VALORISATION OF DATE WASTE FOR THE PRODUCTION OF PROBIOTIC BACTERIAL BIOMASS

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Abstract.- *This study aimed to valorise date waste by improving biomass production of* Lactobacillus plantarum *G_1 using date juice as carbon source. The physicochemical analysis indicated that the date juice contained a high amount of sugars easily assimilated which make it favorable for the growth of the bacterial strain. The results showed that the date juice with 12% of total sugar supplemented with salts and organic nitrogen found in MRS medium produced a higher biomass yield when compared to biomass production in MRS medium and in date juice alone. Plackett-Burman statistical experimental design was used to screen the most important nutrients influencing lactic acid biomass production. Seven variables including date extract were screened for their effect on* Lactobacillus plantarum *biomass production. Results from this analysis demonstrated that the date extract, yeast extract and peptone were the most significant factors.*

Key words**:** *Biomass production, date juice,* Lactobacillus plantarum*, Plackett-Burman design, probiotic*

VALORISATION DE DECHETS DE DATTE POUR LA PRODUCTION DE LA BIOMASSE PROBIOTIQUE BACTERIENNE

Résumé.- *Cette étude s'intéresse à valoriser les déchets de dattes dans l'amélioration de la production d'une biomasse bactérienne de* Lactobacillus plantarum *G_1 en utilisant le jus de dattes comme source de carbone. L'analyse physico-chimique révèle que le jus de dattes contient une quantité importante de sucres facilement assimilables ce qui le rend favorable à la croissance de la souche bactérienne. Le jus de dattes à 12% de sucres totaux additionné de sels et d'azote organique du milieu MRS produit un rendement de biomasse plus élevé par rapport à celui de la production dans le milieu MRS et dans le jus de dattes seuls. Le plan expérimental statistique de Plackett-Burman est utilisé pour sélectionner les nutriments les plus importants influençant la production de la biomasse lactique. Sept variables, y compris l'extrait de dattes, sont testés pour leurs effets sur la production de la biomasse de Lactobacillus plantarum. Il est à noter que l'extrait de dattes, l'extrait de levure et la peptone sont les facteurs qui ont les effets les plus significatifs.*

Mots clés: *Production de la biomasse, jus de dattes, Lactobacillus plantarum, matrice de Plackett-Burman, probiotique.*

Introduction

Natural products that promote and protect human health against the increasing number of diseases are gaining an increasing interest. Living microorganisms (probiotics) are widely used for human nutrition [1]. Hence, the industrial production of these bacteria is becoming more and more important, and the design of new growth media is required for production of probiotic biomass to respond to the increasing demand for food containing such microorganisms [2-4]. Therefore, the important step in the preparation of a low cost

industrial process for production of probiotic biomass is the optimization of the growth conditions and medium components [5]. Since most of probiotic microorganisms require a rich medium for their growth, it is necessary to search for some inexpensive alternative substrates, to be used as a part of the growth medium formulation. The cost of nutrient substrates and the processes used play important roles to ensure optimal results [3-4,6].

Lactic acid bacteria grow well on De Man-Rogosa-Sharpe medium (MRS), nevertheless at industrial scale, production of biomass or metabolites requires less expensive media. One of the ways to reduce the production cost is to use low cost growth media by utilizing low cost substrates like food processing wastes [7]. Several studies focused on the optimization of the growth medium of probiotic strain, PATHAK and MARTIROSYAN (2012) conducted a study to modify MRS culture medium, using plant seed powders for the growth of probiotic starter culture of *Lactobacillus lactis* and they found that the growth of probiotic increased significantly when compared with standard MRS medium [8].

The important amount of wastes generated by agricultural and agri-food industries and practices represents which causes serious problem for the environment can be used as substrates for the production of industrially important compounds and microbial biomass, because of their high content of sugars and furthermore they can be easily assimilated by microorganisms [9]. Date fruit waste is an example of agri-food wastes generated in the Arab region. This waste may potentially be a suitable substitute for the glucose required by the lactobacilli during their growth and biomass production because it contains large amounts of sucrose, glucose and fructose and it also contains proteins, lipids, mineral elements and some vitamins [6, 10-11]. Dates syrup was used as a source of sugar for the cultivation of *Lb. plantarum* and for the production of carotenoids [12]. SHAHRAVY *et al.* (2012) used date powder as a low-cost main carbon source during the optimization of culture conditions for the production of a probiotic bacterium, *Lb. casei* ATCC 334 [6].

This study was conducted to analyse the effect of a local date juice containing media on the production of a probiotic lactic acid bacterial biomass, optimization was carried out using Plackett-Burman experimental design.

1.- Materials and methods

1.1.- Bacterial strain and culture media

The bacterial strain used in this study was isolated previously from chicken crop, it was identified by biochemical tests and API gallery system (Biomerieux) for *Lactobacilli* identification as *Lactobacillus plantarum* G_1, the strain was considered as a probiotic bacterium based on its high resistance to bile salts, low pH and simulated intestinal fluid. The strain was further identified by molecular technique using 16S rRNA gene. Partial sequence of 16S rRNA was amplified by PCR technique, sequenced by automate sequencer after isolation and extraction of DNA. 16S rRNA was compared with the sequences deposited in Genbank using BLAST sequence comparisons program. The phylogenetic tree was generated by Tree Dyn program (v198.3) proposed by Methods and Algorithms for Bioinformatics LIRMM (http://phylogeny.lirmm.fr/phylo_cgi/simple_phylogeny.cgi).

The bacterium G_1 was activated by culturing it in Man Rogosa Sharpe (MRS) broth (20 g glucose, 10 g peptone, 10 g meat extract, 5 g yeast extract, 5 g sodium acetate, 2 g bipotassic phosphate, 2 g ammonium citrate, 0.2 g magnesium sulfate, 0.05 g manganese sulfate and 1.08 ml Tween 80, pH 6.5) (Biokar Diagnostics, France) for 24 h at 37°C. The purity of the strain was checked on MRS agar and it was stored at -20°C in 20% (v/v) glycerol.

2.2.- Preparation and analysis of date juice

Date wastes were collected from the city of Ghardaïa located in the northern of Algerian Sahara. Morphological observation indicated that they contained several varieties of dates; they were fallen before and/or during the harvesting operation making them inappropriate for human consumption. To prepare date juice, 160 g of washed and pitted dates were weighed and minced in distilled water and final volume was made up to 1200 ml. The water–date mixture was heated at 80-85°C for 45 min with stirring, filtered through a cloth and sterilized at 121°C during 20 minutes [13-14].

The pH value was measured with a Hanna pH-meter. The titratable acidity (TA) was determined by measuring the citric acid that constitutes the major organic acid of date juice. 40 ml of juice has been titred by a solution of NaOH 1N, in presence of some drops of phenolphthalein indicator. Contents of total soluble solids (°Brix) were determined using a digital refractometer [15]. The dry matter was determined after drying a sample in an isothermal oven at a temperature of 105°C for 24 h until a constant sample weight [16]. The ash content was estimated based on the destruction of all organic matter as a result of combustion in a muffle furnace at 550°C for 3 h (g ash/100 g sample) [17].

The mineral content was determined by atomic absorption spectrophotometry. Samples were mineralized and placed in an acid solutions, and then were atomized in an acetylene flame [18]. Protein content was determined using the Kjeldahl method. The digestion was carried out by boiling with concentrated sulphuric acid using $CuSO_4/TiO_2$ as catalysts. The ammonium salt was then converted to ammonia by the addition of sodium hydroxide; after steam-distillation the ammonia was trapped in a boric acid solution. The quantity of ammonia was determined by titration with a standardized acid solution, and its value expressed using the appropriate calculations [19].

The total sugars content was determined using the method described by Dubois *et al.* (1956). The total sugars are first extracted with distilled water in the presence of concentrated sulfuric acid; the monosaccharides are dehydrated in compounds of the family furfural derivatives. These products are condensed with phenol to give yellow-orange complex monitored by measuring the increase in optical density at 490 nm. A standard curve was previously prepared from 0.01% glucose solution [20].

The total phenolic content (TPC) analysis was carried out according to the method described by JUNTACHOTE *et al.* (2006) using Folin-Ciocalteu reagents. Approximately 0.5 ml of diluted date juice samples was added into test tubes followed by 0.5 ml of Folin-Ciocalteu's reagent and 0.5 ml of sodium carbonate. The blank sample was prepared by replacing 0.5 ml of sample with 0.5 ml of distilled water. The test tubes were vortexed for 10 s and allowed to stand in the dark environment at room temperature for 1h. The optical density of the blue-colored samples was measured and was read in triplicate at 760 nm. The calibration curve was constructed with gallic acid and the total phenolic contents were

expressed as mg equivalent gallic acid (GAE)/100g dry matter basis [21].

1.3.- Biomass estimation

For the inoculum preparation, 1mL from reactivated culture was taken and used to inoculate 100 ml Erlenmeyer flask containing 20 ml of MRS broth. The inoculated flask was incubated at 37°C for 16 h. The biomass concentration (dry weight g/l) was estimated using a calibration curve calculated using dilutions of a biomass suspension with known optical density (OD) [22]. An Erlenmeyer flask containing 50 ml of MRS broth was inoculated with 5 ml of bacterial culture and was incubated at 37°C for 16 h. A fixed volume of the dilutions (10ml) with a measured OD was centrifuged at 6000× *g* for 20 min. The pellet was recovered with 100 µl of distilled water, filtered through a Whatman papers filters and left to dry at 105°C [23]. Filters were weighed before filtration and after drying. A relationship can be established between biomass concentration (g/l) and optical density (600 nm).

1.4.- Growth in presence of date juice

In order to evaluate the efficiency of using date juice as medium for *Lb. plantarum* G1 growth, two Erlenmeyer flasks, one containing 10 ml MRS broth and the other containing 10 ml date juice alone were prepared. They were inoculated with bacterial cells (5%) and then incubated for 24 h at 37°C. The biomass concentration (g/l) was determined. To evaluate the effect of date juice concentration on biomass production, different concentrations of date juice (1%, 2%, 4%, 8%, 10%, 12% and 14%) were added to 100ml Erlenmeyer flasks containing 20ml of MRS broth without glucose. The flasks were inoculated with 5% of a 16 h bacterial culture and were incubated at 37°C for 48h. Twenty ml of MRS broth with glucose inoculated and incubated in the same conditions was used as a control. The optical density was measured at 600 nm after 24 h and 48 h, biomass (g/l) was determined as mentioned before.

1.5.- Plackett-Burman design

To screen medium components that affect the biomass production, Plackett-Burman (PB) statistical design was applied [24]. Seven components were selected for the design including date juice and each factor was represented at two levels, high (+) and low (−) as shown in Table I. A total of 8 sets of experiments (Table I) were carried out in 100 ml Erlenmeyer flask containing 20 ml of each medium in order to determine the significant factors affecting production of biomass. The Erlenmeyer flasks were inoculated with a 16 h bacterial culture and incubated for 24 h. All experiments were carried out in duplicate and the biomass concentration was determined as mentioned above. The variable with confidence levels above 95% is considered the most significant factor that effects the biomass production of *Lb. plantarum* G1. PB statistical design is based on the first-order model as given in the following equation:

$$Y = \beta_0 + \Sigma \beta i \, xi \qquad (1)$$

Where Y is the response value (biomass production), β_0 is the model intercept and β_i is the linear coefficient, X_i represents the level of the independent variable.

Table I.- Plackett-Burman design for biomass production by *Lactobacillus plantarum* G_1

Trials	X1 Date juice (%)	X2 Yeast extract (g/l)	X3 Peptone (g/l)	X4 Tween (g/l)	X5 Ammonium citrate (g/l)	X6 Temperature (°C)	X7 pH	Biomass (g/l)
1	10(-1)	10(+1)	20(+1)	1(-1)	2(+1)	37(+1)	5.5(-1)	22.47
2	10(-1)	5(-1)	20(+1)	6(+1)	0.5(-1)	37(+1)	6.5(+1)	17.74
3	14(+1)	5(-1)	10(-1)	6(+1)	2(+1)	30(-1)	6.5(+1)	19.84
4	14(+1)	10(+1)	10(-1)	1(-1)	2(+1)	37(+1)	5.5(-1)	22.32
5	10(-1)	10(+1)	20(+1)	1(-1)	0.5(-1)	37(+1)	6.5(+1)	23.05
6	14(+1)	5(-1)	20(+1)	6(+1)	0.5(-1)	30(-1)	6.5(+1)	22.07
7	14(+1)	10(+1)	10(-1)	6(+1)	2(+1)	30(-1)	5.5(-1)	22.96
8	10(-1)	5(-1)	10(-1)	1(-1)	0.5(-1)	30(-1)	5.5(-1)	21.6

2.- Results and discussion

2.1.- Molecular identification of the strain

The isolated strain *Lb. plantarum* G_1 was genetically identified and the partial sequence obtained after the isolation of 16S rRNA gene by PCR was analyzed using the BLAST program of the Genbank. The Results indicated that the isolate under study was 99% closer to *Lb. plantarum*. Moreover, the phylogenetic analysis confirmed a closer phylogenetic proximity of the G1 sample with the *Lb. plantarum* species (fig. 1). The nucleotide sequence was deposited in the GenBank sequence database and given the accession number KC965107.

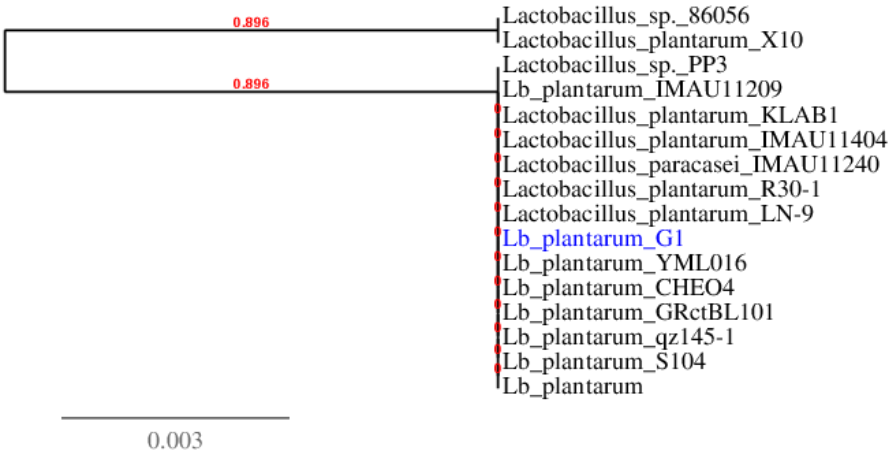

Figure 1.- Phylogenetic relation of *Lb. plantarum* G1 strain sequence with 16S rRNA of the highest 15 similar bacteria. The dendrogram was generated by Tree Dyn program (v198.3)

2.2.- Date juice analysis

Fermentation processes are based on the quality of the used culture medium. Therefore, it is important to determine the biochemical composition of date juice which can vary with the quality of the used dates, the ripening stage of the date, the cultivation conditions such as soil and agronomic practices, etc. [25]. The physicochemical characteristics of our date waste juice are summarized in table II.

From the obtained results (tab. II), it can be observed that the pH value for the tested date juice was 6.35 and its TA value was found to be 0.31% (as citric acid, on wet weight basis). BENCHABANE *et al*. (2012) found that the pH value of date juice prepared from Ghars variety collected in the last stage of maturity was similar to our result (6.2) [14]. The obtained pH is higher than that obtained by Acourene and Tama (2001) and OULD EL HADJ *et al*. (2012), which were 5.9, 5.82, for date juice prepared from Tinissine (Touggourt) and Techerwit varieties, respectively [18,26]. Concerning TA, it is in general an intermediate of the metabolic processes. It influences the growth of the micro-organisms and affects the quality of conservation of the products. According to ACOURENE *et al*. (2007), acidity is dependent on the conditions of fertilization and irrigation on the palm trees [13].

The results showed that the date juice had 2.6 g (9.37%) of dry matter and 90.63% of moisture. This obtained result is comparable with other findings. OULD EL HADJ *et al*. (2006) reported that the dry matter and humidity were 11.2 ± 1.07%, 88.8±2.57%, respectively [27]. BENCHABANE *et al*. (2012) found that dry matter was equals to 10.57% and the humidity was 89.43% [14].

As illustrated in Table II, the total soluble solids (TSS) for the prepared date juice was 61°Brix. TSS depends on several factors such as the variety and the methods of extraction of sugar. ACOURENE and TAMA (2001) reported that the total soluble solids for tested date juice from Deglet Nour, Tantboucht and Tinissine varieties were 21°, 22.5°, 23.5°, respectively [18]. The ash content of the date juice was 1.11% of dry matter. The obtained value is in the same line with that reported by ACOURENE and TAMA (2001) for juice extracted from Deglet Nour and slightly lower than juice from Tinissine (1.34) and Tantboucht (1.49%) varieties [18]. However, OULD EL HADJ *et al*. (2006) reported a concentration of 1.78±0.05 for date juice prepared from date waste (h'chef) [27]. The difference in ash content may be due to the difference in climatic and storage conditions [28].

Date juice contained 0.331 /100 g (0.331% fresh matter FM) of protein; this result is in accordance with those found by OULD EL HADJ *et al*. (2012) in juice of Techerwit variety and lower than in Degla Beida (0.53%) and Hamraya (0.67%) varieties [26]. Acourene and Tama, (2001) reported that Deglet Nour, Tantboucht and Tinissine juice contained 0.24%, 1.05%, 0.80% of protein, respectively [18]. Even if protein content is low, other researchers have reported that it contains 23 types of amino acids, some of which are not present in most popular fruits [16].

The total sugars content was equal to 6.6% on a fresh weight basis. This value was less than that obtained by OULD EL HAJD *et al*. (2006) (16.64±1.08) [27]. Acourene *et al*. (2007) obtained a date juice with a content of total sugars (22.61% of FM) from offal's of Deglet-Nour [13]. In another study, OULD EL HADJ *et al*. (2012) extracted only 11.61± 0.26% and 11.00±0.21 from Degla Beida and Hamraya variety. These findings confirmed that the sugar content and type depend on the date variety, consistence and maturation state [16].

As shown in table II, total phenolic content in date juice was 14.53mg/ml. Mansouri *et al*. (2005) analyzed mature date varieties from valley of Ghardaïa and they showed that date contains overall a low total phenolic rate [29]. Some important minerals were present

in our date juice with significant amounts, results showed that Iron was the predominant mineral (2.53mg/100 ml FM), followed by, zinc (1.5 mg/100 ml FM), copper (0.9 mg/100 ml FM), and manganese (0.8 mg/100 ml FM). Our results were comparable with those reported by ACOURENE *et al*. (2007) where iron content was similar with that obtained in Deglet Nour juice and smaller than that found in Tantboucht juice (5.86 mg/100 ml FM). Manganese, copper and zinc content were higher than that obtained in Deglet Nour juice (0.07, 0.07 and 0.25 mg/100 ml FM) [13].

Table II.- Physicochemical parameters of date juice

Parameters	Date waste juice
pH	6.35
Titratable acidity (%)	0.31
TSS°Brix	61
Dry matter	2.6 g (9.37%)
Humidity (%)	90.63
Ash (%)	1.11
Total sugars	35g/l (6.6g/100g FM[a])
Total nitrogen (%)	0.053
Protein (g/100g)	0.331
Total phenolic (mg/ml)	14.53
Manganese mg/100 ml FM	0.8
Zinc mg/100 ml FM	1.5
Iron mg/100 ml FM	2.53
Copper mg/100 ml FM	0.9

FM: Fresh Matter

2.3.- Production of probiotic biomass in the presence of date juice

The optimization process was carried out using date juice as source of carbon supplemented with other components of MRS for *Lb. plantarum* G1 growth. Biomass was estimated as cell dry mass by converting the optical density (OD) of culture to dry cell mass through a linear correlation standard curve. The use of calibration curve of biomass dry weight and OD to estimate biomass concentration is a simple method compared with other techniques such as the determination of colony forming units (CFU).

According to the obtained results, the moisture and carbohydrate were predominant in date juice, with small amounts of total phenolics, protein and mineral matter. With regard to the total sugar content, the date juice may be used potentially as a suitable and economical alternative to glucose required for the growth of Lactobacilli. In order to compare the *Lb. plantarum* G1 biomass production in MRS standard medium and in date juice alone, two culture media was prepared, the first one containing MRS broth and the second one consisted of date juice alone. The results showed that the date juice is favorable to the growth of the bacterial strain (15.19 g/l biomass) because it contains a high amount of sugars easily assimilated by *Lb. plantarum* whereas, the biomass production in MRS medium (22.85 g/l biomass) is higher than that obtained in date juice alone. These results may be explained by the low protein and mineral content in date juice which is insufficient for Lactobacilli growth compared with MRS broth which is a rich medium. The best culture medium for industrial production is that which provide the best production yield in the shortest time with low cost [13], date fruit may represent one of them if appropriately optimized [3]. Date extract constitutes a good fermentation medium for microbial growth. It is rich in mineral matter including calcium, potassium and phosphorus but poor in

magnesium, thereby the medium needs some additives [26]. BOUDJELAL and NANCIB (2001) reported that date juice alone is a poor nitrogen and salts source for *Lb. rhamnosus* growth and lactic acid production. Consequently, the juice was enriched with different nutriments such as yeast extract, $MgSO_4$, K_2HPO_4, KH_2PO_4 and Tween-80 [30]. In another study, ELSANHOTY *et al.* (2012) used date juice for carotenoid production by *Lb. plantarum* strains, they observed that the use of date juice alone gave significant yield. However, supplementation of MRS medium with salts and organic nitrogen further increased the yield [12].

To evaluate the effect of date juice concentration on biomass production, MRS medium was supplemented with different concentrations of date juice (tab. III). The results showed that the date juice is favorable to the growth of the bacterial strain, the biomass increases after addition of date juice reaching maximum yield (25.38g/l) when the culture medium supplemented with 12% of date juice (35 g/l of total sugar), the same biomass concentration was noted with MRS broth. Below this, the value of biomass concentration decreased. The obtained biomass concentration in the medium containing 12% of date juice instead of glucose and supplemented with salts and organic nitrogen sources was very close to that obtained with MRS standard medium. After 48 h the biomass concentration decreased with all tested date juice concentrations. Thus, date juice may be considered as good alternative to glucose because it is rich in sugars and it provides a good source of energy since most of the carbohydrates are fructose and glucose, which are easily assimilated by *Lb. plantarum* G1 for its growth.

Table III.- Biomass concentration in MRS medium and glucose-free MRS supplemented with different concentrations of date juice

Date juice concentration (%)	1	2	4	8	10	12	14	MRS with glucose
Biomass (g/l) after 24h	14.8	20.8	23.5	24.1	24.4	25.38	24.4	25.2
Biomass (g/l) after 48h	13.86	15.02	21.96	21.07	24.1	23.5	22.65	26.3

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2.4.- Evaluation of the effect of medium components on biomass production

Plackett-Burman design is a screening test that can be used to determine the significant factors and allows selecting which of them can be further investigated to find the optimum values [31]. For this purpose eight medium formulations were examined using Plackett-Burman statistical design to select the factors affecting biomass production. The main effect of each variable was calculated and presented graphically in Figure 1a; it was estimated as the difference between the averages of measurements made at the high level and at the low level of a factor. The data in table I showed a variation of biomass production from 17.74 to 23.05 g/l. This variation showed the strongest influence of medium components on biomass production and reveals the importance of optimization process to reach higher productivity. The analysis of the data from the Plackett–Burman experiments involved a first order (main effects) model. The polynomial model describing the correlation between the factors and biomass production(Y, g/l) could be presented by the following equation:

$$Y_{biomass} = 21{,}506 + 0.67X_1 + 0.74X_2 + 1.51X_3 - 1.18X_4 - 0.33X_5 - 1.5X_6 - 1.19X_7 \quad (2)$$

From the analysis of the regression coefficients of the variables presented in table IV, peptone had the major positive effect on biomass production, followed by yeast extract and

date juice, whereas Tween 80, temperature, pH and ammonium citrate had a significant negative influence. Thereby, variables with positive effect are required with a concentration higher than the high value (+) concentration for further optimization studies and variables with negative effect are required with a concentration lower than the indicated as low level (-) in PB design [32].

The ranking of factor estimates is given in a Pareto chart as illustrated in Figure 1b. The Pareto chart demonstrated the importance of each factor and offers an effective tool to present the results obtained by PB design [33]. SREEKUMAR and KRISHNAN, (2010) applied PB design to enhance probiotic biomass production of *Bacillus subtilis* SK09, they found that pH, ammonium citrate and peptone were the most important variables [31].

Table IV.- The regression analysis of the effect of each variable along with the coefficient level, t and P value

Variables	Coefficient	*t-value*	*P-value*
Date juice (%)	0.67	11.5734	0.0548
Yeast extract (g/l)	0.74	12.8722	0.0493
Peptone (g/l)	1.52	26.3072	0.02418
Tween- 80 (g/l)	-1.18	-20.3762	0.03121
Ammonium citrate (g/l)	-0.33	-5.729	0.11001
T°C	-1.5	-25.9176	0.02455
pH	-1,19	-1.3765	0.21782

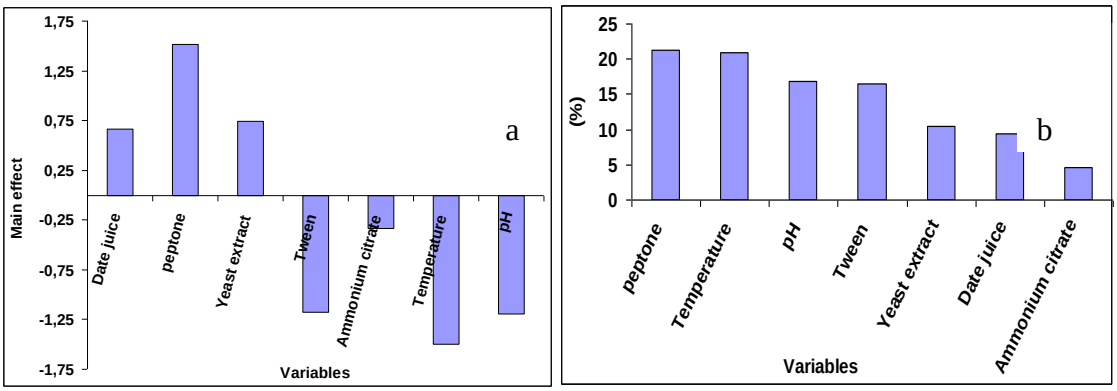

Figure 1 (a,b).- The effect of the studied factor on the production of biomass of *Lb. plantarum* G_1. (a: Main effect of different factors on the biomass production; b: Pareto chart rationalizing the effect of each variable on the biomass production)

Conclusion

Based on the obtained results, date juice could be proposed as an efficient substitute of glucose in MRS medium. The variables with highest confidence levels were considered as most significant on probiotic biomass production. In this study date juice, yeast extract and peptone were found to be the most significant variables affecting biomass production. These factors may be used for further optimization of biomass production of *Lb. plantarum* G_1.

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