

Original Article

DEVELOPMENT OF CULTIVATION MEDIUM FOR HIGH YIELD KEFIRAN PRODUCTION BY  
*LACTOBACILLUS KEFIRANOFACIENS*

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ABSTRACT

**Objective:** Kefiran is one of the important microbial polysaccharides of many pharmaceutical and nutraceutical applications. It shows many potential medical applications as antimicrobial and immunomodulatory compound. The present work focuses on medium optimization for kefiran production using the standard strain *Lactobacillus kefiranofaciens* in submerged cultivation system.

**Methods:** Different types and concentrations of key nutrients such as carbon sources (glucose, mannitol, sucrose, lactose), nitrogen sources (yeast extract, peptone, meat extract, casein hydrolysate) and inorganic phosphate were evaluated to select the most appropriate nutrients and concentrations for kefiran production. Growth curve kinetics were also carried out to compare between the kinetics of cell growth and kefiran production in medium before and after optimization.

**Results:** It was clearly observed that type and concentration of carbon and nitrogen sources showed the significant effect on kefiran production. On the other hand, phosphate showed less effect on this process. The maximal kefiran production of about 1.25 g/l was obtained in medium composed of (g/l): lactose, 50.0; yeast extract, 12.0; KH<sub>2</sub>PO<sub>4</sub>, 0.25; sodium acetate, 5.0; Triammonium citrate, 2.0; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.2; MnSO<sub>4</sub>.5H<sub>2</sub>O, 0.05.

**Conclusion:** The new medium formulation increased the kefiran production from 0.23 g/l upto 1.29 g/l concomitant with the reduction of production time from 72 to 60 h.

**Keywords:** Kefiran, *Lactobacillus kefiranofaciens*, Medium optimization, Submerged cultivation.

INTRODUCTION

Kefir grains were used as the main component in many cultures as healthy yogurt/fermented milk drink with potential nutraceutical activities based on its high content of different types of probiotic bacteria and special type of biopolymer (Kefiran). Kefiran is an exopolysaccharide composed of repeated units of glucose and lactose in an equimolar manner. This carbohydrate polymer was first isolated from kefir grain by La Riviere et al. [1]. Further studies on kefir grain revealed that this water soluble polysaccharide is mainly produced by *Lactobacillus kefir* sp. [2]. Moreover, different studies showed that kefir grain bioflora is composed of a wide range of microorganisms exerting a symbiotic relationship. However, almost 90% of microbiota of grain are composed of bacteria belonging to lactic acid bacteria (LAB) such as (*Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Leuconostoc*) and acetic acid bacteria belonging to genus *Acetobacter* [3,4]. These bacteria exist in a symbiotic relation with different types of yeasts belonging to *Kluyveromyces*, *Saccharomyces*, *Candida* and *Torulaspota* species and fungi from species *Pichia*, *Zygosaccharomyces* and *Yarrowia* [5,6]. Based on its GRAS status (Generally Regarded As Safe) according to FDA (US Food and Drug Administration), kefiran is currently applied in many business sectors such as food, nutraceutical, and cosmeceutical industries [7,8]. In addition, it was also proven to have many potential pharmaceutical applications as antimicrobial and immunostimulant functional polysaccharides [9]. Thus, it became one of the main functional polysaccharides of high economic impact in wellness industries [10]. Recent studies showed also that it has many application in nanotechnology research and application, and was used in the preparation of UV-protective kefiran/ZnO nanocomposite as well as in the fabrication of nanofibers [11,12]. The industrial production of Kefiran in industrial scale is carried out in submerged cultivation using *L. kefiranofaciens* pure culture or in mixed culture system with other bacteria such as *L. acidophilus*, *L.*

*parakefir*, *L. kefirgranum* and/or some yeasts like *Saccharomyces cerevisiae*, and *Candida kefir* [13].

The main challenge in this process is to develop a suitable economic medium and cultivation strategy to produce the EPS in high concentration using suitable medium (in terms of components availability and cost) in shorter production time. Therefore, many studies focus on the development of an industrial medium for this process [14,15]. However, the current data in the literature clearly demonstrate that there is no standard medium developed so far for this process as EPS production is also dependent on the type of strain applied. The present work is focused on the development of a suitable production medium for kefiran production using the standard strain *L. kefiranofaciens* in submerged cultivation system. Studies included the selection of proper type and concentration of carbon, nitrogen, and phosphorus substrates and the finally a comparative cultivation kinetics was carried out to compare the performance of the initial and the modified production media in terms of cell growth, volumetric and specific kefiran production.

MATERIALS AND METHODS

Microorganism

*Lactobacillus kefiranofaciens* ATCC 8007 used in this study was initially obtained in a lyophilized form from American Type Culture Collection (ATCC, University Boulevard, Manassas, VA 20110 USA). The bacterium was first activated in Man-Ragosa-Sharpe (MRS) broth medium consisting of (g/L): 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; di potassium phosphate, 2.0. The pH of the medium was adjusted to 5.5 before sterilization. The grown cells in liquid culture were inoculated into MRS agar medium and were incubated for 48 hours to produce the working cell culture. The arisen colonies were

harvested using 50% glycerol solution, stored in 2 mL cryogen tube and preserved at -78°C.

### Inoculum preparation and kefir production medium

Inoculum was prepared by inoculating 250 mL Erlenmeyer flasks containing 50 mL of MRS liquid medium with 0.5 mL of the frozen working cell bank cultures. The inoculated flasks were incubated at 200 rpm and 30°C for 24 hours in a rotary shaker (Innova 4080, New Brunswick Scientific, NJ, USA). The grown cells were used to inoculate the kefir production medium in a concentration of 5% (v/v). The initial kefir production medium was composed of (g/L): glucose, 20.0; yeast extract, 6.0; KH<sub>2</sub>PO<sub>4</sub>, 0.25; sodium acetate, 5.0; Triammonium citrate, 2.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; MnSO<sub>4</sub>·5H<sub>2</sub>O, 0.05. Glucose, was sterilized separately at 110°C for 20 min and added to the medium before inoculation. The inoculated flasks were incubated on a rotary shaker (Innova 4080, New Brunswick, NJ, USA) at 200 rpm and 30°C.

### Sample preparation and cell dry weight determination

Samples, in the form of two flasks containing 50 mL each, were taken at different time intervals during the cultivation. Immediately after collection, the fermentation broth was centrifuged in 50 mL falcon tubes at 9000 rpm for 15 minutes to precipitate the cells. The supernatant was taken for kefir analysis. Cell pellets were then centrifuged again under the same conditions. After the second centrifugation cycle, the supernatant was discarded and the cells were dried at 65°C in an oven for 48 hours.

### Kefiran determination

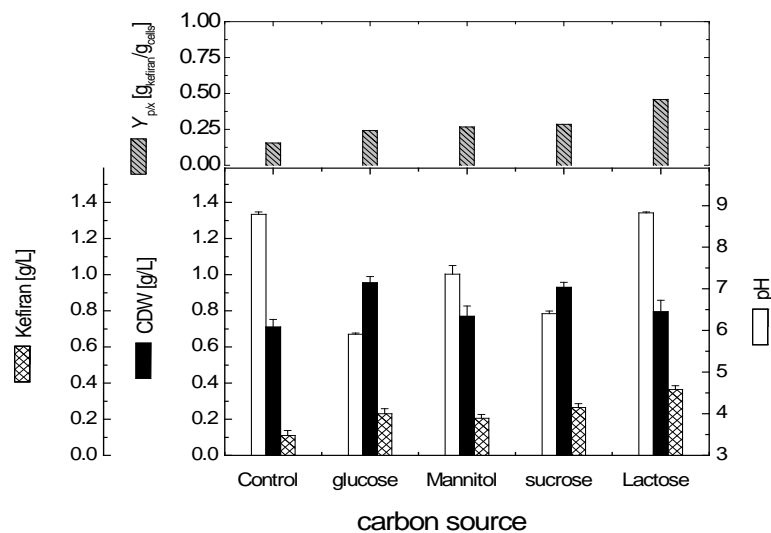
Kefiran was recovered from the culture supernatant by the addition of an equal volume of cold absolute ethanol at 4°C and left overnight for complete precipitation. The resulting precipitate was collected by centrifugation at 9000 rpm for 15 minutes, dissolved in bi-distilled water and re-precipitated with cold absolute ethanol. This step was repeated for three times to obtain pure kefiran. The final precipitate was dried at 65°C in an oven for 48 hours.

## RESULTS AND DISCUSSION

### Effect of difference carbon sources on kefiran production

Carbon source is usually a very important medium component especially for polysaccharide production by microorganisms. Thus, different types of commercially viable carbon sources were studied for their ability to support biomass and kefiran production. Thus, two monosaccharides (glucose and mannose) and two disaccharides (sucrose and lactose) were used in this experiment. All sugars were used in an equal concentration of 20 g/L (autoclaved separately and added after medium sterilization). The results of cell growth and kefiran production after 72 h cultivation in shake flasks are shown in figure-1. As shown, of all sugars tested, the cell growth of about 0.96 g/L was obtained in glucose supported culture followed by sucrose, lactose and mannitol. On the other hand, the maximal volumetric kefiran production of about 0.36 g/L was obtained in the medium containing lactose as the sole carbon source. This value was almost 56% higher compared to the initial medium which included glucose as carbon source. For better understanding the relation between cell growth and kefiran production, the polysaccharide yield based on biomass [ $Y_{p/x}$ ] was calculated. As shown, the maximal value of 0.46 g/g was obtained in lactose culture which was almost 92% higher compared to those obtained in glucose medium.

The cell performance for kefiran production was of the following manner: lactose > sucrose > mannitol > glucose. The superiority of lactose over other carbon sources for supporting kefiran production may be due to the fact that kefiran composed of lactose and glucose in 1:1 ratio [16]. Thus, lactose could act as polymerization ready sugar for kefiran production. For other bacterial polysaccharides such as xanthan, sucrose and glucose were most favorable for biopolymer production as they act as easily assimilated carbon source and ready building blocks for biopolymer biosynthesis [17]. Thus, the cultivation medium supplemented with lactose was employed in all further experiments.



**Fig. 1: Effect of different carbon sources on cell growth and kefiran production by *L. kefiranofaciens*. Data represented are the average of values taken from two shake flasks cultivations for 72 hours under the same conditions (Error bars represent standard error calculated)**

### Effect of different lactose concentrations on kefiran production

Previous experiment has shown that lactose, is the most favorable carbon source for kefiran production when was applied in 20 g/l. Therefore, in this experiment we studied the possibility of increasing kefiran production by optimizing the lactose concentration. Therefore, lactose was supplemented to the culture medium in different concentrations ranging from 0.0 to 100 g/l. The results in figure-2 clearly demonstrate that cell growth increased proportionally with the increase in lactose concentration in all

applied concentrations and reached a maximal value of 1.4 g/l at 100 g/l lactose. On the other hand, the maximal volumetric kefiran production of about 0.76 g/l was obtained in 50 g/l lactose supplemented culture and kept more or less constant in all higher lactose cultures. However, the specific kefiran production increased by increasing lactose concentration up to 50 g/l and reached 0.71 g/g, and gradually decreased thereafter. Thus, we can conclude that the optimum lactose concentration suitable for high volumetric and specific kefiran production was 50 g/l. accordingly; this concentration was used in the subsequent experiments.

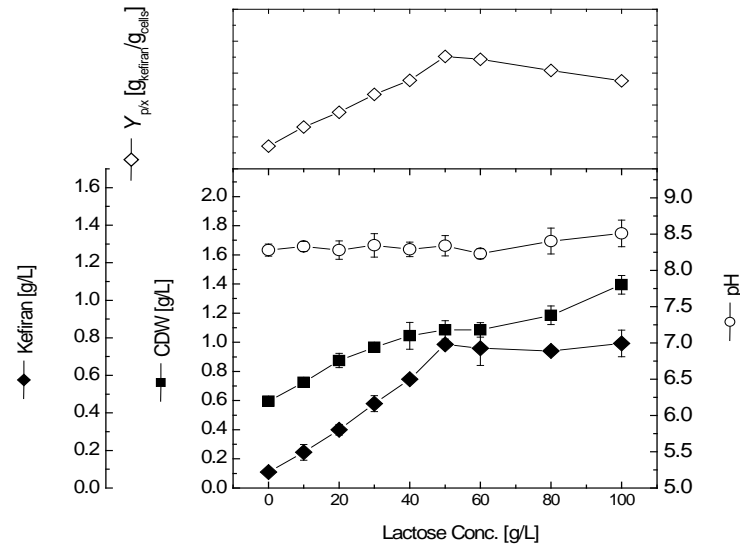


Fig. 2: Effect of different lactose concentrations on cell growth and kefiran production by *L. kefirifaciens*. Data were taken after submerged cultivations for 72 hours

**Effect of the type and concentration of nitrogen source on kefiran production**

Different organic nitrogen sources (yeast extract, peptone, meat extract, and casein hydrolysate) were added to the production medium to evaluate their suitability to support kefiran production. The results in figure-3 demonstrate that the type of nitrogen source has a strong influence on both cell growth and kefiran production. As shown, meat extract was the best nitrogen source to support cell growth reaching about 1.3 g/L biomass (about 79% higher compared to the control culture without nitrogen source). On the other hand, yeast extract was the most suitable nitrogen source for kefiran production followed by peptone and casein hydrolysate. Yeast extracts supplemented culture gave the highest volumetric and specific kefiran production of 0.75 g/L, and 0.71 g/g, respectively. Based on these results, other experiments on the effect of different yeast extract concentrations on cell growth and kefiran production were carried out. As shown in fig. 4, for the range of yeast extract examined in this experiment (0-14 g/L), cell growth increased proportionally with yeast extract and the maximal biomass of about 2 g/L was obtained in 14 g/L culture. On the other

hand, kefiran production was enhanced by the addition of yeast extract when added to the medium in a concentration between 0-12 g/L and kept more or less constant above this value.

The maximal volumetric kefiran production of about 1.25 g/L was obtained in 12 g/L yeast extract supplemented culture. It is also worthy to note that the specific kefiran production yield was almost the same in all concentrations applied. Other recent studies showed that tryptone and meat extract supported kefiran production better than inorganic nitrogen sources [15]. However, they also reported that yeast extract addition stimulated kefiran production and acted as a source of vitamins in addition to other organic nitrogen source. In this study, yeast extract was applied as a dual source of nitrogen and vitamins to enhance kefiran production. In cultivation processes for many primary and secondary metabolites production, yeast extract is usually an important medium component as it acts as a source of nitrogen, vitamins, and many other growth factors such as  $\alpha$ - and  $\beta$ -factors [18,19]. Thus, we can conclude that the addition of yeast extract to the culture medium supported polysaccharide production through the increase of bacterial growth not through the enhancement of cell productivity for kefiran production.

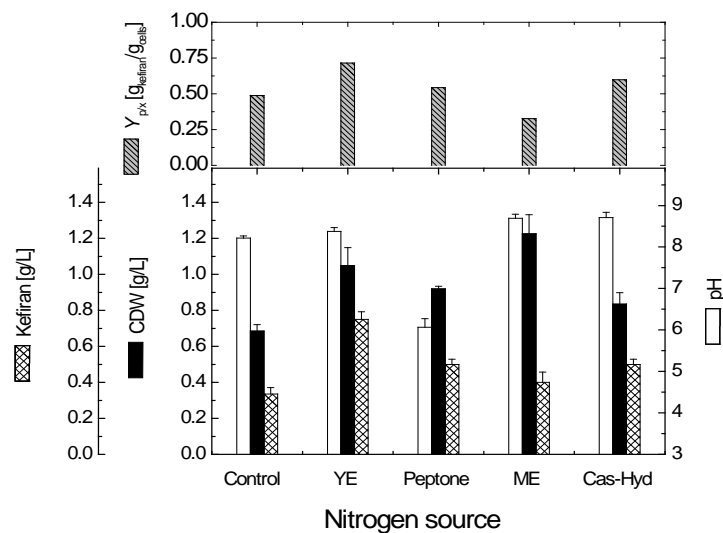


Fig. 3: Effect of different nitrogen sources on kefiran production by *L. kefirifaciens* in shake flask cultures. Data were taken after submerged cultivations for 72 hours

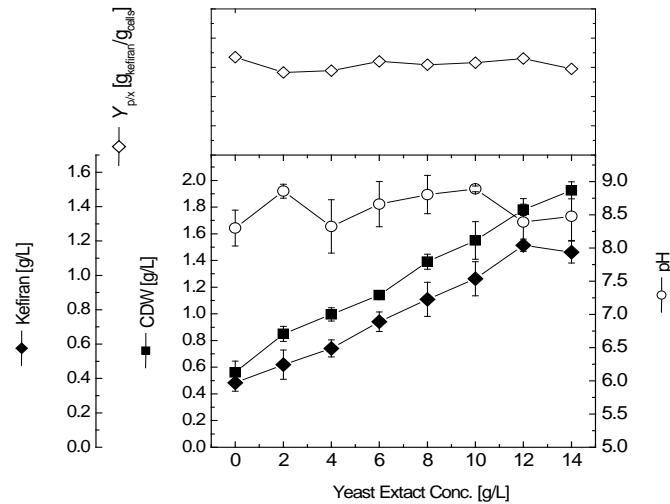


Fig. 4: Effect of different yeast extract concentrations on cell growth and kefiran production by *L. kefiranofaciens*. Data were taken after submerged cultivations for 72 hours

**Effect of phosphate concentration on kefiran production**

To investigate the effect of phosphate on bacterial growth and polysaccharide production, cells were cultivated in different media having an increased concentration of inorganic phosphate (in the form of potassium dihydrogen phosphate) ranging between 0 to 2 g/L. As shown in figure-5, increasing the concentration from 0 to 0.75 g/L resulted in a significant increase in cell growth. However, further increase in phosphate concentration didn't show any further influence on cell growth. On the other hand, the maximal volumetric kefiran production of about 1.25 g/L was obtained in the medium

supplemented with 0.25 g/L inorganic phosphate. Additionally, the maximal value of specific kefiran production of about 0.69 g/g was also obtained at this concentration. These results clearly demonstrate that phosphate is important for kefiran production, but should be applied at low concentrations not exceeding 0.25 g/L. Higher phosphate concentrations will only increase the biomass and will exhibit a negative effect on kefiran biosynthesis. However, it is also worthy to note that phosphate concentration in the culture did not show any significant influence on the final pH of medium. The values of the pH of the media having different phosphate concentrations ranged between 8.2-8.6.

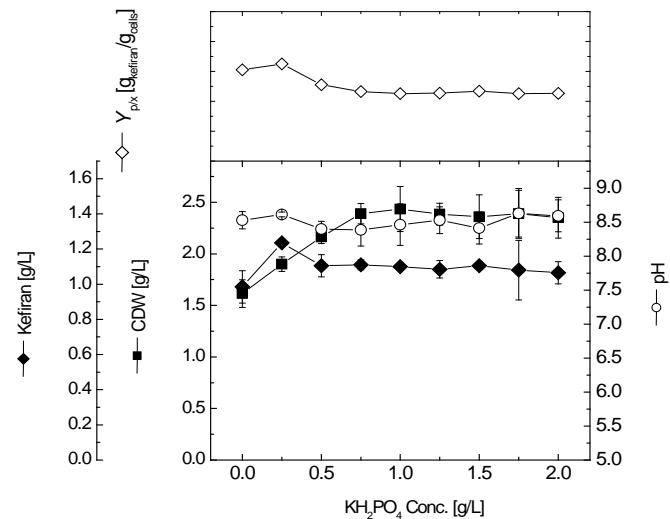


Fig. 5: Effect of different phosphate concentrations on cell growth and kefiran production by *L. kefiranofaciens*. Data were taken after submerged cultivations for 72 hours

**Kinetics of cell growth and kefiran production in medium before and after medium optimization**

This experiment was conducted to compare the kinetics of cell growth and kefiran production in medium before and after optimization. Thus, two parallel sets of cultivations were run simultaneously for this comparative study. As shown in figure-6, cells grew exponentially in both cultures, however with different growth rates. The maximal biomass obtained in the optimized medium was about 1.92 g/L (about 90% higher compared to the

biomass obtained in the parallel cultivation using the un-optimized medium). Kefiran started to be produced in both cultures after a lag phase of about 20 h. In the initial medium, kefiran was produced in the culture with a low rate of about 0.004 g/L/h and reached its maximal value of 0.26 g/L after 72 hours. In the other parallel experiment using the optimized medium, the kefiran production rate was 0.027 g/L/h and the maximal volumetric kefiran production of 1.29 g/L was achieved after only 60 hours. This polysaccharide volumetric yield was almost 5 times higher compared to the yield obtained in the medium before optimization.

For better understanding the cell performance and kefiran production in both media, specific kefiran production was also calculated. As shown in figure-6, the maximal kefiran yield of 0.71 g kefiran/g cells was obtained in the optimized medium formulation. This value was almost three folds higher than those obtained in the initial medium. This directly indicates that the high volumetric kefiran production was

mainly due to higher cell performance rather than the increase in the biomass. Thus, we can conclude that the new medium formulation not only resulted in a significant increase in both volumetric and specific kefiran production by about 390% and 220%, respectively, but also reduced the production time from 72 to only 60 hours. Thus, this new formulation is promising for industrial production of kefiran.

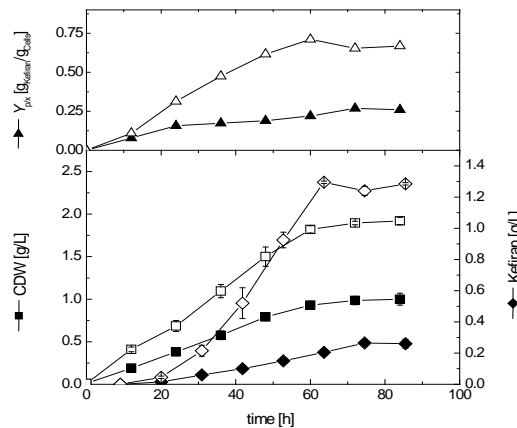


Fig. 6: Kinetics of cell growth and kefiran production by *L. kefiranofaciens* shake flask cultivations using un-optimized and optimized medium (closed and opened symbols represent the data for un-optimized and optimized medium, respectively)

## CONCLUSION

Optimization of the cultivation medium for kefiran production was successfully achieved in this work. The optimum medium for production was composed of (g/L): lactose, 50.0; yeast extract, 12.0;  $\text{KH}_2\text{PO}_4$ , 0.25; sodium acetate, 5.0; Triammonium citrate, 2.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2;  $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.05. This new medium formula not only increased the kefiran volumetric production from 0.23 up to 1.29 g/L but also shorten the production time from 72 hours to only 60 hours. The significant increase in both volumetric and specific kefiran production using the new medium formulation, make it attractive for further studies on production in industrial scale.

## AKNOWLEDGEMENT

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## CONFLICT OF INTERESTS

Declared None

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