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EFFECT OF FREEZE-DRYING PROCESS ON THE PHYSICOCHEMICAL AND MICROBIOLOGICAL PROPERTIES OF MEXICAN KEFIR GRAINS

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Abstract: The aim of this research was to investigate how the operating parameters of freeze-drying affected properties of Mexican kefir grains. The factors investigated were the freezing time (3-9h), freezing temperature (-20 to -80°C), pressure (0.2-0.8mbar), and lyophilization time (5-20h). Max changing range and one-way analysis of variance showed that lyophilization time and freezing time had significant effects ($p < 0.05$) on the response variables, i.e., residual moisture content and water activity, and the pressure had a significant effect on the color difference and survival of probiotic microorganisms. The best drying conditions were a freezing time of 3 h, freezing temperature of -20°C, pressure of 0.6 mbar and lyophilization time of 15 h. Under these conditions, it was obtained a product with residual moisture content < 6, water activity < 0.2, survival rate of lactic acid bacteria > 8.5 Log *colony-forming unit per gram* and yeasts > 8.6 Log.

Keywords: freeze-drying, operating parameters, properties, kefir grains, probiotic

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1. Introduction

Kefir grains are a starter culture used to elaborate kefir, a probiotic and popular beverage in the functional food markets. This culture consists of a complex mixture of bacteria and yeast that live in a symbiotic relationship [1-3]. These grains have irregular form with similar shape to the cauliflower, yellow or white color and variable size, from 0.3 to 3.5 cm in diameter [4-6]. However, this microbiota can only grow from pre-existing grains [7] and given the wide variation in their complex microbiological mixture which, depends on their source, quality and type of milk used for its reproduction, it is difficult to obtain a defined and uniform starter culture for industrial kefir market [8] and dehydration of kefir may be an option to extend the market values of this product [9].

Probiotic foods are increasing in popularity in the marketplace [10] and they are defined as "live organisms" which, when administered in adequate amounts, give health benefits to the host and in order to provide this, their probiotic must contain at least 10 million colony-forming units per gram (CFU/g) [11]. This implies that microorganism must remain viable and maintain their characteristics from the beginning of the production process until they are consumed, because one of the most important requisites is that probiotic microorganisms survive throughout the production process and storage [12,13].

Freeze-drying is a well-known dehydration process in the food and dairy industry where, the major applications are preservation of probiotics, starter cultures and biological materials. And although it is true that, compared to other drying techniques such as spray drying freeze drying is an expensive process due to its relatively high investment, operation and maintenance costs [14], it is also true the advantages freeze-drying offered due to its results in terms of viability, high quality and long storage time in dairy bacterial cultures [15-19] The advantages of freeze-drying include, the nutrients in food will stay intact, the drying at low temperatures reduces degradation of heat-sensitive products, chemical decomposition is minimized, the final moisture can be controlled during the process and the water content of the dried product can be reduced to very low levels [20], enhanced product stability in a dry state [21,22]. Freeze-drying is a process where water or another solvent is frozen, followed by its removal removed from the sample in final steps of the lyophilization. This process combines in three steps freezing and drying: 1)freezing where the sample is frozen at low temperature, 2)primary drying is the phase where the ice is sublimated under reduced pressure and 3)secondary drying is the last stage of the freeze-drying process which is a desorption step where the residual moisture is removal or reduced to a low level because, the objective of any kind of microbiological drying process is to enable cell survival and long storage [23-25]. Although some studies [26-30] have been carried out on freeze drying of kefir grains the effect of process variables to obtain a stable defined and uniform starter culture with suitable characteristics in terms of viability, % RMC, aw and color for the kefir market have not been considered.

2. Materials and Methods

2.1. *Kefir grains preparation*

Fresh kefir grain biomass was obtained from local sources in Orizaba, Veracruz, Mexico. Propagation, activation and adaptation to the medium was performed in a laboratory for 60 days. UHT milk samples were inoculated with kefir grains 10:90 (w/v) by 24-hour cycles to temperature 26°C. Prior freeze-drying kefir grains were selected and washed with distilled water until all residues of milk were removed [31]. Kefir grain biomass viability was important for experimental data validity [32].

2.2. *Experimental design and freeze-drying*

Freeze-drying of Mexican kefir grains (MKG) was carried out in a freeze dryer (Labconco Equipment Co., Kansas, USA) with capacity of 12 liters and ultra-low freezer (Environmental equipment Cincinnati, Ohio, U.S.A). The selected levels for each affecting factor are shown in Table 1. The freezing time was set at 3, 5, 7 and 9 h; freezing temperature rate of -20, -40, -

60 and -80°C; pressure 0.2, 0.4, 0.6 and 0.8 mbar. For this study 5, 10, 15 and 20 h were selected as lyophilization time. The $L_{16}4^4$ orthogonal design using is shown in Table 2.

Table 1. Factors and levels used in the freeze-drying experiments

Levels	**Factors**			
	Ft **(h)**	***Fte*** **(°C)**	***P*** **(mbar)**	***Lt*** **(h)**
1	3	-20	0.2	5
2	5	-40	0.4	10
3	7	-60	0.6	15
4	9	-80	0.8	20

Ft: freezing time; Fte: freezing temperature; P: pressure; Lt: lyophilization time

Table 2. $L_{16}4^4$ orthogonal experimental design sheet

Experiments	**Factors**			
	Ft **(h)**	***Fte*** **(°C)**	***P*** **(mbar)**	***Lt*** **(h)**
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	1	4	4	4
5	2	1	2	3
6	2	2	1	4
7	2	3	4	1
8	2	4	3	2
9	3	1	3	4
10	3	2	4	3
11	3	3	1	2
12	3	4	2	1
13	4	1	4	2
14	4	2	3	1
15	4	3	2	4
16	4	4	1	3

Ft: freezing time; *Fte*: freezing temperature; *P*: pressure; *Lt*: lyophilization time

2.3. Analytical methods

The residual moisture content (% RMC) of dried kefir grains was measured using the MA35 moisture analyzer (Sartorius AG, Göttingen, Germany). Samples of 0.5 g were heated to 65 °C until constant weight. Water activity (a_w) was determined at 25°C using an Aqualab water activity meter (series 3, Decagon Devices Inc, Pullman, WA). Color measurements were carried out using a Hunter Lab MiniScan XE Plus (Hunter Associates Laboratory Inc., Reston, VA) and results were expressed in Hunter Lab Units *L**(Lightness/darkness), *a**(redness/greenness) and *b** (yellowness/blueness). Survival rate of lactic acid bacteria (LAB) and yeasts before and after lyophilization were enumerated as Log (CFU/g) by an inverted plate method. Decimal dilutions with peptone water were prepared from the kefir grains and LAB were enumerated on MRS agar (Dibico, Mexico) at 35°C for 3 days. Yeasts were enumerated on yeast peptone dextrose medium (YPD, Difco, USA) after incubation at 25°C for 5 days.

2.4. Statistical analysis

Significance of the affecting factor was determined by an analysis of variance at $p < 0.1$ and $F > F\text{crit} = 2.605525$, and at $p < 0.05$ and $F > F\text{crit} = 3.490295$, respectively. In addition, the importance of the affecting factor in range analysis was determined by max changing range of dried final product properties [33]. Multiple regression was used to predict the behaviour between final properties of the dried product and the affecting factors; i.e., final properties as dependent variable and four parameters, i.e., freezing time (*Ft*), freezing temperature (*Fte*), pressure (*P*), lyophilization time (*Lt*) as independent variables in the analysis.

3. Results and Discussion

3.1. Residual moisture content of dried Mexican kefir grains

Residual moisture content of dried MKG is an important factor that affects the long-term stability of the product and its shelf life. Analysis of the residual moisture content of dried MKG is shown in Table 4. The results show that factors such as freezing time, freezing temperature and pressure in the four selected levels did not have a significant effect on this response variable but, it was significant ($p = 0.00163 < 0.1$ and $F = 9.62136 > F\text{crit} = 2.605525$ and $p = 0.00163 < 0.05$ and $F = 9.62136 > F\text{crit}\ 3.490295$) for four levels of lyophilization time, which is consistent with the conclusions of other researchers [34-38] which indicate that secondary drying (desorption) in the freeze-drying process plays a significant role in the desired moisture content of the final product.

Table 4. Data analysis of dried MKG residual moisture content (% RMC)

Data analysis for % RMC	**Factors**			
	Ft **(h)**	***Fte*** **(°C)**	***P*** **(mbar)**	***Lt*** **(h)**
K_1	23.01	30.55	21.46	188.69
K_2	99.40	62.26	77.36	46.07
K_3	70.77	79.11	83.95	15.66
K_4	73.06	94.32	83.46	15.82
R'	76.39	63.78	62.49	173.03
F	0.46713	0.33514	0.41678	9.62136
p Value	0.71063	0.80025	0.74417	0.00163

MGK: Mexican kefir grains; *Ft*: freezing time; *Fte*: freezing temperature; *P*: pressure; *Lt*: lyophilization time; K_1: level 1; K_2: level 2; K_3: level 3; K_4: level 4; R': changing range

The max changing range (173.03) of the lyophilization time in this analysis was the largest among of the range of the four variables. This indicates that the lyophilization time plays an important role in affecting the % RMC of dried MKG. On the other hand, the range (76.39) for freezing time was larger than those of the remaining two factors. This also shows that the freezing time is the second important factor that affects the residual moisture in the dried product.

According to the multiple regression analysis of the $L_{16}4^4$ orthogonal experiments results, the regression equation for the residual moisture content of dried MKG is obtained in equation (1) as follows:

$$\text{RMC}(\%)=12.3+12.7X_1-10.59X_2+36.60X_3-38.85X_4-2.74X_1^2+4.29X_1X_2-0.97X_1X_3 -2.460X_1X_4+1.143X_2^2-0.80X_2X_4-3.12X_3^2-4.348X_3X_4+8.924X_4^2 \quad (1)$$

In this equation, the square of the correlation coefficient (R^2) was 0.9974. The coefficient (38.85) of lyophilization time was the largest among the coefficients of the four variables. This also indicates that the lyophilization time plays an important role in affecting the % RMC of dried MKG. On the other hand, the coefficients (36.60 and 12.70) for pressure and freezing time respectively were the largest than those of the remaining factors it can be showed with p values for each coefficient.

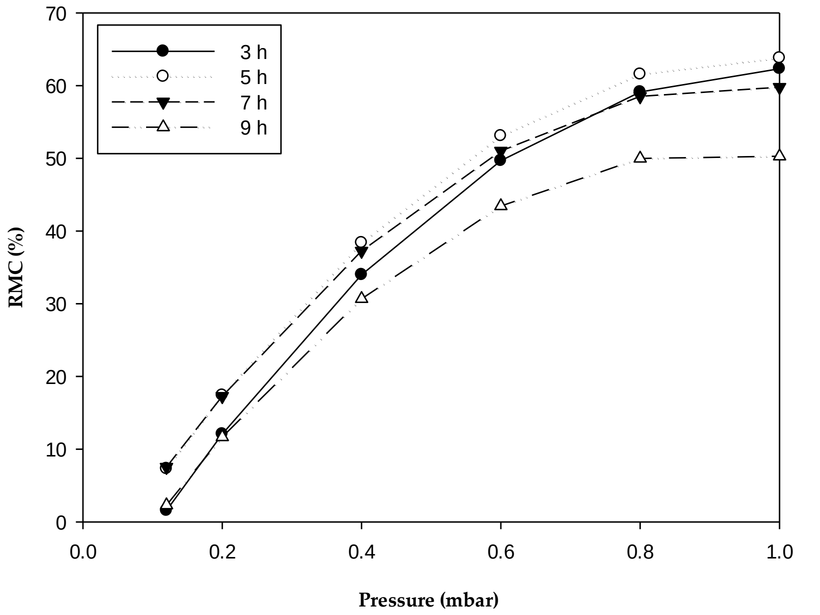

Figure 1. Predicted residual moisture content of dried MKG at different freezing time and pressure

According to equation (1), the predict % RMC of dried MKG with different freezing time and pressure is given in Figure 1. The lyophilization time and freezing temperature in the equation were considered at 5 h and -20°C, respectively. Figure 1 depicts that % RMC has a greater increase when increased pressure in a freezing time is 5 h. However, it was also observed that as the values of pressure were less than 0.1, the %RMC decreased. In Table 3 shows the experimental and predicted % RMC of dried MKG and the error differences. The least value obtained in the experimental results was 2.38 a freezing time 5 h, freezing temperature -40°C, pressure 0.2 mbar, and lyophilization time was 20 h. The error relative difference between the experimental and predicted % RMC was -15.29%.

3.2 Water activity of dried Mexican kefir grains

Water activity (a_w) in the dried MKG is one the most important factor that affect the quality and probiotic viability during the storage time of the dried product, because during the dried processes, the decrease of water activity produces damages on the bacterial structures, decreasing their viability [39]. If the a_w is < 0.60, it assures complete inhibition of microbial growth and lipid oxidation in food products during storage [40] The water activity is given in Table 3. It is seen than the most of the experiments the water activity is below 0.6.

The analysis of the data obtained is this study is show in Table 5. It shows that the water activity of the dried MKG is significantly influenced ($p < 0.1$ and $F = 6.64924 > F\text{crit} = 2.605525$ and $p = 0.00163 < 0.05$ and $F = 6.64924 > F\text{crit}$ 3.490295) for four levels in the lyophilization time. This is consistent with the findings of facts reported that during the freeze-drying, extremely low water activity values are obtained with equilibrium sorption behavior for solid materials in contact with humid gas and free water is frozen and low drying temperatures prevent thermal and enzymatic degradation [41,42].

The max changing range (2.603) of the lyophilization time in this analysis was the largest among the range of the four variables. This also indicates that the lyophilization time plays an important role in affecting the a_w of dried MKG. On the other hand, the range (1.402) obtained of pressure was larger than those of the remaining two factors showing, which means that pressure is another factor that significantly affects this response variable.

Table 5. Data analysis of dried MGK water activity (a_w)

Data analysis for a_w	Factors			
	Ft **(h)**	***Fte*** **(°C)**	***P*** **(mbar)**	***Lt*** **(h)**
K_1	0.529	0.977	0.478	3.114
K_2	1.725	1.430	1.315	1.254
K_3	1.424	1.295	1.716	0.511
K_4	1.712	1.687	1.880	0.511
R′	1.196	0.711	1.402	2.603
F	0.60391	0.15021	0.77428	6.64924
p Value	0.62489	0.92756	0.53045	0.00678

MGK*:* Mexican kefir grains; *Ft*: freezing time; *Fte*: freezing temperature; *P*: pressure; *Lt*: lyophilization time; K_1: level 1; K_2: level 2; K_3: level 3; K_4: level 4; *R′*: changing range

The equation for the water activity using multiple regression is as follows (Equation 2):

$$a_w=0.246+0.033X_1-0.098X_2+0.319X_3-0.3560X_4-0.0074X_1^2+0.0405X_1X_2+0.0441X_1X_3 -0.0640X_1X_4+0.0307X_2^2-0.0321X_2X_4-0.0260X_3^2-0.0692X_3X_4+0.1162X_4^2 \quad (2)$$

Where the R^2 for equation (2) was 0.9979 coefficient of the lyophilization time (0.3560) indicates that the lyophilization time plays the most important role in affecting water activity of the dried MKG. The relationship between freezing time and pressure was obtained using equation (2). When the freezing temperature and lyophilization time were kept at -20°C and 5 h, respectively. This is show in Figure 2. It was found that the water activity decreased almost to zero whit the low pressure and freezing time by 3 h when the other two factors were kept constant.

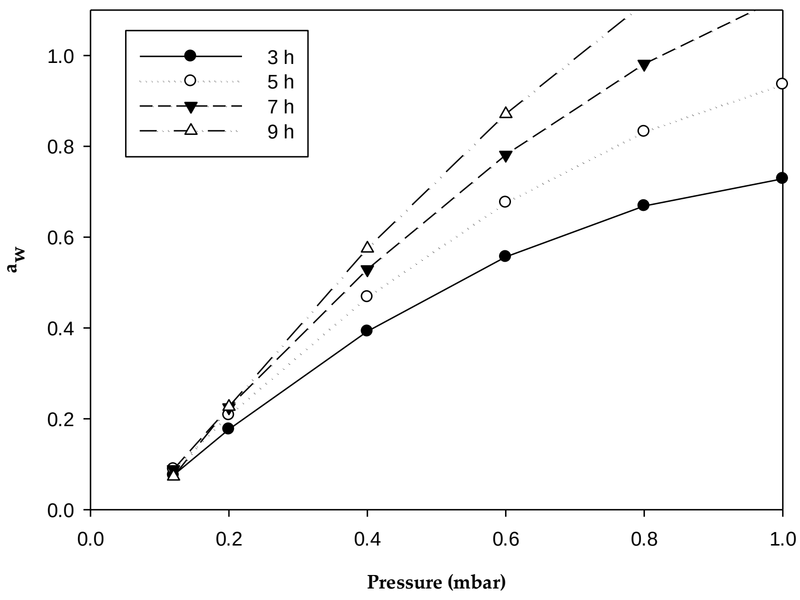

Figure 2. Predicted water activity of dried MKG at different freezing time and pressure

In Table 3, the experimental water activity is compared with the predicted water activity. It is seen that the least value obtained in the experimental result was 0.076 a freezing time 9 h, freezing temperature -80°C, pressure 0.2 mbar, and lyophilization time was 15 h. The error relative difference between the experimental and predicter a_w was -22.63% in test number 16.

Table 3. Experimental (Exp-R) and predicted results (Pre-R) of dried MKG properties

Experiments	Factors																	
	Residual moisture content (% RMC)			Water activity (a_w)			Color difference Exp-R						Log (CFU/g) of LAB			Log (CFU /g) of Yeasts		
	Exp-R	Pre-R	Error (%)	Exp-R	Pre-R	Error (%)	*L**	*a**	*b**	ΔE	Pre-R	Error (%)	Exp-R	Pre-R	Error (%)	Exp-R	Pre-R	Error (%)
1	11.84	12.08	-2.02	0.173	0.177	-2.20	44.02	-1.13	6.40	15.5	14.77	4.73	4.99	4.72	5.37	5.22	4.64	11.24
2	4.84	5.50	-13.55	0.111	0.121	-9.19	38.42	-0.98	5.58	6.23	7.66	-22.92	8.03	8.27	-2.94	5.63	6.94	-23.24
3	3.08	2.51	18.47	0.114	0.105	8.07	39.96	-1.12	6.43	7.92	6.5	17.89	8.56	8.32	2.85	8.67	7.44	14.24
4	3.25	3.12	3.88	0.131	0.128	2.60	50.58	-1.44	7.49	10.55	11.3	-7.13	4.61	4.87	-5.68	5.44	6.11	-12.42
5	4.84	3.23	33.24	0.111	0.089	20.00	36.73	-1.34	4.85	3.93	4.86	-23.59	6.81	7.45	-9.43	8.44	9.10	-7.81
6	2.38	2.74	-15.29	0.137	0.142	-3.65	42.30	-1.45	6.79	10.98	10.82	1.48	8.20	8.06	1.76	8.27	8.19	1.03
7	65.33	65.08	0.38	0.983	0.98	0.35	39.11	-1.43	5.76	7.92	8.05	-1.68	8.35	8.47	-1.49	8.4	8.55	-1.71
8	26.85	28.56	-6.35	0.494	0.518	-4.86	42.60	-1.30	5.71	8.95	8.00	10.59	8.42	7.76	7.86	8.68	8.08	6.94
9	2.94	4.64	-57.86	0.136	0.161	-18.31	38.65	-0.91	5.53	12.74	11.77	7.61	8.64	7.99	7.56	8.67	8.03	7.36
10	3.95	4.36	-10.43	0.210	0.216	-2.95	35.46	-1.08	4.80	7.13	6.89	3.37	6.49	6.31	2.85	6.20	6.08	1.92
11	3.45	3.18	7.91	0.092	0.088	4.02	44.60	-1.11	6.61	22.17	22.36	-0.83	6.59	6.75	-2.35	7.43	7.59	-2.04
12	60.43	58.85	2.62	0.986	0.964	2.27	37.98	-1.51	5.57	9.34	10.25	-9.70	8.48	9.10	-7.35	8.48	9.14	-7.77
13	10.93	9.86	9.74	0.557	0.543	2.53	49.33	-1.52	7.93	15.60	16.17	-3.62	7.13	7.59	-6.47	4.29	4.71	-9.82
14	51.09	52.64	-3.04	0.972	0.995	-2.37	41.17	-1.17	6.78	8.80	7.89	10.3	7.54	6.91	8.34	8.14	7.54	7.42
15	7.25	5.86	19.21	0.107	0.087	18.60	42.62	-1.42	5.84	3.80	4.65	-22.34	5.04	5.63	-11.61	5.95	6.55	-10.15
16	3.79	5.03	-32.72	0.076	0.093	-22.63	38.92	-1.09	5.16	8.27	7.63	7.74	7.90	7.40	6.37	8.02	7.60	5.22

MGK: Mexican kefir grains; Exp-R: experimental results; Pre-R: predicted results

3.3. Color difference of dried Mexican kefir grains

Color is the first judgment and major quality attribute in dried foods because its change indicates that certain components in the product might have deteriorated[43-45]. In this study, the reference standard was a fresh sample of MKG. L^*, a^*, and b^* values were 35.28, -1.15 and 2.84, respectively. The analysis of color data is shown in Table 6. In this table R' is the maximum difference among the four levels for each factor. The larger R' the more significant the effects are. It can be seen from Table 6 that the lyophilization time affects the lightness (L^* value), in terms of redness (a^* value) it is observed that the pressure affects more significantly and the lyophilization time and freezing time affects yellowness (b^* value) opposite to other researches which report that color parameters are affected by freezing and drying time [46].

From Table 6**,** results revealed that L^*, a^*, b^*, and ΔE of dried MKG are affected by pressure, it can also be noted that pressure affect in color difference (ΔE) of dried MKG (p = 0.07176 < 0.1 and F = 3.01767 > Fcrit = 2.605525) but the other response variables were not significant (p < 0.1 and F > Fcrit = 2.605525, and at p < 0.05 and F > Fcrit = 3.490295), the change is seen from R'. Because it is obtained via equation (3), the L^*, a^*, and b^* make contribution to ΔE.

Table 6. Data analysis of MKG color difference (ΔE)

Data analysis for ΔE		**Factors**			
		***Ft* (h)**	***Fte* (°C)**	***P* (mbar)**	***Lt* (h)**
L^*	K_{L1}	172.98	168.72	169.84	162.28
	K_{L2}	160.74	157.35	155.75	174.95
	K_{L3}	156.69	166.28	162.37	151.07
	K_{L4}	172.04	170.08	174.47	174.15
	R'_L	16.29	12.73	18.72	23.08
	F	0.91679	0.40606	0.94622	2.23689
	p Value	0.46201	0.75143	0.44902	0.13646
a^*	K_{a1}	-4.67	-4.90	-4.78	-5.24
	K_{a2}	-5.52	-4.68	-5.25	-4.91
	K_{a3}	-4.61	-5.08	-4.50	-4.63
	K_{a4}	-5.20	-5.34	-5.47	-5.22
	R'_a	0.91	0.66	0.97	0.61
	F	1.28314	0.44649	1.30616	0.48155
	p Value	0.32468	0.72427	0.31764	0.70120
b^*	K_{b1}	25.90	24.71	24.96	24.51
	K_{b2}	23.11	23.95	21.84	25.83
	K_{b3}	22.51	24.64	24.45	21.24
	K_{b4}	25.71	23.93	25.97	25.65
	R'_b	3.39	0.78	4.13	4.59
	F	0.94204	0.04580	0.96255	1.57480
	p Value	0.45084	0.98634	0.44197	0.24680
ΔE	$K_{\Delta E1}$	40.20	47.77	56.92	41.56
	$K_{\Delta E2}$	31.78	33.14	23.30	52.95
	$K_{\Delta E3}$	51.38	41.80	38.41	27.25
	$K_{\Delta E4}$	36.47	37.11	41.20	38.07

$R'_{\Delta E}$	19.61	14.63	33.62	25.71
F	0.75283	0.39523	3.01767	1.36381
p Value	0.54157	0.75880	0.07176	0.30074

MGK: Mexican kefir grains; K_{L1}: lightness level 1; K_{L2}: lightness level 2; K_{L3}: lightness level 3; K_{L4}: lightness level 4; R'_{L}: lightness changing range; K_{a1}: redness level 1; K_{a2}: redness level 2; K_{a3}: redness level 3; K_{a4}: redness level 4; R'_{a}: redness changing range; K_{b1}: yellowness level 1; K_{b2}: yellowness level 2; K_{b3}: yellowness level 3; K_{b4}: yellowness level 4; R'_{b}: yellowness changing range; $K_{\Delta E1}$: color difference level 1; $K_{\Delta E2}$: color difference level 2; $K_{\Delta E3}$: color difference level 3; $K_{\Delta E4}$: color difference level 4; $R'_{\Delta E}$: color difference changing range

The equation for color difference (ΔE) of the dried product using the multiple regression analysis is obtained as follows (Equation 3):

$$\Delta E=16.2+11.02X_1+18.15X_2-21.51X_3-7.27X_4+0.61X_1^2-5.77X_1X_2-1.23X_1X_3 \\ +1.590X_1X_4-0.257X_2^2-0.07X_2X_3-1.45X_2X_4+3.0X_3^2+1.754X_3X_4 \quad (3)$$

Where R^2 for the equation (3) is 0.9666. Figure 3 provides the relationship among color difference (ΔE), freezing time and pressure at freezing temperature -20°C and lyophilization time 5 h. It is seen that at the same freezing time, the predicted color difference decreases with the pressure increase until a pressure of 0.6 mbar and stays constant up to a pressure of 0.8. The minimum ΔE is obtained with freezing time from 3 h. Coefficient of pressure, i.e., 21.51, indicates that the pressure plays the most important role in affecting color difference of the dried MKG.

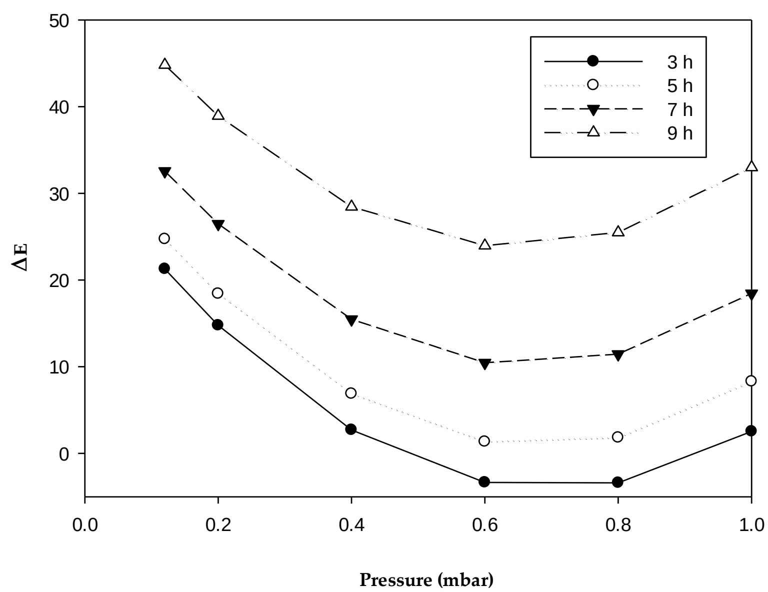

Figure 3. Predicted color difference (ΔE) of dried MKG at different freezing time and pressure

In Table 3, the experimental ΔE of dried MKG is compared with the predicted ΔE. It is seen that the minimum ΔE was 3.80 at freezing time 9 h, freezing temperature -40°C, pressure 0.6 mbar, and lyophilization time 5 h, and the maximum ΔE was 22.34 %.

3.4. Survival of probiotic microorganism of dried Mexican kefir grains

The survival rate of microorganisms after freeze-drying requires adequate stabilization processes to avoid bacterial damager or death [47], and the most obvious factor in the final product to be considered probiotic, because the survival rate of microorganisms is easy to lose due to the stress to which bacteria are subjected during freezing-drying. It is reported that for a food to be considered probiotic it must contain at least 10^6 colony forming units per gram (7 Log CFU/g) of food. Counts > 8 Log (CFU/g) have been suggested to ensure probiotic effects [48-50]. In this research, the survival rate of microorganisms was investigated under different experimental drying conditions. In this analysis, the initial value of survival of probiotic microorganism in the fresh sample of MKG was 9.05 and 8.90 Log (CFU/g) of LAB and Log (CFU/g) of yeasts, respectively.

From the results presented in Table 7, it is noted that *R'* is the maximum difference among the four levels for each factor. The larger *R'* the more significant the effects are. The max changing range (6.57) of pressure was the largest among the range of the four variables. This also indicates that the pressure plays an important role un affecting the survival LAB of dried MKG. On the other hand, the range (5.60) for freezing time was larger than those of the remaining two factors. This also shows that the freezing time is another important factor that affects the survival LAB in the dried product, different results from other researchers that indicates a highest value survival rate for LAB frozen at -196°C [51].

Table 7. Data analysis of dried MGK Log (CFU/g) of LAB

Data analysis for Log (CFU/g) of LAB	**Factors**			
	***Ft* (h)**	***Fte* (°C)**	***P* (mbar)**	***Lt* (h)**
K_1	26.19	27.57	27.67	29.37
K_2	31.79	30.27	28.36	30.17
K_3	30.20	28.54	33.16	29.76
K_4	27.61	29.40	26.59	26.48
R'	5.60	2.69	6.57	3.69
F	0.81998	0.14854	1.16898	0.32509
p Value	0.50748	0.92864	0.36213	0.80726

MGK: Mexican kefir grains; *Ft*: freezing time; *Fte*: freezing temperature; *P*: pressure; *Lt*: lyophilization time; K_1: level 1; K_2: level 2; K_3: level 3; K_4: level 4; *R'*: changing range

From Table 7 it can also be noted that although the influence of four factor is not significant ($p > 0.1$ and $F < Fcrit = 2.605525$ neither $p > 0.05$ and $F < Fcrit = 3.490295$) in survival Log (CFU/g) LAB of dried MKG, the changed is seen from *R'*, and the survival of dried MKG is affected mostly by pressure and freezing time. The equation for log (CFU/g) LAB of the dried MKG using multiple regression analysis is obtained as Equation (4):

$$\text{Log(CFU/g) of LAB} = 0.06 - 2.88X_1 + 3.09X_2 + 2.332X_3 + 3.52X_4 + 0.504X_1^2 - 0.157X_1X_4 + 0.103X_2^2 - 0.510X_2X_3 - 0.907X_2X_4 - 0.433X_3X_4 \quad (4)$$

In this equation, the square of the correlation coefficient (R^2) was 0.8851. The relationship between the log (CFU/g) LAB and pressure was obtained using Eq. (4) when freezing temperature and lyophilization time was kept at -20°C and 5 h, respectively. The predicted Log (CFU/g) of LAB is showed in Figure 4. It can be seen that the Log (CFU/g) of LAB increases when the pressure increases and freezing time decreases. The maximum value obtained was a pressure 1 mbar and freezing time 3 h. On the other hand, with higher values of pressure the survival of LAB increases more than two logarithmic cycles.

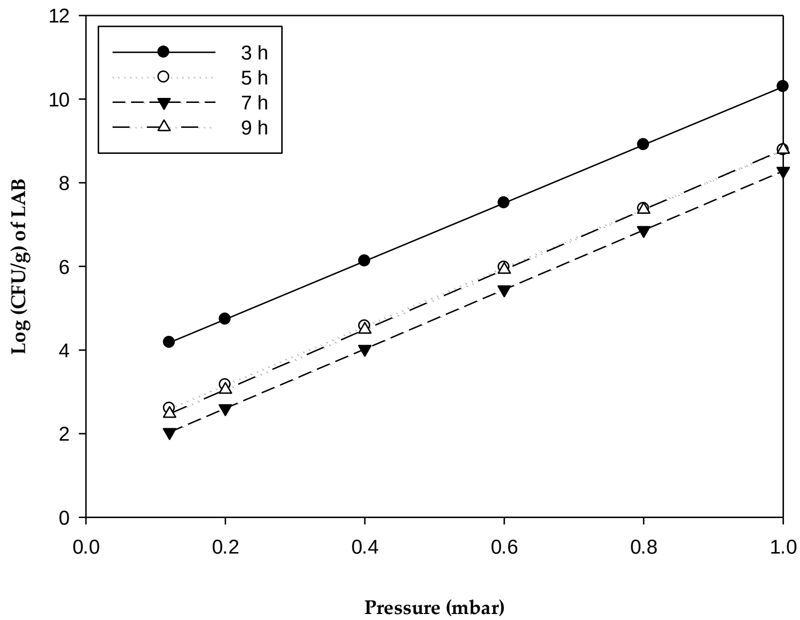

Figure 4. Predicted Log (CFU/g) of LAB of dried MKG at different freezing time and pressure

The experimental and predicted Log (CFU/g) of LAB values of dried MKG are shown in Table 3. It should be noted that the highest value is 8.64 Log (CFU/g) of LAB less than 0.5 cycle logarithmic that fresh sample before dried (9.05 Log (CFU/g) of LAB). This was at a freezing time 7 h, freezing temperature -20°C, pressure 0.6 mbar and lyophilization time 20 h. The relative error difference between the experimental and predicted Log (CFU/g) of LAB was 7.56%. Freeze drying had a negative effect on the survival rate of 4.53%. This result is different from other researchers that report a negative effect on the viability of all probiotic organisms nearly 7% in LAB without use to support materials and 16.4% with the use to support materials during freeze-drying [52-53].

From the results presented in Table 8 for yeasts, is noted that *R'* is the maximum difference among the four levels for each factor. The larger *R'* the more significant the effects are. The max changing range (9.83) of pressure was the largest among the range of the four variables. This also indicates that the pressure plays an important role affecting the survival yeast of dried MKG. On the other hand, the range 8.83 for freezing time was larger than those of the remaining two factors. This also shows that the freezing time is another important factor that affects the survival yeast in the dried product.

Table 8. Data analysis of dried MGK Log (CFU/g) of Yeasts

Data analysis for Log (CFU/g) of Yeasts	Factors			
	***Ft* (h)**	***Fte* (°C)**	***P* (mbar)**	***Lt* (h)**
K_1	24.97	26.63	28.94	25.02
K_2	33.79	28.25	28.51	21.75
K_3	30.78	30.46	34.16	31.48
K_4	26.40	30.61	24.33	28.33
R'	8.83	3.98	9.83	6.58
F	2.23175	0.34546	2.19270	0.53571
p Value	0.13706	0.79307	0.14177	0.66659

MGK: Mexican kefir grains; *Ft*: freezing time; *Fte*: freezing temperature; *P*: pressure; *Lt*: lyophilization time; K_1: level 1; K_2: level 2; K_3: level 3; K_4: level 4; R': changing range

From Table 8 it can also be noted that although the influence of four factor is not significant ($p > 0.1$ and $F < Fcrit = 2.605525$ neither $p > 0.05$ and $F < Fcrit = 3.490295$) in survival Log (CFU/g) of yeasts of dried MKG, the changed is seen from R' and the survival of dried MKG is affected mostly by pressure and freezing time.

Multiple regression analysis for Log (CFU/g) of yeasts is follows (Equation 5):

$$\begin{aligned}\text{Log}(\text{CFU/g})\text{of yeasts} = {} & 2.52+3.35X_1-0.71X_2+3.90X_3+2.57X_4-0.32X_1^2+2.77X_1X_2-0.656X_1X_3 \\ & -0.348X_1X_4+0.080X_2^2+0.150X_2X_3-0.42X_2X_4-0.375X_3^2-0.343X_3X_4\end{aligned} \quad (5)$$

In this equation, the square of the correlation coefficient (R^2) was 0.8017 and the Log (CFU/g) of yeast by Equation (5) is showed in Figure 5. The freezing temperature was set at -20°C and lyophilization time 5 h. It can be seen from Figure 5 than the maximum survival of yeast value predicted is reached when the pressure increases to 0.8 mbar and freezing temperature was 3 h. On the other hand, with higher values of pressure and higher freezing times of 3 h, the survival of yeast decreases more than two logarithmic cycles.

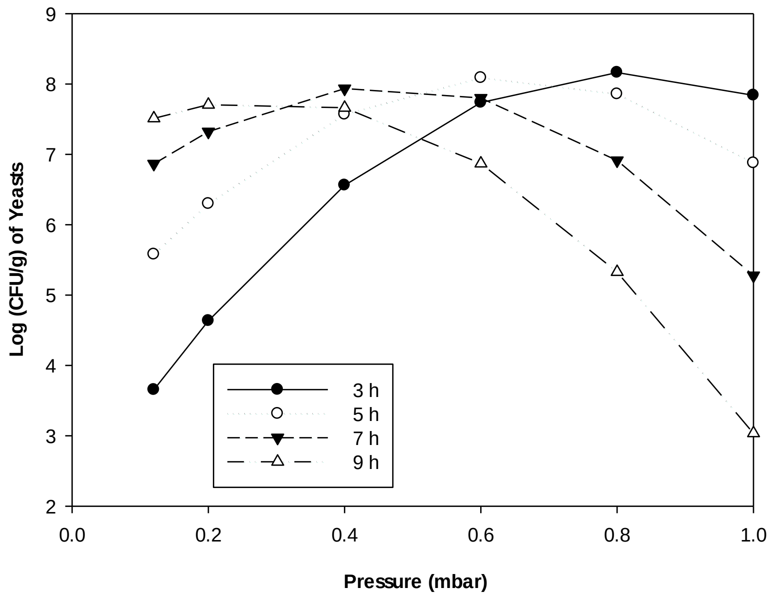

Figure 5. Predicted Log (CFU/g) of yeasts of dried MKG at different freezing time and pressure

The experimental and predicted Log (CFU/g) of yeasts values of dried MKG are shown in Table 3. It should be noted that the highest value is 8.67 Log (CFU/g) of yeasts less than 0.5 cycle logarithmic that fresh sample before dried (8.90 Log (CFU/g) of yeasts). This was at a freezing time 3 to 7 h, freezing temperature -20 to -60°C, pressure 0.6 mbar, and lyophilization time was 15 to 20 h. The relative error difference between the experimental and predicter Log (CFU/g) of yeasts was 14.24 and 7.36% in the test No. 3 and 9 respectively. Freeze drying had a negative effect on the survival rate of 2.60 %, this result is different from other researchers that report a negative effect on the viability of yeasts after freeze-drying, with the use to support materials were between 2.8 and 10.5%[54-55].

3.5. Optimization of the freeze-drying parameters for dried Mexican kefir grains

Because the residual moisture, water activity and survival probiotic bacteria are the most important factors for dried MKG, optimization of the freeze-drying considers these three important factors, looking for values for % RMC < 6; a_w is < 0.60 and at least 10^6 colony forming units per gram (7 Log CFU/g) of probiotic microorganism. The optimum process for desirable moisture, water activity, and survival probiotic microorganism is shown in Table 9. Optimal levels for them are the same, freezing time of 3 h, freezing temperature of -20°C, pressure 0.6 mbar and lyophilization time 15 h. The residual moisture in the dried product needs to below 6% [56]. In this study, less than 6% of residual moisture content in dried MKG was used. In Table 3, the pressure of 0.6 mbar was chosen as the optimal one to prevent survival probiotic bacteria loss. It is seen that the average Log (CFU/g) of LAB and yeasts is 3.24 and 3.45 logarithmic cycles less respectively than that of 0.2 mbar. Therefore, the pressure of 0.6 mbar was selected as the optimal level. Under the optimal conditions given, the properties of dried MKG were residual moisture 5.03%; a_w 0.167; ΔE 7.79; LAB 8.5 Log (CFU/g); and yeasts 8.6 Log (CFU/g).

Table 9. Optimal factors for each properties of dried MKG

Properties of dried MKG	**Factors**			
	***Ft* (h)**	***Fte* (°C)**	***P* (mbar)**	***Lt* (h)**
RMC	Level 1	Level 1	Level 3	Level 3
a_w	Level 1	Level 1	Level 3	Level 3
Log (CFU/g) of LAB	Level 1	Level 1	Level 3	Level 3
Log (CFU/g) of yeast	Level 1	Level 1	Level 3	Level 3

MGK: Mexican kefir grains; *Ft*: freezing time; *Fte*: freezing temperature; *P*: pressure; *Lt*: lyophilization time.

4. Conclusions

Freeze-drying of Mexican kefir grains was carried out in a compact freeze dryer. The L_{16} orthogonal experimental design was applied to find the optimal operating conditions for obtaining a product suitable for the market. The residual moisture and water activity were mainly influenced by the operating lyophilization time ($p < 0.05$). These responses variables decrease when the lyophilization time also decreases. On the other hand, the color difference and survival probiotic bacteria were significantly affected by pressure. The optimal conditions for dried MKG freeze-drying are freezing time of 3 h, freezing temperature of -20°C, pressure of 0.6 mbar, and lyophilization time of 15 h. Under the optimal conditions given, the properties of dried MKG were residual moisture 5.03%; a_w 0.167; ΔE 7.79; survival of LAB 8.5 Log (CFU/g) and yeasts 8.6 Log (CFU/g) which are physicochemical and microbiological characteristics suitable as a functional and probiotic food for the market.

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