Processing optimization of probiotic yogurt containing glucose oxidase using response surface methodology

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ABSTRACT

Exposure to oxygen may induce a lack of functionality of probiotic dairy foods because the anaerobic metabolism of probiotic bacteria compromises during storage the maintenance of their viability to provide benefits to consumer health. Glucose oxidase can constitute a potential alternative to increase the survival of probiotic bacteria in yogurt because it consumes the oxygen permeating to the inside of the pot during storage, thus making it possible to avoid the use of chemical additives. This research aimed to optimize the processing of probiotic yogurt supplemented with glucose oxidase using response surface methodology and to determine the levels of glucose and glucose oxidase that minimize the concentration of dissolved oxygen and maximize the *Bifidobacterium longum* count by the desirability function. Response surface methodology mathematical models adequately described the process, with adjusted determination coefficients of 83% for the oxygen and 94% for the *B. longum*. Linear and quadratic effects of the glucose oxidase were reported for the oxygen model, whereas for the *B. longum* count model an influence of the glucose oxidase at the linear level was observed followed by the quadratic influence of glucose and quadratic effect of glucose oxidase. The desirability function indicated that 62.32 ppm of glucose oxidase and 4.35 ppm of glucose was the best combination of these components for optimization of probiotic yogurt processing. An additional validation experiment was performed and results showed acceptable error between the predicted and experimental results.

Key words: oxygen stress, probiotic yogurt, glucose oxidase, response surface methodology

INTRODUCTION

A relationship was established between dairy products and probiotic bacteria more than 2,000 yr ago when people consumed large amounts of fermented milks such as kefir and yogurt. More recently scientists have established a positive connection between these products and health. Yogurt is indeed recognized as a healthy food with a positive health appeal, and for this reason the consumer market for yogurt is increasing throughout the world. In Brazil an increase in its consumption of 2.4% was registered in 2008, involving US\$5.3 billion in sales (Rocha and Madureira, 2009). Yogurt remains one of the main vehicles for probiotic cultures in the preference of the consumer (Siegrist et al., 2008; Hailu et al., 2009), even though the technological advantages of other dairy products such as cheeses and ice creams have been highlighted (Cruz et al., 2009a,b), and various studies examine the development of yogurt and nondairy products supplemented with probiotic cultures (Dave and Shah, 1998; Antunes et al., 2005; Mortazavian et al., 2006, 2007; Aryana et al., 2007; Almeida et al., 2008; Ramasubramanian et al., 2008; Korbekandi et al., 2009; Saccaro et al., 2009; Granato et al., 2010b).

The incorporation of probiotic bacteria into foods and the maintenance throughout storage of their viability to benefit consumer health presents a constant challenge to the food industry, requiring an understanding of intrinsic and extrinsic factors related to processing. The viability of probiotic microorganisms in yogurt depends on the strains used, the interaction between the species, cultivation conditions, production of hydrogen peroxide attributable to the bacterial metabolism, pH value and pH decrease rate during the fermentation, final product acidity, and lactic and acetic acid concentrations (Shah, 2000). It has also been reported that the packaging materials used and the storage conditions are important

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factors for the quality of products containing probiotic microorganisms because the metabolism of this microbial group is essentially anaerobic or microaerophilic (Mattila-Sandholm et al., 2002). Thus, the oxygen level throughout storage of the product should be as low as possible to avoid toxicity and death of the microorganism and consequent loss of functionality of the product.

Glucose oxidase is an enzyme produced by fungi such as *Aspergillus* and *Penicillium*, produced both intra- and extracellularly, catalyzing the formation of gluconic acid and hydrogen peroxide as from glucose and oxygen (Leskovac et al., 2005). It has been used to retard lipid oxidation, prevent off-flavors in various types of products such as mayonnaise (Isaksen and Adler-Nissen, 1997), control enzymatic browning in fruit purees (Parpinello et al., 2002), stabilize the color in grape juice (Castellari et al., 2000), stabilize alcoholic beverages such as beer, and conserve strawberry preserves and fruit juices, among other uses (Isaksen and Adler-Nissen, 1997). It has an isoelectric point between 4.2 and 4.3 and an optimum pH for activity between 3.5 and 6.5 (Labuza and Breene, 1989). Glucose oxidase can constitute a potential alternative to increase the survival of probiotic bacteria in yogurt because it consumes the oxygen permeating to the inside of the pot during storage, thus making it possible to avoid the use of chemical additives (Meyer and Isaksen, 1995). In addition, it does not require any changes in the original product processing steps and is easy to implement in the industrial routine of a dairy plant; thus, it can add value to the yogurt produced without overburdening production.

In the present study the processing of probiotic yogurt with added glucose oxidase was optimized using response surface methodology and the desirability function method. To the best of the authors' knowledge this is the first report on the use of a biotechnological option as a tool to minimize the sensitivity to oxygen suffered by probiotic bacteria.

MATERIALS AND METHODS

Growth of Cultures

Pure cultures of *Streptococcus salivarius* ssp. *thermophilus* TA 040, *Lactobacillus delbrueckii* ssp. *bulgaricus* LB 340, and *Bifidobacterium longum* BL 05 were obtained from Danisco (Copenhagen, Denmark). Each strain was maintained at -80°C . Skim milk powder (SMP; Molico, São Paulo, Brazil) reconstituted at 11% (wt/vol) was used to prepare the starter lactic cultures. For *B. longum* BL 05, SMP was used with the addition of 0.5% (wt/vol) yeast extract (Oxoid, Bas-

ingstoke, UK) and 0.05% (wt/vol) cysteine (Sigma, St. Louis, MO), whereas for the other lactic cultures, SMP was supplemented with 2% (wt/vol) glucose (Synth, São Paulo, Brazil). The steps involving handling of the microorganisms were carried out in a laminar flow chamber.

Yogurt Processing

The probiotic yogurts were processed using traditional methodology (Tamine and Robinson, 2007). Raw milk, standardized with skim milk powder at 3.5% (wt/vol; Atilatti, Itatiba, Brazil) was submitted to heat treatment (95°C for 15 min), cooled to 45°C , and inoculated with 1% (vol/vol) *Strep. salivarius thermophilus* ssp. TA 040, 1% (vol/vol) *L. delbrueckii* ssp. *bulgaricus* LB 340, and 2% (vol/vol) *B. longum* BL 05. The inoculated milk was subsequently submitted to fermentation at 45°C , and pH was monitored until it reached values of 4.6 ± 0.05 . The mixture was then cooled to 10°C and the gel was broken by stirring with the simultaneous addition of glucose oxidase (Glucamax CO, Prozyn, São Paulo, Brazil; mixture of glucose oxidase and catalase) and glucose (Synth) in the concentrations indicated in Table 1. The final product was then filled into 100-mL polypropylene pots (permeability of $0.20 \text{ cm}^3/\text{pot}$ per day; Dix Toga, São Paulo, Brazil, the packaging system used for the probiotic yogurts available on the Brazilian market) and stored under refrigeration at an average temperature of 5°C until analyzed after 15 d of storage.

B. longum Count

One milliliter of yogurt was transferred to a screw-capped test tube containing 9 mL of 0.1% (wt/vol) peptone water, and various dilutions were made from this dilution. The *B. longum* count was carried out in duplicate by deep plating in sodium lithium-propionate chloride agar with 0.5 g/L of LiCl and 0.75 g/L of sodium propionate and incubating under anaerobic conditions at 37°C for 3 d (Zacarchenco and Massaguier-Roig, 2004).

Dissolved Oxygen

Dissolved oxygen was quantified at 2 moments: in the yogurt immediately after stirring, and in the final product supplemented with glucose oxidase and glucose, after 15 d of refrigerated storage. In the first case, a quantity of 200 mL of the product was removed; in the second case, the measurements were carried out in the product contained in the plastic pots. An MO128 O_2 analyzer (Mettler Toledo, Columbus, OH) was used,

Table 1. Experimental design and levels of factors in actual and coded values (ppm)

Assay	Glucose		Glucose oxidase	
	Coded independent variable	Real value	Coded independent variable	Real value
1	-1	9.4	-1	31.6
2	1	30.6	-1	31.6
3	-1	9.4	1	88.4
4	1	30.6	1	88.4
5	-1.41	5	0	60
6	1.41	35	0	60
7	0	20	-1.41	20
8	0	20	1.41	100
9 ¹	0	20	0	60
10 ¹	0	20	0	60
11 ¹	0	20	0	60

¹Central point.

obtaining the results for the oxygen concentration of the sample in terms of parts per million (ppm). The apparatus was calibrated according to the manufacturer's instructions using distilled water (100% dissolved oxygen) and saturated sodium metabisulfite solution (0% dissolved oxygen) as standards. These values correspond to approximately 10 and 0 ppm of dissolved oxygen, respectively, according to the automatic conversion of the equipment. Measurements were performed 5 times for each sample.

Experimental Design and Statistical Analyses

A planned sequence of experiments is important to generate optimum responses for the variables of interest. In the present study, response surface methodology was used, where glucose oxidase and glucose concentrations were the independent variables used, whereas the optimal conditions of the system were determined by means of the desirability function, illustrated below by way of a short theoretical introduction.

Response surface methodology was used to optimize the processing of probiotic yogurt containing *B. longum* BL 05 supplemented with glucose oxidase. The independent variables used were the concentrations of the enzyme glucose oxidase and its substrate glucose, whereas the dependent variables (responses) were the dissolved oxygen concentration and the *B. longum* BL 05 count. A central compound rotational design with 2 independent variables and 2 levels was used, with 4 assays under axial conditions ($\alpha = 1.414$) and 3 repetitions at the central point, representing a total of 11 assays. One control assay, without the addition of glucose or glucose oxidase, was made and analyzed simultaneously, giving an overall total of 12 assays.

The levels of the independent variables were chosen from the preliminary tests, taking the economic feasi-

bility of the process into consideration (in the case of adding the enzyme) and the values for glucose reported for yogurts found on the market (Mannino et al., 1999). The choice of *B. longum* was related to the greater sensitivity of this strain to exposure to oxygen compared with other species of bifidobacteria (Kawasaki et al., 2006), making it more suitable for the present study.

Table 1 shows the experimental design used and the coded and real values for the variables. The statistical analysis was carried out using the software Statistica 7.1 (Statsoft, Tulsa, OK). Initially, a complete second-order polynomial quadratic model was adjusted to each of the responses based on the following:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2, [1]$$

where β_0 , β_1 , β_2 , β_{11} , β_{22} , and β_{12} represent the regression coefficients (β_0 represents the term for the intersection, β_1 and β_2 represent the linear effects, β_{11} and β_{22} represent the quadratic effects, and β_{12} represents the interaction effect) and X_1 and X_2 are the coded independent variables (glucose oxidase and glucose, respectively). The analysis was carried out using the coded variables, and the removal of nonsignificant terms ($P > 0.05$) was applied when necessary. The quality of the fitted models was evaluated by ANOVA, based on the F -test and on the percentage of explained variance (R^2_{adj}), which provides a measurement of how much of the variability in the observed response values could be explained by the experimental factors and their interactions (Granato et al., 2010c). The data used for modeling were the means of 2 replicates in the case of microbiological analyses and 5 replicates in the case of dissolved oxygen. To visualize the relationships between the responses and the independent variables, surface response plots of the fitted regression equations were generated using the statistical package Statistica 7.1.

RESULTS AND DISCUSSION

To build a response surface from experimental data, at least one of the assays must be different from the other formulations, and this requirement was initially fulfilled (Table 2). From this, linear and quadratic models were tested to verify which best fitted the data. The ANOVA showed that both the model proposed for dissolved oxygen and that proposed for the total *B. longum* count were significant ($P < 0.001$) and presented a low experimental error (mean square pure error < 0.003), and high determination coefficients ($R^2_{\text{adj}} = 0.83$ and 0.94 for dissolved oxygen and *B. longum* count, respectively) were obtained, which validated the experimental data.

Table 2 summarizes the estimated regression coefficients of the quadratic polynomial models for the response variables along with the corresponding R^2_{adj} values and F -ratio values. The lack of fit, which measures the fit of the experimental data to the model obtained, did not result in a significant value for the *B. longum* count, but for oxygen this value was significant ($P = 0.01$). This means that the mathematical model for *B. longum* count can be used for prediction purposes whereas the model for dissolved oxygen can be used only to have an idea about the response. However, the significant lack of fit can be considered marginal once the experimental error was low ($P < 0.001$).

It is important to point out that high fitted coefficients of determination were found (0.83 for the concentration of dissolved oxygen and 0.94 for the *B. longum* count), which means that the models explained 83 and 94% of the variation in the experimental data. The fitted coefficient of determination is defined as the ratio between the explained variability and the experimental data with respect to the total variation and is a measure

of the fit of the model to the experimental data. Small values for coefficients of determination suggest that the dependent variables chosen were not very relevant to the model in question, fitting the experimental data well when the value for R^2_{adj} is close to 1 (Granato et al., 2010a). In practice, high values for R^2_{adj} ($> 70\%$) suggest that the models obtained are adequate to describe the influence of the dependent variables studied, oxygen concentration and *B. longum* count.

Oxygen Content

The toxicity of oxygen constitutes a technological hurdle in the development of probiotic yogurts because exposure to oxygen during probiotic yogurt storage is highly significant for the probiotic bacteria. *Bifidobacterium* spp. is a microorganism of intestinal origin with an anaerobic metabolism, incapable of synthesizing ATP by way of its respiratory metabolic pathways and exclusively dependent on a fermentative metabolism (Talwalkar and Kailasapathy, 2004). In these organisms, the oxygen sequestering system is reduced or absent, resulting in the incomplete reduction of oxygen to hydrogen peroxide and consequent accumulation of oxygen-derived metabolites that are toxic to the cell (O_2^- , OH^- , and H_2O_2), eventually causing cell death (Vasiljevic and Shah, 2008). High intracellular H_2O_2 concentrations inhibit fructose-6-phosphofructokinolase, a key enzyme in the metabolism of bifidobacteria (Shah, 1997). In this sense, a positive correlation has been reported between the levels of 2 enzymes (NAD-oxidase and NAD-peroxidase) and the susceptibility of bifidobacteria to oxygen; these enzymes present relevant activity in most of *Bifidobacterium* spp. that are aerotolerant (Roy, 2005).

In the present study, low levels of dissolved oxygen were noted in the yogurts after 15 d of refrigerated storage, varying from 0.93 to 1.84 ppm, representing a reduction of 69.02 to 86.03% in the value of oxygen found in the control yogurt (5.94 ppm) to which glucose oxidase and glucose were not added. The model proposed fitted the data in an adequate way ($R^2 = 0.83$) and a quadratic effect of the glucose, followed by the interaction effect of the glucose and glucose oxidase, a linear effect of the glucose, and a quadratic effect of the glucose oxidase were obtained, resulting in a surface with a minimum point (Figure 1). These effects indicate how much each factor influences the response under study; in this case, the concentration of dissolved oxygen, its influence is proportional to its value. A positive effect indicates that when passing from a minimum value to a maximum value, the response will increase. On the other hand, a negative effect indicates that on passing from a minimum value

Table 2. Regression coefficients of the second-order polynomial models¹

Factor ²	O ₂ (ppm)	<i>Bifidobacterium longum</i> (log cfu/mL)
Constant	0.94	8.44
GOX	NS	0.56
(GOX) ²	0.12	−0.35
GLU	0.13	−0.18
(GLU) ²	0.29	−0.47
GLU × GOX	0.14	−0.20
R^2_{adj}	0.83	0.94
F	9.13	21.92
P -value (model)	< 0.001	< 0.001
P -value (lack of fit)	0.01	0.06
Pure error	< 0.001	0.002

¹Analysis was performed using coded units.

²GOX = glucose-oxidase; GLU = glucose; R^2_{adj} = percentage of explained variance.

Figure 1. Response surface plot for the dissolved oxygen. GLU = glucose; GOX = glucose oxidase. Color version available in the online PDF.

to a maximum value, the response decreases (Ribeiro et al., 2008). The findings emphasize the importance of the substrate, suggesting that an additional amount of substrate is required for the action of the enzyme along the shelf life of the product. Indeed, Vroemen (2003) warned that for certain food applications, such as low pH applications, a certain amount of glucose must be added for the enzyme to remain active.

Oxygen along the probiotic yogurt storage presents a dynamic behavior because it can permeate into the plastic pot throughout storage of the probiotic yogurts. Indeed, a great variation in probiotic yogurts and fermented milks in the values for dissolved oxygen found throughout storage has been reported (Miller et al., 2002, 2003). These results are related to factors inherent to the methodology, such as different procedures and sensitivities of the instruments used, expressed at the locations where the values for this parameter were registered (i.e., in the center, or extremity, or both, of the yogurt). In this context, it is noteworthy that

the use of a biotechnological tool such as the enzyme glucose oxidase could be an alternative to minimize this drawback, as are the addition of ascorbic acid (Dave and Shah, 1997), milk electroreduction (Bolduc et al., 2006), the use of polystyrene laminated with high gas barrier material (HIPS/tie/EVOH/tie/PE, Nupak, Visypak, Melbourne, Australia; Miller et al., 2002), active packaging systems (Miller et al., 2003), microencapsulation (Talwalkar and Kailasapathy, 2003), the use of plastic packaging systems with different polarities and crystallinities (Jasson et al., 2001), and the use of Oxyrase (which reduces fractions from bacterial cell membranes; Oxyrase Inc., Mansfield, OH; Ordonez et al., 2000), producing results similar to or even better than previously studied technological solutions without the need to change the plastic packaging system used. It also must be pointed out that the addition of glucose oxidase does not demand any marked change in the yogurt processing procedure or, consequently, in the operational routine of the dairy plant. This is impor-

tant for small-scale industrial units, which frequently do not have a specialized technical team. Cruz et al. (2007) pointed out that technological options aimed at minimizing the stress caused by exposure to oxygen in probiotic dairy products should recommend the use of appropriate materials and be economically feasible.

Another factor that should be taken into consideration is that there is no need for addition of chemical compounds, which may be rejected by potential consumers, to yogurts. In this regard, food companies aim to develop products that are as natural as possible, which means that companies are avoiding the use of chemical compounds, as is well demonstrated in a recent focus group study in São Paulo, Brazil (Behrens et al., 2010).

***B. longum* Viable Count**

Throughout their shelf life, probiotic yogurts should present certain minimum viable counts of probiotic bacteria to exert a beneficial effect on consumer health. It has been suggested that these microorganisms should be present with a minimum count of 10^6 cfu/g or mL, the daily ingestion dose indicated being 10^8 cfu/g or mL, implying a continuous ingestion of 100 mL or 100 g of yogurt. Such values are necessary to compensate for possible reductions during their passage through the gastrointestinal tract, represented by the acidic pH of the stomach and of the bile salts of the intestine and during the processing and storage of the product (Vasiljevic and Shah, 2008). Bacteria of the genera *Bifidobacterium* are inhabitants of the human intestinal tract and are relatively stable during the adult phase of life, declining with advancing age (Arunachalam, 1999). Considering their complex metabolic conditions, including their sensitivity to oxygen exposure, maintenance of viable counts capable of exerting a therapeutic function is a continuous technological challenge for probiotic dairy product processors, especially for yogurts. In fact, a loss of viability has been observed for various strains of bifidobacteria during the storage of probiotic yogurts available for commercialization in various countries throughout the world, such as Australia (Shah et al., 1995), Argentina (Vinderola et al., 2000), Brazil (Barreto et al., 2003), Spain (Gueimonde et al., 2004), the United States (Ibrahim and Carr, 2006), and Saudi Arabia (Al-Otaibi, 2009), and oxygen toxicity has been presented as a possible reason, among others, for these results.

Oxygen tolerance is dependent on the strain used (Shimamura et al., 1992). In general, *B. longum* has been reported as presenting unsatisfactory performance in the presence of high oxygen concentrations, being extremely sensitive to this adverse reaction condition,

and showing a loss of viability (Simpson et al., 2005; Jayamanne and Adams, 2009). Although alternatives such as prior adaptation to high oxygen concentrations have been considered promising as a solution to this question (Talwalkar and Kailasapathy, 2004), they demand additional work that could interfere with the routine of a small-scale dairy plant and have economic implications. Thus, technological solutions are required that demand little or no alteration in the processing of the probiotic yogurt and that have little effect on production costs.

In the present work, viable *B. longum* counts varying from 6.9 to 8.7 log cfu/mL were found, representing increases of 11.29 to 40.32% in relation to the log count found in the control yogurt without the addition of glucose oxidase and glucose, which was 6.2 log cfu/g. This represents high values for the count of this microorganism, considering that the yogurt was already halfway through its commercial shelf life (i.e., after 15 d of refrigerated storage). The model proposed fitted the data in an excellent way ($R^2_{\text{adj}} = 0.94$), and a linear effect of the glucose oxidase and the quadratic influence of glucose followed by the quadratic effect of glucose oxidase and linear effect of the glucose were attained, resulting in a surface with a maximum point (Figure 2).

Overall, the results report a positive effect of glucose oxidase to predict the *B. longum* count, indicating its influence in increasing the amount of viable probiotic strain. However, a certain dependence on glucose can be noted for complete efficiency, considering a determined limit, because glucose presented a negative effect. This could be related to substrate inhibition, a phenomenon that normally occurs in enzymology, and would impede the glucose oxidase from exerting its function of sequestering the oxygen that permeates into the plastic pots. Mirón et al. (2004) described a mathematical model for the acting kinetics of the glucose oxidase and showed that substrate inhibition really is a factor that alters the activity of the enzyme, causing a decrease in the reaction rate because of restrictions in diffusion caused by the increase in viscosity attributed to the gluconic acid produced.

The predominance of this enzyme can be explained on the basis of its function in the product: that of sequestering oxygen, creating an anaerobic environment, and facilitating growth of the probiotic microorganism. However, it is important to investigate the maximum limit of its addition both for economic and technological reasons because it could have some adverse effect on product quality, as reported for salad dressing (Min et al., 2003) and bread (Bonet et al., 2006).

It should be pointed out that even species of *Bifidobacterium* can metabolize glucose (Gomes and Mal-

Figure 2. Response surface plot for *Bifidobacterium longum*. GLU = glucose; GOX = glucose oxidase. Color version available in the online PDF.

cata, 1999), which could contribute to an increase in viability of the microorganism. However, the findings of this work indicated that the exposure to oxygen has a fundamental role in the maintenance of the functionality for probiotic yogurts. The control sample, to which the enzyme and glucose were not added, showed a *B. longum* count of 5.9 log cfu/g and oxygen concentration of 6.35 ppm, which are lower than all values found in the probiotic yogurts with added enzyme and glucose. It suggests that *B. longum* did not use the glucose for its growth.

Simultaneous Optimization

Optimization is the choice of a best alternative from a specified set of alternatives. It therefore requires some way of describing the potential alternatives and some way of deciding which of the alternatives is best (Granato et al., 2010c). In process engineering,

it may be the process of maximizing a desired quantity or minimizing an undesired one. The conditions or constraints (values of the processing variables) that produce the desired optimum value are called optimum conditions, and the best of all the feasible designs is called the optimal design (Tzia, 2003). If a particular specification of a variable satisfies all the constraints, it is called feasible, and this specification will determine a value for the objective function or objective value. Any function defined in the entire feasible set could be considered to be an objective function at any time (Granato et al., 2010a).

Finding the overall optimal conditions in food development is not straightforward or easy, and most researchers use the graphical approach of superimposing the different response surfaces and finding the experimental region that would give the desired values for the responses. This method, although visually attractive, requires the generation of a large number of graphs,

Profiles for Predicted Values and Desirability

Figure 3. Simultaneous optimization of the dissolved oxygen concentration (O_2) and the *Bifidobacterium longum* (BL) count. GOX = glucose oxidase; GLU = glucose. Color version available in the online PDF.

even when only 2 or 3 responses are involved, and fails to determine the real optimum point. To overcome this limitation, simultaneous optimization has recently been used in optimizing food products and processes (Granato et al., 2010c).

The desirability function combines all the responses into a single measurement, and it has been used for the development of processed food products such as chocolate (Alamprese et al., 2007) and prebiotic desserts (Granato et al., 2010c). It involves transformation of each response variable (Y_i) into a desirability value (d_i), where $0 \leq d_i \leq 1$, and the value of d_i increases as the desirability of the corresponding response increases. Depending on whether a particular response (Y_i) is to be maximized or minimized, different desirability functions [$d_i(Y_i)$] can be used. The individual desirability functions are then combined using the geometric mean

according to the following equation to arrive at the overall desirability D:

$$D = (d_1 \times d_2 \times \dots \times d_k)^{1/k}, \quad [2]$$

where k is the number of responses.

This single value for D gives the overall assessment of the desirability of the combined response levels, and clearly the range of D will fall in the interval 0,1. A high value for D in equation [1] indicates the more desirable and best function of the system and is considered to be the optimal solution of this system. These optimum values of the factors are determined from the value of individual desired functions that maximize D . The variable D also has the property that if any $d_i = 0$ (i.e., if one of the response variables is unacceptable), then $D = 0$ (i.e., the overall product is unacceptable). Because

of this, the geometric mean of the expression for D was used (Derringer and Suich, 1980).

Simultaneous optimization using the desirability function was carried out using the following independent variables for the probiotic yogurt: dissolved oxygen concentration and *B. longum* count (Figure 3). A value equal to 0 was imposed for the minimum value of the desirability function, and a value equal to 1 was imposed for the maximum value of the desirability function (i.e., the greatest possible value for the *B. longum* count). The software executes millions of interactions and calculations and finally informs the maximum value of the desirability function and the conditions under which it was obtained (i.e., which values for the glucose and glucose oxidase concentrations will result in the respective desirability function), which should provide the minimum dissolved oxygen concentration and maximum *B. longum* count. Based on this methodology a desirability function of 0.956 was found when the glucose oxidase and glucose concentrations were 62.32 and 4.35 ppm, respectively (Figure 3).

An additional experiment was carried out to validate these data. Yogurts were produced using the same methodology described above and with the optimum values for glucose oxidase and glucose previously enumerated. After 15 d of refrigerated storage, the analysis for dissolved oxygen and the *B. longum* count were carried out on 3 samples removed at random. The mean values found for these analyses were 0.59 ppm of dissolved oxygen and 8.69 log cfu/mL of *B. longum*, whereas the values predicted by the model were 0.52 ppm of dissolved oxygen and 8.74 log cfu/mL of *B. longum*, resulting in deviations of 12.96 and 0.57%, respectively. This reinforced the good results found for the experimental design, principally for *B. longum*.

In general, the results of this study suggest that glucose oxidase could be a viable alternative to minimize the toxicity caused by oxygen in probiotic microorganisms. Further studies are required to investigate the effect and stability of the enzyme up to the end of the shelf life of the probiotic yogurt and its influence on intrinsic quality parameters such as pH, proteolysis of the cultures, and metabolism of the yogurt and probiotic cultures. In addition, it is important to study the sensory aspects to verify the acceptability of these products by the consumers.

CONCLUSIONS

In the present study, the processing of probiotic yogurt supplemented with glucose oxidase was optimized using response surface methodology. Low values for dissolved oxygen and high *B. longum* counts were observed, suggesting the utility of the alternative technology in

question. The models generated were adequate to predict and check the tendencies for the variables studied. Simultaneous optimization by means of the desirability function indicated that the optimum glucose oxidase and glucose concentrations were 62.32 and 4.35 ppm, respectively, generating values of 0.52 ppm for oxygen and 8.74 log cfu/mL for *B. longum*, which were proven by an additional validation experiment with acceptable errors between the predicted and experimental results.

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