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Small-Scale Process for the Production of Kefiran through Culture Optimization by Use of Central Composite Design from Whey and Kefir Granules

José Manuel Pais-Chanfrau, Lorena D. Carrera Acosta, Paola M. Alvarado Córdor, Jimmy Núñez Pérez and Milton J. Cuaran Guerrero

Abstract

Cheese is one of the most demanded dairy products worldwide. However, during the conversion of milk to cheese, about 10 liters of milk are employed and about 9 liters of whey are generated for each 1 kg of cheese produced. The whey has traditionally been used for animal feed and as starting material for obtaining whey proteins. Furthermore, whey has the significant values of BOD and COD, becoming the most important contaminant in the dairy industry. For this reason, further growth of cheese sector is being limited by the surplus of whey as a by-product of the production of the cheeses. One of the many possibilities offered by the whey is its use as a starting material to produce many biotech products with a higher added value. The kefir is a degradable biopolymer and is formed by galactose and glucose units, in almost similar proportions, which have been found with numerous benefits for human health. It is produced by a consortium of acid-lactic bacteria and yeasts, which coexist within the kefir granules, which are able to grow and multiply using the lactose present in the whey. The objective of the present study is to establish a small-scale process that allows the obtaining of kefir.

Keywords: kefir, central composite design, statistical optimization, *Lactobacillus kefirianofaciens*, kefir production process

1. Introduction

Although it is still below the per capita consumption level recommended by FAO, milk production in Ecuador has been growing for some years now [1]. The stimulus of milk consumption and its derivatives was a state policy in recent years [2]. Within Ecuador, the Andean region marches at the head of production levels, accounting for about 77% of the country's production [3].

Of the 5.5 million liters of milk produced daily in Ecuador, 48% is sent to the industry, 35% of this goes to the industrial production of cheese [4], and about 12% of the total milk goes to the artisan cheese production. Therefore, around 47% of the total daily production of fresh milk is destined to produce different types of cheeses.

To produce 100 kg of cheese, about 1000 liters of fresh milk are used and about 900 liters of liquid milk whey (WL) are generated [5]. The WL is a yellowish liquid, whose characteristics vary with the region, the time of year, and the forage used in cattle feed. The majority composition is formed by 4.5–5% (m/v) of lactose, 0.6–0.8% (m/v) of whey proteins, 0.5% (m/v) fat, and 8–10% (m/v) (dry basis) mineral salts [6]. Its own composition gives the WL high values of COD and BOD, reporting values of 60–80 and 30–50 kg/m³, for the COD and BOD₅, respectively [7, 8]. This by-product of the dairy industry, therefore, classifies as the most polluting effluent of this industry and constitutes the main impediment, for the further growth of the dairy industry [9].

There are numerous reviews on the use of whey as a raw material to obtain products with a higher added value than cheese or other dairy products [5, 10–12].

One of the products that can be obtained from WL by fermentation is the exopolysaccharides (EPS), which can be formed by sugars of equal or lower sweetening level than commercial sugar, but which can provide other beneficial effects for human health, classifying many of them as prebiotic substances [13].

Kefiran is an EPS, soluble in water, edible, and biodegradable, which is part of the structure of the kefir granule [14–17], which forms a symbiotic consortium of lactic acid bacteria (LAB), fungi, and yeasts, which can transform fresh milk in a fermented and effervescent milk drink, with a certain ethanol content, called kefir, very popular in central-eastern Europe and in Asia [18–21].

The role of kefir seems to protect the microbiota inside the kefir granules [22]. Its synthesis is attributed to the LAB *Lactobacillus kefiranoformis*, but it has been proven that its levels depend on the interactions that this bacterium establishes with others and the presence of some yeasts of the consortium present in the kefir granule [23, 24].

Kefiran, like kefir, has been attributed to several beneficial properties for human health, such as promotion of the growth of *Bifidobacterium bifidum* and modulating its genetic expression [25], its healing capacity [26, 27], hypotensive [28], hypercholesterolemia lowering [29, 30], anti-inflammatory agent [31], body weight regulator [32], immunoenhancer [33], and it has some anticancer effects [18, 34, 35]. It is for this reason that interest has recently grown to produce this EPS [24, 36, 37].

The production of kefir goes through a fermentative phase where the polysaccharide is synthesized by *L. kefiranoformis*, using a suitable carbon source such as lactose, present in the WL [38]. There are numerous reports suggesting that the temperature, the amount of inoculum, and the lactose concentration determine the amount and productivity of this phase [23, 24, 39]. However, the diversity of origins of the kefir granules and the logical variation of the amounts and types of microorganisms present in the granule mean that the optimal conditions may vary in each case and region.

2. Statistical optimization of kefir production from whey and kefir granules by using response surface methodology

Response surface methodology (RSM) has been extensively used to find optimal fermentation conditions for the production of metabolites and chemicals from different microorganisms [40, 41].

Certain response (i.e., product concentration or productivity) depending for many well defines cultivation variables (like pH, temperature, substrate concentration, etc.) can be related via experimentation through a mathematical model with certain response like concentration or productivity of a metabolite produce by microorganism. Such a model can be used, after its verification, to “navigate” inside defined region of variables, to find the combination of such a variable able to maximize or minimize the response function [42–45].

Some designs of experimental (DOE) arrangements have been employed in RSM. One of the most popular is the central composite design (CCD), which the experimental points surrounded certain central point in equidistance from them [46].

In CCD, the extreme values of the response are searching inside a square inscribed in the sphere formed by the experimental points. In the CCD, in its minimal expression, only the central point needs to be replicated, assumed that the statistical variation of the rest of all experimental points is the same that in the central one. Additional point, however, can enhance the accuracy of the model. With this kind of DOE, a quadratic model of response could be obtained. A second degree of a polynomial can be used to find the extreme values (minimum or maximum) inside the experimental surface. Before that, however, it can be demonstrated that the quadratic model of response is suitable to navigate inside his values to find the optima. Analysis of variance (ANOVA) of the mathematical model and the analysis of his residuals can be served for this purpose.

Several professional statistical software can be used to perform the planning and analysis of the mathematical model. Design-Expert 11, from the Stat-Easy, Inn. (Minneapolis, USA) was used through this work.

In previous work, whey powder (WP) and kefir grains have been used to find the suitable conditions for the WP concentration and temperature to maximize kefir production [37] or maximize certain desirability function formed by equally weighed kefir as a prebiotic, and the cell concentration of LAB and yeast in a functional drink [47].

In those works, however, some inconveniences associated to cost of WP as a starting raw material has been detected when the preliminarily economic analysis was performed, suggesting the direct use of a cheaper sweet or acidic whey instead of the WP for similar purposes.

The way of optimization passes for a well-designed experiment where the response (concentration of kefir in the fermentation broth) is correlated with relevant independent variables, like concentration of liquid sweet whey (WL) in the culture medium and temperature. The optimization algorithm used is shown in **Figure 1**.

For its simplicity, a central composite design was selected, bringing as a central point 25°C and 42% (m/m), for temperature and WL, respectively. A temperature of 25°C brings a maximum value of kefir concentration in previous work with the same origin kefir grains (“Yogurt-Kombucha-Tibicos en Ecuador,” Quito, Pichincha, Ecuador, www.kefir.ec), but employing whey powder as a lactose source [37].

Kefir was determined according to phenol-sulfuric acid method [48] which employed the glucose as a reference sugar similar as it had been determined elsewhere [24, 37].

Due to a complexity of the consortia of microorganism presented in the kefir grain, a culture medium is supplemented either with glucose or sucrose, adjusted the brix of the medium at a constant value of 14%, as was suggested elsewhere [43, 44], and it was performed in previous work [37].

Figure 1. Flow-chart to find the maximum of kefir production by using CCD in RSM.

The starting CCD experiments for two independent variables (A: Temp and B: %WL) at a minimum of 13 points experiments are recommended [46], five of which corresponds to the central point and the rest of eight surrounding the central point. Due to the existence of position in a shaker, however, two experiments per point surrounding four experiments for a central experiments points were performed (Figure 2).

Table 1 shows the coded and actual values of the independent variables and the corresponding value for the response variable.

As a response range was a ratio of max to min of 12.4082, the response could be transformed. In this case, a power transformation was selected, which brings a model for coded variables of:

$$(Kefiran + 10.0)^{0.28} = 4.33 + 0.2231 A + 0.0665 B + 0.2322 AB - 0.2233 A^2 - 0.2107 B^2 + 0.3001 A^2B - 0.7354 AB^2 - 0.2848 A^2B^2 \quad (1)$$

Figure 2.
Original surface data for independent variables.

Std	Code variables		Actual variables		Response
	A: Temp.	B: WL	A: T, °C	B: WL, % (m/m)	Kefiran, g Glu/ml
1	−1.000	−1.000	17.0	17.3	130.0
2	1.000	−1.000	33.0	17.3	17.8
3	−1.000	1.000	17.0	66.7	171.0
4	1.000	1.000	33.0	66.7	105.5
5	0.000	−1.414	25.0	7.1	109.0
6	0.000	1.414	25.0	76.9	129.7
7	0.000	0.000	25.0	42.0	180.7
8	0.000	0.000	25.0	42.0	182.4
9	0.000	0.000	25.0	42.0	173.3
10	0.000	0.000	25.0	42.0	171.4
11	−1.414	0.000	13.7	42.0	84.7
12	−1.414	0.000	13.7	42.0	82.4
13	−1.000	1.000	17.0	66.7	161.1
14	−1.000	−1.000	17.0	17.3	128.8
15	0.000	1.414	25.0	76.9	132.5
16	0.000	−1.414	25.0	7.1	108.6
17	1.000	1.000	33.0	66.7	87.6
18	1.000	−1.000	33.0	17.3	14.7
19	1.414	0.000	36.3	42.0	169.7
20	1.414	0.000	36.3	42.0	145.8

Table 1.
Coded and actual values of independent variables and its corresponding values of response.

ANOVA for reduced quadratic model for the transformed response of kefir is shown in **Table 2**.

The model *F-value* of 190.66 implies that the model is significant. There is only a 0.01% chance that an *F-value* that is large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB, A², B², A²B, AB², A²B² are significant model terms.

The accuracy of the model can be judged by the plot of the actual responses *versus* predicted responses (**Figure 3**), and the normal plot of residuals (**Figure 4**).

The *predicted R²* of 0.9729 is in reasonable agreement with the *adjusted R²* of 0.9876; i.e., the difference is less than 0.2.

Adeq. precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 45.727 indicates an adequate signal. This model can be used to navigate the design space.

The *fit statistics* of the model is:

Std. dev.	0.0597	R ²	0.9928
Mean	3.86	Adjusted R ²	0.9876
C.V. %	1.54	Predicted R ²	0.9729
		Adeq. precision	45.7268

After checking the model for kefir, an optimization procedure to find the maximum of kefir was performed. A wide range of region for temperature and WL concentration was found, and suggested a combination of 90 points of temperature and WL concentration for the maximum of kefir concentration.

Taking one of this point is to perform the validation experiments and confirm or refuse the model for the kefir. This point corresponds with a combination of temperature and WL concentration of 30.5°C and 47.8% (m/m), respectively (**Figure 5**).

Three experiments at the selected condition were performed, reaching values of kefir concentration of 181.1, 146.6, and 167.9 g Glu/ml. As can be seen, only the first are located inside the range suggested by the model, whilst two are outside. These results cannot confirm the validity of the model of kefir.

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	5.43	8	0.6786	190.66	<0.0001	Significant
A-T	0.3981	1	0.3981	111.86	<0.0001	
B-WL	0.0353	1	0.0353	9.93	0.0092	
AB	0.4312	1	0.4312	121.16	<0.0001	
A ²	0.3990	1	0.3990	112.11	<0.0001	
B ²	0.3553	1	0.3553	99.84	<0.0001	
A ² B	0.3602	1	0.3602	101.22	<0.0001	
AB ²	2.16	1	2.16	607.79	<0.0001	
A ² B ²	0.3245	1	0.3245	91.19	<0.0001	
Pure error	0.0391	11	0.0036			
Cor total	5.47	19				

Table 2.
ANOVA for the kefir model.

To check the usefullness of the kefiran model (Eq.1), one of the suggest combination of temperature (30.5°C) and sweet liquid whey (47.8% (m/m)) that should maximize the kefiran concentration was used to perform three validation experiments. Those experiments could not validate the model (**Table 3**).

According to the flow-chart shown in **Figure 1**, at this point could be evaluated another of 90 data points suggest in the optimization procedure to maximize kefiran for this model and perform with him the additional validation experiments or, alternatively, can be add the values of validation experiments to the originally data and find a new, more robust, suitable cuadratic model for determination of kefiran. The second alternative was followed, and a new set of experimental points was obtained (**Figure 6**).

The coded and actual quadratic model for kefiran were, respectively:

$$Kefiran = 176.75 - 8.56 A + 7.88 B + 10.91 AB - 28.15 A^2 - 28.50 B^2 + 21.35 A^2B - 54.50 AB^2 + 17.40A^3 - 18.24 A^2B^2 \tag{2}$$

$$Kefiran = - 1017.04387 + 132.1778 T + 12.3776 WL - 1.6439 T \cdot WL - 4.3795 T^2 - 0.059484 WL^2 + 0.052743 T^2 \cdot WL + 0.012188 T \cdot WL^2 + 0.033978 T^3 - 0.000467 T^2 \cdot WL^2 \tag{3}$$

ANOVA, the model for kefiran concentration (**Table 4**), its fit statistics (**Table 5**), and the plot of predicted *vs* actual kefiran concentration values (**Figure 7**) suggests that the model could be used to find inside the space of independent variables the maximum value of kefiran.

Response	Predicted mean	Predicted median [*]	Std. dev.	n	95% PI low	Data mean [†]	95% PI high
Kefiran	190.9	190.7	9.7	3	175.5	164.8	206.9

^{*}For transformed responses, the predicted mean and median may differ on the original scale.
[†]For transformed responses, the data mean is calculated on the transformed scale.

Table 3.
Confirmation experiments for the first kefiran model.

Figure 6.
New design space data of independent variables.

The *model F-value* of 60.14 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. *P-values* less than 0.0500 indicate model terms are significant. In this case, B, AB, A², B², A²B, AB², A³, A²B² are significant model terms. The model term A is insignificant, but it counts to support hierarchy of the model (**Table 4**).

The fit statistics of the quadratic model for the kefiran concentration is shown in **Table 5**. The *Predicted R²* of 0.9307 is in reasonable agreement with the *Adjusted R²*

Source	Sum of squares	df	Mean square	F-value	P-value	
Model	50004.24	9	5556.03	60.14	< 0.0001	significant
A-T	39.19	1	39.19	0.4242	0.5262	
B-WL	497.29	1	497.29	5.38	0.0372	
AB	952.66	1	952.66	10.31	0.0068	
A ²	6339.38	1	6339.38	68.62	< 0.0001	
B ²	6498.00	1	6498.00	70.34	< 0.0001	
A ² B	1823.85	1	1823.85	19.74	0.0007	
AB ²	4845.51	1	4845.51	52.45	< 0.0001	
A ³	584.72	1	584.72	6.33	0.0258	
A ² B ²	1330.43	1	1330.43	14.40	0.0022	
Pure error	1200.93	13	92.38			
Cor total	51205.17	22				

Table 4.
ANOVA of the quadratic model for kefiran concentration.

Std. dev.	9.61	R ²	0.9765
Mean	129.67	Adjusted R ²	0.9603
C.V. %	7.41	Predicted R ²	0.9307
		Adeq. precision	25.3566

Table 5.
Fit statistics of the quadratic model for kefiran concentration.

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Figure 7.
Predicted versus actual values of kefiran concentration.

of 0.9603; i.e., the difference is less than 0.2 and *Adequate precision* measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 25.357 indicates an adequate signal. This model can be used to navigate the design space.

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
A:T	Is in range	17	33	1	1	3
B:WL	Is in range	17.3	66.7	1	1	3
Kefiran	Maximize	14.7	182.4	1	1	5

Table 6.
Constrains to find the maximum of kefiran concentration.

Number	T, °C	WL, %(m/m)	Kefiran, mg Glu/ml	Desirability
1	23.864	45.932	178.090	0.974
2	19.962	66.700	174.901	0.955

Table 7.
Solution candidates to values for independent variables to reach maximum for the kefiran concentration.

Figure 8.
Contour plots of solution values for independent variables and candidates to reach a maximum for kefiran concentration.

Response	Predicted mean	Std. dev.	n	SE Pred	95% PI low	Data mean	95% PI high
Kefiran	178.1	9.6	3	8.1	160.7	177.5	195.5

Table 8.
Value of the validation experiments for two-side confidence of 95%.

A numerical optimization to find the maximum of kefiran concentration inside the space of independent variables with constraints shown in **Table 6** was performed.

And two, equally in possibilities, solution candidates to reach a maximum for the kefiran concentration were obtained (**Table 7**).

The first suggested solution (23.9°C and 45.9% (m/m) for temperature and whey liquid, respectively) was selected to perform the three validation experiments.

The validation experiments for the kefiran concentration were 175.5, 184.5, and 172.6 mg Glu/ml, reaching a mean of 177.5 ± 6.2 mg Glu/ml, which is inside the probability interval of the model. In this manner, the validation experiments confirm the adequateness of the kefiran model (Table 8).

Further experiments to establish the small-scale production of kefiran were conducted starting with a fermentation stage with the same media, inoculum concentration, and a temperature and concentration of whey at the optimal conditions find here.

3. Small-scale production of kefiran

The soluble exopolysaccharide kefiran, synthesized by *Lactobacillus kefiranofaciens* as a part of kefir granules, could be isolated and purified from the culture broth after the lactose-fermentation from whey [37, 49]. To achieve this, several isolated steps can be executed like filtration, homogenization, thermal inactivation of glycolytic enzymes, polysaccharide precipitation, separation, and washing the precipitate (**Figure 9**).

Due to kefiran representing 50% (dry basis) of a granule [50], after fermentation, the broth could be homogenized to use all the granules to isolate the kefiran. However, together with kefiran, certain contaminants and glycolytic enzymes that eventually could degrade the kefiran itself will be aggregated. For this reason, it could be convenient to ask the question if it is necessary to employ the disrupted whole fermentation broth including in it the kefir granules to isolate kefiran, or only use the supernatant of the culture after separate the kefir granules from the broth of culture.

Another question to ask is, if the kefiran grains is separated from the supernatant is the option choice, how many times the kefiran granules can be reused? Will it be convenient to reuse many times and replace the inoculum at all or to refresh inoculum partially with a fresh grain?

At this point, at least until three times was reused, the same kefiran granules with the washing the granules with abundant sterilized distillate water between batches, with no significant difference in the final kefiran concentration (results not shown). Further experiments, however, should be performed to know how many times this process could be repeated.

Either two alternatives can be implemented instead, but only if the homogenization of whole fermentation broth brings a significant increment in the kefiran concentration in respect to use the supernatant. Additionally, in the case that only the culture supernatant was used, less time and energy would be used, and the kefir

Figure 9.
Lab-scale procedure for the kefiran isolation from the fermentation of whey with kefir granules.

Figure 10. Experiments to compare supernatant versus homogenate, and ethanol absolute (anhydrous) versus technical in the yield of kefiran. Legend: Sup—supernatant, Hom—homogenate, Abs—EtOH-absolute ethanol, and Techn. EtOH—technical ethanol (96% (v/v)).

Figure 11. Two possible flow diagrams for the kefiran production, by using: (A) the homogenization of whole fermentation broth and (B) the supernatant of fermentation broth. Legend: SW/AW: sweet or acidic whey, EtOH: ethanol, and TK: tank.

grains can be reused inside the process and wash residuals should be less contaminated than the similar is obtained for the homogenization variant.

Another question is associated to the use of solvent precipitation. Several reports referred the use of absolute (anhydrous) ethanol [24, 38], with a higher price respect his counterpart, the technical ethyl alcohol at 96% (v/v). If the technical ethyl alcohol at 96% (v/v) could be employed instead the more expensive absolute (anhydrous) one during the solvent precipitation of kefiran, a significant impact over the manufacturing cost of kefiran could be obtained.

At this point, three independent experiments were conducted to compare homogenization *versus* supernatant alternatives, and the utilization of absolute (anhydrous) *versus* technical ethyl alcohol as the precipitation agent. When the precipitation experiments were performed using absolute alcohol (anhydrous) a proportion of 50:50 with a material was employed; and when technical alcohol at 96% was used instead the anhydrous ethanol, the technical alcohol: material was mixed in a proportion of 60:40. Each experiment was started with the same fresh whey and inoculum and was performed as shown in **Figure 9**.

The alternatives shown in **Figure 10** do not have significant differences ($p > 95\%$) between them, suggesting that the more suitable and economic way for production of kefiran is to use the supernatant of fermenting broth and technical ethanol as a precipitation solvent (**Figure 11B**), instead of the homogenization of whole broth and using absolute ethanol as a solvent (**Figure 11A**).

Kefiran isolation process was carried out from three 100 ml batches each, yielding 5.2 ± 0.1 g/l of kefiran in the culture supernatant.

These results are slightly higher than the 3.1 ± 1.3 [37] or 1.91 g/l of kefiran [24] reported recently, and higher to the values between 1.5–3.7 g/l reported previously [38, 51].

4. Conclusions

The usefulness of the RSM was demonstrated in the search for a condition where the concentration of kefiran is maximized from liquid sweet whey, significantly cheaper than whey powder. It was also possible to satisfactorily replace the “absolute” (anhydrous) ethyl alcohol with the technical-grade ethyl alcohol at 96%(v/v). Both facts represent a remarkable financial saving in the production of the kefiran, which would allow to begin the studies to scale-up the production technology of this attractive EPS.

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Author details

José Manuel Pais-Chanfrau*, Lorena D. Carrera Acosta, Paola M. Alvarado Córdor,
Jimmy Núñez Pérez and Milton J. Cuaran Guerrero
North-Technical University (Universidad Técnica del Norte, UTN), FICAYA,
Ibarra, Imbabura, Ecuador

*Address all correspondence to: jmpais@utn.edu.ec

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