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Screening of some factors affecting bacteriocin production from *Lactobacillus curvatus* G6 using Plackett-Burman design

Mohamed Sifour[1,2,*], Houria Ouled-Haddar[1,2], Tayeb Idoui[1,3], Salima Aissaoui[1], Hiba Namous[1]

[1]Department of Applied Microbiology and Food Sciences, Faculty of Nature and Life Sciences, University of Jijel, (ALGERIA)

[2]Laboratory of Molecular Toxicology, University of Jijel, (ALGERIA)

[3]Laboratory of Biotechnology, Environment and Health, University of Jijel, (ALGERIA)

E-mail : sifourm@yahoo.fr

ABSTRACT

Lactobacillus curvatus G6 isolated from newborn feces is capable of producing significant amounts of antimicrobial compounds, especially bacteriocin. Bacteriocin of *Lb. curvatus* G6 is active against *Listeria monocytogenes* and methycilin resistant *Staphylococcus aureus*. The produced bacteriocin showed stability in acidic and neutral pH with optimal activity at pH 6.0, sensitivity to proteolytic enzyme, and heat stability at different temperatures (from 40°C to 100°C). Bacteriocin production started at the beginning of the log phase of the bacterial growth with maximum level after 28 hours (stationary phase). The optimum temperature for the growth (37°C) did correspond with the temperature requirement for the maximum bacteriocin activity. Plackett-Burman design was used to screen factors affecting bacteriocin production by *Lb. curvatus* G6. Results demonstrated that glucose seems to be the most significant factor affecting bacteriocin production followed by ammonium citrate and peptone.

KEYWORDS

Antimicrobial activity;
Bacteriocin production;
Heat stability;
Lactic acid bacteria;
Lactobacillus curvatus;
Listeria monocytogenes;
Plackett-Burman design.

INTRODUCTION

Lactic acid bacteria (LAB) represent a heterogeneous group of microorganisms that are naturally present in many foods and in the gastrointestinal and urogenital tract of animals[1]. Some strains of LAB possess the potential to combat gastrointestinal pathogenic bacteria such as *Helicobacter pylori*, *Escherichia coli* and *Salmonella* and spoilage organisms and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus*[2]. It has been shown that these microorganisms can produce antimicrobial compounds, such as bacteriocins or bacteriocin-like inhibitory substances[1]. Bacteriocins become a current subject for several researches. These bacteriocins are now being explored for their potential utility in human and animal health applications, food biopreservation and agricultural uses[3,4]. An experimental focus on bacteriocin production by probiotics LAB strains has indicated that this potential might play a considerable role during in vivo interactions occurring in the human gastrointestinal tract, for instance towards *H. pylori*. Whereas bacteriocins in food are degraded by the proteolytic enzymes of the stomach, probiotic bacteria may lead to in situ

production of bacteriocins in the gastrointestinal tract[2, 5].

Bacteriocins are ribosomally synthesized peptides, and this fact creates the possibility of improving their characteristics to enhance their activity and spectra of action. Production of bacteriocins by LAB is growth associated: it usually occurs throughout the growth phase and stopped at the end of the exponential phase (or sometimes before the end of growth)[3, 6,7], but the yield of bacteriocin per unit biomass is affected by several factors and the most important are media (carbohydrate and nitrogen sources, cations) and fermentation conditions (pH, temperature, agitation and aeration and dilution rate in continuous fermentation Bacteriocin production is deeply affected by type and level of the carbon, nitrogen and phosphate sources, cations, surfactants and inhibitors[6,8,9]. Different studies have shown that type of carbon source and its concentration represents a crucial factor in optimization of bacteriocins. On the other hand, some studies demonstrated that also nitrogen source plays an important role in the optimization of bacteriocin production. Many studies have shown that highest bacteriocin titres usually are obtained at pH and temperature values lower than the optimum ones for growth[6,8,10]. Since pH control improves the growth of LAB, it also results in improved bacteriocin production. However, the optimal pH for bacteriocin production is usually 5.5-6.0. Growth at optimal temperature usually results in optimal bacteriocin production but temperature stress and growth at sub-optimal temperature may result in an increase of bacteriocin production[6]. This was also observed by Lim[9], when he found that the bacteriocin production during the growth period of *Lb. plantarum* KC21 was higher at 30°C than at 37°C. It seems that agitation and aeration affect bacteriocin production. In fermentations at pH 5.5 using glucose media with *Lc. lactis* IO-1, maximum nisin Z concentration was obtained at 320 rpm and only a small decrease of nisin concentration was obtained at 1000 rpm. On the other hand agitation at >540rpm resulted in inhibition of growth and nisin production in xylose media[6]. There is a general practice of determining optimal concentration of media components by varying one factor at a time. However, this method is extremely time consuming and expensive for a large number of variables and may result in wrong conclusion. Experimental design techniques present a more balanced alternative to the one-factor-at-a time approach to fermentation improvement. The factorial design of a limited set of variables is advantageous in relation to the conventional method of manipulation of a single parameter per trial, as the latter approach frequently fails to locate the optimal conditions for the process, because of its failure to consider the effect of possible interactions between factors[11,12].

The aim of the present study was to characterize and determine the antimicrobial spectrum of a bacteriocin produced by the *Lactobacillus curvatus* G6 isolate and to select the factors affecting culture conditions for bacteriocins production using the Plackett-Burman design.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Lactobacillus curvatus G6 used in this study was previously isolated and identified from newborn feces[13]. Isolate was grown at 37°C in Man Rogosa Sharpe (MRS) broth (Biokar Diagnostics, France). Indicator strains used for determining antimicrobial activity were grown on nutrient agar. The antimicrobial activity and bacteriocin assay were realized on Muller-Hinton agar.

Determination of bacteriocin activity

Lb. curvatus G6 was grown in MRS broth (pH 6.5) inoculated with 1% of an overnight culture and incubated overnight at 37°C for 24 h. After incubation, cells were removed by centrifugation (6000×g for 20 min at 4°C). The cell-free supernatant was sterilized by filtering through a 0.22 µm Millipore filter. The antimicrobial activity was determined using the well diffusion method (WDM)[14]. The indicator bacteria were cultured on nutrient agar for 24 h at 37°C, and used to prepare cell suspensions in 9 ml normal saline. Twenty ml of Muller Hinton agar cooled to 45°C was mixed with 110µl of the indicator strain suspension, pooled in a Petri dish and incubated aerobically for 2 to 4h at 37°C. A 6 mm wells were made and were filled with 100µl of the supernatants. Plates were incubated at 37°C for 24 h. Inhibition zones were determined by measuring the diameter of the clear zones around the well. Bacteriocin activity was assayed as follow; the antimicrobial effect of organic acids was eliminated by adjusting the pH to 6.5 with NaOH 5N. Inhibitory effect

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of hydrogen peroxide was eliminated by the addition of 1mg/ml catalase. The catalase-treated samples were incubated for 2 h at 37°C, after incubation the treated and neutralized cell-free supernatants were then tested for antagonistic activity against indicator bacteria by the WDM[15]. Bacteriocin activity was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition[16]. Zone of 1 mm and above was considered as inhibition.

Effect of temperature, pH and enzymes on bacteriocin activity

In order to characterize the produced bacteriocin, the effect of temperature, pH and hydrolytic enzymes was studied. The effect of temperature on the bacteriocin stability was tested by heating the cell-free supernatant samples of *Lb. curvatus* G6 at 40, 60, 80 and 100°C. Residual activity was determined after 15, 30 and 60 min by the WDM using methycilin resistant *Staphylococcus aureus* (MRSA) as indicator organism. For pH effect, cell-free supernatant of an overnight culture of *Lb. curvatus* G6 was adjusted to pH 2.0 to12.0 with HCl 1N or NaOH 5N. After 4 h of incubation at room temperature, the samples were readjusted to pH 6.5 and the activity was determined by the WDM using MRSA as indicator organism. To determine the effect the enzymatic treatment, supernatant was treated with the following enzymes at a final concentration of 1 mg/ml: lipase (Sigma), trypsine (Sigma), α-chymotrypsin (Merck), pronase E (Merck), α- amylase (Fluka). Samples and the controls (consisted of only cell-free supernatant and tris-HCl buffer) were incubated at 37ºC for 2 h and heated in boiling water for 5 min to inactivate the enzymes. The remaining activity was determined by the WDM using the MRSA as indicator organism.

Dynamics of bacteriocin production

The growth and the production of bacteriocin by *Lb. curvatus* G6 through 48 h was carried out. 100 ml MRS broth was inoculated with an 18 h-old culture (1 %, v/v) of *Lb. curvatus* G6 and incubated at 37°C for 48 h. Bacterial growth (OD at 660nm), changes in culture pH, and antimicrobial activity of bacteriocin against MRSA were determined at time interval. The WDM was used and the activity expressed as AU/ml as described previously.

Optimum temperature of bacteriocin production

MRS broth in Erlenmeyer flasks was inoculated with an 18 h-old culture (1 %, v/v) of *Lb. curvatus* G6 and incubated at different temperatures: 30, 37 and 40°C. Samples were collected after 24 h and examined for bacteriocin production (AU/ml) as described earlier.

Screening of factors affecting production using Plackett-Burman design

In order to determine which nutrients and physical conditions affect bacteriocin production, a screening experiment was applied using a fractional two-level factorial design according to Plackett and Burman[17]. For screening purposes, various medium components as well as environmental factors have been evaluated. The different factors were prepared in two levels: -1 for low level and +1 for high level (TABLE 1), based on Plackett-Burman statistical design. This is a two–level fraction factorial design that allows the investigation of n-1 variables in at least n experiments. Seven independent variables were screened in 08 combinations. All trials were done in 100 ml Erlenmeyer flask containing 50 ml of the medium. Each Erlenmeyer flask was inoculated with 1% v/v of an 18 h bacterial culture (A660 = 0.58 corresponding to approximately 24×10^7 CFU/ml) grown on MRS medium. After 24 hours of incubation, cells were removed by centrifugation at 6000 rpm for 20 min. The supernatants were neutralized with NaOH 5N and filtered through a 0.22 µm Millipore filter. The WDM was used and the activity was expressed as AU/ml as described previously.

The main effect and statistical t-value of each variable were calculated. The calculated effect can be positive, negative or neutral depending on the overall influence of the variable upon the measured response. Also, the variable significance was determined by using probability table depending on P value, the variables which have more significant effects were used for further optimization. Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \Sigma \beta i\, xi$$

where Y is the response (bacteriocin production), β_0 is the model intercept at center point and βi is the variable coefficient for the independent variable and xi are the independent variables. The values of the coefficients were calculated by data regression using Microsoft Excel 7.0 software. The model describes no interac-

tion among factors and is used to screen and evaluate the important factors that influence bacteriocin production. The variables whose confidence levels were higher than 95% were considered to significantly influence the measured responses.

Statistical analysis of the data

The data were subjected to multiple linear regressions using MICROSOFT EXCEL 2007 to estimate t -value, P-value and confidence level. The significance level (P-value) was determined using the Students t -test. The t -test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. If this probability is sufficiently small, the idea that the effect was caused by varying the level of the variable under test is accepted. Confidence level is an expression of the P-value in percent.

TABLE 1 : Media components and test levels for Plackett–Burman experiment

Variable	Variable code	Low level (-1)	High level (+1)
Glucose (g/l)	*X1*	0.25	0.5
Beef extract (g/l)	*X2*	0.25	0.5
Yeast extract (g/l)	*X3*	0.125	0.25
Peptone (g/l)	*X4*	0.25	0.5
Tween (g/l)	*X5*	0.025	0.05
Ammonium citrate (g/l)	*X6*	0.05	0.1
pH	*X7*	5.5	6.5

RESULTS AND DISCUSSION

Bacteriocin production by *Lb. curvatus* G6

Cell fee supernatant of *Lb. curvatus* G6 showed antimicrobial activity against several bacteria such as MRSA, *B. subtilis*, *E. coli* ATCC28484, pathogenic *Klebsiella* sp. 111 and *L. monocytogenes*. When the neutralized cell-free supernatant was treated with catalase (1mg/ml) the strain confirmed its activity only against three indicator strains (*L. monocytogenes*, MRSA and *B. subtilis*) (TABLE 2). The treated cell-free supernatants effectively inhibited *B. subtilis* with a maximum inhibitory activity (1667 AU/ml), MRSA (1500 AU/ml) and *L. monocytogenes* (1000 AU/ml). The neutralized cell-free supernatant exposed to the action of catalase inhibited the growth of the indicator strains (*L. monocytogenes*, MRSA and *B. subtilis*) which gives evidence that the inhibitory activity is due to the production of bacteriocins[18]. Bacteriocins produced by LAB have a broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria. Bacteriocins have bactericidal or bacteriostatic activity towards closely related Gram-positive bacteria. The antimicrobial spectrum frequently includes spoilage organisms and food-borne pathogens such as *L. monocytogenes* and *Staphylococcus*. The activity against Gram-negative bacteria such as *E. coli* and *Salmonella* has been shown, but usually only when the integrity of the outer membrane has been compromised, for example after osmotic shock or low pH treatment, in the presence of a detergent or a chelating agent, or after a pulsed electric chock or high-pressure treatment[2,19]. Singh and Prakash[20] found that, several LAB strains isolated from cottage cheese are capable of inhibiting pathogenic microorganisms in the food environment and display crucial antimicrobial properties with respect to food preservation and safety.

Effect of temperature, pH and enzymes on bacteriocin activity

Based on the results showed in Figure 1 bacteriocin produced by *Lb. curvatus* G6 was considered to be heat stable, as the activity remained constant after heating at 100°C for 60 min (Figure 1). Ogunbanwo et al.[21] recorded during the characterization of bacteriocin produced by *Lb. plantarum* F1 and *Lb. brevis* OG1, that bacteriocin produced by *Lb. brevis* OG1 was considered to be the most heat stable, as the activity remained constant after heating at 121°C for 60 minutes, but declined thereafter, while bacteriocin produced by *Lb. plantarum* F1 activity remained constant after heating at 121°C for 10 min followed by subsequent decline. Other results were obtained by Belguesmia et al.[22] through a characterization of enterocin S37: a bacteriocin produced by *Enterococcus faecalis* S37, isolated from poultry feces. The results showed that, the

TABLE 2 : Antimicrobial activity of supernatant of *Lb. curvatus* G6 against some indicator strains

Indicator strains	MRSA	*B. subtilis*	*E. coli* ATCC 28404	*Klebsiella sp.* 111	*L. monocytogenes*
CFS	+	+	+	+	+
Neutralized CFS	+	+	-	+	+
Neutralized CFS treated with catalase	+	+	-	-	+

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stability of antibacterial activity of enterocin S37 remained intact after heating treatments (80°C for 1h and 90°C for 15 min). Figure 2 showed that the antimicrobial activity of *Lb. curvatus* G6 was significantly influenced by pH; it was observed that the residual activity was stable in the range of pH 6.0 to pH 10.0 with maximum activity at pH 6.0. Kumar et al.[23] showed that the antimicrobial property of enterocin LR/6, a bacteriocin from *Enterococcus faecium* LR/6, remained unaffected in the pH range of 2-6. At pH 7 and 8, the activity was reduced by 20%. This property has been considered highly useful for their application as food preservative.

The bacteriocin of *Lb. curvatus* G6 was exposed to the action of various enzymes (lipase, trypsine, α-chymotrypsin, pronase E and α-amylase). Complete inactivation or significant reduction in activity was observed after treatment of the cell-free supernatant with chymotrypsine, trypsine and pronase which confirmed the proteinaceous nature of the active agent. The other enzymes (amylase and lipase) did not cause inactivation. This confirmed that carbohydrate and lipid moieties if existing were not required for the inhibitory activity. It is well established now that all the bacteriocins are of proteinaceous nature, or at least contain a peptide which is responsible for their bactericidal function. However, their sensitivity to the proteolytic enzymes is variable[24]. Ivanova et al.[25] observed that trypsine, chymotrypsine and rennin had no effect on bacteriocin produced by *Lc. lactis* subsp.*lactis* b14.

Dynamics of bacteriocin production

Growth and bacteriocin production by *Lb. curvatus* G6 was monitored during 48 hours of growth in MRS broth. Production of bacteriocin started after 4h of incubation at the beginning of the log phase of the bacterial growth (Figure 3), and increased gradually with bacterial growth till it reached its maximal level (2416.66 AU/ml) after about 30h of incubation (in stationary phase). The bacterial growth increased during the same period of incubation and remained more or less constant after 32 h. This result suggests that bacteriocin production is growth associated. Several Authors have noted that bacteriocin production is dependent on biomass concentration. The optimal level of plantaricin ST194BZ, produced by *Lb. plantarum* ST194BZ, was obtained in growth media that supported high biomass production, such as MRS[26]. After about 30 h of incubation a decrease in

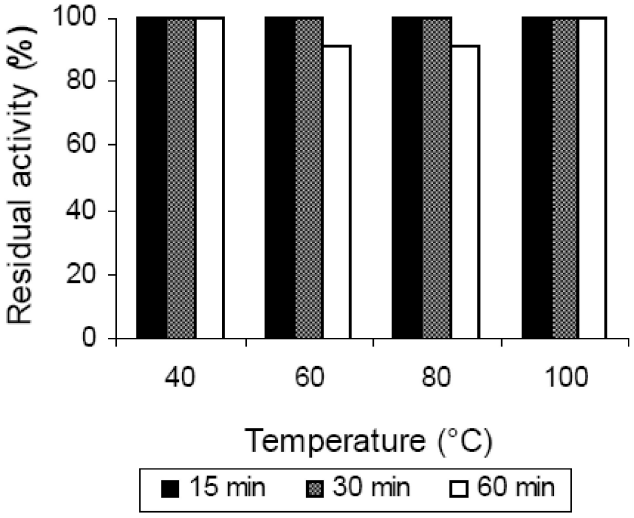

Figure 1 : Effect of temperature on activity of bacteriocin produced by *Lb. curvatus* G6.

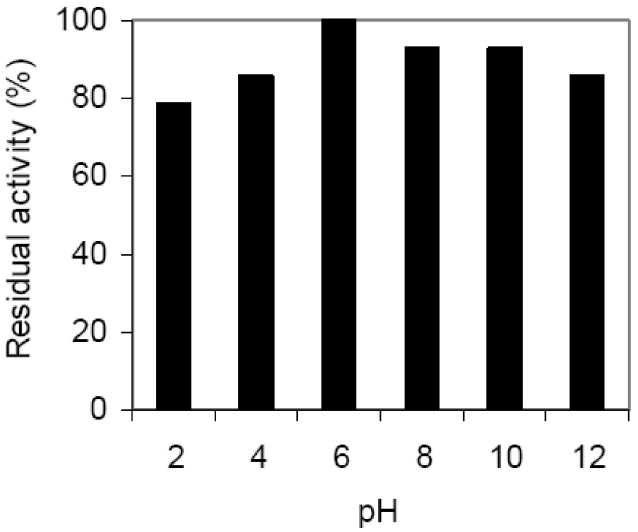

Figure 2 : Effect of pH on bacteriocin activity produced by *Lb. curvatus* G6.

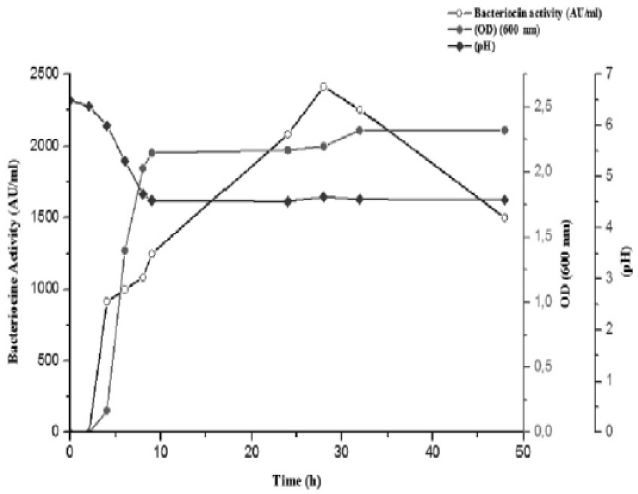

Figure 3 : Monitoring of bacteriocin production from *Lb. curvatus* G6 in MRS medium at 37°C during 48 h.

bacteriocin production to 2250 AU/ml was observed. The decrease in activity of bacteriocins at the end of the monitored period could be explained by the degradation of the bacteriocin by extracellular proteolysis enzymes produced by the producer organism in the medium[1,11], similar decreases have also been observed for bacteriocin produced by *Enterococcus faecium* ST311LD[27]. During the period of growth the pH of the medium decreased from 6.5 to about 4.6 especially in the 10 first hours. The decrease of pH is due the production of acids such as lactic acid. From a metabolic point of view, this trend would be a characteristic of primary metabolite production as observed for several bacteriocins such as bacteriocin produced by *Lb. acidophilus* La-14[28].

Optimum temperature for bacteriocin production

Figure 4 shows the effect of different temperatures (30, 37 and 40°C) on bacteriocin production by *Lb. curvatus* G6. The optimum temperature for the production of bacteriocins was 37°C, but the inhibitory activity was detected when the isolate was incubated at 30 and 40°C, which suggests that growth temperature, plays an important role in bacteriocin production. Growth temperature and bacteriocin production are often correlated as reported for several bacteriocin[29]. Mataragas et al.[8] found that the optimum temperature for the production of bacteriocins produced by *Leuconostoc mesenteroides* L124 and *Lb. curvatus* L442 was 25°C and was lower than that of growth (30°C).

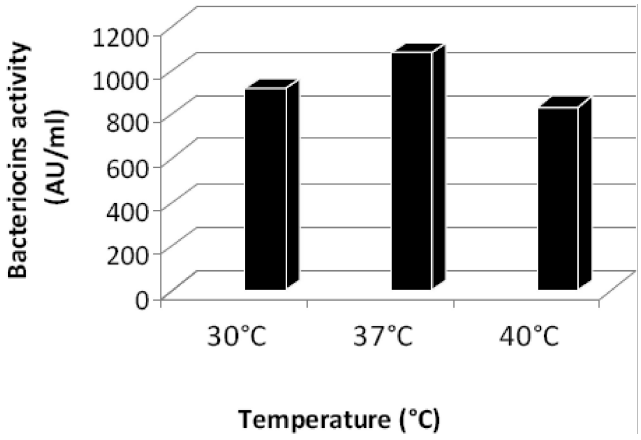

Figure 4 : Effect of temperature on bacteriocins production

Plackett-Burman design

In order to enhance *Lb. curvatus* bacteriocin production, experiments were designed to select factors affecting this production using Plackett-Burman experimental design. TABLE 3 presents the Plackett-Burman design for 7 culture variables and their corresponding response in terms of bacteriocin production (Figure 5). TABLE 4 shows the regression analysis of the effect of each variable along with the coefficient, t and P value.

TABLE 3 : Plackett-Burman design for bacteriocin production by *Lb. curvatus* G6

Trials	X1	X2	X3	X4	X5	X6	X7	Bacteriocin (UA/ml)
1	-1	+1	+1	-1	+1	+1	-1	0
2	-1	-1	+1	+1	-1	+1	+1	0
3	+1	-1	-1	+1	+1	-1	+1	1250
4	+1	+1	-1	-1	+1	+1	-1	1666.66
5	-1	+1	+1	-1	-1	+1	+1	0
6	+1	-1	+1	+1	-1	-1	+1	1083.33
7	+1	+1	-1	+1	+1	-1	-1	1083.33
8	-1	-1	-1	-1	-1	-1	-1	1250

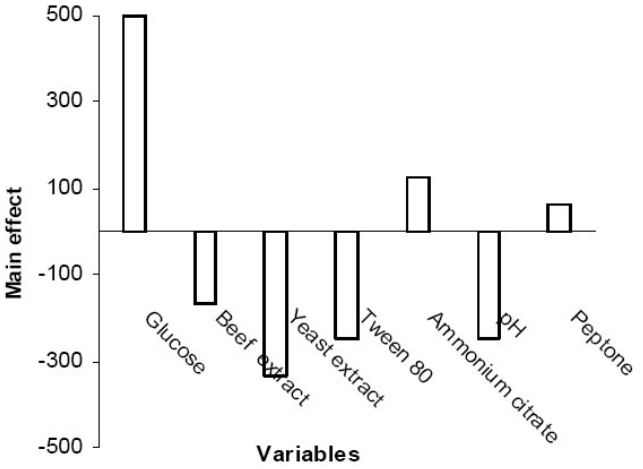

Figure 5 : Effect of different factors on bacteriocin production *Lb. curvatus* G6 as screened with Plackett–Burman design

TABLE 4 : The regression analysis of the effect of each variable along with the coefficient, t and P value.

Variables	Coefficients	*t*- Statistics	*P*-value
Glucose	499,99	2,12132034	0,2804378
Beef extract	-166,66	-0,70710678	0,60817345
Yeast extract	-333,33	-1,41421356	0,39182655
Tween 80	-249,99	-1,06066017	0,48126507
Ammonium citrate	124,99	0,53033009	0,68957386
pH	-249,99	-1,06066017	0,48126507
Peptone	62,5	0,24187781	0,81692974

When the concentration effect value of the tested variable was positive, the influence of the variables was greater

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at the high concentration tested, and when negative, the influence of the variables was greater at low concentration. The variation in bacteriocin production in different sets ranged from 0 to 1666.66 UA/ml reiterating the importance of selection and identification of important factors, the Pareto graph was drawn to show the effect of all variables on bacteriocin production (Figure 6).

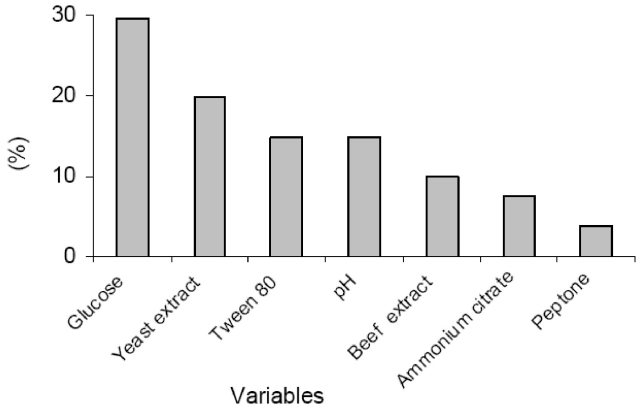

Figure 6 : Pareto chart rationalizing the effect of each variable on the bacteriocin production produced by *Lb.curvatus* G6

As it is clearly shown in (Figure 5), glucose had the major influence on bacteriocin production, followed by ammonium citrate and peptone. Beef extract, yeast extract, Tween 80 and pH on the other hand, had a significant negative influence. These variables had confidence level above 95% in comparison to other variables and therefore, were considered to be highly significant for bacteriocin production by *Lb. curvatus* G6. In the study of kumar and Srivastava[30] on screening of factors affecting production of bacteriocin by *Enterococcus faecium* LR/6, they observed that sodium acetate had the major influence on the bacteriocin production, followed by dipotassium hydrogen phosphate, and triammonium citrate but Tween 80 and tryptone had a significant negative influence. In the other hand, Han et al.[31] found that Tween 80 did not significantly affect bacteriocin production by *Lb. plantarum* YJG using Plackett-Burman design. Tween 80 decreased production of *L. mesenteroides* subsp. *mesenteroides* bacteriocin by more than 50%[26].

Figure 6 shows the ranking of factors estimates in a Pareto chart. The Pareto chart displayed the magnitude of each factor estimate and it is a convenient way to view the results of a Plackett-Burman design. The polynomial model describing the correlation between the 7 factors and the bacteriocin activity could be presented as follows:

$$Y_{activity} = 791{,}66 + 499{,}99X_1 - 166{,}66X_2 - 333{,}33X_3 + 62{,}5X_4 - 249{,}99X_5 + 124{,}99X_6 - 249{,}99X_7$$

The most significant variables affecting bacteriocin production can be used for further studies on the optimization of bacteriocin production, while variables of negative significant effect will be not eliminated but used in all trials at their low level (-1), because if they do not affect bacteriocin production they can affect bacterial growth. As it mentioned by Parente and Ricciardi[6] the optimization of growth conditions does not necessarily result in optimization of bacteriocin production. Todorov and dicks[26] have recorded a good growth of *L. mesenteroides* subsp. *mesenteroides* in the presence of 10% (w/v) soy milk or 10% (w/v) molasses, but there was no bacteriocin production.

CONCLUSIONS

According to these results, we can say that these isolated strains can have positive impact on their use as starter cultures for fermented foods, with a view to improve the quality and microbiological safety of these products. Furthermore, due to their inhibitory effect on *L. monocytogenes*, they can also be used more specially to selectively inhibit food spoilage by this high-risk pathogen. Finally, we suggest that further studies could be carried out on the characterization of amino acid and nucleotide sequences of these antimicrobial compounds, and also combination between Plackett-Burman design and other approaches of optimization such as RSM (Response Surface Methodology) for enhancing bacteriocin production.

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