

# Influence of Different Growth Conditions on the Kefir Grains Production, used in the Kefiran Synthesis

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## Abstract

The purpose of this study was to optimize the kefir grains biomass production, using milk as culture media. The kefir grains were cultured at different changed conditions (temperature, time, shaker rotating speed, culture media supplemented) to evaluate their effects. Results showed that optimal culture conditions were using the organic skim milk, incubated at 25°C for 24 hours with a rotation rate of 125 rpm. According to results, the growth rate was 38.9 g/L for 24 h, at 25°C using the organic milk - OSM, 36.87 g/L during 24 hours, optimal time for propagation process gave 37.93 g/L kefir grains biomass when the effect of temperature level was tested. The homogenization of medium with shaker rotating induced a greater growth rate, it was obtained 38.9 g/L for 24 h, at 25°C using rotation rate at 125 rpm. The growing medium (conventional milk) supplemented with different minerals and vitamins may lead to improve the growth conditions of kefir grains biomass. The optimization of the growth environment is very important for achieving the maximum production of kefir grains biomass, substrate necessary to obtain the polysaccharide kefiran.

**Keywords:** *kefir grains, growth rate, biomass production*

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## INTRODUCTION

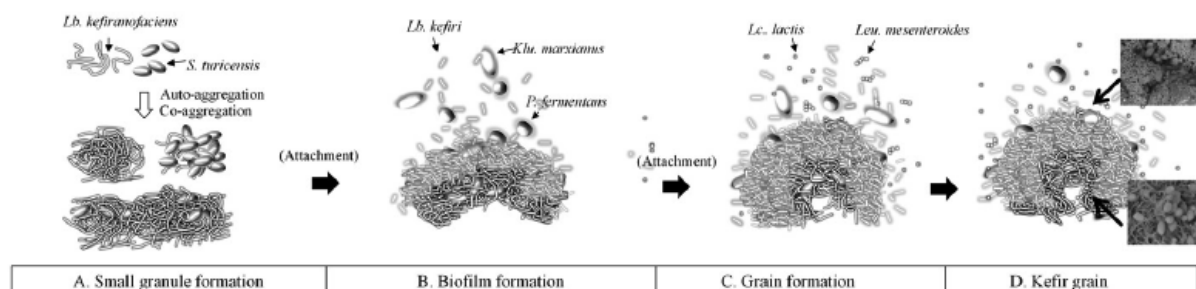
Kefir grains resemble to small cauliflower florets: they measure 1 – 3 cm in length, are lobed, irregularly shaped, white to yellow-white in colour, and have a slimy but firm texture (La Rivière *et al.* 1967; Farnworth, 2005). Grains are kept viable when transferred daily into fresh milk, grow for approximately 20 hours, during this time, increasing their mass by 25% (Farnworth, 2005).

The mechanism formation of kefir grains remains still unknown, but recently, Wang *et al.* (2012) presented a schematic model of grains formation (Fig.1).

The microflora of kefir grains is variable, depending on the source of the grain. The grains

contain a balance of specific microbes that co-exist in a complex symbiotic relationship and include species of yeasts, lactic acid bacteria (lactobacillus and lactococcus), acetic bacteria and mycelial fungi (Garbers *et al.*, 2004). Lactic bacteria that exist in the kefir grains have attracted considerable attention because of their ability to compete and inhibit the development of spoilage and pathogenic microorganisms, either by the production of lactic acid or by increasing the expression of antimicrobial agents (Kourkoutas *et al.*, 2007).

The chemical composition of kefir grains is 890–900 g kg<sup>-1</sup> water, 2 g kg<sup>-1</sup> lipid, 30 g kg<sup>-1</sup> protein, 60 g kg<sup>-1</sup> sugars and 7 g kg<sup>-1</sup> ash (Garrote



**Fig. 1.** The schematic model of kefir grains formation. (Source: Wang *et al.*, 2012)

*et al.*, 1997). The matrix of kefir grains contains a specific polysaccharide, which wasn't isolated from other substrates, and for this reason called original kefiran. The first author who identified this polysaccharide was La Rivière *et al.* (1967), who estimated that kefiran, represents almost half of the substance cohesive grains. This polysaccharide has an important potential as a food gum in food industry, in innovative packaging material productions, or as a fortification agent in foods because of its well-known health benefits (Piermaria *et al.*, 2009).

Reproductive capacity of the grains is significantly influenced by growth conditions, Kořakowski and Ozimkiewicz (2012) demonstrated through their studies that in unfavourable conditions, kefir grain growth is disturbed, their appearance deteriorates and they lose their resilience. They shrink, and their microbiological balance is disrupted, whereas in favourable conditions, after multiple passages into milk, they retrieve their typical appearance, physiological functions and technological properties.

There are several methods of grains' activation before inoculation in growth medium, as described in the literature (Zajšek and Goršek, 2010). However, information on the possible factors which influences the kefir quality and kefiran production, due to different activation times for kefir grains, has not been too often reported up to date.

The kefir grains growth curve is essential for optimisation, control and monitoring purposes and the effect of process parameters on the kefir grains' growth rate has been studied. The main objectives of this study aimed to investigate the effects of temperature, time, shaker rotating speed and culture media supplementation on the propagation of kefir grains.

## MATERIALS AND METHODS

The samples of kefir grains (KG) were obtained from the collection of the Walloon Agricultural Research Centre (CRA-W, Gembloux, Belgium). The microflora identified in the kefir grain KJ included *Acetobacter* sp., *Kazachstania exigua*, *Lactobacillus kefirifaciens* subsp. *kefirgranum*, *Lb. kefirifaciens*, *Lb. parakefirifaciens*, *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* (Ninane, 2008).

For activation, the fresh kefir grains that were washed with sterile water were inoculated (15 g) in skim milk ultra-high temperature-treated (300 ml) at room temperature for short period (24 – 48 h) and the medium was exchanged daily, this process being necessary to maintain the grains' viability.

In this study, the kefir grains (4.5% w/v) were cultured in skim milk at different conditions (temperature, time, shaker rotating speed, culture media supplemented) to evaluate their effects during bioprocess. The kefir grains mass concentration was determined by weighting on a SHIMADZU analytical balance (AX 120) (SHIMADZU Corporation), it was applied the gravimetric procedure according the literature, with a slight adjustment. Biomass growth rate was according to the formula below, which was used to evaluate effects of culture conditions. All results

$$\%G = \frac{X_{n+1} - X_n}{X_n} * 100$$

were carried out 3 times for means.

Where:

G = growth rate;

X<sub>n</sub> = biomass weight after n days (g);

X<sub>n+1</sub> = biomass weight after (n+1) days (g)

At the end of each experiment the grains were separated from the fermented product by filtration through a plastic sieve, and the grains

were washed with water at room temperature for kefiran isolation procedure and washed with milk at 22 – 25°C, for a new passage (Zajšek and Goršek, 2010).

For this experiment each of the factors were changed separately when the other variables, which have been found to be optimal were left unchanged. Therefore the kefir grains were cultured at different changed conditions to evaluate the effects, as shown in Tab. 1. Regarding the Factor 1, incubation process was performed at 25°C for 72 h.

The titratable acidity and pH values were measured during the propagation. Total titratable acidity determined according by method of Official Methods of Analysis (2005). The results are expressed in lactic acid g/l, considering that 1 ml 0,1 N NaOH used for neutralization corresponded to 0,0090 g Lactic acid. The pH value was determined by using a digital pH meter model Consort C532, Sn 84219 (Consort n.v. Parklaan 36, B-2300 Turnhout, Belgium). Prior to use, the pH meter was standardized with standard buffer solution of pH 4 and 7.35002 produced by Funke Gerber, Berlin, Germany.

The chemical composition of milk was analyzed with LactoStar, Milk analyzer, Type 3560-0. This is a rapid analysis which can be applied for measurement of fat, solids non-fat, proteins and lactose. The samples were analyzed by automatic dosage and the results of the parameters analyzed have a high accuracy.

The data were expressed as mean  $\pm$  standard deviation (SD) from two replicates for each sample. An analysis of variance (ANOVA) of the data was performed using the SPSS 19.0 statistical analysis system, and a TurkeyHSD test with a confidence interval of 95 or 99% was used to compare the means. Differences were considered significant at  $P < 0.05$ .

## RESULTS AND DISCUSSION

The effect of growing medium composition on kefir grain growth

Milk is the main medium for kefir grains growth; therefore its major components significantly influenced the growth rate. It was used two type of milk: Conventional milk - CSM and Organic milk - OSM, the value of major components composition (Fat - lipids, proteins-PROT, lactose-LAC and SNF-non-fat-dry matter) of milk medium is shown in Tab. 2.

The quantities of the various main constituents of milk can vary considerably between cows of different breeds and between individual cows of the same breed, and depending on the type of feed (Bylund, 1995). The sample OSM shows higher values of protein and lactose, substrates necessary for the growth of kefir grains. The results of chemical determinations for the milk medium were within the maximum permissible by data legislation (SR 2418:2008).

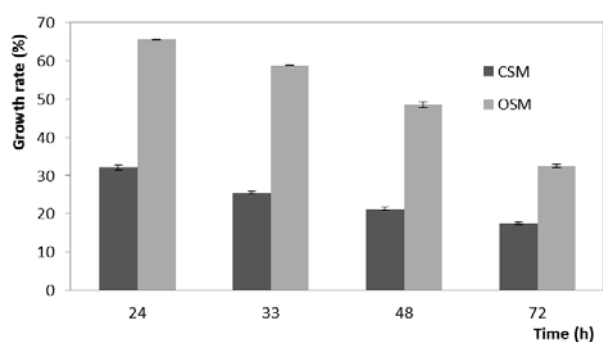
The results obtained in the study of Florence *et al.* (2012) propose organic milk as a raw

**Tab.1.** Six different factors used in kefir grain propagation experiments

Factor 1 Type growing medium	Factor 2 (h) Time	Factor 3 (°C) Temperature	Factor 4 Stirring type	Factor 5 (rpm) Shaker rotating speed	Factor 6 (g/L) Culture media supplemented
Conventional milk(CSM)	4	20	Static	100	Carbohydrate
Organic milk (OSM)	8	25	Shaker rotating	125	substrates
	12	28	speed (100 rpm)	150	Minerals
	16	32	Magnetic stirrer	200	Vitamins
	24		bar		Nitrogen source
	33				
	48				
	72				

**Tab. 2.** Chemical composition of skim milk used as culture media

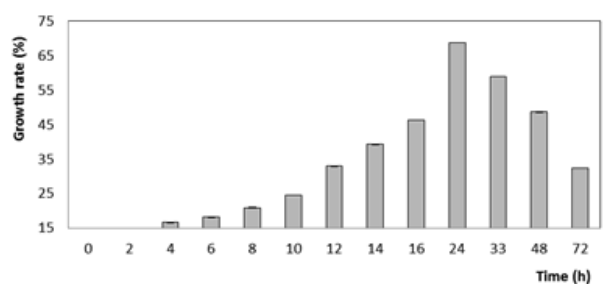
Samples	Chemical composition (%)			
	FAT	PROT	LAC	SNF
CSM	0.16 $\pm$ 0.0070	3.22 $\pm$ 0.0141	4.55 $\pm$ 0.0070	8.42 $\pm$ 0.0070
OSM	0.13 $\pm$ 0.0070	3.48 $\pm$ 0.0141	4.94 $\pm$ 0.0141	9.25 $\pm$ 0.0423



**Fig. 2.** The growth medium - dependent kefir grains biomass

material to produce fermented milks, thanks to its technological characteristics evidenced by the improvement in lactic acid bacteria counts and superior levels of conjugated linoleic acid (CLA) and  $\alpha$ -linoleic acid (ALA).

The results (Fig. 2) indicated that the growth rate of grains is directly affected by type of milk. Using OSM the KG biomass increase was with 38.9 g/L for 24 h, at 25°C, with agitation at 125 rpm, in this experiment was achieved a prediction of the



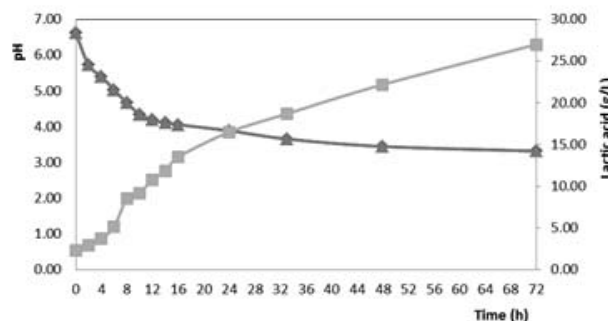
**Fig. 3.** The effect of time on the kefir grains biomass growth rate

optimum parameters of the propagation process, the parameters will be presented below.

#### Influence of incubation time on kefir grain growth

In order to monitor the grains growth in skim milk standard, it can be noticed that the mass increases more rapidly in the first 24 hours, after which it shows a significant decrease in the next 48, 72 hours at 25°C (Fig. 3). Thereafter, it appears that the rate of grains growth slows due to the nutrient depletion or because of the excessive acidity for activity biological. The pH and lactic acid (g/L) values of kefir liquid, during this propagation process was shown in Fig. 4.

By monitoring this parameters, it was aimed the indirectly measuring of biological activity the grains. It can be observed a decrease over time to 72 hours, the pH stabilizes at pH 3.3, this



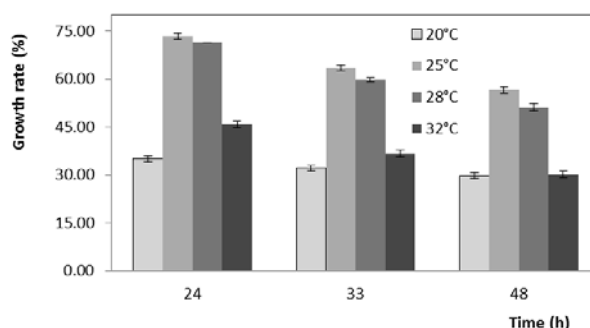
**Fig. 4.** Time-dependent dynamics of pH and lactic acid level

decrease could be dependent on the increase in number of micro-organisms capable to acidify the milk (Irigoyen *et al.* 2003), and the kefir grains biomass significantly increases until to 24 hours of propagation.

The pH value of kefir at 24-h of incubation reached of 3.89, that was in agreement with the pH values obtained by Demirhan *et al.* (2011), Demirhan *et al.* (2013), Zajsek and Gorsek (2010), and by Zajsek and Gorsek (2011).

#### Influence of temperature on kefir grain growth

In our experiments, the optimal temperature for kefir grains growth was observed to be 25 °C, with the highest growth rate, of 37.93 g/L for 24 h (Fig. 5). It was observed that between 25°C and 28°C, no significant differences ( $p>0,005$ ) between



**Fig. 5.** The effect of temperature on the kefir grains biomass growth rate

growth rates as indicated by microorganisms distribution in grains. Therefore, the yeast coloni-

zation is on the surface and middle part of the grains and the lactic acid bacteria is inside the grains.

Rimada and Abraham (2001) studied the influence of temperature on the kefir and kefir grains production using whey as growing medium and it reported that kefir grains growth and kefir yield are maximal at 43°C. Zajsek and Gorsek (2010) based on results by Rimada and Abraham (2001), assume that due to high temperature, kefir is dissolved and transferred into medium.

Zajsek and Gorsek (2011) reported that the highest kefir grains biomass increase was 18.8 g/L and kefir production at 25°C, using conventional milk, also, the kefir production is the highest (2.79 % w/w) mainly due to the fact that microorganisms protect themselves against environmental influences by increasing the kefir production.

De Vuyst and Degeest (1999) explained that the 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 exopolysaccharides biosynthesis.

Impact of the stirring type and speed on kefir grain growth

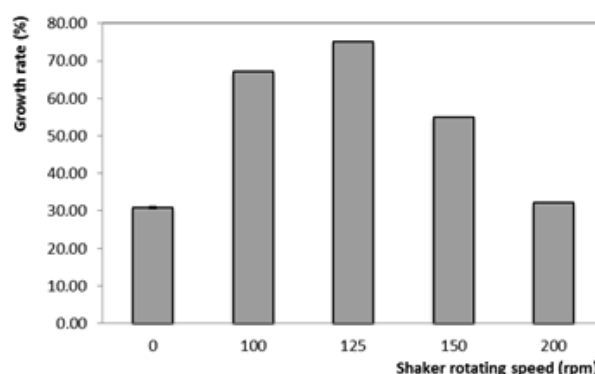
In Tab. 3 is shown the effect of agitation rate on kefir grains biomass, pH and lactic acid values. This test shows that the agitation with shaker rotating induced a greater growth rate, probably ensuring a better homogeneity of the test medium and therefore a greater availability of nutrients and air for microbial colonies by grains. The

**Tab. 3.** The effect of rotation type on the kefir grains biomass ( $\Delta Y_{KG}$ ), pH and lactic acid

Type of homogenization		Time (h) of incubation	
		1	24
$\Delta Y_{KG}$ (g)	Shaker rotation	15.00	23.67±0.01a
	Static	15.03	20.15±0.01b
	Magnetic rotating	14.98	19.69±0.01c
	Shaker rotation	6.25	3.72±0.005b
Ph	Static	6.26	3.7±0.005a
	Magnetic rotating	6.24	3.64±0.005c
Lactic acid (g/L)	Shaker rotation	2.55	17.25±0.707b
	Static	2.35	13.05±0.707c
	Magnetic rotating	2.20	17.75±0.707a

Each value represents the mean of two measurements± standard deviation.

Different letters within a column indicate significant differences among formulation ( $P < 0.05$ ).



**Fig. 6.** Growth rate to the kefir grains biomass, dependent on the shaker rotating speed

significant differences were observed for pH and lactic acid values, the lowest value was recorded using magnetic rotating, as homogeneous method of the environment. Use of the magnetic stirring bar causes loss of integrity of the grains, causing their crumbling.

The highest biomass was produced using shaker rotating; therefore we investigated the agitation rate at 100, 150 and 200 rpm (Fig. 6). In their studies, Zajšek and Gorsek (2011), they reported that the agitation rate of 80 rpm produced highest biomass (7.14 g/L), using mKG<sub>0</sub>=42 g/L and Guzel-Seydim *et al.* (2005) recommended during the fermentation medium as to shake the kefir at a speed between 70 and 100 rpm, the propagation time of 24 h.

According to results, rotation rate at 125 rpm gave the highest growth rate, kefir grains biomass increase with 38.1 g/L for 24 h, at 25°C from mKG<sub>0</sub>=50 g/L kefir grains biomass.

#### Influence of different nutrients added to the culture medium

The addition of different nutrients and their effects on growth rate, lactic acid production and pH is presented in Tab. 4. We expected that addition of certain nutrients in the test medium would compensate for the eventual exhaustion of nutrients from a few hours of testing, or increase the biological activity of grains.

Among the carbon sources tested, the maximum of kefir biomass  $\Delta Y_{KG}$ =23.23 g/L, were obtained using the mixture Glucose:Galactose

Zajšek and Goršek (2011) reported that using the carbon sources for production of kefir biomass, they obtained the maximum of biomass 12.36 g/L when the lactose concentration was 5% (w/v). Harta *et al.* (2004) also examined the influence

**Tab. 4.** The effect of different nutrients on the kefir grains biomass ( $\Delta Y_{KG}$ )

Sources (w/v)	$\Delta Y_{KG}$ (g/L)	Growth rate (%)	Lactic acid (g/L)	pH
Control	38.90±0.00141a	74.55	16.94	3.67
Lactose 50 g/L	20.52±0.0212g	41.28	13.55	3.93
Glucose : Galactose 25:25 g/L	23.23±0.000e	46.22	13.00	3.80
Glucose 20 g/L	17.47±0.0141k	35.07	12.00	3.74
Glucose : Sucrose 1:3, 20 g/L	22.50±0.2828f	44.91	11.90	3.59
Minerals:				
MgSO <sub>4</sub> H <sub>2</sub> O: MnSO <sub>4</sub> H <sub>2</sub> O (1:1)				
0.05 g/L	14.00±0.000m	28.03	10.85	4.11
0.1 g/L	19.95±0.0141h	39.74	12.55	3.82
0.15 g/L	13.05±0.0282n	26.11	11.60	3.89
0.25 g/L	30.33±0.0141c	60.62	10.40	4.09
0.4 g/L	18.42±0.0282i	36.61	11.50	4.06
0.75 g/L	17.97±0.0282j	35.96	12.70	3.86
Yeast extract 5 g/L	19.65±0.0141h	39.21	16.95	3.64
Yeast extract 10 g/L	35.70±0.000b	71.33	16.97	3.61
Yeast extract 15 g/L	20.73±0.0424g	41.38	16.6	3.66
B-Complex 0.05 g/L	24.10±0.0141d	48.10	10.20	4.08
B-Complex 0.107 g/L	11.50±0.1414o	22.97	10.50	4.01
Tryptone 1g/L	16.43±0.0141l	32.82	12.70	3.71

Values represent the mean of two experiments± standard deviation.

Means within each column with same letters are not significantly different ( $P > 0.05$ ).

Different letters within a column indicate very significant differences among formulation ( $P < 0.01$ ).

\*Control = KG grown in milk bio

of different carbohydrates on the kefir biomass production at 30°C, 24h in synthetic medium. They obtained the highest biomass (27.25 g) using a mixture Glucose:Sucrose at a fixed-ratio of 1:3. Therefore, the different carbon sources might have different effects of catabolic repression on the cellular secondary metabolism (Zajšek and Goršek, 2011).

The addition of different mineral can influence the kefir biomass increase. By adding 0.25 g/L Mn<sup>2+</sup> and Mg<sup>2+</sup>, the amount of biomass at the end of propagation time was 30.33 g/L (growth rate was 60.62%), higher than when using carbon source.

Demirhan *et al.* (2011) also found that the kefir biomass was the highest when added MgO, containing 0.30 g/L with 46.3% increase.

The presence of different vitamins usually affects the rates of biosynthesis of many metabolites, in this study the influence of vitamins on biomass increase was tested by adding the B-complex and yeast extract.

The results show that the supply of growing medium with 10 g/L yeast extract determines an increase of kefir grains biomass with 71.33%, lower growth rate (48.1%) was reported by adding B-Complex 0.05 g/L concentration. Also,

they suggest that the supply of vitamins is an absolute requirement for the growth of kefir grains microbiota because those microorganisms of kefir grains are capable of synthesizing these vitamins.

The mass of grains increased very significantly ( $p < 0.01$ ) from 11.50 g/L to 35.70 g/L at the different nutrients used which indicate the links between nutritional substrates and growth rate of grains.

The addition of nitrogen source was not so effective for increasing the KG biomass, although, Wang and Bi (2008) indicated that the yield of kefir was the highest when casein was added into the medium, followed by addition of peptone, tryptone, yeast extract, and yeast powder.

We conclude that the addition of yeast extract gives the highest rate of growth using the conventional milk, but using organic milk a higher growth rate was obtained and with a medium cost of production, and the kefir production is dependent on the kefir grains growth rate.

## CONCLUSION

Parametric analysis was used to define the important kinetic parameters of the process and to establish the most adequate growth model. In recent decades, for economic as well for

environmental reasons, there has been a continuous and growing pressure to recover and exploit the agro-food wastes, therefore the potential application of kefir grain biomass in obtaining the amount of polysaccharide kefiran has increased.

The highest KG biomass increase was obtained using organic milk as culture medium and incubation parameters: 24 hours at 25°C with agitation rate at 125 rpm. Also, the age of the kefir grains, the conditions of production and handling can influence the kefir grains propagation.

We observed that the kefir grains biomass increased significantly by addition of mineral sources and vitamins in conventional medium. This environment has proven to be low in carbon sources and vitamins, necessary elements for growth of the microorganisms. The high growth activity of lactobacilli is affected by medium formulation and minerals are considered to be a limiting factor

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