

## Lactic acid production by immobilization of *Lactobacillus* sp. isolated from olive mill wastewater

Khadidja Ayadi<sup>a,b,\*</sup>, Malika Meziane<sup>b</sup>, Khadidja Bounedjar<sup>b</sup>, Djamila Tahraoui Douma<sup>b</sup>, Souhila Bensouna<sup>b</sup>, Mohammed Fellah<sup>b</sup>, Khaled El-Miloudi<sup>c</sup>

<sup>a</sup>Laboratoire Eau et Environnement, Faculté de Technologie, Université Hassiba Benbouali de Chlef, Algérie, email: k.ayadi@univ-chlef.dz (K. Ayadi)

<sup>b</sup>Laboratoire Bio-Ressources Naturelles, Faculté des Sciences de la Nature et de la Vie, Université Hassiba Benbouali de Chlef, Algérie

<sup>c</sup>Laboratoire Physique Théorique et Physique des Matériaux, Faculté de Technologie, Université Hassiba Benbouali de Chlef, Algérie

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### ABSTRACT

The aim was to investigate the production of lactic acid by *Lactobacillus* sp. isolated from olive mill wastewater (OMW). The bacterial strains were immobilized on bovine bone and pozzolan supports. The best acidifying activity was detected in the strain *Lb16* (*Lb. rhamnosus*) with an acidity equal to 45°D. All fermentations exhibited a biphasic growth (diauxie). The best lactic acid production was noted in MRS (de Man, Rogosa and Sharpe) broth with the strain immobilized in continuous culture on pozzolan (17 g L<sup>-1</sup>) and bovine bone (11 g L<sup>-1</sup>), in non-supplemented OMW medium with bovine bone support in batch mode (6.2 g L<sup>-1</sup>), 4.5 g L<sup>-1</sup> on the pozzolan support and 3.8 g L<sup>-1</sup> on the bovine bone support at continuous mode. For OMW medium supplemented with 25% whey, the best lactic acid production by *Lb. rhamnosus* was seen in a discontinuous batch with 8.1 g L<sup>-1</sup> on bovine bone support, followed by 7.4 and 6.4 g L<sup>-1</sup> respectively on the pozzolan and bovine bone in continuous mode. The study has demonstrated that it is possible for an agri-food effluent (i.e., olive mill wastewater) to produce a valuable biochemical product (i.e., lactic acid). Further work is needed to optimize process parameters such as the optimal OMW/whey ratio and nutrient supplementation.

**Keywords:** Lactic acid; *Lb. rhamnosus*; Olive mill wastewater; Physicochemical parameters; Microbial composition; Immobilized cells fermentation

### 1. Introduction

Lactic acid is a naturally occurring organic acid that can be used in a wide variety of applications, such as cosmetics, pharmaceuticals, chemicals, food, and medicine [1]. It can be obtained eco-friendly by the fermentation of sugars found in renewable resources [1,2]. Furthermore,

*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Pediococcus* have been employed as starter cultures in industrial food fermentations. Among these, *Lactobacillus* strains are of great commercial importance due to their high acid tolerance, high yield, and high productivity [3]. They can be engineered for the selective production of L/D-lactic acid [1,2]. Many studies have been reported on

\* Corresponding author.

lactic acid production by the fermentation of starchy materials, such as corn, rice, potato, and sweet sorghum juice [4]. Renewable lignocellulosic biomass materials have been described to be low-cost carbon sources and as feedstock for lactic acid fermentation, including waste sugarcane, corn stover, apple pomace, wheat straw, and wood waste [4].

According to their composition, the residues used for lactic acid production can be divided into three groups: lignocellulosic waste, starchy waste, and residues from dairy processes [5]. Some studies were carried out using olive mill wastewater (OMW) to produce methane, butanol, butanediol, and other alcohols [6]. OMW was also employed as a feed or as an organic fertilizer for the soil [7]. Several fermentation processes have been investigated to improve lactic acid productivity, including repeated batch fermentation, continuous fermentation, cell immobilization, and co-cultivation of microbial producers [8].

The current study was aimed at investigating the production of lactic acid by *Lactobacillus* sp. isolated from olive mill wastewater (OMW). The bacterial strains were immobilized on bovine bone and pozzolan. The feeding media were pure OMW and OMW supplemented with 25% whey. MRS (de Man, Rogosa and Sharpe) medium was used as a control. The productivity of lactic acid was monitored by the measurement of the pH and the acidity of the fermentation products.

## 2. Materials and methods

### 2.1. Olive mill wastewater and whey sampling

Olive mill wastewater (OMW) samples were collected from a mill at en Nakhla, Chlef located in northern Algeria (36°15'36"N 1°24'50"E). To our knowledge, no chemical additives were used during olive oil production. Samples of freshly produced OMW were taken from the storage ponds, placed into sterilized bottles, and kept at 4°C until employed for physicochemical and microbiological analysis.

Native whey was produced from raw milk in the laboratory. The milk was placed in a glass jar which was covered loosely to protect against dust and other debris. The milk was left on a laboratory bench until the pH dropped to 4.6 during the coagulation process. The warmer the room, the faster it would separate. The mixture was placed in a colander containing a cheesecloth to separate the curd from the liquid. The whey was refrigerated at 4°C until use.

### 2.2. Physicochemical and microbiological characterization of olive mill wastewater

The physicochemical characterization of three samples each of olive mill wastewater (OMW) stock was carried out according to AFNOR Standards (Association Française de Normalisation). The pH and the conductivity of OMW were measured at ambient temperature using calibrated Hanna Instruments pH and conductivity meters, respectively. The acidity (the equivalent of oleic acid) was evaluated by titrating 10 mL OMW with 0.1 N NaOH solution. The turbidity was evaluated by a Hanna Instruments turbidimeter. Dry matter was determined after desiccation of 25 mL of OMW samples at 105°C and expressed in g L<sup>-1</sup> of the initial volume of OMW. Suspended matter (SM) in

a volume of OMW sample was determined by filtration through a 0.45 μ pore membrane. The mass of the residue was weighted as the SM in g mL<sup>-1</sup>. The chemical oxygen demand (COD) was determined by the dichromate method [9]. The biological oxygen demand (BOD<sub>5</sub>) was determined by a BOD-meter after incubating a dilute solution of OMW giving the amount of oxygen consumed by microorganisms for 5 d at 20°C in the dark. The other physicochemical parameters: orthophosphates, chlorides, nitrites, nitrates, calcium, magnesium, ammonium, sulfate contents were measured according to Rodier et al. [9]. The salinity expressed in mg L<sup>-1</sup> of OMW was determined by Hanna Instruments portable salinometer. Total phenolic contents were assessed according to the Folin–Ciocalteu method [10] with 0.5 mL of sample solution added to 2.5 mL of Folin–Ciocalteu reagent and diluted 10 times with distilled water followed by the addition of 4 mL of Na<sub>2</sub>CO<sub>3</sub>. The mixture was then incubated in a water bath at 45°C for 30 min and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer against a blank sample. The total phenolic content was measured as gallic acid equivalents (mg GAE/g of extract). The determination of residual oil and fats was realized by hexane extraction.

The microbiological composition was determined to evaluate the presence of germs indicative of pollution. Samples were collected in sterile bottles for microbiological analysis and transported immediately in thermos-cool boxes at 4°C to the laboratory.

The recording of bacteria reflecting hygienic degrees such as total aerobic flora (count on plate count agar, Difco at 22°C and 35°C), total and fecal coliforms (at 37°C and 44°C, respectively on VRBL agar, Difco), fecal streptococci (at 37°C on Rothe media presumptive test and Litsky media confirmative test), yeasts and molds, (on the Potato Dextrose Agar plates used for the viable counts) were carried out according to international standards [11].

### 2.3. Isolation of lactic acid bacteria from olive mill wastewater

Grown colonies of lactic acid bacteria especially *Lactobacilli* on de Man, Rogosa and Sharpe agar (MRS) agar for 48–72 h at 37°C were checked for gram and catalase reactions before counting [12]. Serial decimal dilutions were prepared in the same diluent, and 0.1 mL was inoculated in triplicates by surface spreading on de Man, Rogosa and Sharpe agar (MRS) (Merck, Darmstadt, Germany) [13]. The pH of the media was adjusted to 6.5. The plates were anaerobically incubated at 37°C for 48 h. Finally, a single colony of *Lactobacillus* was isolated according to the colony morphology and applying biochemical tests such as gram staining, catalase, and oxidase. Well-isolated colonies were picked and transferred to MRS broth for the enrichment of *Lactobacillus* at 37°C [13]. After 48 h of incubation, the resulting colonies were counted, and the counts were expressed as the decimal logarithms of the colony-forming units per millilitre (log CFU/mL). Isolated colonies were cultivated in MRS broth (Merck, Darmstadt, Germany) at 37°C for 48 h. Colonies were selected randomly (based on their differences in color, shape, elevation, and size) and were streaked onto MRS agar medium. A single colony was then sub-cultured twice to ensure the purity of the culture.

### 2.3.1. Gram staining and cell morphology

Identification of the isolates was performed by standard staining procedure. The shape morphology of the freshly grown cells was viewed under a light microscope.

### 2.3.2. Catalase and oxidase tests

A drop of hydrogen peroxide (3% v/v) solution was placed on a pure single colony. The immediate formation of bubbles (gas production) was considered to be the positive test of catalase [14]. A well-isolated colony is chosen from a fresh culture to be tested for oxidase. A small portion of microorganisms is removed using a sterile swab and rubbed on an oxidase disc. A purple color appears and turns black if the oxidase test is positive [14].

### 2.3.3. Carbon fermentation test

In the carbon fermentation test, nutrient agar was prepared with 1% (w/v) glucose and 0.004% (w/v) bromocresol purple (as a pH indicator). About 10  $\mu$ L of culture was then spotted on the agar. After incubation at 37°C for 24 h, a positive result was shown by a change from a purple color to a yellow zone around the culture as a result of reduced pH by acid production through the fermentation of glucose by the bacteria. Taxonomic identification was carried out by API 50 CH (bioMérieux, Craponne, France) [15].

## 2.4. Treatment of inorganic and organic supports

Pozzolan and bovine bone were used as inorganic and organic supports, respectively.

### 2.4.1. Pozzolan

Pozzolan from the quarry of Béni Saf, Tlemcen (Algeria) was washed with tap water to remove impurities. The support was cured at a high temperature before its use. Granular pozzolan of 6 mm diameter was obtained by size reduction of the rocks using sieving. Particles were 65% porosity and 1.029 density [16].

### 2.4.2. Bovine bone

Both cancellous and cortical portions of cow bone (bones collected from university canteens) were employed [17]. After freezing, soft tissues such as muscles and tendons were removed. The bones were then cut into small pieces with less than 2 cm diameter. These pieces were boiled in water for 10 h for deproteinization before their immersion in a 1% sodium hydroxide plus 1% hydrogen peroxide solution for 1 h [17]. They were then washed under flowing water and dried in an oven (MEMMERT) at 80°C for 1 h. The pieces were shaped into small blocks of 6 mm diameter and sterilized in an autoclave. Density, porosity, and volumetric masses were determined.

### 2.4.3. Inoculum preparation

In each medium of MRS, OMW without supplementation, OMW supplemented with 25% of whey, the strain

*Lactobacillus* ssp. was cultivated and incubated at 37°C for 24 h. After, a viable cell count was performed with the plating technique on bilayer MRS agar medium in sterile disposable Petri plates and incubated at 37°C for 24 h. The count was expressed in CFU/mL. In all performed experiments, the stain had the maximum cell concentration of 10<sup>8</sup> CFU/mL, after incubation for 18 h, which was then treated as the inoculum condition for other experiments.

### 2.4.4. Stationary phase determination of strain

Experiments with pure strain culture were carried out in 250 mL flasks with a volume of 200 mL MRS broth and 1% (v/v) inoculum. The initial concentration was 10<sup>7</sup> CFU/mL and the initial pH was 6.5. The flasks were incubated at 37°C and growth curves were observed until the stationary phase.

The increase in cell concentration was monitored by counting viable cells by the plating method as described above and by measuring absorbance by a UV-spectrophotometer (Optizen) at a wavelength of 600 nm, at predetermined time intervals.

## 2.5. Experimental set-up of the fermentation process

### 2.5.1. Immobilization cells on supports

One colony was placed in a test tube containing 10 mL of the fermentation solution and incubated at 37°C for 18 h and then each tube was placed in 250 mL of the substrate with initial pH of 6.5. 100 g of bovine bone and pozzolan were used for cell fixation. When immobilization was complete, the supports were washed twice, and then the fermentation reaction was carried out under uncontrolled pH [18].

Free batch fermentation was conducted under the anaerobic condition at 37°C using 1% (v/v) of the isolates in the presence of three media: MRS broth, OMW and OMW supplemented with 25% whey. The best fixations and productions were chosen according to the differences between the initial and final biomass, initial and final pH as well as the initial and final Dornic degree (as a measure of lactic acid concentration) after 72 h of fermentation.

#### 2.5.1.1. Batch culture

The bioreactor was placed on an orbital shaker (100 rpm) for 72 h at 37°C. The flask of batch culture with immobilized cells contained a bed of pozzolan or bovine bone as schemed by Fig. 1.

#### 2.5.1.2. Continuous culture

The support was placed in a lab-scale fermenter of 80 mm diameter and 200 mm height. A sampling device was placed at the top of the fermenter (Fig. 1). The media containing the biomass in the stationary phase was periodically re-injected into the reactor by a circulation pump collecting the liquid from the top through the distributor. The temperature was maintained at 37°C by a water jacket. The inlet flow was maintained from 40 to 50 mL h<sup>-1</sup> for 3 d for better bacterial fixation.

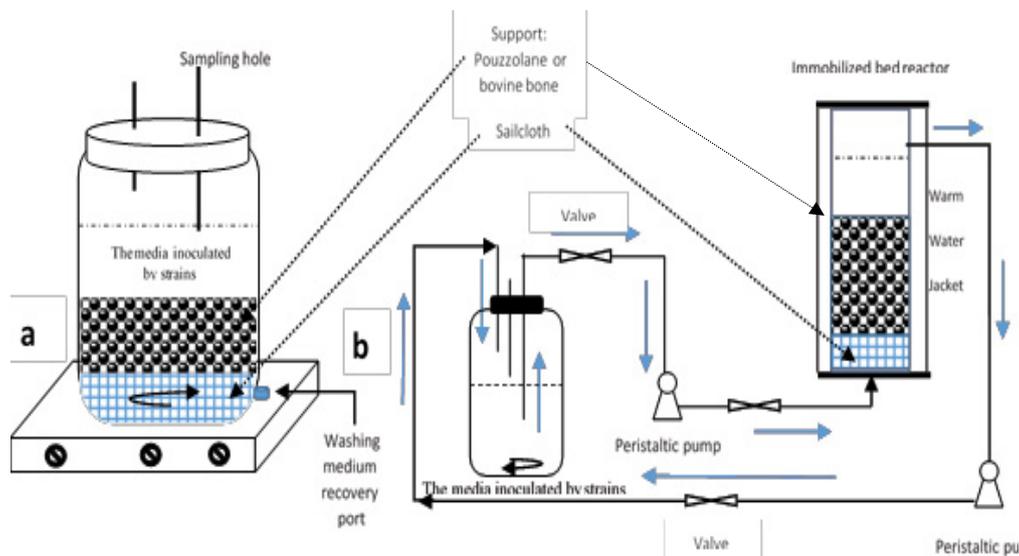


Fig. 1. Immobilization of cells on supports: (a) batch and (b) continuous immobilization.

## 2.5.2. Experimental fermentation

### 2.5.2.1. Batch culture with free and immobilized cells

Two batch cultures with free and immobilized cells were carried out in 1-L flasks with a working volume of 800 mL, inoculated in the presence of media, and incubated at 37°C for 96 h at 200 rpm. The initial pH was fixed at 6.5.

### 2.5.2.2. Continuous culture immobilized cells

The fermentation broth was pumped from a bottom sample port of the fermenter using a peristaltic pump. The substrate was passed through sailcloth that was located at the bottom of the reactor to prevent the support from being dragged towards the medium distribution conduit (as indicated in Fig. 2). The reaction was conducted with uncontrolled pH, the inlet flow was 90 mL h<sup>-1</sup>, the dilution rate was  $0.36 \pm 0.01$  h<sup>-1</sup>, the residence times of 3 h 45 min. The contact time was  $33 \pm 0.1$  min for pouzzolan and  $43 \pm 0.3$  min for bovine bone [18].

## 2.6. Quantification of organic acid and pH determination

### 2.6.1. Cell leakage estimation

The free cells and cells leaked from the support matrix were collected by centrifugation at 10,000 rpm for 20 min and dried at 90°C. The initial weight of each support matrix was determined by drying a specified quantity to a constant weight. The support matrices with cells were carefully washed with distilled water and dried again. The difference between the weights of the support matrices before and after cell adsorption was considered the weight of adsorbed cells [19].

### 2.6.2. Organic acids produced and pH values

Quantification of organic acids produced by the isolate and determination of pH values were performed. The three

media MRS broth, OMW, OMW supplemented with 25% whey inoculated with 1% (v/v) of the isolate and incubated in anaerobic condition at 37°C for 18 h were used for lactic acid production in immobilization cultures (batch and continuous methods) compared with free batch fermentation [20]. Fermented samples were collected at 0, 1, 3, 6, 9, 15, 24, 48, 76 and 92 h. The pH of the cultures was determined using a pH meter and the quantification of the organic acid was achieved by titration with 0.1 N NaOH using phenolphthalein as a pH indicator [21].

### 2.6.3. Biomass determination

Biomass was monitored by measurement of optical density (OD) (UV-spectrophotometer Optizen). Cell dry weight was calculated from the optical density using a calibration curve for the strain. *Lactobacillus* strains were enumerated by the simpler pour plate technique on MRS media [22].

## 3. Results and discussion

### 3.1. Physicochemical characteristics of olive mill wastewater samples

Organoleptic characterization showed that olive mill wastewater (OMW) samples were generally dark brown to intense black in appearance, with a smooth texture and ranging from a fresh taste of olive oil to rancid odor. Table 1 presents the physicochemical characteristics of the OMW samples. All the data were averaged over at least three measurements.

In this study, the measured pH value of 4.88 is within the range of 4.4–5.4 reported in the literature [23,24]. The increase in the acidity of the OMW is probably due to the increase of free fatty acids concentration by oxidation in the storage ponds [25].

The electrical conductivity of 34 mS cm<sup>-1</sup> is related to the concentration of dissolved ionic substances and their nature [24]. This is indicative of the high salt content present

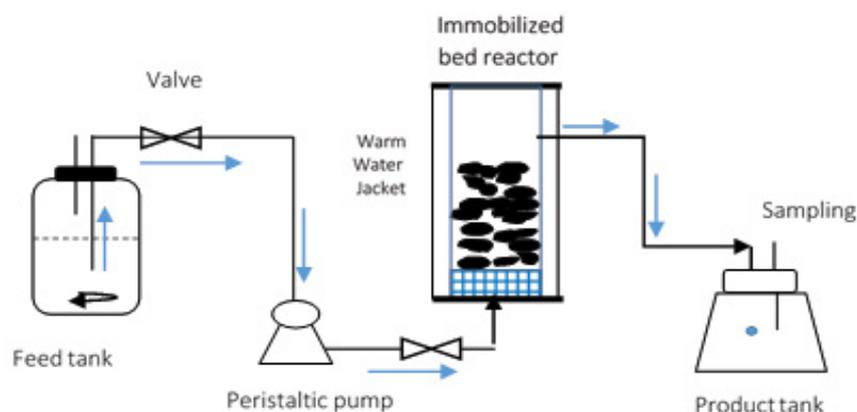


Fig. 2. Lab scale fermenter in continuous culture with immobilized cells on supports pozzolan and bovine bone.

in these effluents due to salting practices for the preservation of olives and the natural presence of minerals in olives. The high content of chlorides of  $3.9 \text{ g L}^{-1}$  confirms this observation. According to Table 1, turbidity and suspended matter (SM) values were 138.9 NTU and  $0.385 \text{ g L}^{-1}$ , respectively. Because of the settling in the storage ponds during a few days, low values of turbidity and SM were obtained.

The OMW is very rich in organic matter expressed in terms of chemical and biological oxygen demands [26]. According to Table 1, the values of COD and  $\text{BOD}_5$  are 183 and  $7 \text{ g L}^{-1}$ , respectively. However, the very low  $\text{BOD}_5/\text{COD}$  ratio indicates a poor biodegradability level of the OMW. When discharged without proper treatment into natural water and soil, OMW causes organic pollution and asphyxiation to microorganisms and plants. Most authors attribute this high toxicity to the presence of phenolic compounds [27,28].

Table 1  
Physicochemical characteristics of the OMW samples [6]

Characteristics	Value
pH	4.88
Acidity (%)	0.65
Electrical conductivity ( $\text{mS cm}^{-1}$ )	34.00
Turbidity ( $\text{NTU}^{-1}$ )	138.90
Dry matter ( $\text{g L}^{-1}$ )	22.60
Suspended matter ( $\text{g L}^{-1}$ )	0.39
COD ( $\text{g L}^{-1}$ )	183.00
$\text{BOD}_5$ ( $\text{g L}^{-1}$ )	7.00
Orthophosphates ( $\text{mg L}^{-1}$ )	1.31
Chlorides ( $\text{g L}^{-1}$ )	3.90
Nitrites ( $\text{mg L}^{-1}$ )	2.08
Nitrates ( $\text{mg L}^{-1}$ )	1.95
Calcium ( $\text{mg L}^{-1}$ )	7.04
Magnesium ( $\text{mg L}^{-1}$ )	19.82
Ammonium ( $\text{mg L}^{-1}$ )	1.55
Sulfate ( $\text{mg L}^{-1}$ )	1.49
Salinity ( $\text{mg L}^{-1}$ )	1.50
Total phenolic contents ( $\text{g L}^{-1}$ )	1.72
Residual oil and fats ( $\text{mg L}^{-1}$ )	0.16

In our study, the content of polyphenolics  $1.72 \text{ g L}^{-1}$  is lower than that reported in the literature [29] due probably to the origin of the OMW, the variety and the maturity of olive, the climatic conditions, and the technological process used to oil extraction.

As well, residual fat content depends on the efficiency of the olive oil extraction system. The fat content, linked to the presence of an oily fraction, gives the OMW a viscous appearance. It forms a lipid layer on the surface of the OMW at the level of the storage ponds limiting natural evaporation.

### 3.2. Microbial communities of OMW samples

The microbiota of the olive mill wastewater is reported in Table 2. The standard plate count values for OMW samples were related to the environmental conditions.

Although OMW is not adequate for microorganism growth, certain bacteria, yeasts, and fungi are resistant to acidic conditions and tolerant to the presence of phenolic compounds [10]. According to Table 2, the count of total aerobic mesophilic flora count was lower than that reported by others [10,28]. It can be argued that it was dependent on the extraction process, the operating conditions, the region, and the storage conditions, and on the physicochemical characteristics of the OMW (i.e., pH, phenolic compounds, mineral salts, fatty acids).

Yeasts, molds, and fungi can grow better than bacteria in olive mill wastewater (OMW) [10,28]. The tolerance of these groups to polyphenols is a genetic trait. Lactic acid bacteria, relevant to this work, were present in the studied OMW. The observed lactic acid bacterial count was  $4.2 \times 10^4 \text{ CFU/mL}$ . Their presence is indicative of their resistance to the aggressive medium of OMW [10]. However, their disappearance could occur over time due to

Table 2  
Microbial communities identified in OMW samples [6]

Microbiota	Bacterial count ( $\ln 10^4 \text{ CFU/mL}$ )
Total aerobic mesophilic flora	0.55
Yeasts and moulds	1.4
Fungi	10.8
Lactic acid bacteria	4.2

a resistance decrease. It is noted that fecal bacteria responsible for sanitary and hygienic problems, were not detected in this study. The absence of these human pathogens could be attributed to their sensitivity to low pH and the antibacterial activity of polyphenolics.

### 3.3. Characterization and identification of isolates

The collected samples were inoculated in de Man, Rogosa and Sharpe agar (MRS) broth in conical flasks and incubated at 37°C overnight. After incubation at 37°C, opaque white-colored cultures were chosen for the growth of *Lactobacillus* and *Lactococcus*. The culture from the MRS broth was then inoculated on MRS agar plates. After overnight incubation at 37°C, pure, white-colored colonies were identified to be *Lactobacillus* and *Lactococcus* by observing their colony morphology, physiological, and their biochemical characteristics. Microscopically they were Gram-positive, rod or cocci-shaped non-motile, catalase-negative, and absence of Endospores. Sixteen strains belonging to the genus *Lactobacillus* were isolated. The biochemical, physiological characteristics of isolated *Lactobacillus* strains are shown in Table 3.

The isolated bacteria were identified through bacteriological and biochemical tests. The sixteen isolates were grown in de Man, Rogosa and Sharpe (MRS) medium at pH 5.6. The isolates were found catalase and oxidase negative; thereby this might confirm that the isolates were *Lactobacillus* spp. [30]. Most of the strains were  $\gamma$ -hemolytic (absence of hemolytic zones around the colonies).

In the current investigation, all sixteen isolates were able to ferment eight carbohydrates, (i.e., fructose, lactose, raffinose, maltose, galactose, D-maltose monohydrate, rhamnose, and D-mannitol) indicating that they can grow in a variety of habitats utilizing different types of carbohydrates. The results of bacteriological and biochemical tests are summarized in Table 3. The conclusions were similar to the findings of Chowdhury et al. [31]. The characteristics of the sixteen (16) isolates indicated that the strains could be *Lactobacillus manihotivorans* (1, 2, 3, 4, 9, and Lb10) and *Lactobacillus rhamnosus* (5, 6, 7, 8, 11, 12, 13, 14, 15 and 16).

### 3.4. Analysis of acidifying power of isolates

The results of the acidifying power of lactic acid bacteria isolates showed that Lb2 (*Lactobacillus manihotivorans*),

Lb5, and Lb16 (*Lactobacillus rhamnosus*) gradually produced lactic acid, and this was simultaneously accompanied by a lowering of the pH in the MRS broth. According to Fig. 3 and Table 4, the best acidifying activity was observed for the strain Lb16 (*Lb. rhamnosus*) with an acidity equal to 45°D after 24 h compared to both isolates and on OMW/25% whey.

The visual inspection of both the supports, before and after the cell immobilization, suggests the biofilm formation on the surface, as shown in Fig. 4. According to Fig. 5, results showed that the growth of the strain isolated from OMW and identified as *Lactobacillus rhamnosus* was better under anaerobic conditions in MRS broth compared to OMW without or with supplementation of whey. Greater quantities of *L. rhamnosus* cells were achieved at lower costs using a medium containing milk whey (MMW) (9.84 log CFU/mL) when compared to the MRS broth (9.63 log CFU/mL), a medium specifically developed for growing of *Lactobacillus* bacteria [32]. Thus, MMW can perfectly replace the MRS broth for *L. rhamnosus* production, without affecting cell production [32].

The conventional batch process of fermentation has some disadvantages such as inhibition by the substrate or the final product. To avoid these problems, this study was focused on improving the production of lactic acid through different feeding strategies using OMW supplemented or non-supplemented with whey [33]. The downstream process is a significant bottleneck because cost-effective methods of producing high-purity lactic acid are lacking [33]. Thus, the investigation of different mode cultures of lactic acid production was one of the aims of this work.

Figs. 6–10 depict the results of fermentation for to up to 96 h at 37°C under anaerobic conditions with initial pH = 6.5. It can be seen that the production of lactic acid by *Lactobacillus rhamnosus* exhibited 2–3 phases. This is observed for the case where the bacterial strain is immobilized on both the supports whatever the culture method. It can be argued, that during the first phase, the cells preferentially degraded the sugar on which they could grow more rapidly. Once the first sugar was used up, the cells move on to consume the second sugar. Upon transition to the second phase, the cells produced the enzymes necessary for the metabolism of the second sugar and so on during a latent period. This phenomenon is called diauxie. It was first described by Monod [34] as glucose–lactose diauxic

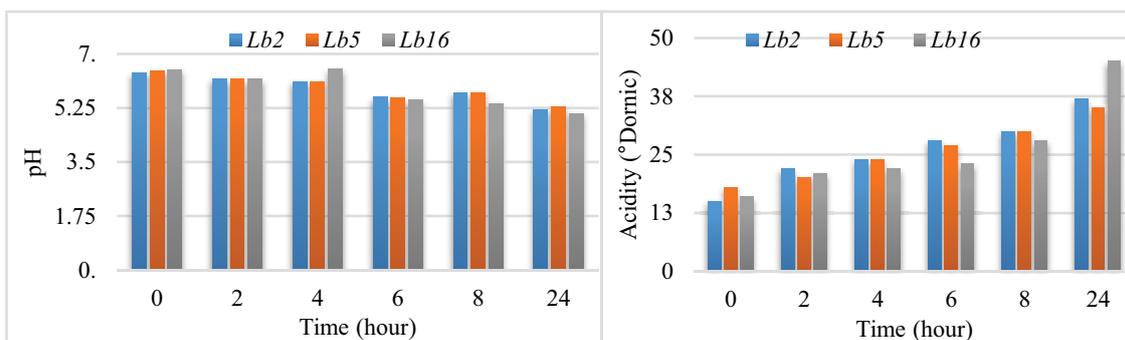


Fig. 3. Results of pH and acidity evolution for 24 h at 37°C and initial pH 6.5 of three isolates Lb2, Lb5 and Lb16.

Table 3  
Biochemical and physiological characteristics of the isolated Lactobacilli strains

Strain	Lb1	Lb2	Lb3	Lb4	Lb5	Lb6	Lb7	Lb8	Lb9	Lb10	Lb11	Lb12	Lb13	Lb14	Lb15	Lb16
Gram	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation type	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T°15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T°37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T°45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 4.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pH 9.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hemolytic	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ
NaCl 2%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaCl 4%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaCl 6.5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NaCl 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ADH	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dextran production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetoin production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteolytic activity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipolytic activity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid production à partir du Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+/-	+	-	-	-	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-maltose monohydrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
D-mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) Positive result or growth;

(-) Negative result or no growth.

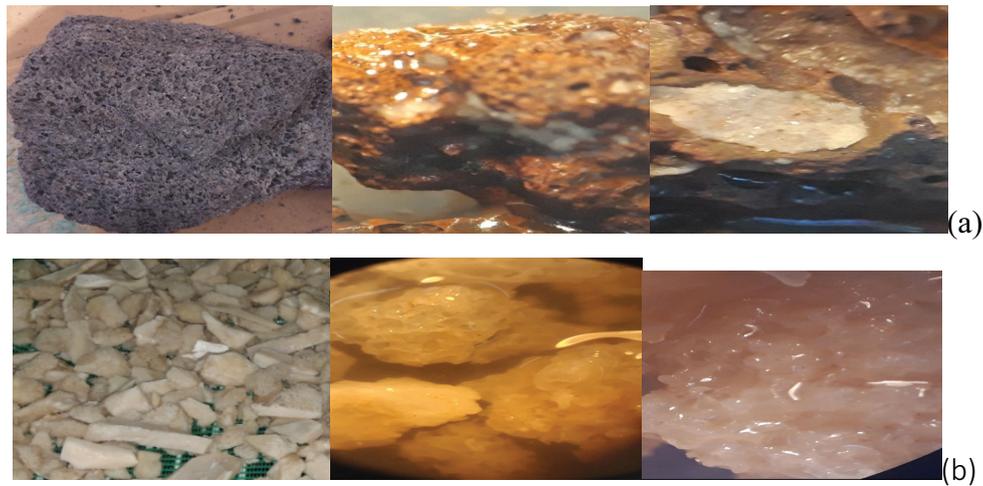


Fig. 4. Results of isolate *Lb16* immobilization on pozzolan (a) and bovine bone (b) for 72 h at 37°C and initial pH = 6.5.

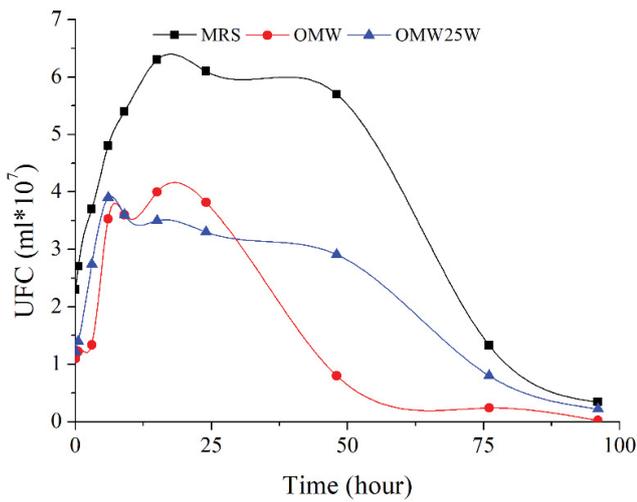


Fig. 5. Effect of medium on *Lb16* growth for 96 h at 37°C and pH = 6.5 in free batch mode.

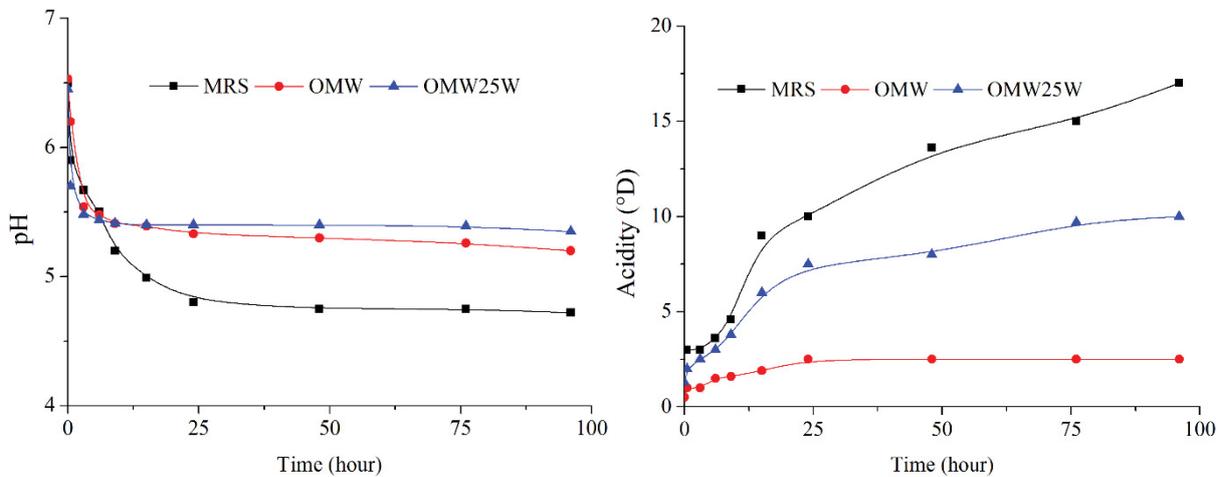


Fig. 6. Effect of medium on pH and acidity production by *Lb16* for 96 h at 37°C and initial pH 6.5 in free batch mode.

growth, which led to a paradigm shift in the understanding of gene regulation [35].

For the MRS broth, the profile of the immobilized cell continuous culture curve showed lactic acid production by *Lactobacillus rhamnosus* in three phases: from 1 to 4.5 g L<sup>-1</sup>, from 4.5 to 5.8 g L<sup>-1</sup>, and from 5.8 to 17 g L<sup>-1</sup>, then the production remained constant on the pozzolan support (Fig. 9) compared to the bovine bone support (Fig. 10) from which it was produced as noted from 0.8 to 3.7 g L<sup>-1</sup>, from 3.7 to 5 g L<sup>-1</sup> and from 5 to 11 g L<sup>-1</sup>.

For the non-supplemented OMW medium, the best lactic acid production by *Lactobacillus rhamnosus* was noted on the bovine bone support in batch mode (Fig. 8) with 6.2 g L<sup>-1</sup>, followed by the continuous mode (Fig. 10) with 4.5 g L<sup>-1</sup> on the pozzolan support and 3.8 g L<sup>-1</sup> on the bovine bone support.

For OMW medium supplemented with 25% whey, the best lactic acid production by *Lactobacillus rhamnosus* was noted in batch mode with 8.1 g L<sup>-1</sup> on the bovine bone support (Fig. 8), followed by 7.4 and 6.4 g L<sup>-1</sup> on the support pozzolan (Fig. 9) and bovine bone (Fig. 10) in continuous mode, respectively.

Table 4  
Acidifying activity for the strain *Lb16* (*Lb. rhamnosus*) after 24 h for isolates and OMW/25% whey

	MRS		OMW		Whey		OMW25W	
	$t_{0h}$	$t_{24h}$	$t_{0h}$	$t_{24h}$	$t_{0h}$	$t_{24h}$	$t_{0h}$	$t_{24h}$
Growth (UFC/mL) ( $\times 10^7$ )	0.54	13	0.49	1	0.51	9	0.66	5
pH	6.5	3.45	6.5	6.3	6.5	3.55	6.5	3.79
Acidity ( $^{\circ}$ Dornic)	73	110	10	1	65	90	30	65

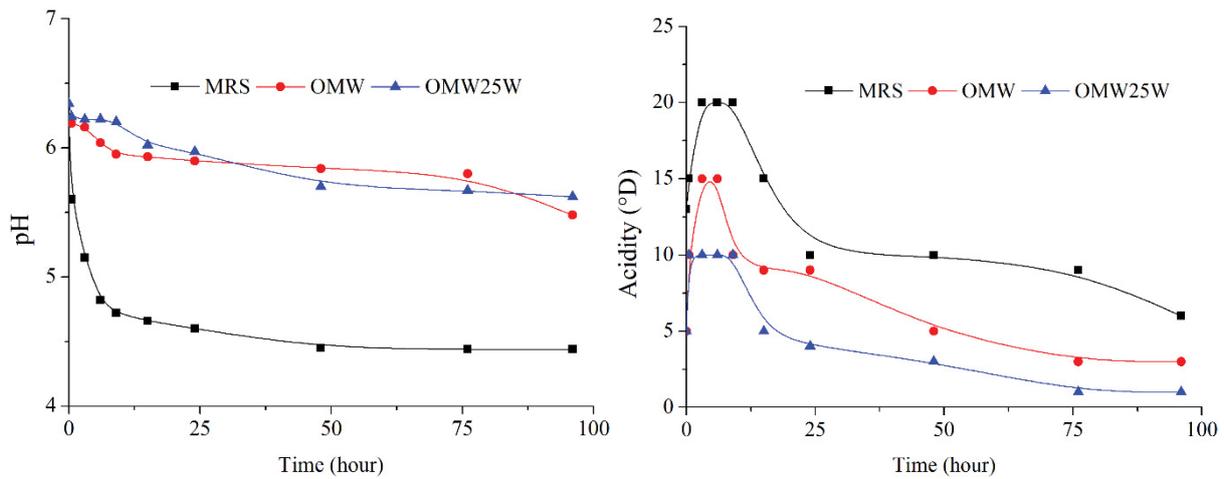


Fig. 7. Effect of medium on pH and acidity production by *Lb16* immobilized on pozzolan for 96 h at 37°C and initial pH 6.5 in immobilized batch mode.

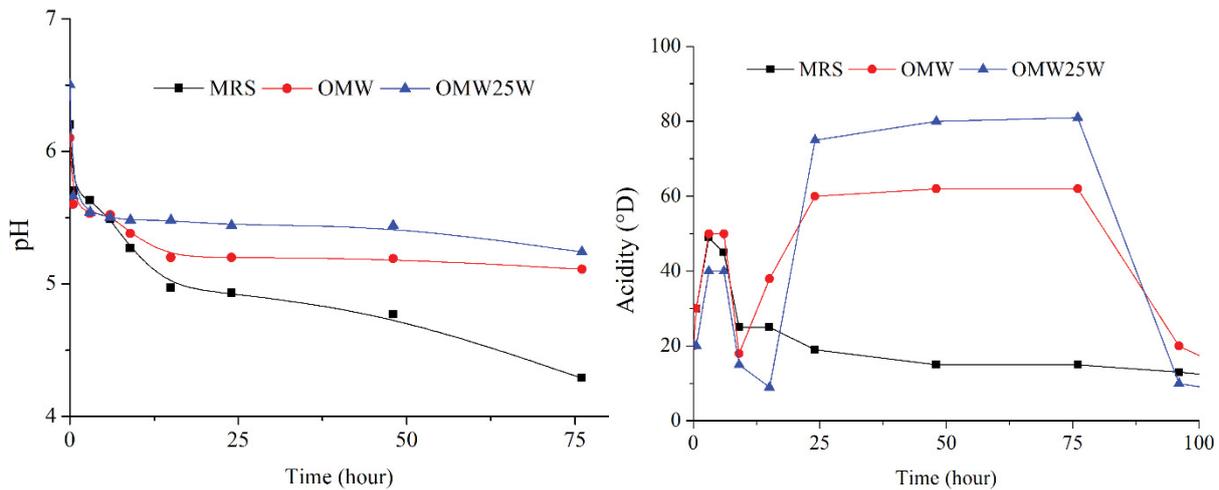


Fig. 8. Effect of medium on pH and acidity production by *Lb16* immobilized on bovine bone for 96 h at 37°C and initial pH 6.5 in immobilized batch mode.

The comparison of the results achieved in this work with those obtained from literature reveals the dependence of the type of bacteria on sugar conversion and lactic acid production [36,37].

L-lactic acid was produced in batch and fed-batch fermentation processes using the strain *L. rhamnosus* ATCC 10863. No cell inhibition was detected. A total of 16.5 g L<sup>-1</sup> of

lactic acid was produced at the batch mode and 22.0 g L<sup>-1</sup> at the fed-batch mode [36].

Srivastava et al. [37] reported on an *L. rhamnosus* ATCC 10863 (called *L. casei* subsp. *rhamnosus* NRRL B-445) fermentation at 39°C using sucrose (105.75 g L<sup>-1</sup>) as substrate supplemented with 30.0 g L<sup>-1</sup> of yeast extract and other compounds. The lactic acid production was 80.0 g L<sup>-1</sup> and the

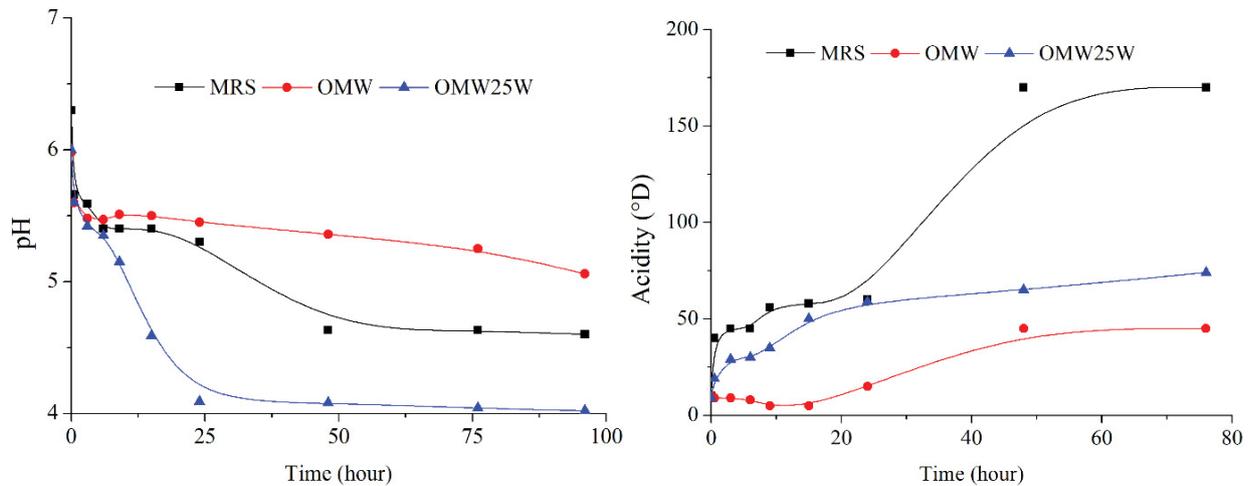


Fig. 9. Effect of medium on pH and acidity production by *Lb16* immobilized on pozzolan for 96 h at 37°C and initial pH 6.5 in immobilized continuous mode.

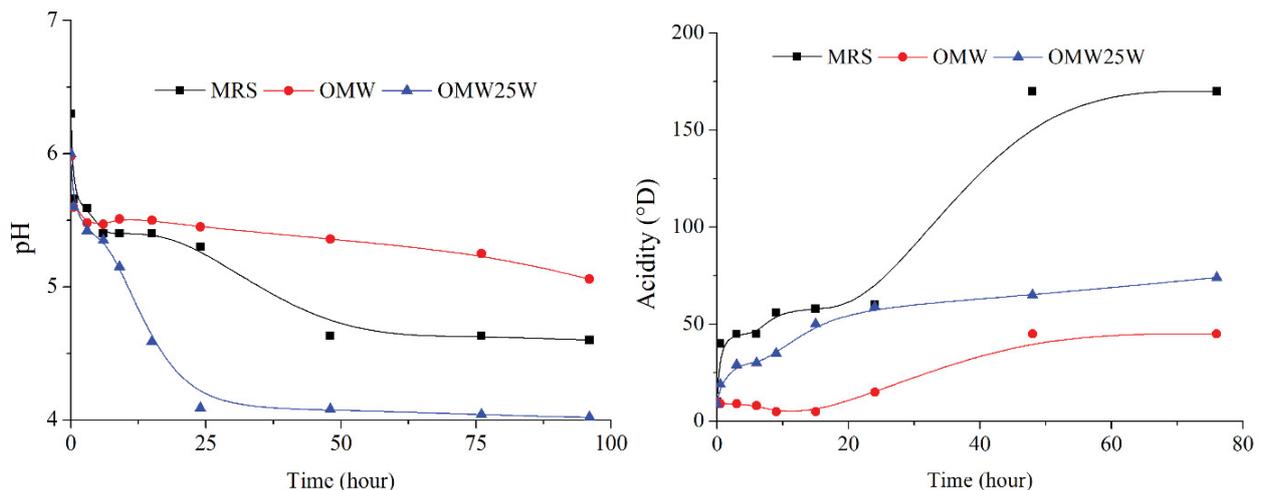


Fig. 10. Effect of medium on pH and acidity production by *Lb16* immobilized on bovine bone for 96 h at 37°C and initial pH 6.5 in immobilized continuous mode.

batch fermentation time was ~220 h. It was observed that up to ~40 h of fermentation, the lactic acid production was ~20.0 g L<sup>-1</sup>.

#### 4. Conclusions

This study has demonstrated that lactic acid can be produced in both batch and continuous fermentation by *Lactobacillus rhamnosus* cells isolated from olive mill wastewater (OMW) with the bacterial strains immobilized on bovine bone and pozzolan supports. The best acidifying activity was detected in the strain *Lb16* (*Lb. rhamnosus*) with an acidity equal to 45°D. All fermentations exhibited a biphasic growth (diauxie). The greatest production was seen in MRS (de Man, Rogosa and Sharpe) broth with the strain immobilized in continuous culture on pozzolan (17 g L<sup>-1</sup>) and bovine bone (11 g L<sup>-1</sup>), in non-supplemented OMW medium with bovine bone support in batch mode (6.2 g L<sup>-1</sup>), 4.5 g L<sup>-1</sup> on the pozzolan support and 3.8 g L<sup>-1</sup>

on the bovine bone support at continuous mode. For OMW medium supplemented with 25% whey, the optimum lactic acid production by *Lb. rhamnosus* was seen in a discontinuous batch with 8.1 g L<sup>-1</sup> on bovine bone support, followed by 7.4 and 6.4 g L<sup>-1</sup> respectively on the support pozzolan and bovine bone in continuous mode. This investigation has confirmed that it is possible for an agri-food effluent (i.e., olive mill wastewater) to produce a valuable biochemical product (i.e., lactic acid). Further work is needed to optimize process parameters such as the optimal OMW/whey ratio and nutrient supplementation.

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