CHEMICAL ENGINEERING TRANSACTIONS Volume 24, 2011
Editor Sauro Pierucci

 ISBN 978-88-95608-15-0 ISSN 1974-9791
DOI: 10.3303/CET1124081

Experimental Assessment of the Impact of Cultivation Conditions on Kefiran Production by the Mixed Microflora Imbedded in Kefir Grains

Katja Zajšek and Andreja Goršek*

University of Maribor, Faculty of Chemistry and Chemical Engineering, Smetanova 17, SI-2000 Maribor, Slovenia
*andreja.gorsek@uni-mb.si

The aim of this work was to investigate the environmental requirements for the production of specific exopolysaccharide (EPS) kefiran from natural starter culture – kefir grains. We have found that the temperature and agitation rate are critical for kefiran production during the 24 h cultivation of kefir grains in full fat cow's milk; our optimized conditions were 25 °C and 80 rpm, respectively. In addition, when optimizing the effects of additional nutrition, we found that 5 % (w/v) lactose and 0.1 % (w/v) thiamine led to the highest production of kefiran from kefir grains. The kefir grains biomass increase, kefiran mass fraction in the grains and mass ratio of glucose and galactose in kefiran reached 11.62 g/L, 1.65 % (w/w) and 1:0.82, respectively.

1. Introduction

Exopolysaccharides produced by LABs have gained the attention of food researchers because of its food grade status and the textural properties they impart to dairy products. Indeed, polysaccharides are used as thickeners, emulsifiers and gelling agents.
Kefiran is produced in kefir grains which consist of a complex population of LABs and yeasts firmly embedded. According to other authors, the principal producer of the kefiran polymer in kefir grains is *Lactobacillus kefiranofaciens* and several other unidentified species of *Lactobacilli* (Frengova et. al., 2002). It has been reported that kefiran is composed of a branched hexa- or heptasaccharide repeating unit containing approximately equal amounts of D-glucose and D-galactose residues. This polymer has an antibacterial and antitumor activity, modulates gut immune system and protects epithelial cells against *Bacillus cereus* exocellular factors (Wang et al., 2008). It can also be used as a food grade additive for fermented product since it enhances the rheological properties of chemically acidified skim milk gels increasing their apparent viscosity and the storage and loos modulus of these gels.
In this study, we tested various culturing (i.e., temperature and agitation rate) and nutritional (i.e., carbon, nitrogen and vitamins) factors that might influence the production of kefiran from the kefir grains. In the continuation we determined optimal conditions for its production, and finally characterized the monosaccharide composition of the isolated EPS.

Please cite this article as: Zajsek K. and Gorsek A., 2011, Experimental assessment of the impact of cultivation conditions on kefiran production by the mixed microflora imbedded in kefir grains, Chemical Engineering Transactions, 24, 481-486
DOI: 10.3303/CET1124081

2. Materials and Methods

2.1 Kefir grains

Kefir grains were obtained from the existing local dairy Kele & Kele d.o.o. The grains were grown at room temperature in 1 L of UHT full fat cow milk without stirring. The medium was changed daily and the grains were washed with cold running water. The milk of the same origin was also used as fermentation medium.

2.2 Fermentations

Batch fermentations were carried out in a 2 L RC1 reaction calorimeter (Mettler Toledo) for 24 h. Fermentations (V = 1 L) were performed at 25 °C and agitation rate of 80 rpm. To optimize the composition of fermentation medium, different kinds of carbon and nitrogen sources and vitamins were added into the 1 L of basal milk medium inoculated with 42 g of kefir grains.

2.3 Assays

2.3.1 Determination of kefir grians biomass increase

At the end of each fermentation kefir grains were separated from the fermentation product by filtration using a plastic household sieve, washed with cold water and then dried carefully on paper towelling. Kefir grains mass concentration was determined by weighting on Mettler Toledo analytical balance. After determining the grains mass, the grains were used for the kefiran isolation procedure.

2.3.2 Isolation of kefiran from kefir grains

The content of kefiran in kefir grains was determined based on the methods of Wang et. al. (2008) and Lin and Chang Chien (2007), with some modifications. Firstly, the final weighed amount of wet kefir grains was treated in 300 mL of boiling water for 3 h with continuous stirring at 200 rpm. One volume of 20 % TCA solution was added into the cooled mixture, and the overnight precipitated proteins and microbial cells were removed by centrifugation (11 000 rpm, 20 min, and 4 °C). The kefiran in supernatant was precipitated by addition of an equal volume of chilled >99.5 % acetone and left in refrigerator overnight. The mixture was centrifuged at 11 000 rpm for 20 min at 4 °C. An isolated sample was washed with acetone and dried during 48 h at 42 °C.

2.3.3 Standard solutions, calibrations, hydrolysis and derivatization of kefiran

Standard stock solutions of D-glucose (Glc), D-galactose (Gal) and D-xylose (Xyl) were prepared at concentration of 1 g/L. For calibration stock solutions were diluted using Milli Q water. Typical calibration range for monosaccharides was from 10 mg/L to 200 mg/L. Approximately 50 mg of kefiran was diluted in 4 mL of Milli Q water and mixed for 24 h. The solution was sonificated for 30 min at 40 ºC before hydrolysis. Hydrolysis was performed by adding 2 mL of H_2SO_4 (c = 0.5 mol/L) to sample solution, which was heated in autoclave at 120 ºC for 40 min. After that the hydrolysates were cooled down to room temperature and diluted to final volume of 50 mL. Derivatization of hydrolysates was performed through reductive amination, using sodium cyanoborohydride according to Doliška et al. (2009). All buffers, samples, Milli Q water and other solutions were filtrated through syringe filters (0.2 μm) before use.

2.3.4 Analysis of monosaccharide composition of kefiran using capillary zone electrophoresis

Detail explanation and optimisation of capillary electrophoresis experiments was described by Dahlman et al. (2000). In our work separations were carried out using an

Agilent CE3D Instrument G-1600 equiped with DAD (190–600 nm). Separations were performed in borate buffer (c = 0.1 mol/L) at pH = 10.5 using 30 % of acetonitrile as electroosmotic flow modifier. Samples were injected hydrodynamically at 50 mbar for 5 s, followed by a plug of buffer solution at p = 50 mbar for 2 s. A voltage of 25 KV was applied, the temperature was constant at 20 ºC and UV absorbance (A) was measured at 306 nm.

3. Results and Discussion

3.1 The effect of temperature

In order to investigate the effect of culture temperature on kefir grains biomass and kefiran production, kefir grains ($\gamma_{KG,0}$ = 42 g/L) were incubated in the basal milk medium with different temperatures ranging from (25–43) °C (Figure 1). The optimal temperature for kefir grains growth and kefiran production were observed to be 37 °C and 25 °C respectively. Rimada and Abraham (2001) studied the influence of T on the kefiran production with kefir grains, where the whey was used as a medium. They reported that kefiran yield and reduction of kefir grains growth are maximal at 43 °C. Based on their results, we can assume that due to high T, kefiran dissolved and transferred into the whey.

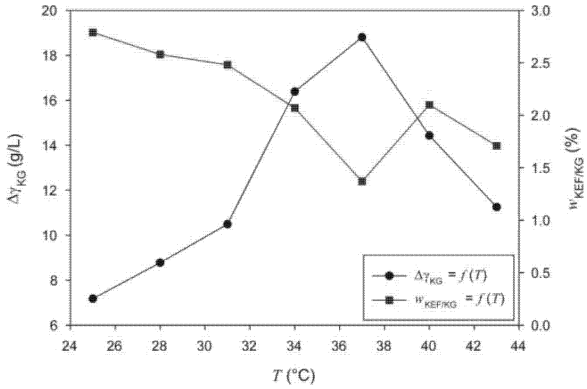

Figure 1: The effect of temperature on the kefir grains biomass increase ($\Delta\gamma_{KG}$) and polysaccharide production ($w_{KEF/KG}$) by mixed microbiota of kefir grains.

The highest kefir grains biomass increase was 18.8 g/L and kefiran production 2.79 % (w/w). We assume that at 25 °C the kefiran production is the highest mainly due to the fact that microorganisms protect themselves against environmental influences by increasing the kefiran production. It is also possible that by increasing temperature, the kefiran content (according to $\Delta\gamma_{KG}$) remains constant and only kefir grains microbiota increases, which causes $\Delta\gamma_{KG}$. A possible explanation was also given by De Vuyst and Degeest (1999). They specify, that slowly-growing cells biosynthesize polymers, needed for building cell walls, much slower, therefore more isoprenoidic lipids, which serve as transport molecules, are at disposal for the EPS biosynthesis.

3.2 The effect of agitation rate

Mixing is provided to keep the fermentation broth homogeneous as well as enhance the mass transfer of nutrients and air. The effect of agitation rate on $\Delta\gamma_{KG}$ and $w_{KEF/KG}$ was investigated in the basal milk medium at 0, 40, 80, 120 and 160 rpm. Fermentation conditions were maintained at the previous determined optimum conditions (T = 25 °C, $\gamma_{KG,0}$ = 42 g/L). The highest kefiran level of 3.16 % was produced from the grains at the

agitation rate of 80 rpm with accompanying highest kefir grains biomass increase concentration (7.14 g/L) (Figure 2).

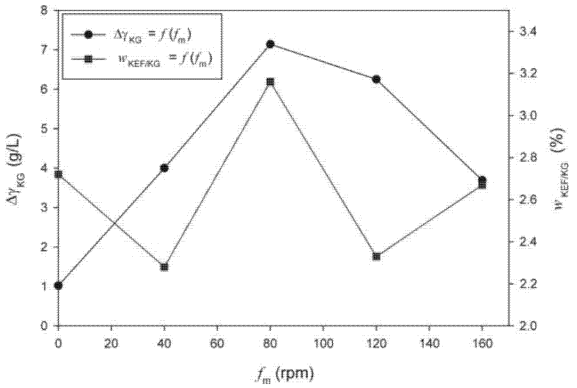

Figure 2: The effect of agitation rate on the kefir grains biomass increase ($\Delta\gamma_{KG}$) and kefiran production ($w_{KEF/KG}$) by kefir grains.

3.3 The effect of carbon sources

To found out the effect of different carbon sources on the $\Delta\gamma_{KG}$ and $w_{KEF/KG}$ by kefir grains ($\gamma_{KG,0}$ = 42 g/L), four carbon sources were compared. Each carbon source was added to the basal milk medium at the concentration of 5 % (w/v) for 24 h (Table 1).

Table 1: The effect of carbon sources on the kefir grains biomass increase ($\Delta\gamma_{KG}$) and kefiran mass fraction in the kefir grains (kefiran production) ($w_{KEF/KG}$).

Carbon source (5 %, w/v)	$\Delta\gamma_{KG}$ (g/L)	m_{KEF} (g)	$w_{KEF/KG}$ (%)	$\zeta_{Glc/Gal}$	Final pH
Control	7.14	1.56	3.16	1 : 1.02	3.78
Fructose	7.62	1.32	2.66	1 : 1.04	3.76
Glucose	11.35	1.15	2.15	1 : 0.82	3.76
Sucrose	8.83	1.19	3.76	1 : 0.99	3.77
Lactose	12.36	2.35	4.33	1 : 1.16	3.67

Among the carbon sources tested, the maximum $\Delta\gamma_{KG}$ (12.36 g/L) and maximum $w_{KEF/KG}$ (4.33 %) were achieved when lactose was added as the carbon source. However, the differences in kefiran production between lactose (4.33 %) and sucrose (3.76 %) and between fructose (2.66 %) and glucose (2.15 %) were not significant. It is possible that different carbon sources might have different effects of catabolic repression on the cellular secondary metabolism. Wang and Bi (2008) examined the influence of different carbohydrates (γ = 100 g/L) on the kefiran production in the MRSL medium, using bacterium *Lactobacillus kefiranofaciens* JCM6985. They indicate that mass concentration of bacteria in the medium is maximal in case of maltose addition, followed by addition of sucrose, lactose, glucose and fructose. At the end of fermentation, the kefiran mass concentration in the medium was also maximal in case of maltose addition, followed by addition of sucrose, lactose, glucose and fructose. High mass concentration of kefiran in the fermentation medium (or in our case in kefir grans) enriched with lactose, is probably the result of galactose consumption for the biosynthesis of kefiran, performed by LABs. Harta et al. (2004) also found that addition of carbohydrate significantly increases the $\Delta\gamma_{KG}$. 24 h fermentations were carried out at 30 °C. In all fermentations they used a synthetic fermentation media (V = 1 L), that was inoculated with 5 g of grains. They found that grains production is maximal when fructose was added (20.75 g), followed by addition of sucrose (19.75 g), glucose (17.50

g), lactose (13.87 g) and maltose (6.37 g). When they used different mixtures of the named carbohydrates, the mixture of glucose and sucrose in the ratio of 1:3 gave the highest $\Delta\gamma_{KG}$. The results in Table 1 show that the average $\zeta_{Glc/Gal}$ is around 1:1, which is in accordance with the literature data (Farnworth, 2005).

3.4 The effect of nitrogen sources

To investigate the influence of nitrogen source on $\Delta\gamma_{KG}$ and $w_{KEF/KG}$, we cultivated 42 g of grains in milk medium containing 50 g/L of lactose and different nitrogen sources, which were added at a concentration of 0.5 % (w/v) for 24 h. Among the four different examined nitrogen sources, no addition of the nitrogen source was the most effective for enhancing the kefiran production (1.84 %) by kefir grains (Table 2). There are few studies on the impact of nitrogen compounds on the yield of kefiran, isolated from the kefir grains. Wang and Bi (2008) indicated that the yield of kefiran was the highest when casein was added into the medium, followed by addition of peptone, tryptone, yeast extract, and yeast powder. The yield of kefiran was lower when urea, ammonium chloride and ammonium sulphate were added.

Table 2: The effect of nitrogen sources on the kefir grains biomass increase ($\Delta\gamma_{KG}$) and kefiran mass fraction in the kefir grains (kefiran production) ($w_{KEF/KG}$).

Nitrogen source (0.5 %, w/v)	$\Delta\gamma_{KG}$ (g/L)	m_{KEF} (g)	$w_{KEF/KG}$ (%)	$\zeta_{Glc/Gal}$	Final pH
Control	8.52	0.93	1.84	1 : 0.85	4.03
Tryptone	15.97	0.82	1.41	1 : 0.88	3.86
Meat extract	13.80	0.79	1.42	1 : 0.74	3.83
Ammonium nitrate	3.69	0.72	1.58	1 : 0.89	3.97
Ammonium chloride	8.54	0.64	1.27	1 : 0.76	4.07

The kefir grains biomass increase appeared to be stimulated by the organic nitrogen sources we tested, with little difference among them. Relative to the organic nitrogen sources, however, the use of inorganic nitrogen sources led to relatively lower kefir grains growths.

3.5 The effect of added vitamins

Although the presence of vitamins usually affects the rates of biosynthesis of many metabolites, the influences that vitamins have on kefir grains growth and kefiran production in kefir grains, however, remained unevaluated until now. We studied the effect of vitamins on $\Delta\gamma_{KG}$, $w_{KEF/KG}$ and $\zeta_{Glc/Gal}$. For that purpose, each vitamin was added to the basal milk medium at a concentration of 0.1% (w/v) (Table 3).

Table 3: The effect of vitamins on the kefir grains biomass increase ($\Delta\gamma_{KG}$) and kefiran mass fraction in the kefir grains (kefiran production) ($w_{KEF/KG}$).

Vitamin (0.1 %, w/v)	$\Delta\gamma_{KG}$ (g/L)	m_{KEF} (g)	$w_{KEF/KG}$ (%)	$\zeta_{Glc/Gal}$	Final pH
Control	11.87	0.88	1.63	1 : 0.84	4.04
Yeast extract	11.63	0.78	1.46	1 : 0.87	3.90
Ascorbic acid	9.63	0.74	1.44	1 : 0.86	3.98
Nicotinic acid	11.26	0.67	1.26	1 : 0.86	3.93
Thiamine	11.62	0.89	1.65	1 : 0.82	4.03

Thiamine was the best vitamin source for the $w_{KEF/KG}$ (1.65 %) by kefir grains lactobacilli. In addition, of the four vitamins we tested, only ascorbic acid gave the low

$\Delta\gamma_{KG}$. Vitamin-free medium supported slightly more growth than did the yeast extract, nicotinic acid, and thiamine (Table 3). These results suggest that the supply of vitamins is not an absolute requirement for the growth of kefir grains microbiota. It is possible that microorganisms of kefir grains are capable of synthesizing the listed vitamins. For example, some bacteria do synthesize their own vitamins, and, therefore, the presence of those organic compounds in the growth medium may be unnecessary.

4. Conclusions

The potential application of kefiran in food industry has increased the interest for the study of the production of this molecule. The optimization of the growth environment is important for achieving its maximal production from kefir grains. We determined that the temperature, agitation rate, and the choices of carbon sources, nitrogen sources and vitamins are the factors that most affect the production and the physico-chemical composition of kefiran. The kefiran production from the grains was maximized after 24 h at 25 °C and 80 rpm. A supply of lactose and thiamine sustains a good degree of kefiran production. Our results prove that the kefiran production can be enhanced dramatically by means of controlling the culture conditions and modifying the medium's composition. We also expect that the kefir grains biomass increase (γ_{KG}) and kefiran production ($w_{KEF/KG}$) are stimulated by addition of mineral sources.

References

Dahlaman O., Jacobs A., Liljenberg A. and Olsson A.I., 2000, Analysis of carbohydrates in wood and pulps employing enzymatic hydrolysis and subsequent capillary zone electrophoresis, Journal of Chromatography A, 891, 157-174

De Vuyst L. and Degeest B., 1999, Heteropolysaccharides from lactic acid bavteria, FEMS Microbiology Reviews, 23, 153-177

Doliška A., Strnad S., Ribitsch V., Stana-Kleinschek K., Willfor S. and Saake, B., 2009, Analysis of galactoglucomannans from spruce wood by capillary electrophoresis, Cellulose, 16, 1089-1097

Farnworth E.R., 2005,Kefir-a complex probiotic, Food Science and Technology, 2, 1-17

Frengova G.I., Simova E.D., Beshkova D.M. and Simov, Z.I., 2002, Exopolysaccharides produced by lactic acid bacteria of kefir grains, Zeitschrift für Naturforschung, 57c, 805-810

Harta O., Iconomopoulou M., Bekatorou A., Nigam P., Kontominas M. and Koutinas A.A., 2004, Effect of various carbohydrate substrates on the production of kefir grains for use as a novel baking starter, Food Chemistry., 88, 237-242

Lin T.Y. and Chang Chien M.F., 2007, Exopolysaccharides production as affected by lactic acid bacteria and fermentation time, Food Chemistry, 100, 1419-1423

Rimada P.S. and Abraham A.G., 2001, Polysaccharide production by kefir grains during whey fermentation, Journal of Dairy Research, 68, 653-661

Wang Y., Ahmed Z., Feng W., Li C. and Song S., 2008, Physicochemical properties of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir, International Journal of Biological Macromolecules, 43, 283-288

Wang M. and Bi J., 2008, Modification of characteristic s of kefiran by changing the carbon source of *Lactobacillus kefiranofaciens,* Journal of the Science of Food and Agriculture, 88, 763-769