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Production of Unique Naturally Immobilized Starter: A Fractional Factorial Design Approach Towards the Bioprocess Parameters Evaluation

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1. Introduction

Pure and/or mixed isolated microbial cultures, in the dairy sector known as starters, are widely used in the manufacture of numerous fermented (cultured) milk products as well as in butter and cheese making (Bylund, 1995). The starter is added to the sterilized milk-based fermentation media and allowed to grow under controlled and, if necessary, on-line regulated process conditions. During the fermentation, the pure or diversified microbial community produces organic substances which give the cultured milk products their characteristic organoleptic properties such as acidity (pH), flavour, aroma, colour and odour as well as consistency.

According to the basic definition known from the literature, the probiotics are food products and nutritional supplements containing live microorganisms and other components of microbial cells that have an extremely beneficial impact on the citizen's live and well-being of the host (Lahteenmaki & Ledeboer, 2006; Salminen et al., 1999). Therefore, it is not surprising that during the last few years, there has been a significantly increase in the worldwide sales of cultured products containing probiotic bacteria (Ostlie et al., 2005).

One of the dairy cultured products is also kefir (known also as kephir, kiaphur, kefer knapon, kipi and kippi), i.e. unique self-carbonated viscous dairy beverage with small quantities of alcohol and can be made with any kind of animal milk, such as those of cows, goats, sheep, camels and buffalos as well as coconut, rice and soy milk (Abraham & De Antoni, 1999; Farnworth, 1999; Koroleva, 1988; Kwak et al., 1996; Loretan et al., 2003; Otles & Cagandi, 2003). Original kefir contains among others also numerous bioactive ingredients that give its unique health benefits, such as, for instance, strengthening immune system (Vinderola et al., 2005), antitumor activity (Liu et al., 2002), improving intestinal immunity (Thoreux & Schmucker, 2001), antimicrobial activity (Garrote et al., 2000; Rodriguez et al., 2005), regulation of cholesterol metabolism (Liu et al., 2006a), improving anti-allergic resistance (Liu et al., 2006b), improving sugars digestion (Hetzler & Clancy, 2003) and antioxidant activity (Liu et al., 2005). Those kefir's health properties indicate that kefir may be an important, high quality and price-competitive targeted probiotic product.

Several methods for kefir production, which use pure and isolated starters, can be found in the literature (Assadi et al., 2000; Beshkova et al., 2003; Fontan et al., 2006). Nevertheless, the real and original kefir can only be produced using traditional methods of adding kefir

grains to a quantity of milk (Otles & Cagandi, 2003; Tamine et al., 1999). Kefir grains are complex natural microbial community entrapped into matrix of protein and polysaccharide (kefiran) and is believed to have its origin in the Caucasian mountains (Bosch et al., 2006; Farnworth, 2005). They are white to light yellowish globular particles (masses) with a diameter (5–35) mm (Bosch et al., 2006; Garrote et al., 1997; Marshall, 1993). The shape of the grains is irregular. Plainly, they are similar to a piece of cauliflower. On the other side, their microflora is much more diverse and complex and therefore difficult to understand and scientifically prove.

During the last two decades, many studies have been focused on thorough analysis of kefir grains microbial composition (Angulo et al., 1993; Garrote et al., 2001; Irigoyen et al., 2005; Kwak et al., Loretan et al., 2003; 1996; Mainville et al., 2006; Marshall, 1993; Simova et al., 2002; Takizawa et al., 1998; Vancanneyt et al., 2004; Witthuhn et al., 2005; Witthuhn et al., 2004). Summarily, kefir grains contain gram-positive homo-fermentative and hetero-fermentative lactic and acetic acid bacteria (*Lactobacillus caucasicus*, *Lactobacillus brevis*, *Lactobacillus bulgaricum*, *Lactobacillus casei*, *Lactobacilus kefir*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus kefiranofaciens*, *Lactobacilus kefigranu, Lactobacillus helveticus* ssp. *jogurti*, *Lactubacillus lactis* ssp. *lactis*, *Lactobacillus fermentum*, *Lactobacillus cellobiosuss*, *Lactococci lactis* ssp. *lactis 1*, *Lactococci lactis* ssp. *lactis 2*, *Lactococcus lactis* ssp. *lactis* var. *diacetylactis*, *Lactococcus lactis* ssp. *cremoris*, *Streptococcus thermophilus*, *Lactococcus filant*, *Streptococcus durans*, *Leuconostoc dextranicum*, *Leuconostoc kefir*, *Leuconostoc lactis*, *Leuconostoc mesenteroides* ssp. *mesenteroides* and *Leuconostoc mesenteroides* ssp. *Cremoris*) gram-negative acetic acid bacteria (*Acetobacter* spp.) and both lactose fermenting and non-fermenting yeasts (*Kluyveromyces lactis, Kluyveromyces marxianus, Torula kefir, Saccharomyces cerevisiae, Saccharomyces unisporus, Candida keyfr, Saccharomyces rouxii, Torulaspora delbrueckii, Debaryomyces hansenii, Candida holmii, Zygosaccharomyces* sp., *Candida lipolytica* and *Cryptococcus humicolus*). Mentionable, the variegated natural microbial population found in kefir grains represent a pattern of symbiotic community (Lopitz-Otsoa et al., 2006).

The unique variegated microbial composition of kefir grains enables their application not only in large-scale kefir production but potentially also in another novel industrial food manufacturing bioprocesses or even in some specific innovative and visionary eco-efficient bioprocesses in sustainable production of safe, efficient as well as high quality fine biochemicals with the highest added value. For instance, different studies indicate that kefir grains can be used in bread production as a substitute for baker's yeast (Plessas et al. 2005) polysaccharide production as a natural source of exopolysacharide (kefiran) (Rimada and Abraham, 2001; Rimada and Abraham, 2003) and bioalcohol production as a natural immobilized kefir yeast cells (Athanasiadis et al., 1999). Moreover, they can also be used as natural variegated microbial starter in production of fermented soy milk powder (Kubow, S. & Sheppard, WO/2007/087722 A1) as well as in production of novel fermented low-alcoholics drink from mixture of whey and raisin extract (Athanasiadis et al., 2004; Koutinas et al., 2007).

Considering abovementioned scientifically proven potential industrial applications as well as other emerging innovative visionary applications which are currently under thorough screening, evaluation and assessment, it is realistic to expect that in the near future the global demand for grains will extremely increase. Therefore, the classical batch production of kefir grains using traditional propagation in milk with relatively low daily kefir grain increase mass fraction, $w_{KG,di}$ = (5–7) %/d, (Libudzisz & Piatkiewicz, 1990) has to be optimized and improved. When grains are produced commercially, it is critically important

for optimization, as well as for monitoring and control of their batch production to know the impact of different bioprocess parameters on daily kefir grains increase mass and mass fraction.

Traditionally, the impact of various significant bioprocess parameters on batch bioprocess performance has been determined experimentally using through planning and time consuming as well as cost ineffective implementing experiments on large industrial scale. With the technological development and growth of the society, however, the bioprocess parameters assessment has been progressively transferred to laboratory scale, which resulted in increased effectiveness and reduced planning cost. Consequently, today almost all bioprocess development activities, which among others include also determination of the relative impact of various significant bioprocess parameters, are practically carried out in laboratory or pilot scale and afterwards, only scale up and tech-transfer into production line is performed.

The technique for the determination and investigation of the influential experiment (bioprocess) parameters at different levels is called the 'design of experiments' (DoE) (Ranjit, 1990). The selection of relevant DoE technique depends especially on the number of parameters influencing the product quality, and the type of the investigated problem. However, conventional full factorial DoE techniques involve altering of one parameter at a time keeping all other parameters constant. When we want to study any given system with a set of independent variables (bioprocess parameters) over a specific region of interest (levels region) and intend to improve the process planning strategy and quality optimization of the bioprocess parameters at the same time, we use the so-called 'Taguchi's approach' (Ranjit, 1990). The use of its algorithm is observed in various optimization problems, starting with optimization of diesel engine parameters (Nataraj et al., 2005), the leaching of non–sulphide zinc ore in the ammonium–sulphate solution (Moghaddam et al., 2005), to the production of clavulanic (Saudagar & Singhal, 2007) and citric acid (Shojaosadati & Babaeipour, 2002) as well as laccase by *Pleurotus ostreatus* 1804 (Prasad et al., 2005), etc. In contrast to the traditional DoE, the standardized Taguchi's experiment design methodology for two independent problem solution plans usually brings the same results, which enables determination of individual bioprocess parameters' relative impact on the final result. This methodology envisages implementation of a minimum number of experiments, which are defined by specific standard orthogonal arrays (OA). Selection of relevant OA is conditioned by the number of parameters and levels.

This chapter examines the traditional batch propagation of kefir grains in fresh high temperature pasteurized (HTP) whole fat cow's milk with some additions (glucose and baker's yeast) under different bioprocess conditions. The main objective of the contribution is to present and describe an experimental determination of the relative impacts of various significant bioprocess parameters that influence traditional batch propagation of kefir grains and daily kefir grain increase mass using the Taguchi's experiment design methodology.

2. Materials and methods

2.1 Equipment

Determination of the relative impact of various significant bioprocess parameters that influence traditional batch propagation of kefir grains and daily kefir grain increase mass using the Taguchi design methodology requires the performance of a series of experiments. In order to ensure the highest quality as well as repeatability of raw experimental data, it is

desired to perform those experiments (batch propagations of kefir grains in enriched milk under different bioprocess conditions) in computer controlled state-of-the-art laboratory reactor or fermentor.

Perhaps one of the most user-friendly and at the same time the most efficient high quality aforementioned equipment is heat flow reaction calorimeter RC1 (Mettler Toledo, Greifensee, Switzerland). Basically, the RC1 system is actually both – state-of-the-art computer controlled, electronically safe-guarded bench-scale 'model' of a batch/semi-batch reactor or fermentor from pilot and/or industrial plant (automated lab reactor (ALR)) and at the same time a heat-flow reaction calorimeter. The RC1 system allows real time measurement, monitor and control of all important bioprocess parameters such as rotational frequency of the stirrer, temperature of reaction or fermentation media, reactor jacket temperature, pH value of reaction or fermentation media, mass concentration of dissolved oxygen, amount of added (dosed) material, etc. Primarily, it is designed for determination of the complete mass and heat balance over the course of the entire chemical reaction or physical transformation (e.g. crystallization, dissolution, etc.). In addition, using specific modifications, it can be employed for investigating thermal effects during bioprocess (Marison et al., 1998). This means that by using RC1 system it is possible to gain and/or determine wide range of process thermal data and constants such as specific heat capacity of reaction mixture, heat flow profile of the reaction or physical transformation, reaction enthalpy, maximum heat flow due to reaction or physical transformation, potential adiabatic temperature increase in case of cooling failure, heat accumulation, etc.. All obtained time-depended calorimetric data (heat flow data) can be further used for kinetic studies, etc. The RC1 system enables performance of chemical and also bio(chemical) reactions or physical transformation under different modes such as isothermal conditions, adiabatic conditions, etc. Using RC1 it is possible to perform distillations and reactions (transformations) under reflux with heat balancing. Last but not least, the RC1 system is a recipe driven (managed) which means that all process operations can be programmed or written by recipe beforehand and thus its maximum flexibility is assured. Finally, it is worldwide recognized as an industrial standard to gain safety data for a later scale-up to pilot or production plant.

2.2 Chemicals, kefir culture and culture medium

Daily kefir grain increase mass was studied using fresh HTP whole fat cow's milk (Ljubljanske mlekarne d.d.) as a culture medium. Its chemical composition is 3.2 % proteins, 4.6 % carbohydrates, 3.5 % fat and 0.13 % calcium. 3D-(+) Glucose anhydrous (Fluka) was obtained from commercial sources. Kefir grains, used as inocolum in this study, originate from Caucasian Mountain and were acquired from an internationally recognized local dairy (Kele & Kele d.o.o.). Their detailed microbial composition was not analyzed. Importantly, the microbial population (bacteria and yeasts) of kefir grains depends on many different factors (age, storage conditions and fermentation medium) and varies with the season. It is almost impossible to assure equal microbial composition during long term period, therefore for sets of experiments within one research, kefir grains with the same viability should be used.

2.3 Kefir grain biomass activation

Kefir grain biomass activation was performed in a glass lab beaker. The collected inactive kefir grains (γ_{KG} = 40 g/L) were inoculated in 1 L of fresh HTP whole fat cow's milk. After

incubation at room temperature (ϑ = (22 ± 2) °C) for 24 h, the grains were separated from the kefir beverages using a household sieve. After washing, they were reinoculated into the fresh milk. The same procedure was repeated over six subsequent days. After this procedure the kefir grains were considered active.

2.4 Analytical determination of kefir grain mass

For the determination of kefir grain mass, the gravimetric method was used. Therefore, kefir grains were separated first from the fermentation medium with plastic household sieve. Then the grains were washed with cold water and dried on filter paper to remove of bulk of adhered water. Finally, kefir grain mass was determined by weighting on Mettler-Toledo analytical balance (PG5002–S).

2.5 Taguchi's experiment design methodology

Dr. Genichi Taguchi has defined the optimization criterion quality as a consistency in achieving the desired or targeted value and minimization of the deviation (Ranjit, 1990). This goal is connected with the performance of a series of experiments with different bioprocess parameters at different levels. The bioprocess parameter is a factor affecting the optimization criterion quality, and its value is called the 'level'. The number of experiments and their sequence are determined by standard OA. When planning the experiments using four bioprocess parameters at four levels, we use the OA L_{16}. Such a plan envisages the performance of 16 experiments, which is significantly less when compared to the full factorial DoE with $4^4 = 128$ experiments.

Due to performing only a part of the envisaged experiments using the traditional full factorial DoE methodology, it is necessary to include an analysis of the results confidence. The standard statistical technique is used for this purpose, the so-called 'analysis of variance' (ANOVA), which recognizes the relative impact of the bioprocess parameters for the optimization criterion (in our case daily kefir grain increase mass) value.

The mathematical algorithm of the ANOVA statistical technique is based on calculation of the variance, which is an indicator of the optimization criterion quality. The ratio between the variance of the bioprocess parameter and the error variance shows whether the parameter affect on the product's quality. The equations required for calculating the relative impact of various significant bioprocess parameters affecting the optimization criterion are presented bellow. The meanings of symbols are described in the sub-chapter "Nomenclature".

$$S_{\mathrm{T}} = \sum_{i=1}^{N} Y_i^2 - \left(\sum_{i=1}^{N} Y_i \right)^2 \Big/ N \tag{1}$$

$$S_j = \sum_{k=1}^{L} \left(\left(\sum_{i=1}^{N_k} Y_i \right)^2 \Big/ N_k \right) - \left(\sum_{i=1}^{N} Y_i \right)^2 \Big/ N \tag{2}$$

$$S_{\mathrm{e}} = S_{\mathrm{T}} - \sum_{j=1}^{M} S_j \tag{3}$$

$$V_j = S_j / f_j \tag{4}$$

$$f_j = L - 1 \tag{5}$$

$$V_e = S_e / f_e \tag{6}$$

$$f_e = f_T - \sum_{j=1}^{M} f_j \tag{7}$$

$$f_T = M - 1 \tag{8}$$

$$F_j = V_j / V_e \tag{9}$$

$$X_j = \left(S_j - f_j V_e\right) 100 \Big/ S_T \tag{10}$$

$$X_e = \left(S_e + \sum_{j=1}^{M} f_j V_e\right) 100 \Big/ S_T \tag{11}$$

We compare variance ratio of bioprocess parameter j, F_j, to the standardized value at defined level of significance, $F_{m,n}$, which is obtained from the standard F tables (Ranjit, 1990), whereby m stands for the degree of freedom of bioprocess parameter j and n means the degree of freedom of error variance, and thus determine the bioprocess parameter impact accordingly. In the case where the variance ratio of bioprocess parameter j falls below $F_{m,n}$, the bioprocess parameter has no impact on the optimization criterion, therefore, it is pooled and ignored in the calculations. Consequently, the variance error changes, as the sum of squares and degree of freedom of the pooled bioprocess parameter are added to the error sum of squares and degree of freedom of error variance, respectively. By using the adjusted variance error, we determine new variance ratio of bioprocess parameter j and compare them again by the $F_{m,n}$. The process of pooling is sequential, which means that the parameter having the smallest impact on the optimization criterion should be pooled first, then we re–calculate the variance ratio of bioprocess parameter j and continue pooling until each bioprocess parameter meets the condition $F_j > F_{m,n}$. If the pooling process begins to perform, Taguchi recommends pooling bioprocess parameters until the degree of freedom of error variance is approximately half the total degree of freedom irrespective of significant test criterion validity $F_j > F_{m,n}$ for all remaining bioprocess parameters (Taguchi, 1987). When the pooling procedure is completed, the relative impact of bioprocess parameter j and error on optimization criterion can be calculated using Eqs. (10) and (11).

3. Experimental work

Experimentally determining the relative impact of various significant bioprocess parameters on the daily kefir grain increase mass, during 24 h incubation in cow's milk, based on Taguchi's fractional factorial design approach, requires the performance of a series experiments. It was established (Harta et al., 2004; Schoevers and Britt, 2003) that culture medium temperature, ϑ, glucose mass concentration, γ_G, baker's yeast mass concentration,

γ_Y, and the rotational frequency of the stirrer, f_m are the main influences bioprocess parameters. The bioprocess parameter in our case is a factor affecting daily kefir grain increase mass and its value is called the 'level'. We examined the relative impact of the selected bioprocess parameters at four different levels, as shown in Table 1.

Bioprocess parameter			Level			
			1	2	3	4
A:	Culture medium temperature	ϑ (°C)	20	22	24	26
B:	Baker's yeast mass concentration	γ_Y (g/L)	0	5	10	15
C:	Glucose mass concentration	γ_G (g/L)	0	10	20	30
D:	Rotational frequency of the stirrer	f_m (1/min)	0	50	70	90

Table 1. Proposed bioprocess parameters and their levels

Experiment	Bioprocess parameter[1]				
	A	B	C	D	E[2]
1	1	1	1	1	1
2	2	1	2	3	4
3	1	2	2	2	2
4	4	1	4	2	3
5	1	4	4	4	4
6	2	2	1	4	3
7	4	2	3	1	4
8	4	4	1	3	2
9	4	3	2	4	1
10	3	1	3	4	2
11	2	3	4	1	2
12	3	4	2	1	3
13	1	3	3	3	3
14	2	4	3	2	1
15	3	3	1	2	4
16	3	2	4	3	1

Table 2. Design of experiments – orthogonal array L_{16}

During the first stage of the experimental work, it is necessary to prepare the design of experiments. The DoE envisages determining the number of experiments, their performance conditions, and their sequence. Based on the assumption that the daily kefir grain increase mass would be affected by four bioprocess parameters being considered at four levels, we chose the L_{16} array as the most adequate OA requiring the performance of 16 experiments (Ranjit, 1990). The OA L_{16} is usually intended for the investigation of five bioprocess

[1] In our case bioprocess parameter E was not considered.
[2] Bioprocess parameters and values of their levels are indicated in Table 1.

parameters at four levels; however, it may also be used in our case (four parameters at four levels) by ignoring the bioprocess parameter E. The DoE is presented in Table 2. The first column presents the experimental serial number. Each experiment was defined by the bioprocess parameters (A, B, C, D and E) marked at specific levels by numbers from 1 to 4.
During the second stage of the experimental work, we implemented the proposed DoE by performing the 24 h kefir grain biomass incubations in the RC1 system. The incubation procedure was the same for all experiments. Individual experiments were implemented by means of first charging the reactor by 1 L of fresh HTP whole fat cow's milk and adding the mass of glucose previously defined by the DoE. This fermentation medium was heated up to working temperature under the defined rotational frequency of the stirrer. After establishing the temperature steady state and dissolved glucose, we inoculated the fermentation medium with the mass of the baker's yeast also defined by DoE and with 40 g of active kefir grains, which corresponds to initial kefir grain mass concentration, γ_{KG} = 40 g/L. After the 24 h incubation was completed, the kefir grain increase mass was determined using the gravimetric method.

4. Results and discussion

The final kefir grain mass concentration in the culture medium, $\gamma_{KG,f}$, daily kefir grain increase mass, $m_{KG,di}$, and daily kefir grain increase mass fraction, $w_{KG,di}$, experimentally determined under different conditions proposed by the DoE (Table 2), are presented in Table 3. Daily kefir grain increase mass fraction, $w_{KG,i}$ is the quotient between the kefir grain increase mass concentration ($\gamma_{KG,f}$ – 40 g/L) and the initial kefir grain mass concentration (γ_{KG} = 40 g/L).

Experiment	$\gamma_{KG,f}$ (g/L)	$m_{KG,di}$ (g)	$w_{KG,di}$ (%)
1	40.40	0.40	1.00
2	45.83	5.83	14.58
3	46.51	6.51	16.28
4	45.44	5.44	13.60
5	43.39	3.39	8.48
6	45.55	5.55	13.88
7	42.06	2.06	5.15
8	53.10	13.10	32.75
9	50.14	10.14	25.35
10	60.62	20.62	51.55
11	41.70	1.70	4.25
12	41.90	1.90	4.75
13	52.60	12.60	31.50
14	58.06	18.06	45.15
15	55.93	15.93	39.83
16	52.56	12.56	31.40

Table 3. Experimental results – orthogonal array L_{16}

Table 3 shows that the highest daily kefir grain increase mass fraction ($w_{KG,i}$ = 51.5 %) was found at the rotational frequency of the stirrer, f_m = 90 (1/min), at culture medium temperature, ϑ = 24 °C, with a glucose mass concentration, γ_G = 20 g/L, and without baker's yeast (γ_Y = 0 g/L).

Moreover, the average impacts of the bioprocess parameters along with interactions at the assigned levels on the daily kefir grain increase mass are shown on Fig. 1. The difference between levels of each bioprocess parameters indicates their relative impact (Prasad et al., 2005). The larger the difference, the stronger is the influence.

It can be observed from Fig.1 that among bioprocess parameters studied rotational frequency of stirrer showed the strongest influence and followed by glucose mass concentration, culture medium temperature and baker's yeast mass concentration. However, the relative impact of the proposed influencing bioprocess parameters on daily kefir grain increase mass were estimated by ANOVA. The sum of squares or deviation, S_j, and the variance of individual bioprocess parameters, V_j, were calculated by equations (2) and (4), and the error value by equations (3) and (6), respectively. The variance ratio, F_j, is the ratio of variance due to the effect of an individual bioprocess parameter and variance due to the error term. It was calculated by equation (9). The results of ANOVA are shown in Table 4.

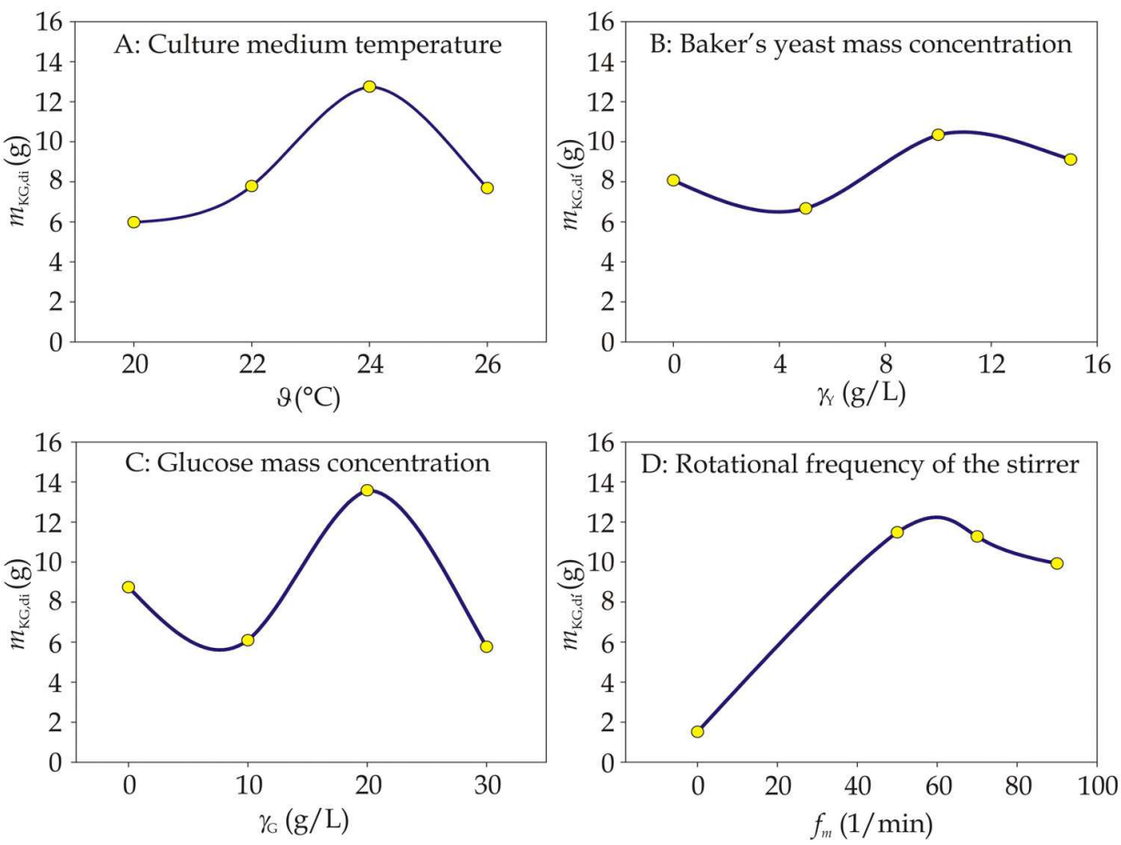

Fig. 1. Individual bioprocess parameters influence at different levels on daily kefir grain increase mass

The degrees of freedom of bioprocess parameter j and error variance equaled ($f_j = f_e = 3$) in all cases. At 90 % confidence (level of importance 0.1), the value $F_{3,3} = 5.3908$ was determined through standardized tables of F–statistics. Table 5 shows that the variance ratio of all bioprocess parameters fell below $F_{3,3}$. In accordance with the Taguchi's method algorithm, we pooled baker's yeast mass concentration from further statistical consideration as the least important bioprocess parameter, i.e., with the lowest variance ratio compared to $F_{3,3}$.

Bioprocess parameter		S_j	f_j	V_j	F_j
A:	ϑ (°C)	102.52	3	34.17	1.893
B:	γ_Y (g/L)	29.18	3	9.73	0.539
C:	γ_G (g/L)	156.58	3	52.19	2.891
D:	f_m (1/min)	269.57	3	89.86	4.978
Error		54.16	3	18.05	1.000
Total		612.01	15	–	–

Table 4. Analysis of variance – orthogonal array L_{16}

Pooling of the baker's yeast as an insignificant bioprocess parameter requires a repeated variance analysis, whereby the sum of squares and the degree of freedom of the pooled bioprocess parameter are added to the error sum of squares and the degree of freedom of error variance, respectively. The results in Table 5 show that, consequently, the variance ratios of the remaining bioprocess parameters increase. In spite of this, a repeated comparison of variance ratio of each bioprocess parameter indicated in Table 5 with the F–statistics value, $F_{3,6} = 3.2888$, shows that culture media temperature does not meets the $F_j > F_{3,8}$ condition. Nevertheless, regarding significant test criterion ($F_j > F_{m,n}$) and especially Taguchi's recommendation, we pooled only baker's yeast mass concentration as insignificant bioprocess parameter on daily kefir grain increase mass. The final results of ANOVA terms, which were modified after pooling baker's yeast mass concentration, are shown in Table 5. The relative influences of the bioprocess parameter j and error on the daily kefir grain increase mass were calculated using equations (10) and (11), respectively.

Bioprocess parameter		S_j	f_j	V_j	F_j	X_j
A:	ϑ (°C)	102.52	3	34.17	2.460	9.9
B:	γ_Y (g/L)	pooled				
C:	γ_G (g/L)	156.58	3	52.19	3.758	18.8
D:	f_m (1/min)	269.57	3	89.86	6.469	37.3
Error		83.34	6	13.89	1.000	34.0
Total		612.01	15	–	–	100.0

Table 5. Final results of variance analysis – orthogonal array L_{16}

The results, shown in Table 5, assign the highest relative influence on the daily kefir grain increase mass (37.3 %) during 24 h incubation to the rotational frequency of the stirrer. The impact of glucose mass contraction and culture medium temperature within the observed ranges (γ_G = (0–30) g/L and ϑ = (20–26) °C) show the lower ones, 18.8 % and 9.9 %, respectively. The remaining fraction represents error influence.
It is well known that kefir grains are bulky and awkward to handle (Bylund, 1994). Despite extensive and careful kefir grain biomass activation, their variegated symbiotic microbial community makes it impossible to retain the constant viability over a long time period. This fact, together with neglecting of possible secondary interactions between bioprocess parameters, mainly explains the relatively high error influence on daily kefir grain increase mass (34.0 %).

5. Conclusion

Using the Taguchi's fractional factorial design approach we analyzed the bioprocess parameters impacts on daily kefir grain increase mass during 24 h incubation in fresh high temperature pasteurized whole fat cow milk. Experiments proposed by the design of experiments (OA L_{16}) were performed in an RC1 reactor system. We determined those conditions which assure the highest kefir grain increase mass fraction and, using analysis of variance, estimated the relative impact of the proposed bioprocess parameters on daily kefir grain increase mass. In the observed bioprocess parameters ranges, we established that the yeast mass concentration was insignificant compared to the other bioprocess parameters. The most influential bioprocess parameter is found to be the rotational frequency of the stirrer (37.3 %), followed by the glucose mass concentration (18.8 %), and the medium temperature (9.9 %), while the remaining share represents an error.
Summarily, this chapter deals with the experimental determination of the relative impacts of various significant bioprocess parameters, that influence one of the most difficult bioprocesses in the dairy industry. The presented results confirm and, even more importantly, upgrade well-known findings about influence of various bioprocess parameters on kefir grain increase mass. On the other side, the presented results also confirm the tremendous importance of optimal kefir grain biomass managements. In addition, the results also clearly verify the fact, that inadequate combination of different significant critical bioprocess parameters has a strong negative influence on daily kefir grain increase mass. For instance, in the worst case the kefir grains growth is almost totally stopped. Last but not least, the presented chapter presents important cutting-edge and, in scientific and commercial society, shortfall basic knowledge needed either for kefir grains mass growth kinetic studies or designing, optimization and commercialization of modern batch or continuous industrial kefir grains production processes.

6. Nomenclature

ALR Automatic Lab Reactor
ANOVA ANalysis Of VAriance
DoE Design of Experiments
f_e degree of freedom of error variance (1)
F_j variance ratio of bioprocess parameter j (1)
f_j degree of freedom of bioprocess parameter j (1)

f_m rotational frequency of the stirrer (1/min)
$F_{m,n}$ standardized value from the F tables at defined level of significance (1)
f_T total degree of freedom of result (1)
HTP High Temperature Pasteurized
L number of levels (1)
M number of bioprocess parameters (1)
$m_{KG,di}$ daily kefir grain increase mass (g)
N total number of experiments (1)
N_k number of experiments on k level (1)
OA Orthogonal Array
S_e error sum of squares (/)
S_j sum of squares of bioprocess parameter j (/)
S_T total sum of squares (/)
V_e variance error (/)
V_j mean square (variance) of bioprocess parameter j (/)
$w_{KG,di}$ daily kefir grain increase mass fraction (%/d)
X_e relative impact of error on optimization criterion (%)
X_j relative impact of bioprocess parameter j on optimization criterion (%)
Y_i i value of optimization criterion (/)
γ_G glucose mass concentration (g/L)
γ_{KG} kefir grain mass concentration (g/L)
$\gamma_{KG,f}$ final kefir grain mass concentration in culture medium (g/L)
γ_Y baker's yeast mass concentration (g/L)
ϑ temperature (°C)

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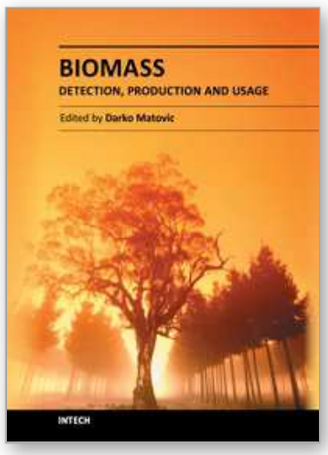

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Biomass has been an intimate companion of humans from the dawn of civilization to the present. Its use as food, energy source, body cover and as construction material established the key areas of biomass usage that extend to this day. Given the complexities of biomass as a source of multiple end products, this volume sheds new light to the whole spectrum of biomass related topics by highlighting the new and reviewing the existing methods of its detection, production and usage. We hope that the readers will find valuable information and exciting new material in its chapters.

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