**A multifactorial design for studying factors influencing the growth and
tyramine production of the lactic acid bacteria *Lactobacillus brevis* CECT
4669 and *Enterococcus faecium* BIFI-58**

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ABSTRACT

A Central Composite Face design was used to study growth and tyramine production by two strains of lactic acid bacteria, *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58. The effects of five physicochemical factors (incubation temperature and time, environmental pH, added tyrosine concentration, and pyridoxal-5-phosphate (PLP) supplementation) on cell growth and tyramine production were analysed under aerobic or anaerobic conditions. The parameters of the quadratic model, for each response variable, were estimated by Multiple Linear Regression (MLR), and the statistical analysis of the results allowed the elucidation of mathematical models capable of predicting the behaviour of the responses as a function of the main variables involved in the process. Incubation time was found to be the most important variable influencing growth in *L. brevis* while pH showed the highest contribution in *E. faecium*. The production of tyramine was dependent on the added tyrosine concentration and incubation time. The proposed MLR model predicted the optimum conditions that gave maximum responses for *L. brevis* and *E. faecium* growth and tyramine production. This model predicted in both strains that anaerobic condition at acidic pH (4.4) favours tyramine production. Thus, a similar behavior was predicted for the two tyramine-producing strains of lactic acid bacteria in relation to tyramine production.

Keywords: biogenic amines; lactic acid bacteria; tyramine; *Lactobacillus brevis*; *Enterococcus faecium*

1.Introduction

During recent years biogenic amines (BA) have been often associated to food intoxications. Histamine and tyramine have been the most studied BA due to their toxicological effects [6]. One of the roles suggested for tyramine is as “false neurotransmitter” or “endogenous amphetamine”. It is known that diet rich in tyramine can increase blood pressure and cause migraine, and that imbalances in the level of tyramine is thought to underlie altered brain function in many pathological conditions, including dystonias, Parkinson´s disease, schizophrenia, drug addiction, and mood disorders [18]. It has also been suggested that tyramine may partake in neurodegenerative disease processes by causing oxidative stress [21].

In fermented foods, BA are mainly generated by decarboxylation of the corresponding amino acids through substrate-specific enzymes of the microorganisms present in the food. The capability of BA formation has been described for several groups of microorganisms, mainly *Enterobacteriaceae*, *Pseudomonas* spp., enterococci and some other lactic acid bacteria (LAB) [17, 19, 22]. The LAB known to possess significant tyrosine decarboxylase activity are enterococci in cheeses [5], lactobacilli in wines [14, 15, 16], *Carnobacterium divergens* in fish [2] and pediococci in beers [7].

Enterococci can be readily isolated from foods, including a number of traditional fermented foods. There are many environmental factors that can affect the amine formation by enterococci. Gardini et al. (2001) have studied the combined effects of temperature, pH and NaCl concentration on the growth dynamics of *Enterococcus faecalis* EF37, its proteolytic activity and its production of BA [4].

A remarkable potential to form BA was observed in some strains of lactobacilli [22]. It was reported that within the *L. brevis* species a high number of tyramine producer strains

could be found. Indeed, only a few reports have described physiological studies of the influence of some physicochemical factors on tyramine production by *L. brevis*. Moreno-Arribas and Lonvaud-Funel (1999) have studied the influence of several effectors common in wines on the tyramine production by an oenological strain of *L. brevis* [14].

Since enterococci and *L. brevis* are usually recognized as tyramine producer LAB in food products and that tyramine is generated by microbial spoilage of food high in protein content or through processing, ripening and storage of fermented foodstuff results evident that the tyramine content of food depends on the biotechnological processes involved in the production procedure as far as food ripening. Therefore, the development of new manufacture or storage techniques (e. g., addition of proteolytic enzymes or proteolytic starter cultures to accelerate cheese ripening, microoxygenation during wine-making process, vacuum or modified atmosphere packaging of meat or fish products,...) made necessary to determine the effects of some technological factors on the kinetics of cell growth and tyramine production by lactic acid bacteria. The influence of the combined effects of these factors was tested with a Central Composite Face design (CCF) by using a quadratic mathematical model. Also, the fitted models were used to predict the optimum growth and tyramine production.

Materials and methods

2.1. LAB strains and growth conditions

The two LAB strains used in this study were of food origin. *Enterococcus faecium* BIFI-58 was isolated from a grape must and *Lactobacillus brevis* CECT 4669 (ATCC 8287) was originally isolated from a green fermenting olive and was provided by the Spanish Type Culture Collection (CECT).

Stocks cultures were kept at -80°C in MRS broth (Pronadisa, Spain) [12] supplemented with 10% glycerol. For inoculum preparation, the LAB strains were subcultured in MRS broth overnight at 30°C in a 5% CO_2 atmosphere. These fresh cultures were used to inoculate the tubes at 0.1% (v/v). The initial population of LAB present on each experimental run was 1×10^6 CFU per ml for both strains.

2.2. Culture medium preparation

The medium used for the tyramine formation was MRS medium with slightly modifications to allow the preparation of the different supplemented media required in the experimental design. The modified medium contained per litre: Peptone (Difco, St. Louis, MO, USA), 10 g; Meat extract (ADSA, Spain), 8 g; Yeast extract (Scharlau, Spain), 4 g; Glucose, 20 g; Tween 80, 1ml; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.2 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.05 g. The ammonium citrate (2 g/l) used in the original media was changed by ammonium acetate (2 g/l) since citric acid was used to adjust the pH. To carry on the experimental design the medium was supplemented with different concentrations of tyrosine and pyridoxal-5-phosphate (PLP), as cofactor for the decarboxylation reaction [14]. To deal with the solubility difficulties of tyrosine we added the corresponding amount of tyrosine *di*-sodium salt (Sigma, USA), with a solubility of 50 g/l. In order to improve the buffer effect and to neutralize the acid produced, we added Na_2HPO_4 to achieve an ionic strength of 100mM. The media was adjusted to the corresponding pH with a solution of 100 mM citric acid. And finally, the different media were distributed in tubes and autoclaved. After autoclaving, the media pH was checked and its variation was lower than 0.2 pH units. All the experiments were performed under non-shaken aerobic and anaerobic conditions in parallel. The anaerobic conditions were achieved by overlaying the tube with paraffin prior autoclave.

2.3. Experimental design

The effects of five physicochemical factors: incubation temperature (Tem) and time (Tim), environmental pH (pH), added tyrosine concentration (Tyr), and PLP supplementation (PLP) on growth and tyramine production by two LAB strains, *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58, were studied using a CCF design. A total of 29 experiments: 2^{5-1} points of a fractional factorial design, 10 star points placed on the faces of the sides, and 3 centre points to estimate the experimental error, were carried out in randomised run order. By using this design, the five factors were tested at 3 different physical levels: incubation temperature (22, 27, and 32 °C), environmental pH (4.4, 6, and 7.6), added tyrosine concentration (0, 1, and 2 g/l), PLP supplementation (0, 200, and 400 µM), and incubation time (1, 4, and 7 days). The coded levels of the factors are -1, 0, and 1.

The response variables were the following: optical density (OD) at 600 nm and tyramine concentration (mg/l), by the two LAB strains, for aerobic or anaerobic conditions. Table 1 shows the experimental matrix design, with the experimental levels of the independent variables (factors), along with the results obtained for each one of the eight response analyzed variables (Y_1 - Y_4 for OD at 600 nm and Y_5 - Y_8 for tyramine concentration).

The full quadratic polynomial model proposed for each response variable (Y_i) was:

$$Y_i = \beta_0 + \sum_{i=1}^5 \beta_i X_i + \sum_{i=1}^5 \beta_{i,i} X_i^2 + \sum_{i=1}^5 \sum_{j=i+1}^5 \beta_{i,j} X_i X_j + \varepsilon, \text{ where } \beta_0 \text{ is the intercept; } \beta_i \text{ the first-order}$$

model coefficient; $\beta_{i,i}$ the quadratic coefficient for i th variable; $\beta_{i,j}$ the interaction coefficients for the interaction of variable i and j ; X_i and X_j the independent variables (physicochemical factors); and ε is the error variable. The parameters of the model, for each of eight response variables (Y_1 - Y_4 for OD at 600 nm and Y_5 - Y_8 for tyramine concentration)

were estimated by multiple linear regression (MLR) using the Statgraphics Plus v.5.1 program (Statistical Graphics Corporation, Manugistics Inc., MD, USA, 2000). This program permits both, the creation and analysis of experimental designs. The effect of the each term in the model and their statistical significance, for each of the response variables Y_1 to Y_8 , was analyzed from the standardized Pareto Chart. The terms not significantly different from zero ($P>0.10$), were excluded of the model and the mathematical model was re-fitted by MLR. The goodness of fit of the model was evaluated by the coefficient of determination (R^2), the residual standard deviation (RSD), and the lack of fit test for the model from the ANOVA table. The lack of fit test is designed to determine whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors. From the new fitted models, the optimum conditions, that maximized each of the responses variables, were obtained.

2.4. Cell growth

The evaluation of bacterial growth was monitored using absorbance readings at 600 nm of MRS cultural broth conditioned according to the CCF. The MRS broth was inoculated with 10^6 cells/ml. The OD was performed by means of a S-22 UV/vis spectrophotometer (Boeco, Germany). When the OD was higher than 0.7, the sample was diluted two- or fourfold in the same media and the resulting absorbance value was multiplied by the corresponding dilution factor to achieve the final OD value.

2.5. Tyramine determination

1
2 Tyramine was determined by reverse-phase high performance liquid chromatography
3 (RP-HPLC) as previously described [17]. A Waters (Waters, Milford, MA) liquid
4 chromatograph consisting of a Waters 600 Controller programmable solvent module, a WISP
5 710B autosampler and a Waters 420 fluorescence detector was used. Chromatographic data
6 were collected and analyzed with the Millenium³² system (Waters, Milford, MA). Samples
7 were submitted to an automatic precolumn derivatization with *o*-phthaldialdehyde (OPA),
8 prior to injection. The reaction solution consisted of 350 mg of OPA and 2.5 ml 2-
9 mercaptoethanol (MCE) in 47.5 ml methanol. All separations were performed on a Waters
10 Nova-Pak C₁₈ column (150 x 3.9 mm i.d., 60 Å, 4 µm) with a matching guard cartridge of the
11 same type. Derivatized amines were detected using a fluorescence detector (excitation
12 wavelength of 340 nm and emission wavelength of 425 nm). Samples were injected in
13 duplicate onto the column after being filtered through a 0.45 µm filter (Millipore, Bedford,
14 MA, USA).

17 3. Results

19 3.1. Effects of physicochemical factors on growth

21 As mentioned in the Materials and Methods section, Table 1 reports the measured
22 responses on bacterial growth expressed in absorbance units by two LAB strains
23 (*Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58) in aerobic and
24 anaerobic conditions, obtained for all the experiments corresponding to the matrix design.

MLR was applied to estimate the parameters of the proposed model to each of the four response variables related to OD at 600 nm (Y_1 to Y_4). Fig. 1 shows the standardized Pareto Charts, that illustrate the dominant terms in the model affecting each of the response variables (Y_1 to Y_4), and their statistical significance. It can easily be seen that the terms, significantly different from zero ($P < 0.05$), that have the strongest influence in the response Y_1 are the incubation time (Tim), having a positive effect, and the interaction incubation temperature*time (Tem*Tim), having a negative effect (Fig. 1). The incubation time (Tim) with a significant positive effect ($p < 0.05$), and the interactions pH*incubation time (pH*Tim), and incubation temperature*time (Tem*Tim), with a significant negative effect ($p < 0.05$), have the strongest influence in Y_2 . Therefore, effects concerning growth of *Lactobacillus brevis* CECT 4669 in aerobic and anaerobic conditions are similar, although in anaerobic condition the interaction pH*Tim exerts a significant negative influence.

As showed in Fig. 1, the terms on the model that mainly influence the responses Y_3 and Y_4 are very similar; so that environmental pH (pH) with a significant positive effect and their quadratic term (pH*pH), with a significant negative effect, have the strongest influence in both variables. Therefore, under aerobic and anaerobic conditions *Enterococcus faecium* BIFI-58 showed a similar behaviour.

From the analysis of the statistical significance of the estimated regression coefficients for each of the response variables Y_1 to Y_4 , the terms in the model not significantly different from zero ($P > 0.10$), were excluded of the same one, and the mathematical model was re-fitted by MLR. The new estimated regression coefficients, for each of the four responses related to OD at 600nm, are listed in Table 2. From the results shown in Table 2, the following conclusions can be drawn: (i) all estimated models did not have lack of fit (P-values of lack of fit test > 0.05), or the model appear to be adequate for the observed data at the 95.0% confidence level; (ii) the fraction of variation of the response explained by the model (R^2) was

> 0.89 for all the responses with exception of Y_1 ; and (iii) the residual standard deviation is smaller to 0.4 in all the cases. All estimated models were found to adequately describe the data except the corresponding one to Y_1 .

Table 3 shows the predicted values, using the fitted models, for the four responses related to OD at 600 nm at the optimum conditions, provided by the statistical program. As can be seen, these predicted optimum conditions were very similar for the responses variables Y_3 and Y_4 , in respect to added tyrosine concentration (0.8 -1.1 g/l), environmental pH (6.7 and 6.4), and incubation time (7 days). Therefore, *E. faecium* BIFI-58 showed a similar behavior in aerobic and anaerobic conditions, with a predicted value of OD 2.2. However, *L. brevis* under anaerobic growth reached the maximal OD (3.08) and was predicted at the most acidic pH (4.4) and the lowest temperature (22 °C) tested. In aerobic conditions, *L. brevis* growth could be predicted to reach only a maximal OD of 2.58 at similar temperatures.

3.2. Effects of physicochemical factors on tyramine production

The same medium on which the optical density has been determined was used for the determination of tyramine produced by *L. brevis* CECT 4669 and *E. faecium* BIFI-58. Table 1 reports the measured responses on tyramine production in aerobic and anaerobic conditions, obtained for all experiments corresponding to the matrix design.

MLR was applied to estimate the parameters of the proposed model to the four response variables related to tyramine production (Y_5 to Y_8 in Table 1). Fig. 2 shows the standardized Pareto Charts for each of the four responses, illustrating the importance and the statistical significance of the different terms in the model. It can easily be seen that, for all response variables, the profile of the coefficients that mainly influence the responses is quite similar, meaning a similar behavior of both LAB strains, *Lactobacillus brevis* CECT 4669

and *Enterococcus faecium* BIFI-58 in aerobic and anaerobic conditions. Added tyrosine concentration (Tyr), incubation time (Tim) and the interaction tyrosine concentration*incubation time (Tyr*Tim), with a significant positive effect ($p < 0.05$), and the quadratic term of tyrosine concentration (Tyr*Tyr), with a significant negative contribution, have the strongest influence in the response variables Y_5 to Y_8 (Fig. 2). The pH (pH) have a significant negative effect in the tyramine production *L. brevis* CECT 4669 in aerobic and anaerobic conditions (variables Y_5 and Y_6), and several additional factors seem to affect significantly tyramine production by *E. faecium* in anaerobic conditions (Fig. 2).

The mathematical model was re-fitted in order to exclude the terms not significantly different from zero ($p > 0.10$), and the estimated regression coefficients for each of the four responses related to tyramine production are also listed in Table 2. From these results, the following conclusions can be drawn: all estimated models were found to adequately describe the data (P-values of lack of fit > 0.05); the fraction of variation of the response explained by the model (R^2) was > 0.82 for all the responses with exception of Y_7 ; the residual standard deviation is smaller to 35 mg/l in all the cases. All the estimated models were found acceptable to describe the data and to know the optimal conditions for tyramine production. Table 3 shows the predicted values, using the fitted models, for the four responses related to tyramine production at the optimum conditions, provided by the statistical program. As can be seen, these optimum conditions were very similar for all responses (1.7-1.9 g/l for added tyrosine concentration, 4.4-4.6 for environmental pH, and 7 days) except the incubation temperature and PLP supplementation. It seems that higher temperatures are needed in aerobic conditions (32 °C) to obtain a maximal tyramine production in both strains. However, during anaerobic growth predicted maximal tyramine production is achieved at 22-24.5 °C. In the same way, both strains seem to require the highest PLP supplementation (400 μ M) during anaerobic growth to obtain maximal tyramine production. The predicted values at these

optimum conditions were 184.9 – 211.7 mg/l during aerobic growth, and 251.5 – 296.5 mg/l in anaerobic growth of *L. brevis* and *E. faecium*, respectively.

4. Discussion

In LAB the environmental control seems to play an important role in tyramine production. Several factors (the incubation temperature, the pH and the growth medium composition) are known to affect bacterial growth and decarboxylase activity [20]. In the results obtained from this study, incubation time was found the most important variable influencing *L. brevis* growth; however, in *E. faecium* growth was mainly influenced by pH (Figure 1).

There are many environmental factors that can affect the tyramine formation by LAB. Some studies have investigated the effects of these factors, but little information exists on their combined or interactive effects. Kimura et al. (2001), described that the halophilic lactic acid bacterium *Tetragenococcus muriaticus* forms histamine at low-pH under O₂-limiting conditions during the stationary growth phase [11]. However, Masson et al. (1997) reported for another lactic acid bacteria, a *Carnobacterium divergens* strain, that oxygen availability did not affect tyramine production and maximal tyramine production occurred during the stationary phase in acidic conditions obtained by low initial pH (<5) [13]. Since both results were obtained for two different amines and different LABs under different conditions, any definite conclusion could be taken. In our study, the proposed MLR model predicted optimal values for tyramine production under anaerobic atmosphere for both LAB strains analyzed, *L. brevis* and *E. faecalis* (Table 3).

Amino acid decarboxylating enzymes show optimum activity at acid pH as a mechanism of environment neutralization against an excessive pH decrease which is incompatible with bacterial growth [3]. Furthermore, according to Chen et al. (1989) [1] and Halász et al. (1994) [6], the growth of bacteria in an acidic medium stimulates the formation of decarboxylases and, therefore, the biosynthesis of amines from amino acids, in order to protect the bacteria against the acidic medium. Similarly in our study, predicted maximal tyramine production will be achieved at the most acidic pH (4.4) tested (Table 3).

Since low pH and anaerobiosis are unavoidable during the fermentation of salted and fermented products, other factors should be considered to prevent tyramine formation in these products. It was previously shown by Joosten (1988) that tyramine formation in cheese was dependent on tyrosine [9]. However, supplementation with tyrosine as the precursor of tyramine had no effect on tyramine formation in bottled beer [10]. Similar information was reported by Izquierdo-Pulido et al. (2000) who did not observe a direct relationship between free tyrosine level in wort (0.1-0.2 g/l) and tyramine formation during fermentation [8]. In both studies tyrosine was added in doses which approximately duplicated or triplicated the usual content of those free amino acids. Levels of free tyrosine thus do not seem to be critical factors in tyramine formation in bottled beer. However, in our study on which higher tyrosine concentrations were assayed, in both strains, the production of tyramine was greatly dependent of the tyrosine added to the culture media (Figure 2). As showed in Fig. 2, the interactions involving added tyrosine concentration are influential factors affecting tyramine production.

Most authors have noted that good cell growth frequently goes hand in hand with tyramine production. Gardini et al. (2001) reported that the production of tyramine under the tested conditions was found to be mainly dependent on the extent of growth of *E. faecalis*. They concluded that the main biological feature influencing tyramine formation was the

1 extent of microbial growth [4]. Nevertheless, optimal cell growth does not always result in
2 high tyramine production levels. The model proposed in this work predicted maximal extent
3 of growth and tyramine production in anaerobic conditions for both strains; however, factors
4 that affect tyramine production are different to those affecting growth (Figures 1 and 2). Our
5 results are in agreement with Connil et al. (2002) who found that the most influential factors
6 on growth did not greatly affect tyramine production by *Carnobacterium divergens* under the
7 experimental conditions used [2]. Taken into account all these results, growth seems to be an
8 essential but insufficient condition for tyramine production and we could conclude that the
9 extent of growth is not the main biological feature influencing tyramine formation.

10 The physicochemical factors analyzed affected tyramine production by different LAB
11 strains, *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58, mainly in the
12 same way. In fact, it seems that the common factors are a high concentration of precursor
13 amino acid, an acidic environmental pH and growth under anaerobic conditions. The model
14 that predicted optimum conditions for tyramine production confirms earlier results showing
15 that amino acid decarboxylases are markedly induced by growth under anaerobic conditions
16 at low pH in rich media containing excess amino acids [3]. Therefore, when possible, these
17 factors should be adjusted in order to minimize tyramine formation risk during food
18 processing.

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Legends of figures

Fig. 1. Standardized Pareto Chart Plot with the effect of each term in the model divided by its standard error, for the four response variables related to OD at 600 nm (Y_1 to Y_4). The vertical line in the chart tests the significance of the effects at 95% confidence level. Positive (+) and negative effects (-) in the response variables are indicated by different bar shading.

Fig. 2. Standardized Pareto Chart Plot with the effect of each term in the model divided by its standard error, for the four response variables related to tyramine production (Y_5 to Y_8). The vertical line in the chart tests the significance of the effects at 95% confidence level. Positive (+) and negative effects (-) in the response variables are indicated by different bar shading.

Table 1. Experimental matrix design and results obtained for each response variable.

Exp No.	Factor					Response variable							
						OD at 600 nm				Tyramine concentration (mg/l)			
						<i>Lactobacillus brevis</i>		<i>Enterococcus faecium</i>		<i>Lactobacillus brevis</i>		<i>Enterococcus faecium</i>	
						Aerobic (Y ₁)	Anaerobic (Y ₂)	Aerobic (Y ₃)	Anaerobic (Y ₄)	Aerobic (Y ₅)	Anaerobic (Y ₆)	Aerobic (Y ₇)	Anaerobic (Y ₈)
1	0	4.4	0	22	7	1.49	1.73	0.20	0.52	0.00	0.00	0.00	0.00
2	2	4.4	0	22	1	0.18	0.22	0.02	0.09	2.98	0.51	22.02	27.97
3	0	7.6	0	22	1	0.40	0.66	1.88	1.40	0.00	0.00	0.00	0.00
4	2	7.6	0	22	7	2.80	0.87	1.04	1.60	0.00	0.00	54.41	127.02
5	0	4.4	400	22	1	0.37	0.38	0.02	0.07	21.93	18.43	7.91	5.55
6	2	4.4	400	22	7	2.13	3.08	0.22	0.56	181.01	281.80	145.99	276.65
7	0	7.6	400	22	7	0.96	1.06	1.90	1.66	4.26	4.78	0.00	8.72
8	2	7.6	400	22	1	0.61	1.08	1.17	1.41	5.63	10.03	60.08	71.38
9	0	4.4	0	32	1	0.62	0.52	0.06	0.13	11.38	6.02	16.02	8.52
10	2	4.4	0	32	7	1.30	1.24	0.66	0.74	210.72	166.77	211.50	174.83
11	0	7.6	0	32	7	0.63	0.86	1.59	1.29	0.00	0.00	14.88	14.66
12	2	7.6	0	32	1	0.84	1.86	1.69	2.01	1.00	5.25	102.93	149.42
13	0	4.4	400	32	7	1.00	0.94	0.23	1.09	8.56	12.25	13.66	22.50
14	2	4.4	400	32	1	0.80	0.78	0.08	0.22	55.69	66.84	43.02	51.85
15	0	7.6	400	32	1	0.64	0.66	1.14	1.55	0.00	1.40	0.00	0.21
16	2	7.6	400	32	7	0.94	1.11	1.15	1.43	165.79	149.76	218.31	193.45
17	0	6	200	27	4	1.75	1.96	1.74	1.63	17.83	5.73	11.98	2.87
18	2	6	200	27	4	1.18	1.52	1.56	2.21	71.59	56.17	68.81	55.17
19	1	4.4	200	27	4	2.11	1.98	0.74	0.78	145.85	177.81	165.39	146.41
20	1	7.6	200	27	4	0.89	1.03	1.77	2.22	125.36	100.51	106.32	90.42
21	1	6	0	27	4	1.52	1.59	1.76	1.83	82.60	76.28	80.23	77.59
22	1	6	400	27	4	1.41	1.73	1.84	2.15	77.30	72.24	70.52	71.77
23	1	6	200	22	4	1.64	1.64	1.49	2.47	70.79	65.76	71.12	142.35
24	1	6	200	32	4	1.52	1.18	1.79	2.06	96.30	63.46	73.05	70.90
25	1	6	200	27	1	0.96	0.77	2.03	1.94	46.11	44.46	135.85	48.00
26	1	6	200	27	7	2.03	1.50	2.21	2.25	160.73	114.71	122.31	98.83
27	1	6	200	27	4	1.88	1.67	1.66	2.17	167.93	145.67	129.95	114.58
28	1	6	200	27	4	1.79	1.88	1.67	1.61	126.71	106.78	104.73	116.58
29	1	6	200	27	4	1.22	1.95	1.60	2.13	136.52	84.69	100.07	73.20

Table 2. Regression coefficients and statistics for the fit, obtained from MLR, for the eight responses.

Regression coefficients	Response variable							
	Related to OD at 600 nm				Related to Tyramine production			
	<i>Lactobacillus brevis</i>		<i>Enterococcus faecium</i>		<i>Lactobacillus brevis</i>		<i>Enterococcus faecium</i>	
	Aerobic (Y ₁)	Anaerobic (Y ₂)	Aerobic (Y ₃)	Anaerobic (Y ₄)	Aerobic (Y ₅)	Anaerobic (Y ₆)	Aerobic (Y ₇)	Anaerobic (Y ₈)
Intercept	-1.2157	-0.3290	-18.7270	-12.2788	25.5447	-115.5650	-1.7840	565.9821
Tyr	0.9037	0.0046	0.2280	0.7853	104.8190	176.7553	28.8449	130.9564
pH		-0.2850	3.7346	4.0798	-2.2800	-243.0052		-194.3033
PLP		0.0058	0.0028	0.0006		0.3661		0.3831
Tem	0.0611	-0.0400	0.5875		-0.2024	57.9395	0.4795	-5.9363
Tim	0.8218	1.4601	-0.0895	0.2615	0.1769	13.7184	-1.0003	16.1544
Tyr*Tyr	-0.4518		-0.2436	-0.3349	-70.2502	-63.0988	-50.3283	-54.2250
pH*pH			-0.2495	-0.2978		17.6216		13.7383
PLP*PLP			-0.0000					
Tem*Tem			-0.0100			-1.1780		
Tim*Tim		-0.0640	0.0253					
Tyr*pH			-0.0759		-9.3895	-12.5137		
Tyr*PLP		0.0008		-0.0006		0.0953		
Tyr*Tem			0.0263		3.1229		3.2076	
Tyr*Tim			-0.0147		10.6815	10.9273	8.2823	9.1613
pH*PLP		-0.0003						
pH*Tem		0.0215	-0.0078			1.4879		1.5867
pH*Tim		-0.0712	-0.0172	-0.0365		-3.0100	-2.0520	-3.3495
PLP*Tem		-0.0002	-0.0001			-0.0164	-0.0130	-0.0179
PLP*Tim		0.0002	0.0001			0.0205		0.0252
Tem*Tim	-0.0243	-0.0170					0.7750	
R ²	0.752	0.892	0.978	0.927	0.827	0.947	0.787	0.888
RSD	0.359	0.147	0.040	0.231	33.730	25.220	30.380	27.890
P	0.735	0.092	0.052	0.579	0.714	0.676	0.258	0.698

Tyr, added tyrosine concentration (g/l); pH, environmental pH; PLP, pyridoxal-5-phosphate supplementation (μM); Tem, incubation temperature (°C); Tim, incubation time (days); R², determination coefficient; RSD, residual standard deviation; P, P-value of lack of fit test.

Table 3. Predicted values for all responses studied at the optimum conditions, obtained from MLR models.

Response variables		Predicted value	Optimum conditions				Time (days)
			Tyrosine (g/l)	Environmental pH	PLP (μ M)	Temperature ($^{\circ}$ C)	
Related to OD at 600 nm	Y ₁	2.58	1.0	*	*	22	7.0
	Y ₂	3.08	2.0	4.4	400	22	6.6
	Y ₃	2.24	0.8	6.7	200	27	7.0
	Y ₄	2.29	1.1	6.4	0.	*	7.0
Related to Tyramine production	Y ₅	211.7	1.7	4.4	*	31.9	7.0
	Y ₆	296.5	1.9	4.4	400	24.5	7.0
	Y ₇	184.9	1.9	4.6	*	32	7.0
	Y ₈	251.5	1.8	4.4	400	22	7.0

*, the factor does not influence the response variables

Figure 1

Figure 2