

Application of Factorial Design for Optimization of the Synthesis of Lactulose Obtained from Whey Permeate

Fernanda C. Zimmer,^a Aloisio H. P. Souza,^b Ana F. C. Silveira,^a Maristela R. Santos,^a
Makoto Matsushita,^c Nilson E. Souza^c and Angela C. Rodrigues^{*a}

^aUniversidade Tecnológica Federal do Paraná (UTFPR), Av. Brasil, 4232, 85884-000 Medianeira-PR, Brazil

^bInstituto Federal do Mato Grosso do Sul (IFMS), Rua Salime Tanure, s/n, Santa Tereza,
79400-000 Coxim-MS, Brazil

^cUniversidade Estadual de Maringá (UEM), Av. Colombo, 5790, 87020-900 Maringá-PR, Brazil

There is a great interest in developing suitable alternatives for the utilization of whey, a by-product from cheese production that contains high concentration of lactose. These alternatives allow the transformation of whey into several products, reducing its environmental impact and providing gains to the dairy industry through the development of new products. The goal of this study was to optimize the synthesis of lactulose from the permeate of bovine whey. The response surface methodology (RSM) was used to investigate the effect of two parameters, time of reaction (90, 135 and 180 min) and isomerization type (sodium sulfite, boric acid and β -galactosidase enzyme), aiming to obtain the highest reaction yield. According to the response surface and desirability function, the best experimental conditions for lactulose synthesis (where it was obtained 50.06 g of lactulose *per* 100 g of whey powder) consisted of a reaction time of 180 min with the use of enzymatic isomerization.

Keywords: prebiotics, experimental design, reuse of industrial by-products

Introduction

Lactulose (4-*O*- β -D-galactopyranosyl-D-fructose) is a ketosis, which is a semi-synthetic disaccharide, consisting of a fructose and a galactose molecule.¹⁻⁵ It is slightly sweeter than lactose and can be used to replace part of the sucrose in some food products.⁶ In addition, lactulose is a functional nutrient with great added value because it is a prebiotic disaccharide that can be widely applied in food and pharmaceutical products.

Lactulose is chemically produced by lactose isomerization through Lobry de Bruyn-Alberda van Ekenstein transformation, using catalysts such as sodium and calcium hydroxides, sulfites, aluminates, phosphates and borates.⁷ The chemical process includes subsequent purification steps to remove high concentrations of by-products. An alternative method for obtaining high purity lactulose is bioconversion; the application of this process to convert lactose into lactulose has been studied using enzymes.⁸

Lactulose has several applications in medicine, including uses in chronic constipation, hepatic encephalopathy, colon carcinogenesis and ischemic stroke. The food industry uses lactulose in infant nutrition, yogurt, fermented soybean extract, fermented skimmed milk and also as a food additive. The European Commission (No. 575/2011)⁹ has approved the use of lactulose as a food ingredient in animal feed production due to several beneficial properties. The main benefits are: reducing antibiotic consumption, presenting prebiotic action, increasing mineral absorption, hyperglycemic and hypocholesterolemic effects, helping to prevent and reduce urinary tract infection, and also reducing intestinal transit time.

Whey is a by-product of cheese production, is a low-cost raw material and has a high concentration of lactose. Large amounts of the whey produced by the dairy industry are used for animal feed and as a food ingredient.¹⁰

However, about 30% of the annual worldwide production of whey are still discarded, and are considered as a water pollutant due to its high chemical and biochemical oxygen demand.^{11,12} The availability and cost-effective use of whey has become very important for dairy industries.

*e-mail: angelac.utfpr@gmail.com

In addition, environmental and economic concerns require the transformation of serum into value-added products.

Whey proteins are its most valuable constituents, and some industries use them as whey protein concentrates. These are obtained by pre-concentration using ultrafiltration followed by spray drying.¹³

Thus, ultrafiltration allows changes in component concentrations and ratios due to the selective retention of proteins and other colloidal materials, the partial retention of nitrogen compounds and the permeation of lactose and mineral salts.

However, the use of whey to obtain whey protein concentrates produces another by-product, the whey permeate, which is also a concern due to its polluting potential.

This by-product is rich in lactose and could be submitted to a spray drying process to obtain powdered lactose for commercial use. However, the atomization of whey permeate becomes unfeasible for industries due to the low commercial value of lactose and the loss of product by crystallization during storage, where part of the amorphous lactose becomes substituted with crystalline lactose.¹⁴

Besides, lactose is little used in the food industry because of its low sweetening power and low solubility.¹⁵⁻¹⁷ However, it can be used as added-value ingredient in many food products, including dairy products, meats, breads, pastries, snacks and beverages, mainly because of its recently discovered functional and bioactive properties.¹⁵

The concentration of lactose in milk and whey varies widely among species. Thus, the lactose content of bovine milk varies with race, other individual factors and especially with the lactation stage of the animal.¹⁸ Despite this variation, lactose is considered the main component of dairy products on a dry basis, and the most abundant solid in the serum, reaching 70% in whey powder.¹⁹

Besides, in optimization experiments studies, the optimal conditions can be achieved by varying the factors, each one in a different experiment.²⁰ However, this kind of approach involves a larger number of experiments, may not guarantee the determination of optimal conditions and does not present the interaction effect of all the factors involved. A good option to overcome this problem is the application of the response surface methodology (RSM). This optimization method is largely used to define the relation between the response and the independent variables. This tool can be applied to develop new products and processes, to improve products and methodologies, to optimize manufacturing processes and to minimize production cost.²¹

Souza *et al.*²² used RSM to optimize the saponification conditions to extract and determine tocopherols contents in peanut samples. A 2² experimental planning was used

to investigate the influence of two factors (concentrations of azuki and chia flour) on the fatty acid composition of chocolate cake.²³ Rodrigues *et al.*²⁴ studied the effect of genotype and roasting conditions on the fatty acid composition of peanut samples with a 3² factorial design.

The objective of this study was to propose an alternative for the reuse of lactose contained in powdered whey permeate by optimizing isomerization methodology for the production of lactulose, thus avoiding the disposal of this polluting by-product in the environment. In this way, the influence of two variables were evaluated: isomerizing type and reaction time of lactulose synthesis, in order to obtain the highest reaction yield, using RSM.

Experimental

Sampling

The powdered whey permeate was obtained from the ultrafiltration of whey to produce whey protein concentrate, followed by spray drying. This by-product was provided by a company from the western region of Paraná State, Brazil.

Reagents and standards

All reagents were of analytical purity and had different commercial origins. The reagents used for chromatographic analyses, such as acetonitrile (CH₃CN), were of HPLC grade, and ultra-pure water (18.2 MΩ cm deionized water, with conductivity 0.054 μS cm⁻¹ at 25 °C) was used. The lactulose standard used to prepare the calibration curve was purchased from Sigma-Aldrich and had a purity greater than 95%.

Isomerization of lactose using sodium sulfite (Na₂SO₃) and sodium hydroxide (NaOH)

The synthesis of lactulose from whey permeate lactose using Na₂SO₃ and NaOH at the concentration of 10% was based on the methodology of Ayder and Halleux²⁵ with some adaptations. It used 44.0 g of whey permeate reconstituted in 100 mL of water, and a mass of Na₂SO₃ (analytical pure) was added according to equation 1.

$$\text{Sodium sulfite mass} = \text{SM} \times \% \text{STS} \times \% \text{SLC} \times \% \text{LCF} \quad (1)$$

where SM is the sample mass, STS the sample total solids, SLC the sample lactose content and LCF the lactose concentration factor.

The pH value was adjusted to 11 using a 10% NaOH solution, and the samples were subjected to isomerization in a thermostatic bath (Tecnal, TE-0532) with stirring at

70 °C, and reaction times of 30, 60, 90, 135 and 180 min. After chilling, NaOH was removed from the reaction product using a chromatographic column with a diameter of 10 mm and a height of 30 cm packed with a cation exchange resin (Purolite SST60). The solution was precipitated with 5 mL of Carrez I solution (potassium ferrocyanide ($K_4[Fe(CN)_6] \cdot 3H_2O$) 15% (m v⁻¹)) and Carrez II solution (zinc sulfate ($ZnSO_4 \cdot 7H_2O$) 30% (m v⁻¹)). After 1 h of phase separation, it was filtered on filter paper and transferred to a 250 mL volumetric flask. Then, 100 mL of ethyl alcohol were added and the volume was adjusted to the meniscus with CH_3CN . The solution was then filtered through a 0.45 μm Millipore filter and analyzed by ultra-high performance liquid chromatography (UHPLC).

Isomerization of lactose using boric acid (H_3BO_3) and triethylamine ($N(CH_2CH_3)_3$)

For the preparation of lactulose from whey permeate lactose using H_3BO_3 and $N(CH_2CH_3)_3$, the methodology of Hicks and Parrish was followed.²⁶ A mixture containing 44.0 g of whey permeate, 0.8900 g of H_3BO_3 and 100 mL of water was placed into 250 mL Erlenmeyer flasks and the pH value was corrected to 11 using $N(CH_2CH_3)_3$ (analytical pure). The samples were immersed in a thermostatic bath (Tecnal, TE-0532) at 70 °C, and when the samples reached the reaction temperature, 10 mL aliquots were collected at time intervals of 30, 60, 90, 135 and 180 min. The amine was removed by a chromatographic column packed with cation exchange resin (Purolite SST60) with a diameter of 10 mm and a height of 30 cm. H_3BO_3 was removed with methyl acetate ($C_3H_6O_2$) by drying the sample in a rotary evaporator (Fisatom 802) under reduced pressure. The sample was washed with 40 mL of methanol and evaporated. This process was repeated three times. The final residue was dissolved in 10 mL of ultra-pure water, 10 mL of CH_3CN , filtered in a 0.45 μm Millipore filter and analyzed by UHPLC.

Isomerization of lactose by the enzymatic method

For the synthesis of lactulose from whey permeate using β -galactosidase enzyme, the methodology described by Adamczak *et al.*²⁷ was applied, with some modifications. An amount of 44.0 g of whey permeate was reconstituted in 100 mL of water, and 2.0 mL of β -galactosidase enzyme (Lactase Maxilact LX 5000, DSM Globalfood, from *Kluyveromyces lactis*) was added. The reaction was allowed to proceed for 5, 10, 20, 30, 40, 50, 60, 90, 135 and 180 min at 35 to 37 °C, with light stirring. At each time point, 1 mL aliquots were collected in Eppendorf tubes and heated at 100 °C for 10 min in order to inactivate the enzyme. After cooling, each sample was centrifuged (Hettich, Rotina 420R). The resulting supernatant was

filtered through a 0.45 μm Millipore filter and analyzed by UHPLC.

Chromatographic conditions for lactulose quantification in whey permeate powder

Chromatographic analyses were performed in UHPLC equipment coupled to a refractive index detector (UHPLC RI, Dionex Corporation, UltiMate 3000, quaternary pump, Sunnyvale, Software Chromeleon, USA). The separation was performed on a RezexTM RPM-monosaccharide Pb⁺² (8%) column (Phenomenex, 300 \times 7.8 mm, 0.8 μm), using ultra-pure water as the mobile phase. The flow rate was 0.40 mL min⁻¹ and the injection volume was 20 μL . The retention time was 17.9 min for lactose and 20.3 min for lactulose. Peaks were identified by comparison of their retention times with lactose and lactulose standards. Quantification was performed using the external standard method. The calibration curves for each sugar were prepared using five concentration levels, from 10 to 80 mg mL⁻¹ for lactose and lactulose, all dissolved in ultra-pure water. The limits of detection (LOD) and of quantification (LOQ) were estimated by triplicate analysis of standard calibration curves for lactose and lactulose, considering the signal-to-noise ratio relative to the background signal as 3 and 10, respectively.²⁸

Experimental design

A 3² full factorial design (two factors at three levels) with duplicates was performed to investigate the influence of two factors on the isomerization reaction of lactose. The two factors were the time of reaction (90, 135 and 180 min) and the type of isomerization (sodium sulfite, boric acid and enzyme), as shown in Table 1. The analyzed response was the lactulose concentration.

Statistical analysis and experimental optimization

All statistical analyses were conducted using Statistica software version 7.0 (StatSoft, USA) with a 5% ($p < 0.05$) significance level for rejection of the null hypothesis. Initially, the values of the main effects, interactions and analysis of variance (ANOVA) were obtained. Thereafter, the normality and homogeneity of variance of all variables was assessed using residual plots. Then, analysis of variance (two-way ANOVA between groups) was performed for all responses. To evaluate the effects of independent variables on the responses, RSM was applied. The basic model equation used to fit the data was (equation 2):

$$E(y) = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_{12}x_1x_2 + \beta_{11}x_1^2 + \beta_{22}x_2^2 \quad (2)$$

The complete model was showing according equation 3:

$$E(y) = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_{12}x_1x_2 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{12}x_1x_2^2 + \beta_{12}x_1^2x_2 + \beta_{12}x_1^2x_2^2 \quad (3)$$

where $E(y)$ is the expected response, β_0 is a constant, $\beta_1, \beta_2, \beta_{11}, \beta_{22}$ and β_{12} are the regression coefficients, and x_1 and x_2 are the levels of the independent variables.²²

The selection of the regression terms that compose the model equation was performed to maintain the mathematical hierarchy. First, the coefficients that correspond to the quadratic interactions were removed, then the linear interaction coefficients were removed, and finally, when necessary, the quadratic main effects were removed.²⁰

The optimization of the experiment consisted in obtaining a global response through the selection of some equations. The desirability function was applied with the transformation of the estimated response (Y_i) to the desired value (d_i), where $0 \leq d_i \leq 1$.

If the objective T is in the response, Y_i is the maximum value (equation 3):

$$d_i = \begin{cases} 0 & Y_i < L \\ \left(\frac{Y_i - L}{T - L}\right)^r & L \leq Y_i \leq T \\ 1 & Y_i > T \end{cases} \quad (4)$$

If the objective T is in the response, Y_i is the minimum value (equation 4):

$$d_i = \begin{cases} 1 & Y_i < T \\ \left(\frac{U - Y_i}{U - T}\right)^r & T \leq Y_i \leq U \\ 0 & Y_i > U \end{cases} \quad (5)$$

In this case, L is the lower limit and U the upper limit.

The convenience function is linear when the weight r is equal to 1. When the chosen $r > 1$, there will be greater emphasis on values close to the target. If the preference is $0 < r < 1$, it will have minor importance.

The individual desirability values (d_i) were combined through a geometric mean to form a global or general convenience (D). This unique value of D (0, 1) provides the overall assessment of combined convenience and the response levels, and D increases as the balance of properties becomes more favorable.²⁹

Results and Discussion

The chromatographic conditions for lactose and lactulose quantification in whey permeate powder were

available although the LOD and LOQ values. Thus, the LOD values were 1.30×10^{-3} and 1.21×10^{-3} mg mL⁻¹ for lactose and lactulose, respectively. The LOQ values were 4.35×10^{-3} and 4.02×10^{-3} mg mL⁻¹ for lactose and lactulose, respectively.

Table 1 presents the conditions of the 3² full factorial model (in duplicate) applied to the tests, as well as the experimental and model-estimated values obtained for the response (lactulose concentration in g 100 g⁻¹ of whey permeate powder). The residuals were obtained by calculating the difference between the experimental value and the value predicted by the model at each experimental point.

The main and interaction effects (linear and quadratic) were evaluated by ANOVA in order to verify their significance in the experimental model. The main quadratic effect of the time factor (x_1^2 , $p = 0.2642$) and the interactions $x_1x_2^2$ ($p = 0.2663$) and $x_1^2x_2$ ($p = 0.1027$) were not significant (Figure 1).

Initially, the 3² full factorial design presented a coefficient of determination equal to 0.9996 and $R_{\text{adjusted}}^2 = 0.9993$ (determination coefficient), which allowed a good explanation of the evaluated parameters to be obtained (time of reaction and type of isomerization). However, it was not possible to calculate the lack of fit since all degrees of freedom were compromised with the main effects and interactions (linear and quadratic). Thus, removing the non-significant effects mentioned above, it was possible to calculate the lack of fit with three degrees of freedom (non-significant effects eliminated from the model) to verify if the model with the main effects and interactions (containing linear and quadratic terms) was able to adequately explain the factors in lactulose synthesis. The withdrawal of the model terms must follow the mathematical hierarchy, the interaction terms with lower significance were removed at first, then, if necessary, the main quadratic terms were removed.

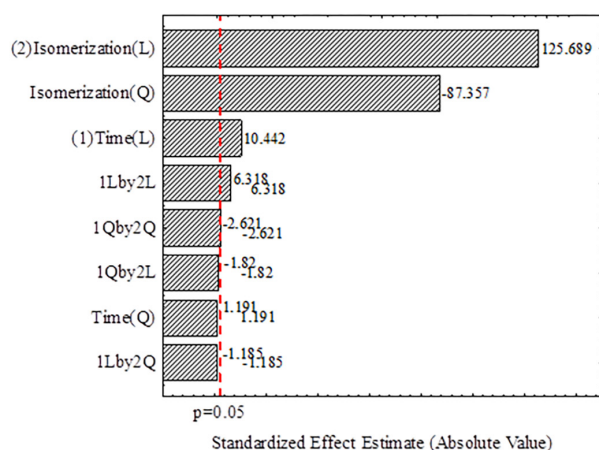
In this study, the interactions $x_1x_2^2$ ($p = 0.2663$) and $x_1^2x_2$ ($p = 0.1027$) were removed, this action allowed to obtain the lack of fit with two degrees of freedom (Table 3). A new Pareto graph (Figure 2) was constructed and the quadratic main effect x_1 (time) remained non-significant, but it was not removed from the model to maintain the mathematical hierarchy. In this process, it was verified that the determination coefficient (Table S1, in the Supplementary Information (SI) section) did not have a marked decrease and R_{adjusted}^2 was 0.9991. These values need to be very close, and in this study, it was possible to explain up to 99% of occurrences through these factors.

There was no lack of fit (Table 2), the residuals had a random distribution, normality and homogeneity in the

Table 1. Parameters of the 3² full factorial design (in duplicate) and the responses obtained in the assays

Assay	Independent variable level		Response		
	time / min	Isomerization	Lactulose / (g 100 g ⁻¹ of sample)	Predicted value ^a / (g 100 g ⁻¹ of sample)	Residual ^b
1	90	sulfite	7.44		-0.56
2	90	sulfite	7.99	8.00	-0.01
3	135	sulfite	9.01	9.33	-0.32
4	135	sulfite	10.30		0.97
5	180	sulfite	8.95	8.84	0.11
6	180	sulfite	8.65		-0.19
7	90	borate	3.90	3.11	0.79
8	90	borate	3.46		0.35
9	135	borate	4.70	5.01	-0.31
10	135	borate	4.01		-1.00
11	180	borate	6.39	6.28	0.11
12	180	borate	6.34		0.06
13	90	enzyme	43.61	44.52	-0.91
14	90	enzyme	44.86		0.34
15	135	enzyme	47.72	47.03	0.69
16	135	enzyme	47.01		-0.02
17	180	enzyme	49.80	50.10	-0.30
18	180	enzyme	50.32		0.22

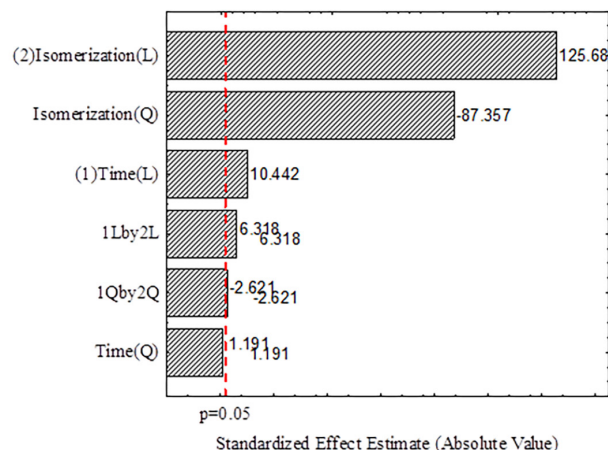
^aPredicted values of the model at each experimental point for the 3² factorial design; ^bresiduals of the model.

**Figure 1.** Pareto graphic representing the level of significance of each main effect and interaction (linear and quadratic), for the full 3² design.

variance, and the values predicted by the model (calculated) were very close to the experimental values (Table 1). All of these factors are important to attest to the quality in obtaining responses from factorial designs.²⁰

After removing the non-significant terms (Figure 1), it was possible to determine the model equation and their determination coefficients (R²) as listed in Table S1 (in the Supplementary Information (SI) section).

The data of the independent variables and the responses were analyzed to obtain the regression equation (Table S1), as well as the values of each main effect and the interaction

**Figure 2.** Pareto graphic representing the level of significance of each main effect and interaction (linear and quadratic) after remove of the non-significant effects, for the full 3² design.

between these effects, and the contribution of these effects to the model, using ANOVA.

Table S1 shows the values of the significant main effects and their interactions for the response, and Table 2 presents the results obtained by ANOVA for the 3² full design in duplicate for the studied response. The response surface was constructed for the levels and independent variables, as shown in Figure 3.

Table S1 and Figure 3 clearly show that the factor that contributed most to the response (concentration

Table 2. ANOVA results for the response studied in the 3² factorial model

Source	Sum of square	Degree of freedom	Mean square	F-test	p value
x ₁ (L)	30.688	1	30.688	109.04	0.000002
x ₁ (Q)	0.399	1	0.399	1.42	0.264230
x ₂ (L)	4445.980	1	4445.980	15797.63	0.000000
x ₂ (Q)	2147.705	1	2147.705	7631.31	0.000000
x ₁ (L) by x ₂ (L)	11.234	1	11.234	39.92	0.000138
x ₁ (Q) by x ₂ (Q)	1.934	1	1.934	6.87	0.027749
Lack of fit	1.324	2	0.662	2.35	0.150815
Pure error	2.533	9	0.281	–	–
Total	6641.796	17	–	–	–

x₁: time; x₂: isomerization; L: linear term; Q: quadratic term.

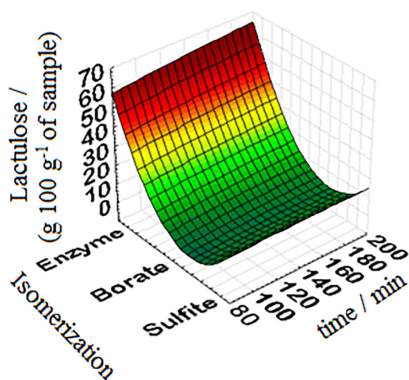


Figure 3. Response surface for the concentration of lactulose according to the type of isomerization and reaction time.

of lactulose) was the isomerization because the points corresponding to this main effect were further from zero (Table S1). It is possible to verify that the quadratic term of the isomerization type factor contributes negatively to the response, since at this level, sodium sulfite is present, which presented intermediate values in the synthesis of lactulose. The linear term obtained a greater and positive contribution to increase of the response, and it is represented by the enzymatic isomerization. Table S1 also shows that there is a positive contribution of the linear term of the time factor in the response, but a small one. The interaction effects among the significant factors presented a smaller contribution compared with the main effects on the response.

Therefore, the response surface (Figure 3) obtained from the 3² full factorial design indicated that the time investigated (three levels) had little influence on the reaction, and the use of β -galactosidase enzyme provided greater efficiency in the isomerization of lactose into lactulose.

The desirability function was used to determine the best conditions for the synthesis of lactulose from whey powder lactose. The investigated factors (reaction time (x_1)

and type of isomerization (x_2)) were evaluated to obtain the highest lactulose content at the end of the process, with the convergence of the target (desirable) to the maximum value (equation 4).

The optimum conditions consisted of a time (x_1) of 180 min and isomerization (x_2) using enzyme (Figure 4). Thus, it was not necessary to perform further experiments different from those proposed by the experimental design since the best working condition was the one of the experimental points.

The best mean result was 50.06 g of lactulose *per* 100 g of whey powder, and the value predicted by the model was 50.10 g 100 g⁻¹ (Table 1). The experimental standard deviation was low (± 0.3677), with a coefficient of variation of 0.7345%. The difference between experimental value and the predicted value was 0.04 g 100 g⁻¹ and the confidence interval was 46.7564 (–95%) to 53.3636 (95%).

Conclusions

The application of the factorial planning enabled the definition of the most adequate levels of both factors studied that produced the best response. It was possible to extract a maximum useful information from the system studied making a minimum number of experiments.

According to the response surface generated by the model, the optimum region of work, where a greater synthesis of lactulose was achieved, was throughout the time range evaluated for the reaction and the enzymatic isomerization with enzyme β -galactosidase. The desirability function indicated the best experimental conditions for lactulose synthesis, which consisted of a time (x_1) of 180 min and the use of enzymatic isomerization (x_2), obtaining a yield of 55.59%. In the optimization process, it is important to emphasize that the optimal point or region of highest efficiency is obtained.

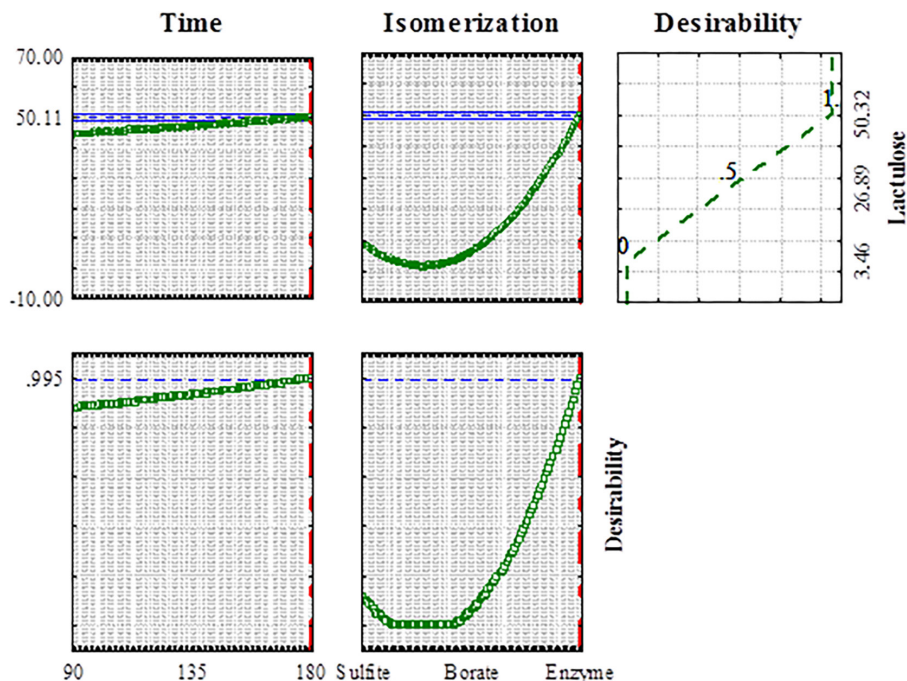


Figure 4. Graphic of the desirability function of the 3^2 design for optimization of lactulose synthesis.

In the present study, the process of isomerization of whey permeate for the production of lactulose was verified. This substance has many applications in the food and pharmaceutical industry, and its production from whey is also a viable method for minimizing the environmental impact of dairy production.

Supplementary Information

Supplementary information (Table S1) is available free of charge at <http://jbcs.org.br> as PDF file.

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