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ORIGINAL PAPER

Optimization of Medium Composition for Enhancing Growth of *Lactobacillus rhamnosus* PEN Using Response Surface Methodology

MAGDALENA POLAK-BERECKA[1*], ADAM WAŚKO[1], MONIKA KORDOWSKA-WIATER[1], MARCIN PODLEŚNY[1], ZDZISŁAW TARGOŃSKI[1] and AGNIESZKA KUBIK-KOMAR[2]

[1] Department of Biotechnology, Human Nutrition and Science of Food Commodities
[2] Department of Applied Mathematics and Computer Science, University of Life Sciences in Lublin, Lublin, Poland

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A b s t r a c t

Response surface methodology was used to optimize media components such as carbon and nitrogen (simple and complex) sources, mineral agents and growth factors (B vitamins, amino acids) for enhancing the biomass production of *Lactobacillus rhamnosus* PEN. For screening experiment the following carbon sources were selected: glucose, glucose+pyruvate, glucose+citrate, glucose+lactate, galactose, fructose, lactose, sucrose, maltose, lactulose, fructooligosaccharides, maltodextrins DP 4–7 and DP 13–17. Nitrogen sources such as yeast extract, meat extract and peptone K were used in lower concentrations than in MRS medium which served as a control. All experiments were run at 37°3C for 24–48 h under stationary conditions. Constituents chosen after the first screening experiments were further screened by the Plackett-Burman design. Glucose and sodium pyruvate, meat extract, potassium phosphate, sodium acetate, and ammonium citrate were chosen as promising medium components for further optimization studies. By solving the regression equation and analyzing the response surface carton, optimal concentrations of the components were determined as: glucose (13.4 g/l), sodium pyruvate (3.4 g/l), meat extract (7.2 g/l), potassium phosphate (2.0 g/l), sodium acetate (5.0 g/l) and ammonium citrate (2.0 g/l). In comparison to MRS broth the optimal medium contained fewer ingredients and in modified amounts but *Lb. rhamnosus* PEN showed better growth activity. Biomass concentration (as dry cell weight) of bacteria cultivated in optimal medium at bioreactor conditions was 5.5 g/l after 16 h of incubation, being higher in comparison with bacterial growth in MRS medium (1.9 g/l) under the same conditions. Moreover, the new medium was less expensive.

K e y w o r d s: *Lactobacillus*, biomass, growth, optimization

Introduction

Lactic acid bacteria are commercially important in many branches of industry *e.g.* pharmaceutical and food industry as dietary supplements and probiotic products. There are many products in the market containing viable LAB (lactic acid bacteria) cells. 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) (Tamime *et al*., 1995; Grönlund *et al.*, 2000; Guandalini *et al.*, 2000; Gupta and Garg 2009). Their positive effect on human health explains the great interest of scientists in their growth and physiology. As *Lactobacilli* are fastidious with respect to nutrient requirements, rich medium is required for good growth (Liew *et al*., 2005). The most common medium for lactic acid bacteria is the Man Rogosa Sharpe medium (MRS) (de Man *et al*., 1960; Rogosa *et al*., 1961). However, high growth activity of lactobacilli is affected by medium formulation. In the past, several studies focused on the optimization of the growth medium of dairy strains of *Lb. rhamnosus* (Liew *et al*., 2005). Research concerned the influence of various carbon and nitrogen sources, other growth factors (amino acids and vitamins) and culture conditions such as temperature, pH, the level of aeration on bacterial growth. Liew *et al*. (2005) found that yeast extract, glucose, vitamins and pH gave higher counts of viable cells than MRS. Another study experimentally verified that co-metabolism of glucose and pyruvate enhances the growth rate of *Lb. rhamnosus* (Bajpaj-Dikshit *et al*., 2003).

Response surface methodology (RSM) is commonly used to explore nonlinear relationships between

* Corresponding author: M. Polak-Berecka, Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Life Sciences in Lublin, Skromna 8, 20-704 Lublin, Poland; phone: (+48) 81 46 23 356; fax: (+48) 81 46 23 400; **e-mail: 3mj@wp.pl**

studied factors and the dependent variables. It also provides information about optimal values of these factors to determine the expected largest (or smallest) values for the dependent variables of interest. This methodology includes factorial design and regression analyses (Xiaobo *et. al*., 2006) and is commonly preceded by primary screening with the "one-variable-at-time" approach or Plackett-Burman design application (Xin *et. al*., 2005; Preetha *et. al*., 2007).

Media formulation and optimization are the primary steps involved in bioprocess technology. The aim of our study was to develop a new medium for cost-effective production of *Lb. rhamnosus* PEN biomass.

Experimental

Materials and Methods

Microorganism. A strain of *Lb. rhamnosus* PEN obtained from Biomed Serum and Vaccine Production Plant Ltd. in Lublin, Poland was used in all experiments.

Culture maintenance and inoculum preparation. Bacteria were stored at –80°C in MRS medium with the addition of 20% (v/v) glycerol. The strain was revitalized in MRS broth at 37°C for 24 h. After two passages the bacterial culture was used to prepare 24 h inoculum in MRS (BTL) medium in the same conditions.

Culture media. a) The influence of different sources of carbon on *Lactobacillus* growth was tested: glucose (POCH), glucose+sodium pyruvate (Sigma), glucose+ammonium citrate (POCH), glucose+sodium lactate (Sigma), galactose (POCH), fructose (POCH), lactose (POCH), sucrose (POCH), maltose (POCH), lactulose (ICN), fructooligosaccharides (Arnaut), maltodextrins DP 4–7 and DP 13–17 (Sigma). The MRS medium containing each carbon source separately in concentration 20 g/l and pH 6.2 was sterilized at 121°C for 15 min in tubes.

b) The influence of optimal concentration of complex nitrogen sources was examined in modified MRS broth, where yeast extract, meat extract and peptone K were used in lower concentrations than in control medium. The concentrations of nitrogen sources are given in Table I. Media prepared in tubes were pasteurized in an autoclave for 20 min.

c) The influence of amino acids and vitamins from B group on bacteria growth was also investigated. Modified MRS medium concerning 100 × lower concentrations of complex nitrogen sources was supplemented with amino acids: L-proline (Pro), L-serine (Ser), L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-histidine (His), L-tryptophan (Trp), L-tyrosine (Tyr), L-threonine (Thr), L-methionine (Met), L-glutamic acid (Glu), L-phenylalanine (Phe) (Sigma) in concentration of 0.1 mg/ml or with vitamins: biotin, folic acid, nicotinic acid, pantothenic acid, pyridoxal, riboflavin, thiamine, cyanocobalamine (Sigma) in concentration 0.001 mg/ml. The media were pasteurized for 20 min. at 90°C. The vitamins solutions were filter-sterilized and added to pasteurized cultivation medium prior to inoculation.

Table I
Concentrations of complex nitrogen sources in modified MRS broth

Complex nitrogen sources	Dilutions of nitrogen sources in relation to MRS broth					
	10 ×	25 ×	50 ×	75 ×	100 ×	500 ×
	Concentrations (g/l)					
Yeast extract	0.40	0.16	0.08	0.053	0.04	0.008
Meat extract	0.80	0.32	0.16	0.107	0.08	0.016
Peptone K	1.00	0.40	0.20	0.133	0.10	0.020
Sum	2.20	0.86	0.44	0.293	0.22	0.044

Stationary cultures in modified MRS broth and biomass analysis. 10 ml aliquots of medium in tubes were inoculated with bacteria at concentration of 2.5% (v/v) and were incubated at 37°C for 24–48 h in relatively anaerobic conditions. Every 2 h samples were collected and analyzed to determine biomass concentration by optical density measurement (OD). Absorbance was measured at 600 nm using Biorad spectrophotometer. Biomass was determined using the standard curve of OD_{600} against dry cell weight.

Verification of statistical model in stationary conditions. On the basis of results obtained in statistical analysis, the optimal medium was composed. It contained glucose (13.4 g/l), sodium pyruvate (3.4 g/l), meat extract (7.2 g/l), potassium phosphate (2.0 g/l), sodium acetate (5.0 g/l) and ammonium citrate (2.0 g/l). 10 ml of the medium was pasteurized in tubes at 90°C for 20 min and then inoculated with *Lactobacillus* strain (2.5% v/v). Incubation in relatively anaerobic conditions was continued at 37°C for 24 h. After 16 h samples were collected to determine biomass concentration. This was done in triplicate.

Influence of pH on bacterial growth in bioreactor conditions. Comparison of *Lactobacillus* growth in optimal and MRS media. Cultures of *Lb. rhamnosus* PEN were run in bioreactor in medium composed on the basis of statistical analysis. pH was adjusted to 4.5, 5.0, 5.5 and 6.0 and was maintained at right level using 30% NaOH. Working volume was 500 ml in 1 l vessel with stirring 100 rpm. The medium was inoculated with 2% (v/v) of 12 h bacterial culture. Incubation temperature and time were 37°C (water bath) and 24 h, respectively. Cultures were run in relatively anaerobic conditions and every 2 h samples were collected for biomass analysis. For comparison study with MRS broth optimal medium with pH 6.0 was chosen and the remaining culture conditions and parameters were as above.

Statistical optimization. The Plackett-Burmann design is a very useful tool that enables us to screen *n* variables using only *n + 1* experiments (Myers and Montgomery, 1995). It was applied to limit the considerate number of media. Then the central composite design was used to estimate response surfaces, following the general model equation:

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

where Y represents response variable, β_0 is the interception, β_i – linear effect, β_{ii} – quadratic effect and β_{ij} – interaction effect coefficients. X_i, X_j are coded values of the factors chosen as the result of the Plackett-Burman design analysis.

The significance of the obtained model was checked by F-test and goodness of fit by multiple correlation R as well as determination R^2 coefficients.

To illustrate the relationships between experimental and predicted values the response surface plots were shown. All design matrices were generated and analyzed using Statistica version 7 software.

Results and Discussion

Screening experiments. Growth activity of *L. rhamnosus* in media containing different carbon sources resulted in different growth profiles. Table II shows the data for growth of *Lb. rhamnosus* PEN in the first set of screening experiments. One-variable-at-a-time approach made it possible to point out the highest yield of biomass and the time after which it was achieved when the medium contained a particular carbon source.

Regarding the effect of carbohydrates, Mataragas *et al*. (2004) reported that glucose concentrations did not improve the total biomass produced by *Leuconostoc mesenteroides* and *Lactobacillus curvatus*, whereas Liew *et al*. (2005) showed a slight effect of glucose on the cell number of *Lactobacillus rhamnosus*. Our study shows that the yield of biomass was considerably higher when the cells were cultured in glucose + sodium pyruvate as compared to the case when the cells were cultured on glucose alone or other examined carbon sources. Bajpaj-Dikshit *et al*. (2003) stated that pyruvate is co-metabolized with glucose by *Lb. rhamnosus* in the medium and the co-metabolism increases the growth rate on glucose 1.4-fold. It appears that among the various carbon sources studied the preferred substrate for *Lb. rhamnosus* is glucose + pyruvate. In medium containing multiple substrates a typical diauxic growth curve was observed.

In the present study it was observed that *Lb. rhamnosus* PEN needs complex nitrogen sources and mineral and organic salts at the level of 0.22 g/l, 2.0 g/l, 7.0 g/l, respectively, for growth. After amino acids supplementation no positive effect on bacterial growth was observed. Analyzed vitamins did not sufficiently influence the growth of the examined bacteria. Data obtained were used for design of response surface methodology.

Table II
Effect of various carbon sources on growth of *L. rhamnosus* PEN

Carbon sources	Maximal value of biomass dry weight (g/l ± s.d.)	Cultivation time (h)
Glucose	5.185 ± 0.177	20
Fructose	5.045 ± 0.215	24
Galactose	4.947 ± 0.15	26
Lactulose	4.034 ± 0.15	26
Lactose	5.113 ± 0.1	28
Saccharose	1.394 ± 0.06	24
Maltose	0.927 ± 0.037	26
Maltodextrins DP 4–7*	0.616 ± 0.039	28
Maltodextrins DP 13–17*	0.749 ± 0.027	28
Fructooligosaccharides	1.434 ± 0.054	26
Glucose + pyruvate	5.219 ± 0.146	16
Glucose + lactate	4.842 ± 0.114	18
Glucose + citrate	4.467 ± 0.416	38

* degree of polymerization

Plackett-Burman design. Central composite design. Medium components chosen at the first screening experiments were further screened by the Plackett-Burman design consisting of 8 experiments. The results of the Plackett-Burman design analysis are presented in Table III with estimation values of factors effect (Table IV).

The yield of biomass was mostly influenced by an increase in the concentration of glucose + sodium pyruvate. The positive effect of yeast extract, organic and inorganic salts (potassium phosphate, sodium acetate, ammonium citrate), meat extract and peptone K was very similar. The increase of NaCl as well as, to a lesser extent, microelements had a negative effect on biomass production.

Despite the fact that yeast extract is used in most fermentation studies as a supplement, we decided to exclude it from further analysis because meat extract gave a slightly higher yield of biomass (Table IV). Moreover, meat extract contains greater amount of total nitrogen (12% w/w) than YE (10% w/w) (Fung *et al*., 2008). Some authors studied the effect of less expensive nitrogen sources such as peptone and malt sprouts. They reported that yeast extract and peptone affect the cell concentration significantly (Manteagudo *et al*., 1995; Hujanen and Linko, 1996). Fung *et al*. (2008) showed that meat extract, vegetable extract and peptone significantly influenced the growth of *Lactobacillus acidophilus.*

Because some authors recommended adding ammonium salts (Heriban *et al*., 1993; Zayed and Winter, 1995) and citrate (Amrane and Prigent, 1998) into the

Table III
Plackett-Burman design for seven variables

Run	Glucose + pyruvate (g/l)	Pepton K (g/l)	Meat extract (g/l)	Yeast extract (g/l)	Organic and inorganic salts (g/l)	Microelements (g/l)	NaCl (g/l)	Biomass (g/l)
1	0.26	0.20	0.16	4.00	9.00	0.238	0.00	1.005
2	20.00	0.20	0.16	0.08	0.90	0.238	30.00	1.229
3	0.26	10.00	0.16	0.08	9.00	0.0238	30.00	0.445
4	20.00	10.00	0.16	4.00	0.90	0.0238	0.00	3.059
5	0.26	0.20	8.00	4.00	0.90	0.0238	30.00	0.691
6	20.00	0.20	8.00	0.08	9.00	0.0238	0.00	5.202
7	0.26	10.00	8.00	0.08	0.90	0.238	0.00	1.009
8	20.00	10.00	8.00	4.00	9.00	0.238	30.00	3.587

Table IV
Variables investigated in the Plackett-Burman design

Medium	Effect Estimate
Glucose + pyruvate	5.322
Peptone K	1.426
Yeast extract	1.505
Meat extract	1.720
Organic and inorganic salts	1.536
Microelements	–0.978
NaCl	–1.571

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

Run	Glucose + pyruvate (X_1) (g/l)	Meat extract (X_2) (g/l)	Organic and inorganic salts (X_3) (g/l)	Biomass (g/l)
1	0.80	0.16	0.90	1.075
2	0.80	0.16	9.00	0.925
3	0.80	8.00	0.90	1.489
4	0.80	8.00	9.00	1.402
5	20.00	0.16	0.90	1.664
6	20.00	0.16	9.00	1.497
7	20.00	8.00	0.90	3.595
8	20.00	8.00	9.00	4.168
9	0.00	4.08	4.95	0.815
10	26.53	4.08	4.95	3.043
11	10.40	0.00	4.95	0.742
12	10.40	10.66	4.95	3.661
13	10.40	4.08	0.00	3.220
14	10.40	4.08	11.75	4.007
15	10.40	4.08	4.95	4.250
16	10.40	4.08	4.95	4.224
17	10.40	4.08	4.95	4.131
18	10.40	4.08	4.95	4.208
19	10.40	4.08	4.95	4.423
20	10.40	4.08	4.95	4.093

medium as they increased the yield of biomass production, organic and inorganic salts were selected for further analysis as the third variable in Central Composite Design.

For these components the full-factorial CCD of RSM was applied to maximize biomass production. This design consisted of five levels – low and high levels, central point and star points with $\alpha = \pm 1.68$. Since the fact that some values of the star points would have been negative the minimum value for the chosen variables assumed to be zero (Preetha *et al*., 2006). The design and observed values of biomass are presented in Table V.

The following quadratic regression function was obtained as a result of the CCD analysis:

$$Y = 0.235316 + 0.269392X_1 - 0.011086X_1^2 + 0.534897X_2 - 0.055169X_2^2 + 0.059668X_3 - 0.007731X_3^2 + 0.012327X_1X_2 + 0.002072X_1X_3 + 0.006323X_2X_3$$

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

The results of ANOVA indicates that the model is statistically significant (Table VI) and the values of

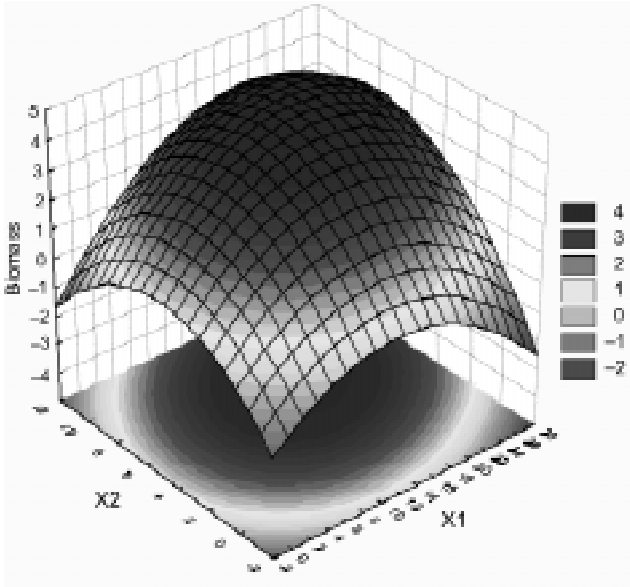

Fig. 1. Effects of glucose + pyruvate (X_1) and meat extract (X_2) on biomass (g/l) production (Y) with organic and inorganic salts (X_3) at its center point level.

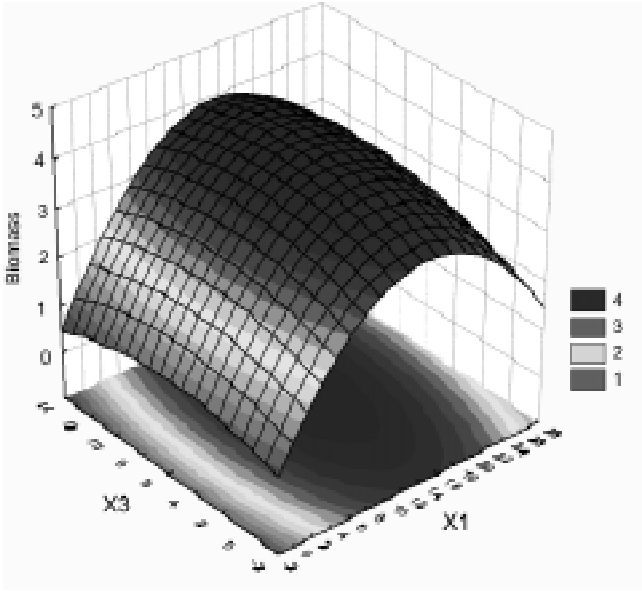

Fig. 2. Effects of glucose + pyruvate (X_1) and organic and inorganic salts (X_3) on biomass (g/l) production (Y) with meat extract (X_2) at its center point level.

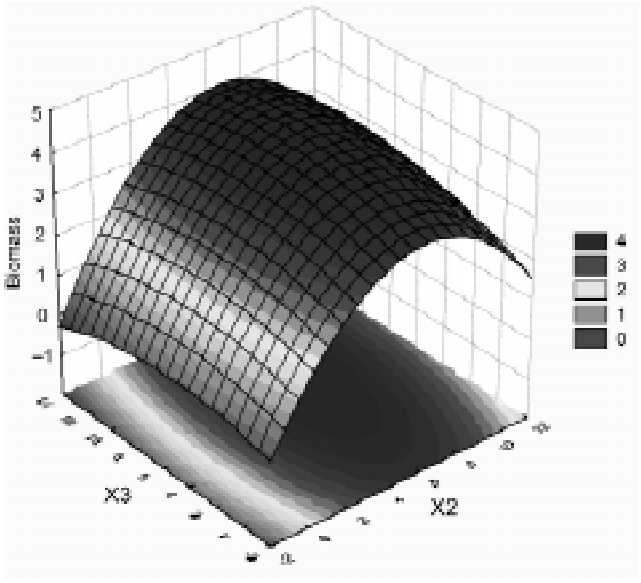

Fig. 3. Effects of meat extract (X_2) and organic and inorganic salts (X_3) on biomass (g/l) production (Y) with glucose + pyruvate (X_1) at its center point level.

$R = 0.94$ and $R^2 = 0.88$ that it is also good fitted to the experimental data and explains 88% of the whole variation of the response. The 3-D response surface plots are shown in Figures 1–3.

As presented above, biomass production is mainly influenced by glucose + pyruvate and meat extract. Based on the regression equation the optimum values of studied ingredients for biomass production, in the low-high level interval, were as follows: glucose + sodium pyruvate – 16.8 g/l, meat extract – 7.2 g/l and organic and inorganic salts – 9 g/l.

Sodium acetate is reported to enhance the growth of the microorganisms (Peters and Snell, 1954).

Verification of the model in bioreactor conditions. In the present study it was observed that maintenance of pH at the constant level is important for biomass production by *Lb. rhamnosus* PEN. The optimum pH for growth of this strain is 6.0, what is illustrated in Fig.4. When this pH value was maintained at the constant level the highest yield of *Lactobacillus* biomass was obtained on optimal medium. The effect of pH was also studied by Guyot *et al*. (2003) with *Lactobacillus manihotivorans* LMG 18010T that grew actively at pH 6.5.

Table VI
Analysis of variance for the current regression model

Source of variation	Sum of square	Degree of freedom	Mean square	F-Value	p-Value
Model	33.80618	9	3.756242	8.128217	0.001488
Error	4.621237	10	0.462124		

Figure 5 illustrates the growth of the analyzed strain in control (MRS) and optimal media. Biomass concentration of the bacteria cultivated in optimal medium in

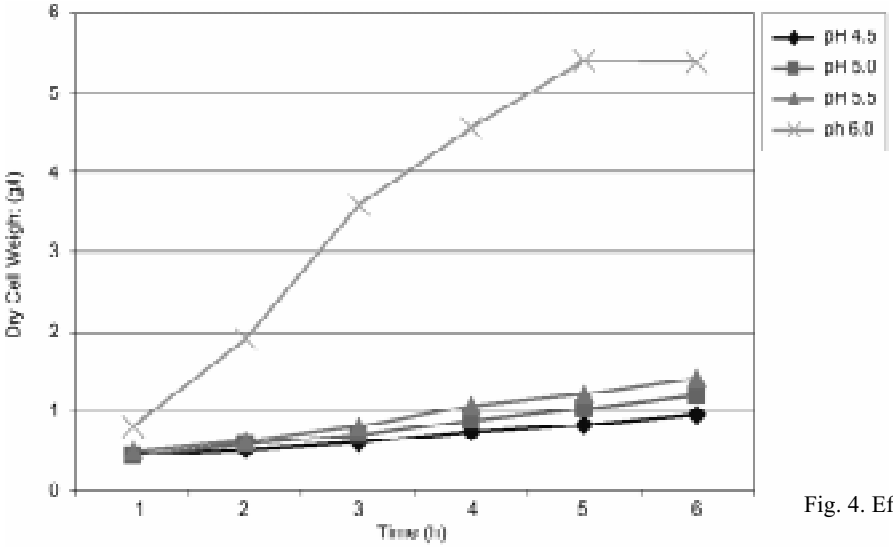

Fig. 4. Effect of pH on *L. rhamnosus* PEN growth in optimal medium.

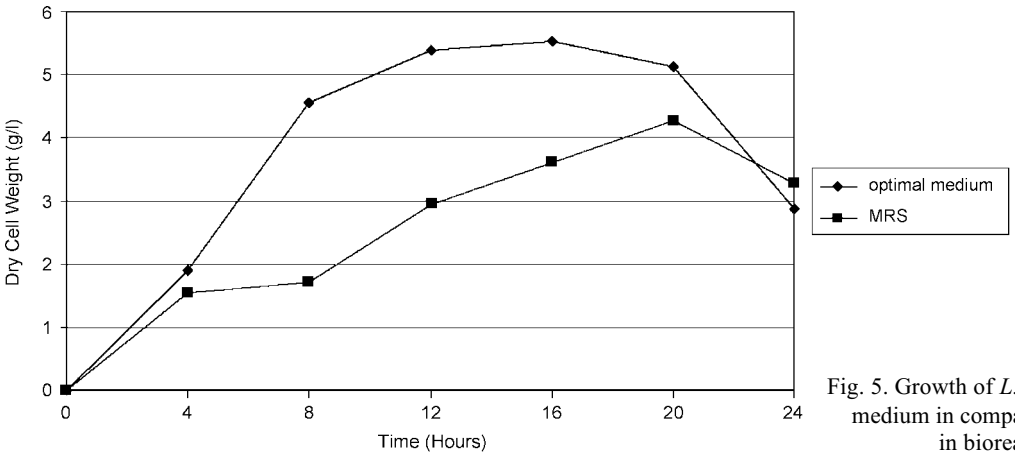

Fig. 5. Growth of *L. rhamnosus* PEN in optimal medium in comparison with MRS medium in bioreactor conditions.

batch culture conditions reached 5.5 g/l after 16 h of incubation, this being higher in comparison with bacterial growth in MRS medium (1.9 g/l) under the same conditions. The presented results confirm the usefulness of the new medium for culture of *Lb. rhamnosus* PEN on a large scale. Moreover, the new medium is less expensive compared to MRS because 1 liter of new medium costs €6.75 and 1 liter of MRS broth costs €9.92 according to our calculation based on Sigma-Aldrich prices. Taking into consideration the high biomass concentration of the examined *Lactobacillus* strain and lower costs of the new, optimal medium production there is a possibility of its large scale application.

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