ISOLATION AND CHARACTERIZATION OF LACTOBACILLUS AMYLOVORUS AS A POTENTIAL STARTER CULTURE

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ABSTRACT

Background Throughout history, lactic acid bacteria were linked to food fermentation. The development of lactic fermentation for bio conservation of food is very promotable. Bacteriocins are produced by several LAB strains and provide an additional hurdle for spoilage and pathogenic microorganisms. They are considered safe additives, useful also for the control of the microorganisms in foods. **Objectives** To determine the abilities as lactic starter for fermented vegetables, we investigated the potency of acid production, salinity tolerance and antibacterial activity of Lactobacillus amylovorus. **Methods** Selection focused on technological criteria, such as rapid acidification, the growth in low pH, at 45°C and tolerance of a high concentration of salt 10%.A total of twenty five indigenous lactic acid bacteria (LAB) were isolated from spontaneous fermented lemons. Among these twenty-five isolates, LBL5 was selected for her significant acidifying activity, antibacterial activity and was identified using API 50CH. **Results** The Lactobacillus amylovorus (LBL5) meets most of the criteria and the strain presents a rapid adaptability to the required conditions. Lactobacillus amylovorus was evaluated for the inhibitory effect toward pathogen and spoilage microorganisms. Furthermore, the strain produces inhibitory substance against Staphylococcus aureus, Klebsiella sp, Pseudomonas aeruginosa and Salmonella sp. Results leads to a large application as a starter culture.

Keywords: *vegetable fermentation's , Lemon, Lactic acid bacteria, lactobacillus amylovorus, bacteriocins.*

1.INTRODUCTION

Controlled fermentation's using starters has become more prevalent in recent years. In fact, for many products this has allowed better control of the fermentation process and has prevented the spoilage of non-desirable microorganisms. The bacteria most commonly used as starters in the fermented foods are Lactobacilli [1,2,3].Isolation of lactic acid bacteria from fruits and vegetables have been frequently reported [4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19].

Lactic acid fermentation represents the most suitable way for increasing the daily consumption of vegetables and fruits. Several selected autochthonous starters adapted to the specific plant matrix were described. In this study, Lemon's fermentation is used in order to isolate strains of lactic acid bacteria. Fermentations are involving the successional activities of a diverse group of microorganisms. Their successions during fermentation varied with the species depending on acid tolerance or growth competitiveness. Vegetables have low sugar content, but are rich in minerals and vitamins and have neutral pH and thus provide a natural medium for LA fermentation. Fruits by a high water-soluble carbohydrates content provide also a medium for proliferation of lactic bacteria [12,13,14,15,16].In previous studies, we isolated lactic acid bacteria (LAB) from fermented vegetables and fruits and then tested their potential as starters of controlled fermentations [7,8,9,10,11,12,13].The purpose of this study was to explore Lactobacillus amylovorus isolated from fermented lemons so as to be used as potential lactic starter. Identification, characterization and evaluation of antimicrobial compounds of LBl5 were investigated. Large numbers of bacteriocin producers have been found among different genera of the lactic acid bacteria [20,21,22,23,24,25,26,27]. A great number of Gram (+) and Gram negative (-) bacteria produce during their growth, substances of protein structure. Bacteriocins can be applied on a purified or on a crude form or through the use of a product previously fermented with a bacteriocin producing strain as an ingredient in food processing or incorporated through a bacteriocin producing strain (starter culture).

2. MATERIALS AND METHODS

2.1 Preparation of biological materiel

Lemons collected from local markets are fermented traditionally at different concentrations of salt. Spontaneous fermentations of lemons are made by immersion of lemons in brine with different salt concentrations (5%, 10%, 15% and 30%).

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2.2 Isolation and purification of strains

Lactic acid bacteria were isolated from naturally fermenting lemons. MRS (de man, Rogosa ,and sharpe) agar plates were used for the isolation of LAB. After incubation and observation gram+ and catalase- colonies are selected and subcultured on MRS medium liquid and solid (pH 5,4). The operation is renewed until pure culture strains of *Lactobacillus sp* retained

2.3 Acidifying capacity

To evaluate the acidifying capacity, the pH was measured after 24 h of incubation at 37°C, by a pH meter previously calibrated using two buffers (pH4.0 and pH 7.0). After each run, the electrodes were disinfected with ethanol and washed with sterile deionized water. Moreover, the acidification rate was calculated as pH = pHf (final value) pH0 (initial value). The experiments were carried out in duplicate.

2.4Identification

Several strains were isolated and studies were focused on strain *lactobacillus amylovorus* lbl5. Identification of LBL5 was done by API 50CH

2.4Effect of pH

The evolution of the growth of lactic acid bacteria according to the pH of the culture medium MRS is assessed by determination of bacterial biomass using a spectro-photometer at 600 nm. The pHs tested are 2.5; 3.5; 4.5 and 7 . The results are monitored after incubation at 30°C for 24 hours.

2.5 Effect of temperature

1ml of overnight cultures was transferred into the tubes which contain 5 ml MRS broth. After inoculation, they were incubated for 24 hours to following temperature: 10ºC, 30ºC and 45ºC. Cells growth was detected by measuring the absorbance at 600 nm.

2.6Tolerance of salinity

The effect of NaCl on the growth of lactic acid bacteria is carried out by inoculation the each bacterial strain in 5 ml MRS broth supplemented with different salt levels (5%, 8% and 10%). They are incubated at 30°C for 24hours.

2.7 Detection of antibacterial activity

To evaluate the Antibacterial activity, the identified strain was tested against some pathogens by the well diffusion assay using cell supernatant. Lactobacillus amylovorus is cultured on liquid MRS medium at 30 ° C, then tested for antibacterial activity using the agar well diffusion method using Mueller Hinton Agar medium. Fresh overnight cultures were centrifuged at 8,000g for 10 minutes, and the cell-free supernatants were used directly or after being filtered aseptically, neutralized with 1 mol / l NaOH (pH 6.5-7) and treated with catalase (0.5 mg / ml). Finally, the bioactive molecule, presumed to be bacteriocins, is ready for testing the antimicrobial activity against the pathogenic bacteria used in this study. The diameters of the inhibition zones were measured. It is noticed that the indicator pathogenic strains included *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli, Salmonella sp*. They were grown on tryptic soy broth overnight at pH 7.0. Wells of 5mm diameter were performed using the top of a Pasteur pipette and were filled with approximately 50µlof *lactobacillus amylovorus*. The plates were then stored at 4 ° C for 4 hours to allow the diffusion of any antimicrobial compound. Following incubation at 37 ° C for 24 h, the plates were monitored for the appearance of clear zones of inhibition. Each test was performed in triplicate.

3. RESULTS

Table 1: Table 1 presents the variation of pH after 24 h of incubation of isolated strains on MRS medium.

Strains	pHi	pHf (24 h)
Lactobacillus sp LBL1	5.88	4.33
Lactobacillus sp LBL2	5.88	4.00
Lactobacillussp LBL3	5.88	3.56
Lactobacillus sp LBL4	5.88	3.23
Lactobacillus amylovorus LBL5	5.88	3.02

Table 2: Table 2 presents the variation of optical density of the isolated strains at different temperatures.

Temperature	LBL1	LBL2	LBL3	LBL4	LBL5
10°C	2.25	1.58	1.09	1.12	0.57
30°C	2.66	2.34	2.14	1.54	1.54
45°C	0.06	0.01	0.01	0.02	2.65

Table 3: Table 3 presents the variation of optical density of isolated strains at different pHs.

PH	2.5	3.5	4.5	7
LBL1	2.05	2.11	0.73	0.92
LBL2	2.02	2.21	0.88	0.82
LBL3	2.23	2.28	1.35	0.87
LBL4	2.33	2.23	1.03	0.52
LBL5	2.46	2.46	0.89	0.37

Table 4: Table 4 presents the variation of optical density at different concentrations of salt.

Salinity%	5	8	10
LBL1	1.52	1.32	0.15
LBL2	1.32	0.54	0.01
LBL3	2.13	1.63	0.05
LBL4	2.45	1.95	0.34
LBL5	1.78	2.02	2.52

Table 5: Table 5 presents the diameter of inhibition zones (mm) of *lactobacillus amylovorus* against pathogenic strains.

Pathogenic strains	*Staphylococcus aureus*	*Klebsiella sp*	*Salmonella sp*	*Pseudomonas aeruginosa*	*Escherichia coli*
Diameter zones(mm)	9	11	8	8	0

4. DISCUSSION

Lactic acid bacteria are a small part of the autochthonous microbiota of vegetables and fruits. The diversity of the microbiota depends on the extrinsic and intrinsic parameters of the plant matrix. Numerous specific aspects have to be considered when selecting strains for the different food fermentations. Therefore selection criteria for LAB depend on the type and the desired characteristics of the final product, the desired metabolic activities. In this context, the screening of the strains which usually occur during the fermentation of some plants was done [7,8,9,10,11,12,13]. The purpose of this study was to select a lactic acid bacterial strain to be used as a starter culture for fermented vegetables and fruits. However, regarding the acidifying activity of the twenty five isolated bacteria (data not published), five bacteria from them showed great acidifying capacity. Our results showed in the table 1 revealed a decline in pH after 24h of incubation due to the metabolism of sugars contained in the MRS medium by lactic acid bacteria. Moreover, the results in the table 1 demonstrate that the *lactobacillus amylovorus* (LBL5) produces acids and the pH reaches 3.02 after 24 h of incubation on MRS medium. This low pH can improve the hygienic properties and storage of the final products [25]. The acidification of food by lactic acid inhibits many pathogenic strains and the decrease of pH is the most important criterion for selection of starter cultures [26,27,28]. It is a crucial factor for reduction of the growth of accidental microflora as revealed by other studies [28,29].

In fact, pH values of the controlled fermentation using *lactobacillus amylovorus* in previous studies showed a rapid drop and the microbial stability of the products [7,8,9,10,11,12,13]. *L. amylovorus* (LBL5) showed excellent production of acid. Moreover, the ability of studied strain to develop in the medium at different initial pH (pH 3.0–pH 5.0) was monitored during the cultivation in MRS medium (Table 3). *Lactobacillus amylovorus* showed great adaptation to pH: 2.5 - 3.5. In many industrial processes the organisms used are often subject to temperatures higher than those most favorable for growth. The effect of temperature on the growth of isolated strains is summarized in table 2. The analysis by the optical density (2.65) showed the capacity of lbl5 cope with high temperature 45°C. The culture of strains in the presence of 5%, 8%, 10% NaCl, allowed us to evaluate their ability to grow in the presence of these different concentrations. Monitoring of bacterial growth at these different concentrations of salt is estimated by measuring Optical Density at 600 nm. The salt tolerance gives lactic bacteria an advantage over other less tolerant species and allows them to begin

metabolism, which produces acid, that inhibits the growth of non-desirable organisms. The results (Table 4) showed two groups of bacteria, lbl1.2.3 and 4 where bacteria stops growth at 10% of salt. The LBL5 Lactobacillus amylovorus indicated to be the most salt tolerant. Also, bacteria stops growth when they feel a saline stress which is due to a strong concentration of NaCl in the medium, and this because of the mechanisms of distress which set up to avoid cellular death [30]. A particular care must be taken in selecting strains to be used as starter culture in high salt containing food systems as vegetables and fruits fermentations. It would be also an advantage for the bio preservation of salted products, allowing them to compete with the flora of halophilic alteration. Several studies confirmed the relevance of the strain as a starter culture for fermented olives, lemons and carrots [7,8,9,10,11,12,13]. Furthermore, the cell-free supernatants of lactobacillus amylovorus gave zone of inhibition against the indicator food pathogenic strains such as *Staphylococcus aureus, Klebsiella sp, Salmonella sp, Pseudomonas aeruginosa*. Several studies showed also that lactobacillus amylovorus produces two types of bacteriocins (actobin A and amylovorin) [31,32]. It is showed also that the factors influencing the production of bacteriocins are mainly the producing strain, the temperature, the pH, the composition of the medium and the fermentation technology used [32].In fact, it seems that the incorporation of 5- 10 g/l of sodium chloride in the water phase of sourdough preparations was beneficial to enhance bacterial growth and amylovorin production [33,34], this results confirmed also the ability of the strain *lactobacillus amylovorus* to grow at moderate to high concentrations of salt. Bibliographical studies showed that *Lactobacillus amylovorus* was selected because of its ability to produce significantly higher concentrations of vitamin [35]. *Lactobacillus amylovorus* meets most of the criteria for industrial application [36,37,38,39,40,41,42,43]. The ability to produce acids, to cope with high NaCl concentration, acidic pH and high temperatures are the most criteria for the selection of *lactobacillus amylovorus* as a starter culture. The growth characteristics of the selected strain lbl5 are very unusual and lead to industrial applications.

5. CONCLUSION

The great challenge for food industry is to produce strain cultures with multiple functions for specific products from specific regions of the world**.** The results of the behavior of the strain (LBL5) isolated showed that *lactobacillus amylovorus* can be recommended to food processing industries and can be employed in bio preservation of food. The culture filtrates from lactic acid bacteria isolated from fermented lemon's exhibited antibacterial activity against indicator test strains. Our results support the potential of *lactobacillus amylovorus* as a starter culture.

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