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THE PLACKETT-BURMAN DESIGN IN OPTIMIZATION OF MEDIA COMPONENTS FOR BIOMASS PRODUCTION OF *LACTOBACILLUS RHAMNOSUS* OXY

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The central composite design was developed to search for an optimal medium for the growth of *Lactobacillus rhamnosus* OXY. The effect of various media components, such as carbon sources, simple and complex nitrogen sources, mineral agents, and growth factors (vitamins B, amino acids) was examined. The first-order model based on Plackett-Burman design showed that glucose, sodium pyruvate, meat extract and mineral salts significantly influenced the growth of the examined bacteria. The second-order polynomial regression confirmed that maximum biomass production could be achieved by the combination of glucose (12.38 g/l), sodium pyruvate (3.15 g/l), meat extract (4.08 g/l), potassium phosphate (1.46 g/l), sodium acetate (3.65 g/l) and ammonium citrate (1.46 g/l).

The validation of the predicted model carried out in bioreactor conditions confirmed the usefulness of the new medium for the culture of *L. rhamnosus* OXY in large scale. The optimal medium makes the culture of the probiotic bacterium *L. rhamnosus* OXY more cost effective.

Keywords: *Lactobacillus* – biomass – growth – optimization – response surface methodology

INTRODUCTION

Lactobacillus are the most common probiotic bacteria used as food adjuncts. They are also extensively used in the pharmaceutical industry. Many pharmaceutical products containing viable cells of LAB (lactic acid bacteria) are widely available. The primary objective of these products is to achieve persistent colonization of the bacteria in the gut during the treatment of a variety of conditions such as gastrointestinal disorders (e.g. post-antibiotic therapy, adjustment of microbial imbalances in the gut, liver diseases) [19]. A number of health benefits have been reported, which explains the great interest of scientists in the growth and physiology of probiotic strains. As *Lactobacilli* are fastidious with respect to nutrient requirements, a rich medium is required for good growth [13]. The most common medium for lactic acid bacteria is the Man Rogosa Sharpe medium (MRS) [5, 18]. However, commercially available probiotic supplements are often costly, mainly due to their expensive production processes and media. It is known that the growth of *Lactobacilli* is affected by medium

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formulation. In the past several studies focused on the optimization of the growth medium of dairy strains *L. rhamnosus* [13]. Researches concern influence of various carbon and nitrogen sources, growth factors (amino acids and vitamins) and culture conditions such as temperature, pH, the level of aeration on bacterial growth. Liew et al. [13] found that yeast extract (6.0% w/v), glucose (5.01% w/v), vitamin solution (1.28 w/v) and pH = 6.9 gave higher counts of viable cells than MRS. Another study experimentally verified that co-metabolism of glucose and sodium pyruvate enhances the growth rate of *L. rhamnosus* [2].

Response surface methodology (RSM) is a multivariate technique that fits the experimental domain studied in the theoretical design through a response function. It also provides an optimum result of studied feature in reduced number of experiments [10].

The most popular approach is based on full factorial central composite design, generation of which is commonly preceded by primary screening of variables. In this procedure Plackett-Burman design is usually applied [4].

Media formulation and optimization are the primary steps involved in bioprocess technology. Thus, the aim of this study was to optimize the growth of the probiotic strain *L. rhamnosus* OXY in a medium containing a modified amount of carbon and nitrogen sources. The addition of mineral salts and growth factors to the optimized medium was further examined in order to investigate if they are pertinent to the improvement of the examined bacteria growth. This work deals with the optimization of the composition of a low-cost medium for biomass production of *L. rhamnosus* OXY.

MATERIALS AND METHODS

Microorganism

The strain of *L. rhamnosus* OXY obtained from Biomed Serum and Vaccine Production Plant Ltd. in Lublin (Poland) was used in all experiments. The bacteria were stored at –80 °C in MRS medium with the addition of 20% (v/v) glycerol. The strain was revitalized in MRS broth (BTL, Poland) at 37 °C for 24 h before use.

Culture media

a) The influence of the following sources of carbon on the growth of *Lactobacillus* was tested: glucose (POCH, Poland) or glucose with sodium pyruvate (Sigma) or with sodium lactate (Sigma) or with ammonium citrate (POCH, Poland), galactose (POCH, Poland), fructose (POCH, Poland), lactose (POCH, Poland), sucrose (POCH, Poland), maltose (POCH, Poland), lactulose (ICN), fructooligosaccharides (Arnaud, Poland), maltodextrins DP 4-7 and 13–17 (Sigma). The MRS medium containing

Table 1
Concentrations of complex nitrogen sources in modified MRS broth

Dilutions of nitrogen sources in relation to MRS broth	Complex nitrogen sources [g/l]		
	Yeast extract	Meat extract	Peptone K
10×	0.40	0.80	1.00
25×	0.16	0.32	0.40
50×	0.08	0.16	0.20
75×	0.05	0.11	0.13
100×	0.04	0.08	0.10
500×	0.01	0.02	0.02

each carbon source separately in the concentration of 20 g/l and pH 6.2 was distributed to tubes and sterilized at 121 °C for 15 min.

b) The effect of complex sources of nitrogen contained in MRS broth (yeast extract, meat extract and peptone K) on the growth of bacterial cells was examined by diluting at various concentrations as shown in Table 1. The media prepared in tubes were pasteurized in a Koch apparatus (100 °C) for 20 min.

c) The influence of amino acids and vitamins from B group on bacterial growth was also investigated. The modified MRS medium containing 100× lower concentrations of complex nitrogen sources was supplemented by L form of amino acids (Pro, Ser, Val, Leu, Ile, His, Trp, Tyr, Thr, Met, Glu, Phe) at the concentration of 0.1 mg/ml or by vitamins (biotin, folic acid, nicotinic acid, pantothenic acid, pyridoxal, riboflavin, thiamine, cyanocobalamine) at the concentration of 0.001 mg/ml. The vitamin solutions were filter-sterilized and added to the pasteurized cultivation medium in tubes prior to inoculation.

Stationary culture conditions and biomass analysis

A revitalized culture of *L. rhamnosus* OXY was used to prepare 24 h inoculum in MRS medium, pH 6.3 ± 0.1. All kinds of media in tubes were inoculated with a 24 h inoculum at the concentration of 2.5% (v/v) and the cultures were incubated at 37 °C for 24–48 h under relative anaerobic conditions. Every 2 h samples were collected and analyse of optical density (OD) were carried out. OD was measured at 600 nm using Biorad spectrophotometer. Biomass concentration was determined using the standard curve of OD_{600} against dry cell weight.

Verification of a statistical model in stationary and bioreactor conditions

On the basis of the results obtained in statistical analysis, the optimal medium was composed. After pasteurization at 100 °C for 20 min, tubes were inoculated with

Lactobacillus strain at the concentration of 2.5% (v/v) and incubated at 37 °C for 18 h. Then samples in triplicate were collected to determine biomass concentration.

The culture of *Lactobacillus rhamnosus* OXY was also run in a bioreactor in the same medium. Four pH values were tested: 6.3 (as a control), 4.5, 5.0, 5.5. The working volume was 500 ml in a 1 l vessel with stirring (100 rpm). The bacterial cells were incubated at 37 °C (use of water coat) for 24 h under relative anaerobic conditions. Samples of the culture were examined for biomass concentration using OD_{600} analysis.

Statistical optimalization

The Plackett-Burman design is a useful tool which enables to screen *n* variables using only $n+1$ experiments [16]. It was applied to evaluate the relative importance of various nutrients for biomass production [8] and to limit their number.

The 2^3 full factorial central composite design for three independent variables with six star points and the same number of replicates at centre points was used to estimate response surfaces, following the general form of the second degree polynomial equation:

$$Y = \beta_0 + \Sigma\, \beta_i X_i + \Sigma\, \beta_{ii} X^2_i + \Sigma\, \beta_{ij} X_i X_j \,,$$

where Y is the predicted response variable, β_0 is the interception, β_i represents a linear coefficient, β_{ii} is a quadratic coefficient and β_{ij} is an interaction coefficient. X_i, X_j, are coded variables [6] influencing Y, chosen as the result of the Plackett-Burman design analysis.

ANOVA was performed and values of the R and R^2 coefficients were calculated to check the significance and the goodness of fit of the obtained regression model.

The relationships between the independent variables and the predicted values were shown as the 2D contour plots. Version 7 Statistica software was applied to obtain figures and present the results of the analyses.

RESULTS AND DISCUSSION

Primary screening of nutrients

Figure 1 presents the results of *L. rhamnosus* OXY biomass formation after 24 h culture on MRS medium containing different carbon sources. Results show that glucose with an addition of sodium pyruvate was the optimal complex carbon source for the growth of the examined strain. Our observation is in agreement with other researchers' statements. As regards the effect of carbohydrates, Mataragas et al. [15] reported that glucose concentration did not improve the total biomass produced by *Leuconostoc mesenteroides* and *Lactobacillus curvatus*, whereas Liew et al. [13] showed a slight effect of glucose on the cell number of *Lactobacillus rhamnosus*.

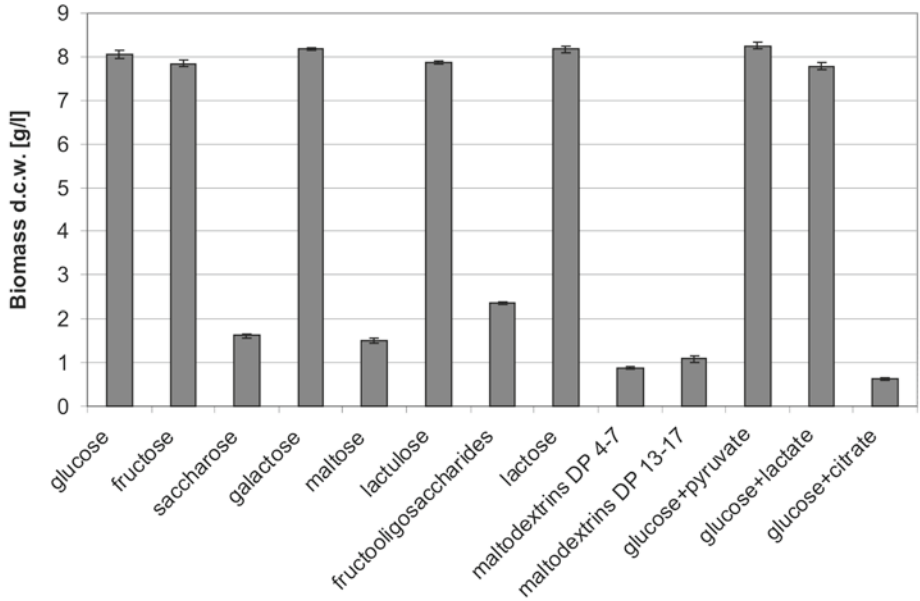

Fig. 1. Effect of various carbon sources on growth of *L. rhamnosus* OXY after 24 h of incubation

Table 2
Plackett-Burman design for seven variables

Run	Glucose + pyruvate	Pepton K	Meat extract	Yeast extract	Organic and inorganic salts	Micro-elements	NaCl	Biomass DCW [g/l]
1	–1	–1	–1	1	1	1	–1	1.6019
2	1	–1	–1	–1	–1	1	1	2.0968
3	–1	1	–1	–1	1	–1	1	0.7143
4	1	1	–1	1	–1	–1	–1	7.7921
5	–1	–1	1	1	–1	–1	1	0.9779
6	1	–1	1	–1	1	–1	–1	7.6873
7	–1	1	1	–1	–1	1	–1	1.2762
8	1	1	1	1	1	1	1	8.2838

Bajpaj-Dikshit et al. [2] reported that co-metabolization of sodium pyruvate with glucose by *L. rhamnosus* increased the maximum growth rate nearly 1.4-fold. It appears that among the various carbon sources studied, glucose + pyruvate is the preferred substrate for *L. rhamnosus*. On a medium containing multiple substrates a typical diauxic growth curve was observed [2].

In the present study it was observed that the examined strain *L. rhamnosus* OXY needs complex nitrogen sources and mineral and organic salts for growth at the level

of 0.22 g/l (which corresponded with 100× dilution of nitrogen sources in relation to MRS broth, Table 1), 2.0 g/l, and 7.0 g/l, respectively. The results of amino acid supplementations into medium showed no positive effect on the bacterial growth. Furthermore, the examined vitamins did not significantly influence the examined bacteria. The data obtained were used to design the response surface methodologies (Placket-Burman and Central Composite Designs).

Statistical optimalization

The minimum and maximum limits of the variables were as follows: glucose + sodium pyruvate: 0.26 g/l; 20 g/l, peptone K: 0.2 g/l; 10 g/l, meat extract: 0.16 g/l; 8 g/l, yeast extract: 0.08 g/l; 4 g/l, organic and inorganic salts (potassium phosphate, sodium acetate, ammonium citrate): 0.9 g/l; 9 g/l, microelements (magnesium sulfate, manganic sulfate (II)): 0.02 g/l; 0.24 g/l, NaCl: 0 g/l; 30 g/l.

The Plackett-Burman design (Table 2) was used for a primary screening of variables. The results presenting the estimated parameters and their ranking are presented in Table 3.

Table 3
Ranking of variables investigated in the Plackett-Burman design

Medium components	Effect	Ranking
Glucose + pyruvate	4.641274	1
Pepton K	0.592906	6
Meat extract	2.346635	2
Yeast extract	0.639662	5
Organic and inorganic salts	2.230552	3
Microelements	–0.388553	7
NaCl	–0.775494	4

Biomass production was most affected by an increase in the concentration of three media components. They were: glucose + pyruvate, meat extract and organic and inorganic salts (potassium phosphate, sodium acetate, ammonium citrate) and these variables were selected for further optimization. An increase in NaCl, and, to a lesser extent, in microelements had a negative effect on biomass production.

Despite the fact that yeast extract is used as a component in most fermentation studies we decided to exclude it from further analysis because meat extract gave a considerably higher yield of biomass. Some authors studied the effect of other nitrogen sources such as peptone and malt sprouts. They reported that yeast extract and peptone affect cell concentration significantly [12, 14]. Fung et al. [7] showed that meat extract, vegetable extract, and peptone significantly influenced the growth of

Lactobacillus acidophilus. Sodium acetate is reported to enhance the growth of microorganisms [17].

Given the fact that the differences between the influence of most of the studied factors on the depended variable (biomass formation) were very similar, and the fact that meat extract contains a greater amount of total nitrogen (12% w/w) than YE (10% w/w) [7], and that some authors recommend ammonium salts [11, 20] and citrate [1] added to the medium, as they increase the yield of biomass production, we choose glucose + pyruvate, meat extract and organic and inorganic salts for further analysis.

The full-factorial CCD (Central Composite Designs) of RSM (Response Surface Methodology) containing six central points and star points was applied to maximize the biomass production. The design with the level of variables and the observed values of biomass are presented in Table 4.

Table 4

Central composite design matrix of the variables along with the experimental values of biomass

Run	Glucose + pyruvate	Meat extract	Organic and inorganic salts	Biomass
1	0.8	0.16	0.9	1.1238
2	0.8	0.16	9	1.0746
3	0.8	8	0.9	2.0396
4	0.8	8	9	2.1903
5	20	0.16	0.9	1.5793
6	20	0.16	9	1.5793
7	20	8	0.9	5.1222
8	20	8	9	5.2834
9	0	4.08	4.95	0.9545
10	26.53	4.08	4.95	5.0867
11	10.4	0	4.95	1.3269
12	10.4	10.66	4.95	6.3048
13	10.4	4.08	0	3.8775
14	10.4	4.08	11.75	5.3261
15	10.4	4.08	4.95	5.2472
16	10.4	4.08	4.95	5.3559
17	10.4	4.08	4.95	5.2979
18	10.4	4.08	4.95	5.3100
19	10.4	4.08	4.95	5.3487
20	10.4	4.08	4.95	5.3898

Table 5
Analysis of variance for the current regression model

Source of variation	Sum of square	Degree of freedom	Mean square	F-Value	p-Value
Model	62.41612	9	6.935125	6.901477	0.002858
Error	10.04875	10	1.004875		

The result of regression analysis showing the relationship between depended and independent variables is presented in the form of the following quadratic regression function:

$$Y = -0.2600 + 0.3289X_1 - 0.0127X_1^2 + 0.6487X_2 - 0.0552X_2^2 + 0.2514X_3 - 0.0204X_3^2 + 0.0173X_1X_2 + 0.0002X_1X_3 + 0.0028X_2X_3$$

where Y is the response value (biomass) and X_1, X_2, X_3 are coded variables of glucose + pyruvate, meat extract as well as organic and inorganic salts, respectively.

The observed results according to the experimental design based on CCD were used to check adequacy of the model using analysis of variance (ANOVA). The model gave goodness of fit with R^2 value of 86% and was highly significant (p-value <0.01) as shown in Table 5.

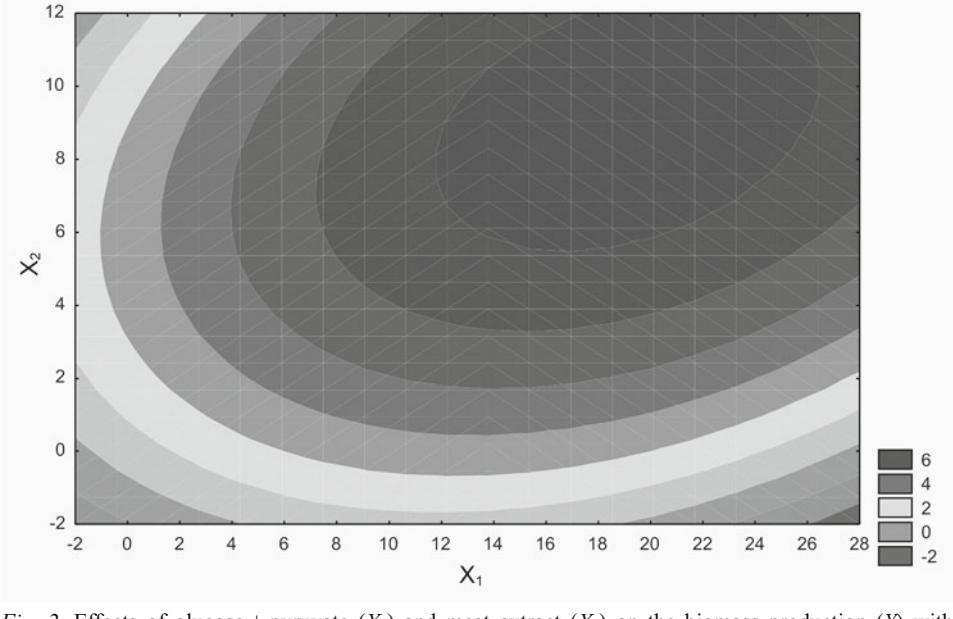

Fig. 2. Effects of glucose + pyruvate (X_1) and meat extract (X_2) on the biomass production (Y) with organic and inorganic salts (X_3) at its center point level

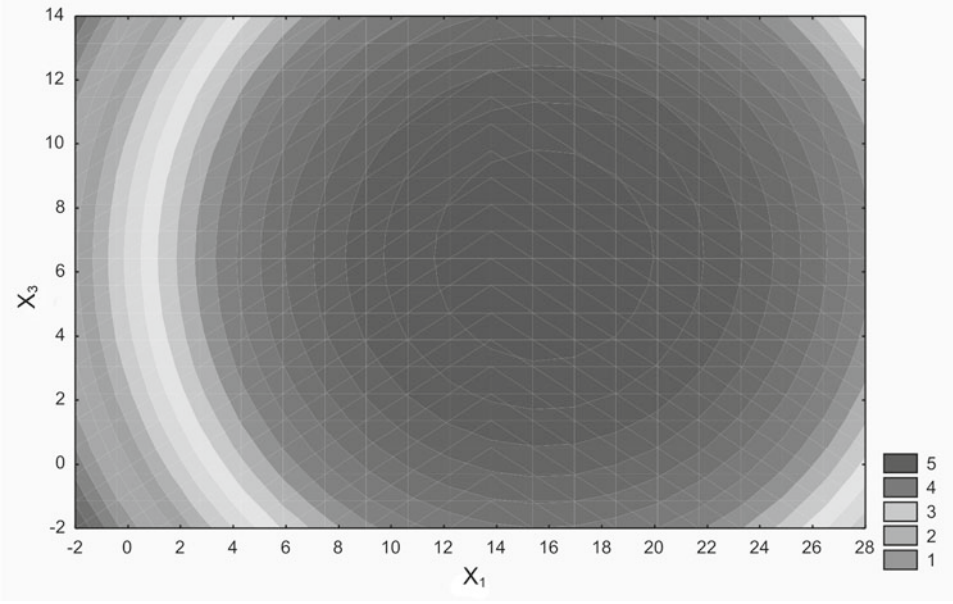

Fig. 3. Effects of glucose + pyruvate (X_1) and organic and inorganic salts (X_3) on the biomass production (Y) with meat extract (X_2) at its center point level

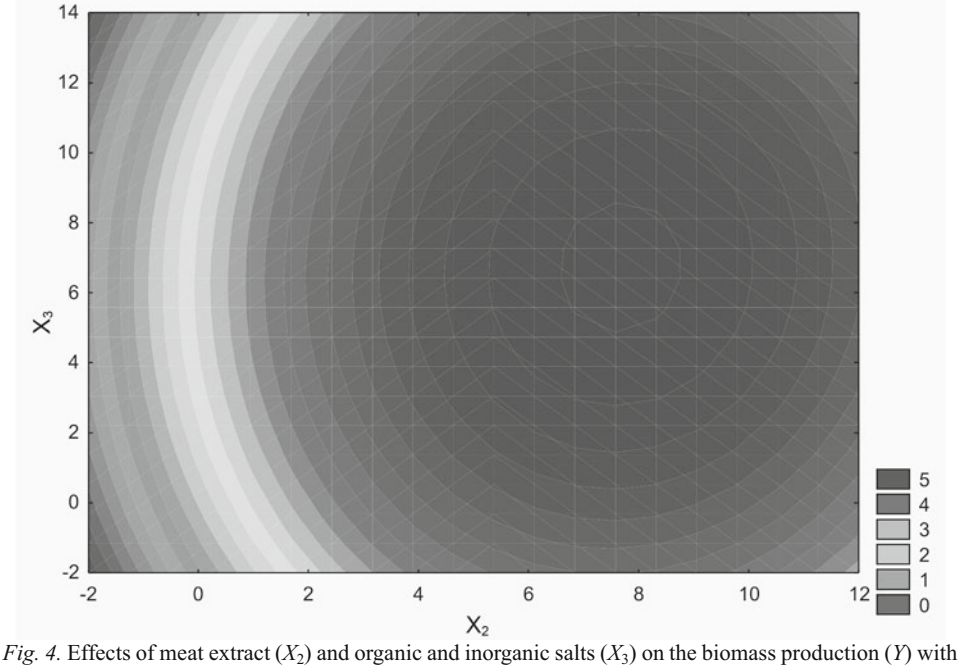

Fig. 4. Effects of meat extract (X_2) and organic and inorganic salts (X_3) on the biomass production (Y) with glucose + pyruvate (X_1) at its center point level

The graphical representations of the regression equation are shown in Figures 2–4. The changes in response value are represented by different intensity of grey colour from bright describing the minimum to dark describing the maximum of the predicted value.

The contour plots in Figure 2 show that there is a significant interaction between glucose + pyruvate and meat extract, while the circular nature of the remaining plots indicates lack of interaction between the rest of the variables [6]. It is also easily seen that the biomass production is mainly influenced by glucose + pyruvate.

Having limited the search for the optimum value to the low-high level interval, the following values of the independent variables were obtained: glucose + pyruvate –15.52 (20.34), meat extract –4.08 (9.95) and organic and inorganic salts –6.57 (10.97). For these values the response was 5.47 (6.32).

Verification of the model in bioreactor

Our results show that the maintenance of pH at a constant level is important for biomass production by *L. rhamnosus* OXY. When pH value was kept constant at the level of 5.5, the highest yield of *Lactobacillus* biomass was obtained on optimal medium (Fig. 5). Other authors claimed that the growth activity of microorganisms depends on the pH of the medium which varies depending on the strain [9].

The results of the analyzed strain growth on optimal medium confirmed the usefulness of the new medium for the culture of *L. rhamnosus* OXY on a large scale. In this medium about 5.2 g/l of dry cell weight after 18 h of incubation was obtained. Costs analysis reveals that the price of 1 l of the optimal medium is 5.34 € and the price of 1 g of biomass was calculated at the level of 1.04 €. The final yield and costs were compared with results of other authors (Table 6). Youssef et al. [20] developed a defined medium composition (33.34 €/l) for the growth of *L. rhamnosus* in which the

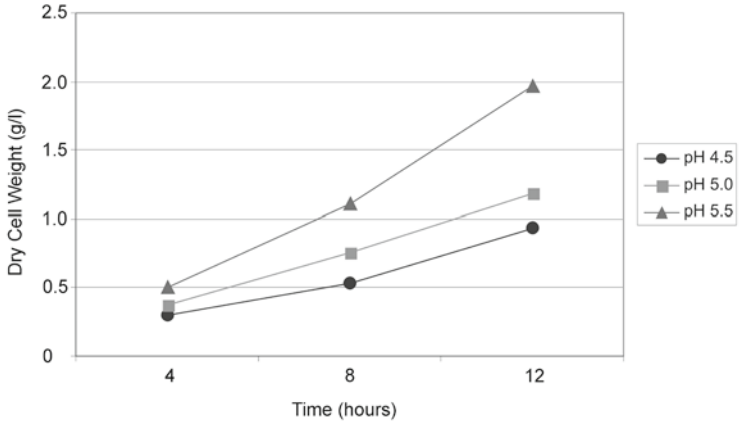

Fig. 5. Influence of pH on *L. rhamnosus* growth in batch culture

Table 6
Comparison of costs (according to Sigma) and biomass production by *L. rhamnosus* in continuous culture using defined media

	Optimized medium (this work)	Youssef et al., 2005 [20]	Berry et al., 1999 [3]
Costs (1 l medium in €)	5.34	33.34	56.76
Cost of 1 g DCW* [€]	1.04	8.34	5.4
Maximum DCW [g/l]	5.143 (t** = 20)	4.0 (t = 26)	10.50 (t = 26)
Productivity [g/l.h]	0.26	0.15	0.40

*DCW – Dry Cell Weight.
**t – time after which max. DCW was achieved.

maximum biomass concentration was 4.0 g/l (dry cell weight) after 26 hours of cultivation. It is clearly seen that the optimal medium in the present study is cheaper and gives better productivity. However, some authors achieved higher yield of the biomass [3] but it must be pointed out that their medium was complex – rich in microelements, vitamins and amino acids (price was ten times higher than that obtained in this study) but productivity was only twice higher (0.4 g/l h) in comparison to the present investigation (0.26 g/l h).

It can be concluded that the optimal medium makes the batch culture of the probiotic bacterium *L. rhamnosus* OXY more cost effective and supports high level of cell growth.

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