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Lactic Acid Bacteria Isolation, Phenotypic Characterization and Growth Kinetics of *Lactobacillus curvatus* from Carob Pods Syrup

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Research Article

Abstract

The main aim of this present study was the isolation and characterization of lactic acid bacteria from carob pods. A total of 6 isolates of lactic acid bacteria were screened and was identified from carob pods as *Lactobacillus curvatus*, *Streptococcus diacetylactis*, *Streptococcus feacalis*, *Streptococcus mitis* and *Streptococcus thermophilus*. This identification was based on phenotypic characters and the API system. *Lactobacillus curvatus* was shown to produce a high amount of acidity, why it is used for studying the kinetics of growth and acidification of carob pods syrup. The carob pods syrup which has been the subject of our work has a high water content 85%, very rich of total sugars 22 g/L and ash 0.8% and the protein fraction is considerable 0.25%. This study was designed not only to evaluate the potential of carob extract for lactic acid production but also to determine the effect of alternative addition of Sodium Acetate and Tween 80 on lactic acid fermentation. The results clearly indicated that the highest amount of biomass and lactic acid were obtained with the carob enriched with sodium Acetate and Tween 80.

Keywords: Carob; Fermentation; *Lactobacillus curvatus.*

1. Introduction

Carob (*Ceratonia siliqua L.*), which has been widely grown in the Mediterranean region, belongs to the Caesalpinaceae subfamily of the family Leguminoseae [1,2]. Carob tree has an economic and environmental importance in Algeria. It is used in reforestation of arid and degraded areas and also as for ornamental purposes [3-5]. However, in recent years, it has been used in the food industry as bioresource and biomass substrate and thus has attracted the attention of producers because of increasing market value. The pulp content in the pod ranges from 73 to 95% [6]. When the fruits are ripe enough, they have 91-92% total dry matter and 62-67% total soluble solids, which consist of 34-42% sucrose, 10-12% fructose, and 7-10% glucose [7]. Carob pods are also characterized by high sugar content 200-500 g/kg [8]. Moreover, carob pods contain appreciable amount of fiber (4.2-39.8%), depending on the type of the extracted fiber [6,9]. Carob pulp is a good source of polyphenols (mainly tannins 16-20%) [10], and protein (2.7-7.6%) but it is poor in lipid (0.4-0.8%). This study was designed not only to evaluate the potential of carob extract for lactic acid production but also to isolation of lactic acid bacteria and determine the effect of alternative addition of Sodium Acetate and Tween 80 on lactic acid fermentation.

2. Material and Methods

2.1 Vegetable material

The carob (*Ceratonia siliqua* L.) used in current experiments was harvested in the month of August 2014 from the region of ELBORDJ (Mascara, Algeria). The choice of this variety is justified by its availability and important nutritive value, especially the one of reducing fermentable sugars such as glucose and sucrose.

2.2 Isolation and characterization

A 25 g sample of carob pods sample was taken aseptically and transferred to sterile plastic bags and then homogenized in 225 mL of sterile buffered peptone water (BPW). Five 10-fold dilutions of the homogenates were then prepared and these were inoculated on plates of MRS agar (Oxoid) acidified with glacial acetic acid to pH 5.4 and M17 medium to pH 7.1 and incubated anaerobically

at 30°C for MRS and 37°C for M17. Colonies with typical characteristics were randomly selected from plates and tested for Gram stain, cell morphology, catalase and oxidase reactions before further sugar fermentation and characterization tests [11]. The short term conservation of the pure isolates was done on solid media [12]. After growth at optimal temperature, the culture was maintained at 4°C and cultures were renewed every month. The long-term conservation of the purified isolates was carried out in MRS broth containing glycerol (Merck, Darmstadt, Germany) (v/v 70/30) and were maintained as frozen stocks at -80°C [13]. Strains were characterized according to the methods and criteria of Buchanan [14] and Klein [15] as follow: They were checked for gas production from glucose in MRS broth containing Durham tubes. Growth at different temperatures was observed in MRS broth after incubation for 5 days at 15, 37, 45°C then 12 days at 5 and 10°C [12]. The ability to grow at pH 3.9 and 9.6 was tested on MRS broth [16]. The tolerance to NaCl was studied by growth in MRS broth containing NaCl at concentrations 2, 4 and 6.5% [17]. Arginine dihydrolase was determined in MRS broth supplemented with 0.3% arginine, which was incubated for 3 days, followed by NH3 detection by addition of Nessler's reagent. Hydrolysis of esculin and the effect of bile salts were observed on bile esculin agar. Heat resistance was determinated in MRS broth at 60 and 65°C for 30 min [18]. Citrate utilization was studied on the media of Kempler and Mc Kay [19]. Production of dextran was recorded on MSE agar [20] and the production of acetoine from glucose was determined by using the Voges–Proskauer test [21]. Carbohydrate fermentation patterns of LAB were determined by means of miniaturized API 50 CH biochemical tests (BioMérieux, Marcy L_Etoile, France).

2.3 Extraction and analysis of carob pods syrup

Carob pods were chopped into small particles (1-3 cm). One liter of hot water at 80-85°C was added to 200 g of carob pods, homogenized and through a cloth. The syrup obtained was centrifuged at 15000 rpm for 10 min to separate the cellulose debris. The collected supernatant was used as culture medium. The syrup is fixed in a pH 6 and sterilized during 20 min at 120°C. The extraction parameters were obtained from method advocated by Turhan et al. [22]. pH is measured using a pH meter and density was determined by density meter. Concentration of lactic acid was determined by acidity titration with NaOH 0.1 N. Total nitrogen of carob and protein content was determined by the method of Kjeldahl digestion and distillation apparatus [23]. Total and reducing sugars were determined colorimetrically at 480 nm by Dubois method [24]. The ash content of the carob was determined according to the AOAC official method 972.15 by incineration one gram of syrup at a temperature of 600°C during 3 h. Moisture and dry matter were determined by drying 10 mL of syrup at 105°C during 18 h. The mineral salts are determined according to the methods advocated by Muhamed [25].

2.4 Fermentation conditions and methods

A total of 6 isolates were isolated from carob pods, of which one isolate (*Lactobacillus curvatus*) was shown to produce a high amount of acidity, why it is used for studying the kinetics of growth and acidification of carob syrup. All experiments were carried out in a 2 L jar fermenter (Applikon Biocontroller ADI1030) with an initial volume of 1 L at 30°C. The agitation speed was set at 300 rpm to insure complete mixing of the fermentation medium. The inocula were incubated at 30°C for 12 h at 300 rpm before their transfert to the fermenter in a 10%. The culture pH was maintained at 6.2 by automatic addition of 25% (w/w) NH_4OH solution during the 30 hours of fermentation. The samples were withdrawn at desired intervals and frozen for further analysis. The biochemical analysis applied on the carob pods syrup shows that it is poor in sodium acetate and fatty-acids, so the addition of these elements (growth factors) is necessary to the syrup in order to import the quantity of lactic acid. Four culture mediums were used: carob syrup (CS), carob syrup with 5 g of Sodium Acetate (CS+SA), carob syrup with 1 mL of Tween80 (CS+T80) and carob syrup with 5 g of Sodium Acetate and 1 mL of Tween 80 (CS+SA+T80). The biomass is determined by measurement of the optical density (OD) at 600 nm by a spectrophotometer HITACHI 4-2000. Culture samples were centrifuged (13200 g at 4°C for 5 min), diluted and filtered. Residual glucose and lactic acid concentrations were determined by Multi parameter Medical Analyzer. The enzymatic kit used for the lactic acid dosage is the PAP Ref-61 192 and for the glucose dosage it is the Elitech diagnosis ref - GPSL-0500. The various analyses carried out allow the following time evolution of the component concentrations present in the culture medium: [Biomass: (OD) =f (t), sugars: S=f (t) and the lactic acid: P=f (t)]. From these raw data it is possible to calculate the fermentation kinetic parameters in the batch culture by the calculation of the specific rate of growth (μ in h^{-1}), of substrate consumption (Qs in $g.g^{-1}.h^{-1}$) and lactic acid production (QL.A in $g.g^{-1}.h^{-1}$).

$$\mu = \frac{r'''_X}{X}, \quad Qs = \frac{r'''_S}{X}, \quad Ql.a = \frac{r'''_P}{X}$$

The maximal specific growth rate (μmax) was determined from the slopes of the plotted linear curve: LnX/X0=f (t). The biomass (Yx/s) and products (Yp/s) yields are defined as the mass ratios in biomass

and metabolites formed per gram of consumed carbonaceous substrate.

3. Result and Discussion

3.1 Isolation and identification of lactic acid bacteria (LAB)

Colonies on MRS medium are whitish, smooth and small. These strains are Gram+ (purple) and bacillary form. The development on M17 gives white colonies, round, opaque, with smooth boundary. These strains are present in the form of cocci in pairs or chains Gram+. Strains were characterized and identified according to the methods and criteria of Buchanan [14] and Klein [15]. A total of 6 isolates were isolated from carob pods, of which 1 isolate (*Lactobacillus curvatus*) was shown to produce a high amount of acidity, why it is used for studying the kinetics acidification of carob syrup. From the results obtained, the strains are *Lactobacillus curvatus*, *Streptococcus diacetylactis*, *Streptococcus feacalis*, *Streptococcus mitis* and *Streptococcus thermophilus* (Table 1).

3.2 Biochemical composition of carob pods syrup

The carob pods syrup which has been the subject of our work has high water content 85% (Table 2), we agree that a product with high water content facilitates lactic acid bacteria proliferation and helps for a better substrate-enzyme contact [26]. Carob pods syrup will be very rich of total sugars 22 g/L. The protein fraction is considerable; therefore it can serve as a nitrogen source (0.25%). An ash content of 0.8% indicates its richness of minerals including potassium (110 mg/100 ml of MF), sodium (80) and calcium (150). Karkacier and Artik report that the fruits are ripe enough; they have 91-92% total dry matter and 62-67% total soluble solids, which consist of 34-42% sucrose, 10-12% fructose, and 7-10% glucose [7].

The content of sugars found a high energy value

Table 1. Identification of lactic acid bacteria (LAB) isolated from carob pods syrup.

Characteristics/ LAB	*L. curvatus*	*St. diacetylactis*	*St. feacalis*	*St. mitis*	*St. mitis*	*St. thermophilus*
Catalase	-	-	-	-	-	-
Growth at 15°C	-	+	+	-	-	-
Growth at 37°C	++	++	+	+	+	+
Growth at 45°C	+	-	+	+	+	+
Growth at pH 9.6	/	+	+	-	+	-
Growth at pH 3.9	+	/	/	/	/	/
Growth at 2% NaCl	+	/	/	/	/	/
Growth at 4% NaCl	+	/	/	/	/	/
Growth at 6.5% NaCl	+	-	-	-	-	-
Resistance at 60°C	+	+	+	+	+	+
Resistance at 65°C	+	+	+	-	-	-
Growth on milk blue Sherman	+	+	+	-	-	-
Fermentative type	homo	homo	homo	homo	homo	homo
Esculin hydrolysis	+	+	+	+	+	+
ADE	-	+	+	+	+	+
Gelatinase	/	-	-	-	-	-
Citratase	/	+	+	+	+	+
VP	/	+	+	-	-	-
Dextran	-	/	/	/	/	/
H_2O_2	-	/	/	/	/	/
Camp test	/	γ	γ	γ	γ	γ
Ribose	+	/	/	/	/	/
Sorbitol	-	/	/	/	/	/
Xylose	-	/	/	/	/	/
Maltose	/	+	+	+	+	-
Arabinose	/	-	-	-	-	-
Rhamnose	/	-	-	-	-	-
TSI	+	+	+	+	+	+
Lipolytic activity	-	/	/	+	/	/

Table 2. Biochemical composition of carob pods syrups.

Biochemical composition of carob pods syrups	Average
Dry Matter (%)	15
Moisture %	85
pH	4.97
Acidities (m.eq %)	30
Density (Kg/m^3)	2.543
Total sugars in g/L	22
Proteins in %	0.25
Ashes in %	0.8
Potassium in mg/100 ml of M.F	110
Sodium mg/100 ml of M.F	80
Calcium in mg/100 ml of M.F	150

17.5 kJ/g D.M. for pod of the carob [5,27,28]. Petit and Pinilla, report that the carob pods are also characterized by high sugar content 200-500 g/kg [8]. According to the results obtained by Yousif and Alghzawi [2] and by Vaheed et al. [29], carob pod contains 45 to 56.10% total sugar and13.60 to19.00% reducing sugar. Vaheed et al. [30] show that the carob pods powder contained 9.09 moisture, 56.10 total sugars, and 19.00 reducing sugars (all as weight %). Chemical composition of carob had been studied extensively for different countries of the Mediterranean area. It had been observed that this composition is depending not only on technological factors such as the extraction and analytical methodologies, but also on the genotype of the plant, the geographical origin, the climate conditions and the harvesting and storage procedures [7,31-33]. The analysis applied on the carob pods syrup shows that it is poor, with minerals such as Magnesium, Manganese and fatty-acids, so the addition of these elements (growth factors) is necessary to the syrup in order to import this quantity of lactic acid. According to Fidan and Sapundzhieva [34], carob fruit (pulps and seeds) and flour are rich in carbohydrates, proteins and also are a good source of K, Ca, Na, Fe, and Mg. According to the literature data, many factors affect the chemical composition of the fruit as well as its mineral content, for example, temperature, dryness [35], irrigation and fertilization [36] and salinity [37]. Finally, the biochemical analysis show that it can constitute a fermentation medium of good quality.

3.3 Lactic acid fermentation

The results clearly indicated that the highest amount of biomass and lactic acid were obtained with the carob enriched with sodium Acetate and Tween 80 (Table 3). The production curves of lactic acid in enriched and un-enriched mediums looked the same, the production in the un-enriched (carob syrups: CS) begins with an initial content of 30 g/L to achieve 50 g/L of lactic acid in 26 h of fermentation (Figure 1).

In parallel, in the medium enriched with sodium acetate (CS with SA), Tween 80 (CS with T80), and

Table 3. Kinetics parameters of carob syrups fermentations.

Fermentations / Parameters	CS	CS +S.A	CS +T80	CS +S.A+T80
OD *i*	0.196	0.193	0.205	0.194
OD *f*	0.512	0.541	0.558	0.695
Sugars *i.* (g/L)	22.8	21.17	22.13	22.20
Sugars *f.* (g/L)	10.098	8.086	8.957	5.613
Sugars consumption (g/L)	12.702	13.084	13.173	16.587
Acidities *i.*	29.547	30	29.245	30
Acidities *f.*	51.594	56.445	54.704	60
µmax (h^{-1})	0.1676	0.179	0.1416	0.2051
Qs max (g/g.h)	10.245	7.282	8.300	10.898
$Q_{L.a}$ max (g/g.h)	15.449	25.235	16.501	25.801
Yx/s (g/g)	0.0146	0.0146	0.0064	0.0077
$Y_{L.a}$/s (g/g)	1.595	3.604	2.056	3.074

i: initial, f: final, S.A: Sodium Acetate, T80: Tween 80, CS: Carob syrups

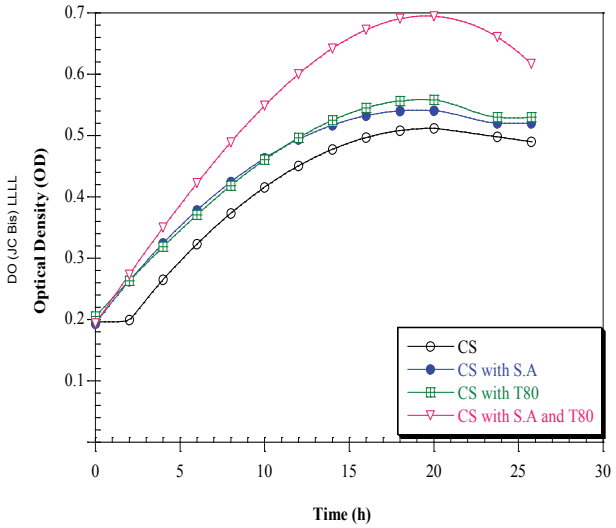

Figure 1. Evolution of optical density (OD) during batch fermentations.

two components (CS with SA and T80), the lactic acid rate evolves gradually to achieve the end of fermentation 60 g/L. The addition of Tween 80 and sodium acetate in carob syrup increase the lactic acid production. On the other hand, the biomass evolution in the un-enriched medium (CS) starts with an initial concentration (OD=0.19) and after 26 h of

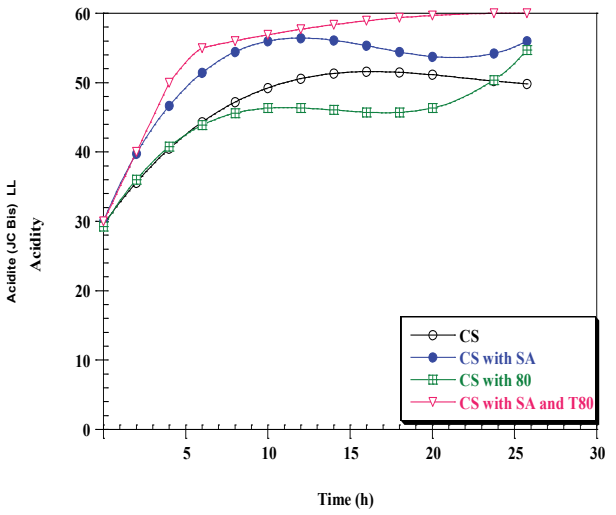

Figure 2. Evolution of lactic acid in g/L for batch fermentations.

fermentation it reaches a maximum value OD=0.5 (Figure 2).

In the case of enriched (CS with SA and T80), we observe a low initial concentration of biomass and in the end of fermentation optical density reaches a maximum value 0.7. Tween 80 (commercial form of oleic acid) is essential for growth of lactic acid bacteria and acetate is a buffer and stimulating [26]. For different fermentations, there is a total absence of the lag phase indicating the perfect adaptation of *Lactobacillus curvatus* to the different medium used. However, decreasing of sugar rates is very faster in the medium supplemented with Sodium acetate and Tween 80. Where the *Lactobacillus curvatus* consumes about half quantity of initial total sugars,

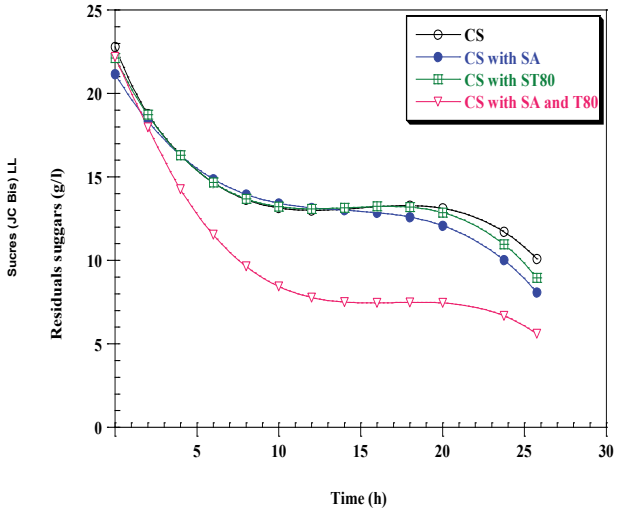

Figure 3. Evolution of residual sugars in g/L for batch fermentations.

during 26 h of fermentation from an initial quantity of 22.2 g/L of sugars, it remains 5.6 g/L which implies an amount of 16.58 g/L (Figure 3).

Comparing the results for both fermentations indicated that the addition of growth factors in culture medium has a positive and beneficial effect on fermentation, since the growth rate and lactic acid production increase after the enrichment of carob pods syrup. The addition of Tween 80 allows better cells excretion of lactic acid by creating pores in the membrane and plays the role of surfactant which makes a good contact between bacteria and nutriments, and for more it is considered as a source of carbon and energy for electrons [26]. From these results, the enriched mediums present an interesting result. Overall, when the carob syrups fermentation medium was compared with carob syrups enriched with SA and T80, in terms of lactic acid produced, maximum specific rate of growth (μmax), maximum consumption specific rate (Qs), maximum production specific rate ($Q_{L.A}$), yield of lactic acid production ($Y_{L.A}$/s), there were significant differences (Table 3). Moreover, kinetic parameters show clear differences between CS and CS with SA and T80. Fermentation CS with SA and T80 resulted in significantly higher lactic acid productivity compared to CS. Overall, the lactic acid yield, the maximum consumption rate, and the specific growth rate were higher in CS with SA and T80 compared to CS.

4. Conclusion

The bioconversion of agricultural by- products mainly the ones rich in fermentable sugars has an economic and strategic interest. The main aim of this study was to isolation, identification and kinetics fermentation of *Lactobacillus curvatus* from carob syrups without and with addition of Sodium Acetate and Tween 80. Six isolates of lactic acid bacteria LAB were screened and five strains were identified as *Lactobacillus curvatus*, *Streptococcus diacetylactis*, *Streptococcus feacalis*, *Streptococcus mitis* and *Streptococcus thermophilus*. This identification was based on phenotypic characters and the API system. By its biochemical composition, the carob syrup is very rich in carbohydrates 22 g/L. which make it a substrate of choice for the development of high value substances. In producing countries, carob pods have traditionally been used as animal and human food and currently the main use is the seed for gum extraction, carob bean gum (CBG) or locust bean gum (LBG) [1]. The carob powder is being acclaimed as an ingredient with a marked nutritional value due to its high levels of dietary fibre (preventative role against heart disease) and phenol compounds (antioxidant activity) [38]. Regarding the high sugar content in carob pod, there have been some studies on the production of the value added product [30,39,40]. Lactic acid can be one of these value added products due to its current and future potentials. Kinetics study of growth of *Lactobacillus curvatus* in carob syrup showed that the addition of sodium acetate combined with Tween 80 to the culture medium increases the yield of lactic acid production.

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