb.* 17, 980–988.
- Kabayama, S., Osada, K., Tachibana, H., Katakura, Y., Shirahata, S., 1997. Enhancing effects of food components on the production of interferon α from animal cells suppressed by stress hormones. *Cytotechnology* 23, 119–125.
- Kandler, O., Kunath, P., 1983. *Lactobacillus kefir sp. Nov.*, a component of the microflora of kefir. *Syst. Appl. Microbiol.* 4, 286–294.
- Kooiman, P., 1968. The chemical structure of kefiran, the water-soluble polysaccharide of the kefir grain. *Carbohydr. Res.* 7, 200–211.
- La Rivière, J.W.M., Kooiman, P., Schmidt, K., 1967. Kefiran, a novel polysaccharide produced in the kefir grain by *Lactobacillus brevis*. *Archiv für Mikrobiol.* 59, 269–278.
- Maeda, H., Zhu, X., Suzuki, S., Suzuki, K., Kitamura, S., 2004. Structural characterization and biological activities of an exopolysaccharide kefiran produced by *Lactobacillus kefiranofaciens* WT-2B^T. *J. Agric. Food Chem.* 52, 5533–5538.
- Maeda, H., Mizumoto, H., Suzuki, M., Tsuji, K., 2005. Effects of kefiran feeding on fecal cholesterol excretion, hepatic injury and intestinal histamine concentration in rats. *Biosci. Microflora* 24, 35–40.
- Medrano, M., Hamet, M.F., Abraham, A.G., Pérez, P.F., 2009. Kefiran protects Caco-2 cells from cytopathic effects induced by *Bacillus cereus* infection. *Antonie Van Leeuwenhoek* 96, 505–513.
- Murofushi, M., Shiomi, M., Aibara, K., 1983. Effect of orally administered polysaccharide from kefir grain on delayed-type hypersensitivity and tumor growth in mice. *Jpn. J. Med. Sci. Biol.* 36, 49–53.
- Pérez, P.F., Medrano, M., Racedo, S.M., Rolny, I.S., Abraham, A.G., 2011. Oral administration of kefiran induces changes in the balance of immune cells in a murine model. *J. Agric. Food Chem.* 59, 5299–5304.
- Piermaria, J.A., Pinotti, A., Garcia, M.A., Abraham, A.G., 2009. Films based on kefiran, an exopolysaccharide obtained from kefir grain: development and characterization. *Food Hydrocolloids* 23, 684–690.
- Schneedorf, J.M., Rodrigues, K.L., Caputo, L.R.G., Carvalho, J.C.T., Evangelista, J., 2005. Antimicrobial and healing activity of kefir and kefiran extract. *Int. J. Antimicrob. Agents* 25, 404–408.
- Shiomi, M., Sasaki, K., Murofushi, M., Aibara, K., 1982. Antitumor activity in mice of orally administered polysaccharide from Kefir grain. *Jpn. J. Med. Sci. Biol.* 35, 75–80.
- Souw, P., Demain, A.L., 1979. Nutritional studies on xanthan production by *Xanthomonas campestris* NRRL B1459. *Appl. Environ. Microbiol.* 37, 1186–1192.
- Sutherland, I.W., 2001. Microbial polysaccharides from Gram-negative bacteria. *Int. Dairy J.* 11, 663–674.
- Taniguchi, M., Nomura, M., Itaya, T., Tanaka, T., 2001. Kefiran production by *Lactobacillus kefiranofaciens* under the culture conditions established by mimicking the existence and activities of yeast in kefir grains. *Food Sci. Technol. Res.* 7, 333–337.
- Then, C., Othman, Z., Mustapha, W.A.W., Sarmidi, M.R., Aziz, R., El Enshasy, H.A., 2012. Production of alginate by *Azotobacter vinelandii* in semi-industrial scale using batch and fed-batch cultivation systems. *J. Adv. Sci. Res.* 3, 45–50.
- Wang, M., Bi, J., 2008. Modification of characteristics of kefiran by changing the carbon source of *Lactobacillus kefiranofaciens*. *J. Sci. Food Agric.* 38, 763–769.
- Yeesang, C., Chanthachum, S., Cheirsilp, B., 2008. Sago starch as a low-cost carbon source for exopolysaccharide production by *Lactobacillus kefiranofaciens*. *World J. Microbiol. Biotechnol.* 24, 1195–1201.
- Yokoi, H., Watanabe, T., 1992. Optimum culture conditions for production of kefiran by *Lactobacillus sp.* KPB-167B isolated from kefir grains. *J. Ferment. Bioeng.* 74, 327–329.