Research Article

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Increasing the fermentation efficiency of Lactobacillus paracasei ssp. paracasei MIUG BL6 in a rye flour sourdough

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Abstract

Background: The present study reports the optimization of the biotechnological conditions in order to improve the lactic acid fermentation process, in a rye flour medium by using Lactobacillus paracasei ssp. paracasei strain, coded MIUG BL6, as a starter culture.

Materials and methods: A sequential statistical methodology, comprised of Plackett-Burman experimental design, central composite design and response surface methodology, was applied to enhance the lactic acid fermentation productivity.

Results and discussion: Among the tested parameters, the milk powder and KH₂PO₄ concentration and also the time of fermentation were identified as the most significant variables that influence the fermentation process. The optimum levels of these significant parameters were determined as follows: 4.21% milk powder, 0.30% KH₂PO₄ and 48 h of fermentation that induced an increase of 1.6 fold of the acidity, compared to the fermentation conducted under the non-optimized conditions. Also, under these optimized fermentation conditions, a good rate of cell multiplication of 10.19 log N/N₀ was achieved.

Keywords: Lactobacillus paracasei ssp. paracasei; Rye flour; Lactic acid fermentation; Plackett-Burman experimental design; Response surface methodology.

Introduction

The production of sourdough bread can be traced back to ancient times. Sourdough is a mixture of flour, mainly wheat or rye [1] and water that is fermented with lactic acid bacteria (LAB), mainly heterofermentative strains, and thus results a mixture that contains lactic and acetic acids. Sourdough plays an important role in the preparation of bread dough to improve the technological properties (for
instance the dough machinability), nutritional properties (through phytate hydrolysis), organoleptic properties (bread volume, crumb texture, and a unique flavor), and storing properties (shelf-life) [2].

In the modern bakery technology, sourdough represents an alternative to baker’s yeast, especially for rye baking [3] (although bakers often use a combination of both of these leavening agents) to manufacture a variety of products such as bread, crackers, snacks, pizza and cakes [1, 4, 5]. The LAB that develop in the dough may originate from the selected flour indigenous microbiota or from a starter culture that contains one or several known species [6].

In order to improve the sourdough acidity, there are many factors to be taken into account, such as the substrate composition, the sourdough fermentation conditions (pH, temperature and fermentation time) and the inoculum concentration of starter [2].

In the last few years, statistical experimental methods such as Plackett-Burman (PB) design and response surface methodology (RSM) have been applied to optimize the fermentation media [7, 8] and the biotechnological conditions for the LAB cultivation [9].

The aim of this study was to evaluate the influence of some parameters, such as carbon and nitrogen sources (qualitative and quantitative influence), pH, temperature and fermentation time, on the metabolic behavior of Lactobacillus paracasei ssp. paracasei MIUG BL6 strain by cultivating in a rye flour medium, in order to improve the biotechnological process. Furthermore, as statistical modern tools, the PB was used to highlight the limiting factors for the lactic acid fermentation productivity whereas the subsequent use of the RSM led to the optimization of the conditions that can assure the highest increase of the acidity during the lactic acid fermentation.

A preinoculum was obtained by cultivating the bacteria in a stationary system on Man, Rogosa and Sharpe (MRS) broth [11], while incubating at 37°C, for 24 h. A rye flour basal medium (BM) containing 8.5 g/L reducing sugars was used for the fermentation. This medium was obtained by mixing 12.5% rye flour with 87.5% tap water, being further supplemented with 2% malt flour. In order to produce the starch gelatinization, the mixture was heated up to 75°C and maintained for 30 min, followed by a decrease of the temperature until 60°C with a maintenance time of 5 h. The BM was sterilized and then supplemented with carbon, nitrogen and mineral sources according to the optimization design variants. For each sample, 1% of the preinoculum was inoculated into 200 mL of liquid BM, with a final optical density of 0.5, measured at the wavelength of 600 nm by using a UV/VIS V-530 spectrophotometer (Jasco, Japan). Afterwards, the samples were homogenized and incubated under stationary conditions at different times and temperatures according to the experimental design variants. The fermented product was considered to be the rye sourdough.

**Materials and methods**

**Microorganism, inoculum preparation and rye medium fermentation**

*Lactobacillus paracasei* ssp. *paracasei* MIUG BL6 isolated from a soil sample (Galați area, Romania) is stored in the Microorganisms collection of Bioaliment Research Platform, “Dunărea de Jos” University of Galați, Romania. The strain was morphologically and biochemically characterized and identified by using the MicroStation™ Microlog System (Biolog, USA). The stock culture was stored in 20% glycerol, at −80°C [10].

PB design and RSM

The fermentation medium composition and the biotechnological conditions were optimized based on mathematical modeling and statistical analysis. Firstly, the most important variables in regards to their main effects on the fermentation behavior of LAB during lactic acid fermentation were identified by using the PB experiment, Design Expert software version 8.0.2.0 (Stat-Ease Inc., MN, USA) [12].

The dextrose, milk powder, yeast extract, KH₂PO₄, lactose, whey powder, soy protein isolate, inuline, inoculum concentration, temperature and time of fermentation were considered as possible factors (independent variables) that can affect the lactic acid fermentation. The titratable acidity was considered as the analyzed response. The experimental matrix used for the design of the independent variables variation is presented in Table 1. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as −1 (low level) and +1 (high level).

A first-order polynomial model (Eq. 1) was used for the mathematical modelling:

$$Y = \beta_0 + \sum \beta_i \chi_i$$

where $Y$ is the predicted response, $\beta_0$ is the model intercept, $\beta_i$ is the linear coefficient and $\chi_i$ is the level of the independent variable [8].
Secondly, the levels of variation of the significant parameters and their interaction effects on the fermentation process were analyzed and optimized by applying the central composite design (CCD), Design Expert software version 8.0.2.0 (Stat-Ease Inc., MN, USA) [12]. The most significant factors were chosen as independent variables, that were previously determined by PB, e.g. milk powder (A), KH₂PO₄ (B) and time of fermentation (C). This procedure involved 17 trials that varied these three independent variables at two different factorial levels (−1, +1), two axial points (α = −1.68179, α = +1.68179) and a center point (0). Thus, the experimental plan was undertaken in order to increase the lactic acid fermentation productivity being strictly correlated to the acidity variation of the fermented product [13]. The variation levels of the independent variables in CCD are given in Table 2.

The experimental data were fitted in accordance to Eq. 2 as a second-order polynomial regression equation.

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i x_i + \sum \beta_{ij} x_i x_j$$  \(2\)

where $$Y_i$$ is the predicted response, $$x_i$$ are the input variables which influence the response variable $$Y$$; $$\beta_0$$ is the offset term; $$\beta_i$$ is the linear coefficient; $$\beta_{ii}$$ the squared effect coefficient and $$\beta_{ij}$$ is the interaction effect [10].

### Table 1: The PB independent variables and the levels of variation.

| Independent variable   | Units | Abbr. | Levels of variation |
|------------------------|-------|-------|---------------------|
| Dextrose               | g%    | A     | −1 0 1              |
| Milk powder            | g%    | B     | 2.0 6.0             |
| Yeast extract          | g%    | C     | 1.0 3.0             |
| KH₂PO₄                | g%    | D     | 0.1 0.3             |
| Lactose               | g%    | E     | 2.0 6.0             |
| Whey powder            | g%    | F     | 1.0 3.0             |
| Soy protein isolate    | g%    | G     | 0.5 2.0             |
| Inuline                | g%    | H     | 0.3 1.0             |
| Temperature            | °C    | I     | 25 37               |
| Time                   | h     | K     | 24 72               |
| Inoculum               | OD₆₀₀nm | L | 0.3 0.7 |

### Analysis

The total titratable acidity (TTA) was chosen as the response for the optimization process. The LAB multiplication rate was also evaluated under the optimized fermentation conditions. All the experiments were done in triplicate.

#### TTA assay

TTA analysis values were evaluated for the fermented products according to the design of the experiments. For the TTA assay, 4 mL of sample was transferred into a 50 mL volume Erlenmeyer flask and diluted with 46 mL distilled water. After mixing, 10 mL of diluted sample was titrated with 0.1 N NaOH, using also 1% phenolphthalein solution (in 70% ethanol) as an indicator at pH 8.5, under shaking conditions [14]. TTA was expressed as the used 0.1 N NaOH volume (mL) [15, 16].

#### LAB counting

Regarding the sourdough, LAB viable cells were determined by using the plate count method [7]. Hereof, serial decimal dilutions of the fermented product were prepared in 0.1% (w/v) peptone water (Merck) and afterwards the cells suspension was subsequently plated on MRS agar supplemented with 1% CaCO₃ on Petri dishes. The plates were incubated for 48 h at 30°C. The plates containing between 30 and 350 colonies were selected for colony counting and the value corresponding to the CFU/mL fermented product was recorded. The rate of LAB multiplication was expressed as log N/N₀, where: N₀ is the CFU/mL after the starter culture inoculation; N is the CFU/mL after 48 h of lactic acid fermentation.

### Statistical analysis

Each experiment was carried out in triplicate and the data represents the mean of replicates. For the statistical analysis and mathematical modelling, PB experimental design and CCD were employed by using the Design Expert software version 8.0.2.0 (Stat-Ease Inc., MN, USA). The models analysis of variance was performed through Fisher test (F-test), by determining its associated probability $$P(F)$$ and the coefficient of determination ($$R^2$$) which measured the goodness of the regression model fitting. The differences with $$p < 0.05$$ were considered significant.
For each variable, the quadratic models were represented as contour plots and response surface curves.

Results and discussion

The parameters screening using the PB design

Due to the fact that the LAB are nutritionally pretentious and require several amino acids and vitamins to grow, it is very important to choose the right nitrogen and carbon sources based on quality and quantity. As carbon sources for the production of the lactic acid, refined sugars, such as glucose, sucrose, maltose, fructose and lactulose [17] are used more frequently than raw starchy substrates, such as barley, corn or wheat. Furthermore, a considerable amount of a complex nitrogen source, such as yeast extract, peptone, beef extract, skim milk, spy (horse serum) peptones, whey protein hydrolysates, must also be added to the medium in order to determine the lactic acid production within a reasonable timeframe [17]. These types of substances can contribute not only to a high biomass but also to a high level of organic acids production through lactic acid fermentation [18]. The nitrogen source is a major factor that influences the growth of Lactobacillus spp. On the other hand, high concentrations of nitrogen can lead to cell death [10]. Several agri-food by-products or wastes (such as sugarcane, molasses or whey as carbon sources) have been evaluated as substrates for the lactic acid production, the main aim being to lower the cost of the process [10].

In this study, the experiments were conducted to evaluate the effect of the selected independent variables, many of them being nutritive sources used for the LAB metabolism during the rye sourdough production.

Table 3 presents the results of the correlative effects of the analyzed variables using the PB.

The regression analysis was performed on the results and a first order polynomial equation was determined. The equation (Eq. 3) calculates the sourdough TTA in accordance to the independent variables.

\[
TTA = 1.46 + 0.23 B + 0.16 D + 0.16 J + 0.29 K
\]  

(3)

Statistical analysis of the analyzed response is presented in Table 4. The F value of 9.78 and the p-values lower than 0.05 denoted that the model terms and also the model are significant. Among the screened variables, the milk powder concentration and the time of fermentation were identified as the most significant variables that influence the organic acid production in regards to L. paracasei ssp. paracasei (Table 4).

These data are in agreement with those reported by Juodeikeiene et al., 2011 [19], who stipulated that the main factor that has an effect on the acidification was the amount of fermentable carbohydrates, although the production of organic acids depended also on other parameters such as temperature, time of fermentation and dough yield. As it can be seen from Table 4, the temperature seems to be a relevant independent variable for the fermentation process, but this parameter is strongly correlated to the bacteria physiology. Due to this fact, it cannot be subjected to a large gradient. However, for the next step of the optimization, the temperature was maintained constant at the value of 31°C.

Table 3: The PB for the screening of the most significant independent variables that influence the lactic acid fermentation of the rye sourdough, obtained with Lactobacillus paracasei ssp. paracasei MIUG BL6.

| Run | Independent variables | Response |
|-----|------------------------|----------|
| A   | B          | C      | D | E   | F | G   | H | J   | K | L | TTA |
| 1   | 6.0  | 3.0  | 3.0  | 0.3  | 6.0  | 3.0  | 0.5  | 0.3  | 25  | 72  | 0.3  | 1.8  |
| 2   | 2.0  | 3.0  | 5.0  | 0.1  | 6.0  | 3.0  | 2.0  | 0.3  | 25  | 24  | 0.7  | 0.9  |
| 3   | 6.0  | 1.0  | 5.0  | 0.3  | 2.0  | 3.0  | 2.0  | 1.0  | 25  | 24  | 0.3  | 1.2  |
| 4   | 2.0  | 3.0  | 3.0  | 0.3  | 6.0  | 1.0  | 2.0  | 1.0  | 37  | 24  | 0.3  | 1.5  |
| 5   | 2.0  | 1.0  | 5.0  | 0.1  | 6.0  | 3.0  | 0.5  | 1.0  | 37  | 72  | 0.3  | 1.5  |
| 6   | 6.0  | 1.0  | 3.0  | 0.1  | 6.0  | 3.0  | 3.0  | 0.5  | 1.0  | 37  | 24  | 0.7  | 1.7  |
| 7   | 6.0  | 3.0  | 3.0  | 0.1  | 2.0  | 3.0  | 0.5  | 1.0  | 37  | 24  | 0.7  | 1.0  |
| 8   | 6.0  | 3.0  | 5.0  | 0.1  | 2.0  | 1.0  | 3.0  | 0.5  | 1.0  | 37  | 24  | 0.7  | 1.4  |
| 9   | 6.0  | 3.0  | 5.0  | 0.1  | 2.0  | 1.0  | 2.0  | 0.3  | 37  | 72  | 0.3  | 2.3  |
| 10  | 2.0  | 3.0  | 5.0  | 0.3  | 2.0  | 1.0  | 3.0  | 0.5  | 1.0  | 25  | 72  | 0.7  | 2.2  |
| 11  | 6.0  | 1.0  | 5.0  | 0.3  | 6.0  | 3.0  | 0.5  | 0.3  | 37  | 24  | 0.7  | 1.3  |
| 12  | 2.0  | 1.0  | 3.0  | 0.1  | 2.0  | 1.0  | 0.5  | 0.3  | 25  | 24  | 0.3  | 0.7  |

*a, dextrose; B, milk powder; C, yeast extract; D, KH2PO4; E, lactose; F, whey powder; G, soy protein isolate; H, inulin; J, temperature; K, time; and L, inoculum.

Table 4: Statistical analysis of the PB mathematical model.

| Source | Sum of squares | Degree of freedom | Mean square | F-value | p-Value |
|--------|---------------|------------------|-------------|---------|---------|
| Model  | 2.23          | 4                | 0.56        | 9.78    | 0.0054  |
| B – Milk powder | 0.61          | 1                | 0.61        | 10.65   | 0.0138  |
| D – KH2PO4 | 0.30          | 1                | 0.30        | 5.28    | 0.0552  |
| J – Temperature | 0.30          | 1                | 0.30        | 5.28    | 0.0552  |
| K – Time | 1.02          | 1                | 1.02        | 17.90   | 0.0039  |
| Residual | 0.40          | 7                | 0.057       |         |         |
| Cor. total | 2.63          | 11               |             | 9.78    | 0.0054  |

$R^2 = 0.8482; \text{Adj } R^2 = 0.7614; \text{coefficient of variance } = 16.37\%.$
Fermentation process optimization by the RSM

CCD and RSM were employed to study the interactions among the significant factors and also to determine their optimal levels. The other variables in the study were maintained at a constant level which gave a maximal acidity in the PB evaluation step [20, 21]. The analyzed response (dependent variable) was the TTA (Table 5).

The numerical optimized values for the insignificant variables so that the yield of organic acids is increased are as following: dextrose 4.0 g%, yeast extract 3.0 g%, lactose 4.0 g%, whey powder 2.0 g%, soy protein isolate 0.5 g%, inuline 0.3 g%, temperature 31 °C and concentration of inoculum of 0.5 at DO600 nm.

Seventeen experiments were performed using different combinations of the variables. Based on the results of the experiments, the following second order polynomial equation that expresses the sourdough acidity in terms of milk powder (A, %), KH2PO4 (B, %) and time of fermentation (C, h) was obtained (Eq. 4):

\[
TTA = +1.67 - 0.24A + 0.089B + 0.36C - 0.038AB - 0.038AC - 0.11BC - 0.024A^2 - 0.17B^2 - 0.15C^2 - 0.012ABC - 0.027A^2B - 0.14A^2C + 0.33AB^2 - 0.33A^2B
\]

(4)

The statistical analysis of the analyzed response (TTA) is presented in Table 6. The F model response value was 51.34 and denoted that the quartic model is significant. There is only a 1.93% chance that the “Model F-Value” could occur due to noise. The values of the “Prob>F” smaller than 0.05 indicate that the factors of the model are significant. In this case A, C, BC, B2, C2, A2C, AB2, A2B2 are significant model terms.

The parity plot showed a satisfactory correlation between the experimental and predictive values (Figure 1). The closer the cluster points are to the diagonal line, the better the model fits, this fact could also be observed from the small difference between the experimental and the predictive values. Moreover, the adequacy of the model was also checked by other different criteria such as the coefficient of determination (R2) that was 0.9972.

Based on the obtained results, the concentration of milk powder and time of fermentation represented the most significant independent variables that improved the rye sourdough acidity with the help of L. paracasei ssp. paracasei strain.

Figures 2–4 show the response surface plots for the sourdough titratable acidity. The synergic effect of the milk powder and KH2PO4 concentrations on the improved sourdough acidity is displayed in Figure 2.

By increasing the milk powder concentration up to a central level of variation (4.21%) and maintaining the concentration of KH2PO4 at a minimum level of variation (0.30%) a positive effect on rye sourdough acidity was highlighted. Under these fermentative conditions the predictive acidity was approximately 2.38.
The interaction effect of the milk powder concentration and the time of fermentation (Figure 3) clearly indicate a proper combination that increases the rye sourdough acidity.

An increase of the milk powder concentration up to a central level of variation (4.21%) and also of the time of fermentation up to a maximum level of variation (48 h) gradually improved the rye sourdough acidity up to 2.38. It can also be observed that the increase of the milk powder concentration led to a different trend of organic acid production that was reversed.

A similar interaction effect was observed between the KH₂PO₄ concentration and the time of fermentation, which finally led to increased rye sourdough acidity. Maintaining the concentration of KH₂PO₄ at a minimum variation level and by increasing the time of fermentation up to 48 h, a final acidity of 2.38 was obtained (Figure 4). Hence, it was demonstrated that the KH₂PO₄ had a positive effect by increasing the rye sourdough acidity. Very good results were obtained at a concentration of 0.3% (w/v) KH₂PO₄ in the fermentation medium. The results are in agreement to Coelho et al., 2011 [10]. It was reported that the addition of phosphate to the culture medium increased the growth of the LAB and enhanced the lactic acid production. Honorato et al., 2007 [22] reports, the highest production of lactic acid was 90.2 g/L, obtained in a medium that contained 220 g/L of molasses, 45 mL/L of CSL, 3 g/L of KH₂PO₄ and 1.5 mL/L of Tween 80. The use of KH₂PO₄ was reported to provide K⁺ and phosphate for the microorganism growth and that it also acts as a buffering agent in the medium [22].
Further, the milk powder, KH$_2$PO$_4$ and the time of fermentation were considered the most important factors that influence the rye sourdough acidity obtained by using as starter *L. paracasei* ssp. *paracasei*. Nonetheless, both the carbon and nitrogen sources cause the inhibition of lactic acid production at higher concentrations due to the repression effect [10]. Honorato et al., 2007 [22] reported that the addition of phosphate to the culture medium increased the growth rate of the microorganism and enhanced the lactic acid production, as this component maintains the pH near the optimal growth value, thereby allowing the conduction of fermentation for a longer period of time. Kishor et al., 2006 [23], reported that when the optimum values of the parameters obtained through RSM (25.0 g/L date sugar, 15.0 g/L sodium acetate, 19.1 g/L peptone, and 4.7 g/L KH$_2$PO$_4$) were applied, the lactic acid production (22.7 g/L KH$_2$PO$_4$) increased by 50.33%, compared to the non-optimized media (15.1 g/L). Instead, according to Kitouni and Oulmi, 2013 [24], K$_2$HPO$_4$ as a source of phosphate appears to have no significant effect on the lactic acid production. Nonetheless, this effect depends on the used starter culture species and the general fermentation conditions that have a strong influence on the fermentation process.

In this study, for the cultivation of *L. paracasei* ssp. *paracasei* MIUG BL6 starter culture on a rye flour medium, the highest level of acidity was obtained by controlling the concentration of milk powder (4.21%) and KH$_2$PO$_4$ (0.30%), after 48 h of fermentation.
Validation of the model

The validation of the mathematical model was carried out under the conditions predicted by the model. Another two randomly picked validation experiments were carried out within the range of the explored experimental parameters. Table 7 presents the experimental design, the actual and the predicted values, and their standard deviations (percentage errors) in regards to the RSM model. The final analysis involved the comparison between the model’s predicted values and the experimentally validated values. Therefore, the average error was below 15% (10.13%, respectively) thus leading to the conclusion that the optimization can be validated and the model can be accepted.

The statistical analysis of the data indicated that the titratable acidity values increased over a period of time (2.20 after 48 h of fermentation), the fermentation time being the most significant variable that influence the acidity of the rye sourdough obtained with \textit{L. paracasei} ssp. \textit{paracasei}. By counting the viable cells from the fermented product, it was observed that the LAB presented the highest microbial count, with a rate of multiplication of 10.19 log N/N\textsubscript{0}, after 48 h of fermentation (Table 7).

These observations are in accordance with those reported by other authors \cite{5, 25} which reported the ability of some LAB strains to multiply on an optimized medium based on rye flour. After 5 days of fermentation, the starter culture cell density reached values ranging from ca. 9.1 to ca. 9.4 log CFU/g rye sourdough.

Conclusions

This study demonstrated the ability of \textit{L. paracasei} ssp. \textit{paracasei} MIUG BL6 to grow and to produce the lactic acid fermentation in a BM based on rye flour and supplemented with carbon and nitrogen sources in an equilibrated ratio.

Through mathematical modeling and statistical analysis, the fermentation conditions were established so that an increasing acidity of 1.6 folds was obtained after 48 h of fermentation. The most important factors concerning the fermentation performance and the sourdough quality (acidity and viable LAB cells) are represented by milk powder, KH\textsubscript{2}PO\textsubscript{4} concentrations and time of fermentation. Nonetheless, the temperature could also have an influence on the lactic acid fermentation productivity, being strongly correlated to the starter culture physiological characteristics.

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