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Optimization of Split Pea and Dry Figs-Based Media for the Growth of *Lactobacillus plantarum* through Plackett-Burman and Response Surface Methodological Approaches

[1]Zohra Kassas, [2]Baida Djeghri-Hocine, [1]Saida Hanoune, [1]Zineb Derradji, [1]Aicha Boudour and [1]Messaouda Boukhemis
[1]Departement of Biochemistry, Faculty of Sciences, University Badji-Mokhtar, Annaba, Algeria
[2]Nationale High Ecole of Marine Science and Coastal Development, Algiers, Algeria

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Corresponding Author:
Zohra Kassas
Department of Biochemistry,
Faculty of Sciences,
University Badji-Mokhtar, Annaba,
Algeria

ABSTRACT
Plackett-Burman design was used to determine the most influential of 9 variables (glucose, lactulose, tween80, K_2HPO_4, sodium acetate, $MgSO_4$, pH, shaking and inoculum size) on the growth of *L. plantarum* BH14. Glucose, lactulose, $MgSO_4$ and shaking had a significant effect on growth of this strain at a 70% confidence level. Glucose, lactulose and $MgSO_4$ showed positive coefficient but shaking had a negative coefficient, thus shaking was avoided in subsequent experiments. Composite design was used to determine the optimum concentrations of the significant variables. An optimized formulation of nutrition levels was suggested from the software at the following concentrations: 11.59 g L^{-1} glucose, 11.59 g L^{-1} lactulose and 0.23 g L^{-1} $MgSO_4$. Growth of *L plantarum* was compared to that recorded on the reference medium 'vegetal MRS'. The results showed that viable counts in the optimized culture medium were significantly higher than those in the vegetal MRS medium.

Key words: Vegetal substrate, *Lactobacillus*, fermentation, experimental design, growth medium

INTRODUCTION

Lactic Acid Bacteria (LAB) play an essential role in biotechnology. They have a wide range application in food, cosmetic and pharmaceutical industry. These are the most important groups of microorganisms used in the food industry. The LAB have contributed in the increased volume of fermented foods worldwide to their roles in biopreservation and in modulating the health of their hosts by their bacteriocin and lactic acid production (Soomro *et al*., 2002).

Therefore, their growth requires rich and complex media based on yeast extract or meat and animal peptones (Ashraf and Shah, 2011). They are also characterized by high polyauxotrophic requirements, for various amino acids, peptides, nucleotides, vitamins and fatty acids, owing to their limited ability to biosynthesize B-vitamins and amino acids (Desmazeaud, 1983; Fitzpatrick and O'keeffe, 2001).

In order to choose appropriate growth medium, different aspect have to be considered: Cost, the ability to reach a high number of cells and harvesting methods (Georgieva *et al*., 2009), Owing to the fastidious growth factor requirements of LAB and since their sensitivity to inhibitors, selective culture media are very difficult to formulate (Djeghri-Hocine *et al*., 2006). Many animal supplementations have been tested such as: ram horn peptone (Kurbanoglu, 2004) and vegetal supplementations as Cassava Bagasse (John *et al*., 2008), potato extracts (Gaudreau *et al*., 2002), molasses (Coelho *et al*., 2011), oilseed crop pea-and chickpea (Djeghri-Hocine *et al*., 2007) cereals (Charalampopoulos *et al*., 2002). Plant extracts are generally more economical than meat extracts and there are reports of their use with LAB (Gaudreau *et al*., 2002).

Nutritional requirement can be determined by the statistical methods. These methods offer several advantages; they are fast, reliable and reduce the number of experiments. They have been used in many optimization processes of fermentation (Coelho *et al*., 2011; Naveena *et al*., 2005). The conventional experimental approaches for the optimization of media by the method "One-variable-at-a-time" requires a large number of experiments and not take account the interactions

between variables while statistical approach can determine the effect of variables and their interactions with a limited number of essays and the results are achieved in an economical manner.

Lactobacillus plantarum is one of the most of lactic acid bacteria used traditionally in fermented food such as meats, vegetables and dairy products (Hwang *et al*., 2012). There are few published literatures undertaking biomass production of LAB. In the present study, we report optimization of growth medium basis on split pea and dry figs for the growth of *Lactobacillus plantarum* BH14 using statistical approach.

MATERIALS AND METHODS

Microorganism: *Lactobacillus plantarum* BH14 isolated from camel milk and pre-identified by the team of Karem NE- Karem-ZADI: Laboratory of Biology of Microorganisms and Biotechnology, Faculty of Science, Department of Biotechnology, University of Es-senia.

Substrate-based fermentation media

Split pea juice: Seeds were manually sorted and rinsed, before being powdered. The 50 g of the powder were then dissolved in 450 mL of distilled water at pH 9, magnetically stirred during 30 min, pH of the resulting slurry were adjusted at 6 to add papaine. This slurry was heated in 60°C water bath for 2 h then centrifuged at 3500 rpm min^{-1} for 15 min. The supernatant was heated in a boiling water bath for 20 min and recentrifuged 4000 rpm min^{-1} for 15 min. The supernatant used as a basis for the preparation.

Dry figs juice: The 250 g of dry figs were rinsed and added to 500 mL of distilled water. The mixture was boiled at 90°C h^{-1}. 100 g of the result was dissolved in 450 mL of distilled water, magnetically stirred during 30 min and centrifuged at 4000 rpm min^{-1} for 20 min. The supernatant was added to split pea juice (v/v) and used as a basis for medium reconstitution.

Inoculum preparation: Colonies isolated from MRS agar were precultured in MRS broth for 18 h at appropriate temperature. A precultured cells with an optical density 0.6 were used to inoculate the fermentation media. Incubation lasted 18 h at 37°C.

Cell enumeration: The growth of the microorganism was estimated by the determination of colony-forming units (CFU mL^{-1}). This procedure involves making decimal serial dilutions of the sample in sterile physiological water. The growth was recorded as Colony Forming Units (CFU) in various media 1 mL of each fermentation sample was decimally diluted in sterile saline solution (9 g L^{-1} of NaCl). One milliliter of dilution 7-8 were pour-plated in the MRS agar media contained 0.05 g of cysteine, to reduce the fraction of oxygen present in the media, thereby promoting higher survival bacteria (Dave and Shah, 1996). After its solidification, petri dishes were incubated for 48 h at 37°C. After incubation, Colony Forming Units (CFU) were counted.

Screening of nutrients and physical parameters using Plackett-Burman design: Plackett-Burman design is an efficient way to screen the important factors "critical factors" among a large number of variables with minimal number of experiments. It allows the investigation of up to N-1 variables with N experiments. This design was used to determine the most influential of 9 variables which includes chemical parameters (glucose, lactulose, tween80, K_2HPO_4, sodium acetate, and $MgSO_4$) and physical parameters (pH, shaking, inoculum size) at two levels (+1 and -1). The low and high levels were shown in Table 1 (Plackett and Burman, 1946). Twelve experiments were generated from those factors. The variables with a confidence level $(1-\alpha) \geq 70\%$ were considered to have a significant influence on the growth of *L. plantarum*. All the ingredients were dissolved in the basis media.

Optimization by response surface methodology: The next step in the formulation of the medium was to determine the optimum levels of significant variables giving the optimal growth. For this purpose, the Response Surface Methodology (RSM), using a Central Composite Design (CCD) of Box and Wilson (1951), was adopted. Three important variables (glucose, lactulose and $MgSO_4$) from the result of Plackett-Burman design were selected for further optimization. Using the CCD method, a total of 20 experiments with various combinations of glucose, lactulose and $MgSO_4$ were conducted. Table 2 displays the range and levels of the variables investigated.

The variables of the experiments were coded according to the following equation:

$$xi = \frac{Xi - X_{CP}}{\Delta Xi} \quad (1)$$

where, x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_{CP} is the real value of an independent variable at the center point and ΔX_i is the step change value.

Table 1: Variables and levels used in Plackett-Burman design

			Experiment values	
Variables	Units	Symbol code	Lower	Higher
Glucose	g L^{-1}	X_1	0	20.0
Lactulose	g L^{-1}	X_2	0	20.0
Tween 80	mL L^{-1}	X_4	0	1.0
K_2HPO_4	g L^{-1}	X_5	0	2.0
Sodium acetate	g L^{-1}	X_6	0	5.0
Mg SO_4	g L^{-1}	X_7	0	0.2
pH	/	X_9	5.5	6.4
Shaking	rpm min^{-1}	X_{10}	0	120.0
Inoculum size	% (v/v)	X_{11}	1	4.0

Table 2: Experimental codes, ranges and levels of the independent variables for response surface methodological experiment

			Levels				
Variables	Units	Symbol code	-1.68179	-1	0	+1	1.68179
Glucose	g L^{-1}	X_1	11.590	15	20	25	28.410
Lactulose	g L^{-1}	X_2	11.590	15	20	25	28.410
$MgSO_4$	g L^{-1}	X_3	0.061	0.100	0.150	0.200	0.234

The behavior of the system was described by the following quadratic equation:

$$Y = b_0 + \Sigma b_i x_i + \Sigma b_{ii} x_i^2 + \Sigma b_{ij} x_i x_j \quad (2)$$

where, Y is the predicted response (log10 CFU mL^{-1}) ; b_0 is the offset term, b_i is the linear effect, b_{ii} is the squared effect; b_{ij} is the interaction effect and x_i is the independent variable. After determining the composition of the optimum fermentation medium, *L. plantarum* was grown in our final medium and in the reference medium (vegetal MRS) for comparison.

Data analysis: Minitab essay version 16 was used to fit the experimental Plackett-Burman design and also the quadratic response surface model to the experimental data through multiple regressions analysis.

RESULTS

Screening of significant variables using Plackett-Burman design: A total of nine variables were analyzed with regard to their effects on growth of *L. plantarum* using a Plackett-Burman design. This last is an efficient way to screen for the important factors "Critical factors" among a large number of variables with minimal number of experiments. According to Paratoe's law, initial screening of the ingredients is done to understand the significance of their effect on the product formation and then a few better ingredients are selected for further optimizations (Naveena *et al*., 2005). The results were analyzed using the software MINITAB 16. Table 3 displays the Plackett-Burman design matrix (real and coded values) of the 12 experiments and the respective results (log 10 CFU mL^{-1}). Whereas, Table 4 represents the coefficient, t-value and p-value of each variable. Factors evidencing p-values of less than 0.3 were considered to have significant and were therefore, selected for further optimization studies effects on the response (cell number).

As can be seen from Table 3, the highest cell number of 10.16 log10 CFU mL^{-1} was obtained in combination N°5.

Glucose, lactulose, $MgSO_4$ and shaking was the influential variables on the growth of *L. plantarum* BH14, those variables had a significant effect on growth of this strain at a 70% confidence level. Glucose, lactulose and $MgSO_4$ showed positive coefficient but shaking had a negative coefficient, these results suggest that *L. plantarum* strain is an anaerobic microorganism. Thus shaking was avoided in subsequent experiments.

While the confidence level of tween 80, K_2HPO_4, sodium acetate, $MnSO_4$, pH and inoculum size was below 70% in the growth, hence, these variables were considered insignificant. Figure 1 (Pareto chart) illustrates the effects of these variables.

The addition of phosphate to the culture medium increases the growth of the microorganism and it maintains the pH near the optimal growth value, thereby allowing the conduction of fermentation for a longer time. (Coelho *et al*., 2011) so, K_2HPO_4 was used at 2 g L^{-1} in the rest of media formulation. The above results indicated that the Plackett-Burman design is a powerful tool for identifying factors, which had significant influence on *L. plantarum* growth.

The critical factors were finding by Plackett-Burman design, the next step was to determine the optima of those variables using a Composite Central Design (CCD).

Response surface methodology: To determine the optimum conditions of fermentation of *L. plantarum* in split pea and dry figs basis medium, the RSM was employed. Table 5 displays the design matrix of the variables in coded units and real values with the respective results. The highest production of biomass was 8.46 log10 CFU mL^{-1}, obtained from combination number 3:20 g L^{-1} glucose, 20 g L^{-1} lactulose and 0.15 g L^{-1} $MgSO_4$.

The application of multiple regression analysis methods yielded the following regression (Eq. 3) for the experimental data and explained the role of each variable and their second order interactions in the growth:

Table 3: Plackett-Burman design (real and coded values) with the respective results

	Independent variables											
Runs	X_1	X_2	X_3	X_4	D_1*	X_6	X_7	X_8	X_9	X_{10}	D_2*	Response log 10 CFU mL^{-1}
1	20	0	1	0	-1	0	0.2	6.4	120	1	1	9.38
2	20	20	0	2	-1	0	0.0	6.4	120	4	-1	9.74
3	0	20	1	0	1	0	0.0	5.5	120	4	1	8.57
4	20	0	1	2	-1	5	0.0	5.5	0	4	1	9.38
5	20	20	0	2	1	0	0.2	5.5	0	1	1	10.16
6	20	20	1	0	1	5	0.0	6.4	0	1	-1	9.76
7	0	20	1	2	-1	5	0.2	5.5	120	1	-1	9.54
8	0	0	1	2	1	0	0.2	6.4	0	4	-1	9.39
9	0	0	0	2	1	5	0.0	6.4	120	1	1	8.67
10	20	0	0	0	1	5	0.2	5.5	120	4	-1	9.48
11	0	20	0	0	-1	5	0.2	6.4	0	4	1	9.48
12	0	0	0	0	-1	0	0.0	5.5	0	1	-1	9.00

*D_1 and D_2 represent dummy variables

Table 4: Estimated effects of the tested variables on growth of *Lactobacillus plantarum* using Plackett-Burman design

Variables	Effect	Coef	SE Coef	t-value	p-value
Glucose	0.5417	0.2708	0.08021	3.38	0.078
Lactulose	0.3250	0.1625	0.08021	2.03	0.180
Tween 80	0.0850	0.0425	0.08021	0.53	0.649
K_2HPO_4	0.2017	0.1008	0.08021	1.26	0.336
Sodium acetate	0.0117	0.0058	0.08021	0.07	0.949
$MgSO_4$	0.3850	0.1925	0.08021	2.40	0.138
pH	0.0483	0.0242	0.08021	0.30	0.792
Shaking	0.2983	0.1492	0.08021	1.86	0.204
Inoculum size	0.0783	0.0392	0.08021	0.49	0.674

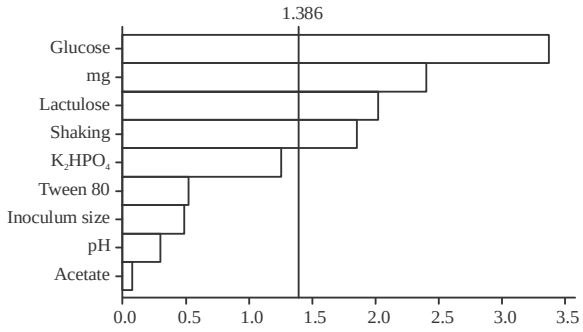

Fig. 1: Pareto chart for the Log10 CFU mL^{-1} of *Lactobacillus plantarum* BH14

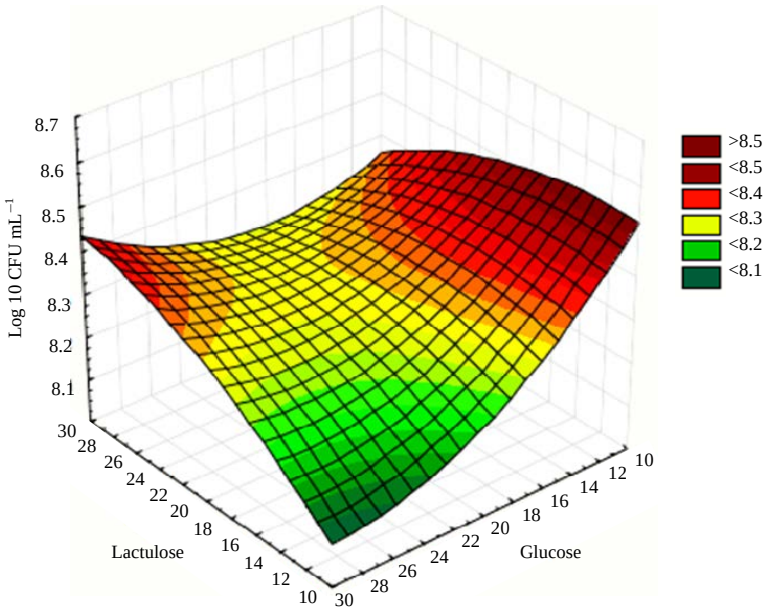

Fig. 2: Response surface of log10 CFU mL^{-1} of *Lactobacillus plantarum* BH14 showing the interaction between glucose and lactulose

Table 5: Central composite design and results

Experiments	X_1 glucose	X_2 lactulose	X_3 $MgSO_4$	Log10 CFU mL^{-1}
1	20	11.5910	0.150000	8.45
2	20	20.0000	0.150000	8.30
3	20	20.0000	0.150000	8.59
4	20	20.0000	0.234090	8.31
5	25	15.0000	0.100000	8.46
6	20	20.0000	0.150000	8.22
7	25	25.0000	0.200000	8.30
8	20	20.0000	0.150000	8.29
9	20	20.0000	0.150000	8.39
10	15	25.0000	0.100000	8.35
11	25	15.0000	0.200000	8.31
12	25	25.0000	0.100000	8.25
13	20	28.4090	0.150000	8.29
14	20	20.0000	0.150000	8.30
15	15	15.0000	0.100000	8.24
16	20	11.5910	0.150000	8.11
17	20	20.0000	0.150000	8.40
18	20	20.0000	0.150000	8.32
19	20	20.0000	0.234090	8.30
20	25	15.0000	0.100000	8.17

Table 6: Analysis of variance for *Lactobacillus plantarum* growth

Sources	DF	Seq SS	Adj SS	Adj MS	F-value	p-value
Regression	9	0.201001	0.201001	0.022333	18.65	0.000
Linear	3	0.043403	0.043403	0.014468	12.08	0.001
Square	3	0.015660	0.015660	0.005220	4.36	0.033
Interaction	3	0.141938	0.141938	0.047313	39.51	0.000
Residual error	10	0.011974	0.011974	0.001197		
Lack-of-fit	5	0.011691	0.011691	0.002338	41.26	0.000
Pure error	5	0.000283	0.000283	0.000057		
Total	19	0.212975				

DF: Degrees of freedom, Seq SS: Sum of squares, Adj SS: Adjusted sum of squares, Adj MS: Adjusted mean sum of squares, F: Variance ratio; p: Probability

$$Y = 8.29873-0.04287X_1+0.02183X_2 +0.02939X_3 + 0.02625X1^2 -0.01264X_{22}+0.01387X_{32}+0.03125X_1X_2 -0.12875X_1X_3-0.01375X_2X_3 \quad (3)$$

The quadratic model in Eq. 3 contains three linear terms, three quadratic terms and three factorial interactions in which Y is the predicted response (log 10 CFU mL^{-1}) and X1, X2 and X3 are the coded values of glucose, lactulose and $MgSO_4$ respectively. Table 6 displays the Student's t-distribution and the probability p-values that used as a tool to check the significance of each of the coefficients.

Our results showed that the effects of glucose (X_1), lactulose (X_2) and $MgSO_4$ (X_3) on the cell number of *L. plantarum* were significant based on p-values lower than 0.05. Also, the squared variable X_{12} and the X_1X_2 interaction, X_1X_3 interaction were also significant for the growth of *L. plantarum*. The Eq. 3 model was modified to the reduced Eq. 4 fitted model:

$$Y = 8.29873-0.04287X_1+0.02183X_2 +0.02939X_3 +0.02625X_{12} +0.03125X_1X_2-0.12875X_1X_3 \quad (4)$$

Analysis of variance for log10 CFU mL^{-1} was done by MINITAB-16. The regression equation obtained from the ANOVA showed that the R^2 (multiple correlation coefficient) was 94.38% (a-value >0.75 indicates fitness of the model). This is an estimate of the fraction of overall variation in the data accounted by the model and thus the model is capable of explaining 95% of the variation in response. In addition a very low p-value (0.000) demonstrated a very high significance for the regression model and smaller p-values denote a more significant corresponding coefficient (Coelho *et al*., 2011).

Graphical representation of response surface shown in Fig. 2-4 help to understand the effect of glucose, lactulose and $MgSO_4$ on the growth, visualize their interactions and locate the optimal level of each variable for maximal response. Three-dimensional response surface plots were constructed by

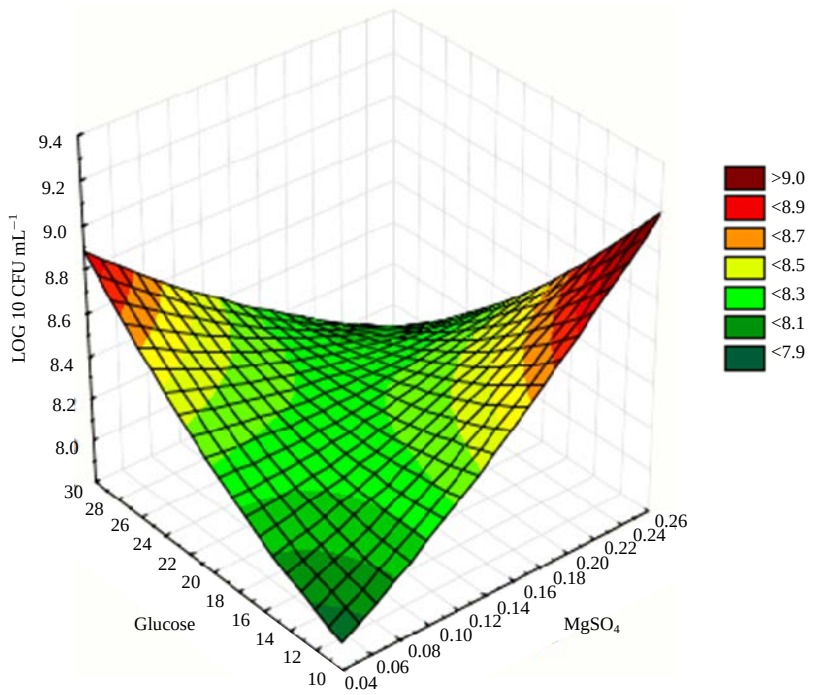

Fig. 3: Response surface of log10 CFU mL^{-1} of *Lactobacillus plantarum* BH14 showing the interaction between glucose and $MgSO_4$

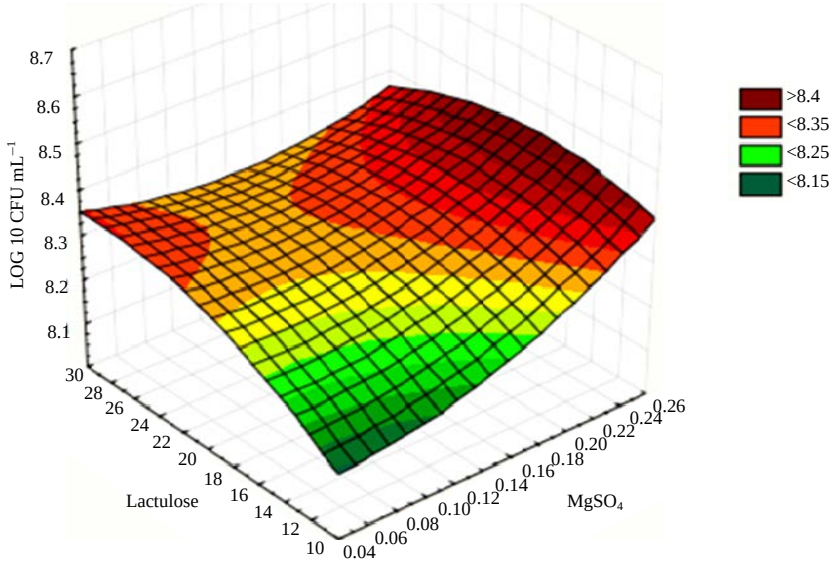

Fig. 4: Response surface of log10 CFU mL^{-1} of *Lactobacillus plantarum* BH14 showing the interaction between lactulose and $MgSO_4$

plotting the response (log10 CFU mL^{-1}) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels.

Figure 2 explains that decrease in concentration of both glucose and lactulose can increase the growth of *L. plantarum.* Figure 3 shows that the response (log10 CFU mL^{-1}) varied significantly when glucose concentration increased and $MgSO_4$ concentration decreased. Figure 4 explains that a maximum cell number was obtained at a high level of $MgSO_4$ and intermediate level of lactulose. An optimized formulation

of nutrition levels was suggested from the software at the following concentrations: 11.59 g L^{-1} glucose, 11.59 g L^{-1} lactulose and 0.23 g L^{-1} $MgSO_4$.

Verification experiment: Growth of *L. plantarum* on our final medium resulted in a higher final cell number than that recorded on vegetal MRS medium, 3.9 10^9 and 2.5 10^9 CFU mL^{-1}, respectively.

DISCUSSION

Lactic acid bacteria are characterized by high polyauxotrophic requirements. Their growth is possible only on rich and complex culture media which must essentially comprise a source of nitrogen (amino acid, peptide), a carbon source, vitamins, minerals and trace elements. These nutrients must be made to optimal concentrations (Djeghri-Hocine *et al*., 2010). Therefore, determining a suitable growth media appears to be difficult.

The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its ready applicability and aptness (Reddy *et al*., 2008).

Most studies on optimizing medium composition for biomass production have been reported. In our study, Plackett-Burman design was used to screen the important factors and central composite design was used to determine their optimum concentrations.

The result showed that glucose, lactulose and $MgSO_4$ were the most significant variables on growth of *L. plantarum* BH14 and their optimal concentrations in the medium were 11.59, 11.59 and 0.23 g L^{-1}, respectively.

The strains of *Lactobacillus* genus are chemotrophic, they acquire the energy necessary to their growth from the oxidation of sugars and other chemical compounds. As shown in Fig. 1, glucose was the most variable influencing the growth of *L. plantarum* BH14. Glucose presented the best carbon source for biomass production of *L. plantarum* YJG (Han *et al*., 2011). It has been reported that a good growth rate of *L. plantarum* was achieved in MRS media supplemented with glucose as carbon source (Georgieva *et al*., 2009). However, a low glucose concentration increased the growth of *L. plantarum* BH14, that can be explained by: A higher glucose concentration may have resulted a cell growth inhibition. Recently, the effect of lactulose on the growth of LAB has been widely studied. *Lactobacillus plantarum* BH14 is able to use lactulose as carbon source, our results are in an agreement with the results of Alejandra Cardelle-Cobas *et al*. (2011), who found that *L. plantarum* CLB7 cell density reached a maximum OD600 value using lactulose as substrate. Saarela *et al*. (2003) are reported that lactulose was the favored lactose derivative used by *Lactobacillus* strains. Also, $MgSO_4$ had a significant effect on growth of *L. plantarum* BH14, it represent a sources of oligoelements serving as cofactors of enzymes involved in the growth of this strain.

Both juices, originated from split pea and dry fig, appeared to be convenient basis for the formulation of specific media for *L. plantarum* growth with low cost. The results showed that viable counts in the optimized culture medium were significantly higher than those in the vegetal MRS medium. This indicates that our newly developed culture medium is more adaptable to the growth of *L. plantarum* than reference medium. That's due to the richness of chemical composition of split pea in protein and starch (Arntfield and Maskus, 2011). Also, dry figs are rich on vitamins, mineral elements and fats. This work confirmed the feasibility of the use of a vegetal substrate to replace the expensive usual nitrogen supplements like yeast extract, meat extract and peptones used on the formulation of culture media for lactic acid bacteria growth.

The optimized medium in this study is more economical and good for the growth of *L. plantarum*. For these reasons, it could be useful in large-scale application.

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