Immobilization Of Lactic Acid Bacteria On Bentonite Clay Particles of Maghnia Region West-Algerian

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Received 28 August 2016; Accepted 18 October 2016; Available online 22 October 2016

ABSTRACT
Microorganisms may attach to the solid surfaces by their bio adhesive properties in order to form a biofilm, which can be used in several areas, including those in the food and the treatment of water. A re-identification of three strains of lactic acid bacteria (Lactobacillus fermentum, Streptococcus thermophilus and Lactococcus lactis subsp. lactis biovar. diacetylactis) have been carried out by its phenotypes characterizations (Macroscopic on culture media MRS  and M17 (liquid and solid), Microscopic after Gram stain, biochemical whose catalase test and fermentation Type before testing their capital assets on clay particles in suspension, thus forming the complex bio sorbents. After centrifuging the suspension, we observed a good fixation of these particles, in particular the Lactococcus lactis subsp. lactis biovar. diacetylactis and Streptococcus thermophilus, respectively with a number of attached cells of 72x10^7 and 70x10^7 cfu/ml, and this after 4 hours of incubation at 30 and 37°C, whereas the Lactobacillus fermentum was less with only 40x10^6 cfu/ml. We have also observed an obvious increase in the growth of the number of cells Lactococcus lactis subsp. lactis biovar. diacetylactis in the presence of clay particles in suspension, from an initial number of 40x10^6 cfu/ml to 85x10^7 cfu/ml after 24 hours of incubation, this is probably due to the catalytic role played by the clay particles in suspension in their multiplication.

KEYWORDS: Clay of Maghnia, Lactic acid bacteria, Streptococcus thermophilus, Lactococcus lactis subsp. lactis biovar. diacetylactis, Lactobacillus fermentum, Bio sorbent.

INTRODUCTION
The industrial developments, technological and agronomic traits have resulted in a great improvement in the economy field. However, they have led to the emergence of a harmful phenomenon, which has severely hurt the humanity, as well as the environment: it is mainly the pollution. To limit or reduce this pollution, it is required to deal with any industrial effluent before its discharge into the natural environment. The treatments used, can be physico-chemical and/or microbiological, involving in some cases, the use of materials that are able to eliminate the micropollutants.

Appeared in recent years a technique named biosorption, technique based on passive taking of various substances: heavy metals [24,25], colors [26] by bacteria mortes / inactivated.

Some microorganisms may attach to the solid surfaces by their bio adhesive properties to form a biofilm, which can be used for example as a bio sorbent in the field of water treatment [10].

We focused on the model of lactic acid bacteria (Lactococcus diacetylactis, Lactobacillus fermentum et Streptococcus thermophilus) because they are used in the dairy industry [17] as a representative example for the
study of the influence exerted by the immobilization process of production on the one hand and, dairy properties and secondly because this group of bacteria has a beneficial effect on the gastro-intestinal tract of man and animals by their capacity for colonization [12].

The formation of a biofilm on a solid surface is a complex phenomenon, so several steps are necessary for this formation, in which physical, chemical and biological processes are involved [19]. In the natural conditions, the bacteria carry out their growth mainly in the form of immobilized communities (films, colonies or the aggregates), their activity is traditionally studied from isolated planktonic cells [12].

In soil, bacteria can develop in association with plants roots and also they can adsorb on inorganic surfaces. The clay in soil plays a central role in influencing its structure, its porosity as well as its ability to exchange. Clay is a simple mineral and natural material, very abundant, consisting of aluminosilicates, the sheet structure is well known, it results from the decomposition of rocks (superficial parts of the earth’s crust, such as granite) that crystallized over time [13].

The aim of the present paper is to test the abilities of accession of lactic acid bacteria to clay particles of Maghnia in suspension to form complexes biosorbents. To do this, we proceeded to the purification of the clay by sedimentation, re-identify of the lactic strains (Lactococcus diacetylactis, Lactobacillus fermentum and Streptococcus thermophilus) isolated from the raw gaot’s milk produced west Algeria, for their phenotypic and biochemical characteristic before securing on clay particle of Maghnia for to get the complex biosorbents, and study their kinetics growth in absence and presence of clay in a suitable culture medium.

**MATERIAL AND METHODS**

**Clay purification:**

We have used a clay placed at our disposal by the company ALFET (Algerian for smellders in Tiaret), from a deposit meadows of Maghnia and marketed by the company ENOF of Maghnia under the name of “bentonite of Foundry”. It is mineral clay containing mainly the montmorillonite, which belongs to the smectite group the Type 2:1, formed by the superposition of three layers, a octahedral layer between two tetrahedral layers. In water, the mineral clays remain in suspension in the form of small particles with a diameter of about 2 µm and possess a lamellar structure (succession of slips) [20]. The chemical composition of elementary clay of Maghnia, is indicated in table 1 [4].

<table>
<thead>
<tr>
<th>Elements</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO₂</td>
<td>669.4</td>
</tr>
<tr>
<td>Al₂O₃</td>
<td>114.7</td>
</tr>
<tr>
<td>Fe₂O₃</td>
<td>11.2</td>
</tr>
<tr>
<td>CaO</td>
<td>0.3</td>
</tr>
<tr>
<td>MgO</td>
<td>11.1</td>
</tr>
<tr>
<td>Na₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>K₂O</td>
<td>0.8</td>
</tr>
<tr>
<td>TiO₂</td>
<td>0.2</td>
</tr>
<tr>
<td>AAs</td>
<td>0.05</td>
</tr>
<tr>
<td>FFAF*</td>
<td>111</td>
</tr>
</tbody>
</table>

*loss to a fire at 900°C.

To rid the clay of Maghnia of all crystalline particles (quartz, feldspar, calcite,…), we conducted firstly a sieving of 40 grams of clay through a sieve of 50 µm, before putting in suspension by agitation during 24 hours in 2 liter of distilled water and then the suspension is put at rest during several days in a test tube to settle. By siphoning it retrieves, the 400 milliliters of the top of the test piece, which constituted a colloidal suspension of clay particles whose size is less than 2 µm, sterilized in an autoclave for 20 minutes at 120°C, before use in our tests. The mass m of the residue obtained after evaporation of a volume V of the suspension in an oven, allows us to determine the concentration of clay particles of the suspension found equal to 0.58 g/l.

**Re-Identification of strains:**

The studied lactic acid bacteria (Lactococcus diacetylactis, Lactobacillus fermentum and Streptococcus thermophilus), are those that the Laboratory of the Applied Microbiology at the University of Oran has kindly put at our disposal. These bacterial species were isolated from the raw milk of cheese goat in the west region of Algeria (Oran, Tiaret).

The activation of lactic strains was performed on MRS environments and M17 Liquid. After growth, it was proceeded to their purification on MRS environments and M17 solids [16]. The cultures were incubated at 30, 37, and 45°C respectively for the Lactococcus diacetylactis, Streptococcus thermophilus and Lactobacillus fermentum, during 24 h to 48 h in order to obtain well isolated colonies [1,18]. The obtaining of pure cultures from colonies well separated, were made by dilution in liquid medium and by stripes in solid medium. The purity of the strains was reviewed by a macroscopic and microscopic control using the Gram stain.

**Macroscopic features:**

The macroscopic study allows us to describe the bacterial cultures on solid and liquid medium. On solid medium, we describe the type of colonies, the color, the rim, the elevation, the form as well as the diameter. In
contrast, on liquid medium, the aspect of micro aerophile culture, which may be of particular interest in the choice of the strain of lactic acid bacteria.

**Microscopic features:**

It is a microscopic examination, which allows you to define the cellular morphological aspects, the form and association type by the Gram stain.

**Biochemical features:**

**Catalase Test:**

The research for the catalase is done by placing in contact bacterial colonies with a few drops of oxygenated water. The gaseous release reflects the positive activity of the catalase enzyme, which decomposes the oxygenated water into water with release of oxygen [15,21].

**Test of fermentative Type:**

This test helps us to evaluate the metabolism type, by which the carbon substrate is transformed. To evaluate their growth, we incubated the tested lactic strains in the mid MRS or M17 liquid containing the bell of Durham, the presence of disorder and gas in the Bell, indicates metabolism heterofermentary bacteria [18].

**Conservation Strains:**

The purified strains must be retained, and according to the period of conservation, we can apply two techniques:

- Strains are preserved in the mid MRS or M17 in solid and Sterilized test tubes. After Plating and incubation in the optimal conditions at 28°C for 24 h, they were stored in the refrigerator at (4°C) during three to four weeks, then they are sub cultured to renew the conservation [14,17].
- We have seeded the strains in a basic liquid medium. After incubation in optimal conditions, we have centrifugal young culture in eppendorfs tubes at 3000 rpm for 5 minutes, then we added to red blood cells we will obtain a mixture of skimmed milk with glycerol (70% skim milk and 30% glycerol), so we must keep it in the freezer to (-20°C) [17].

**Determination of the bacteria growth in time function:**

**Determination of the bacteria growth in suspension in absence of clay:**

To study the growth of bacteria, we began by preparing a young culture of each strain, that we have standardized by using the method of Mc Farland with the standard solution 2.0 (at 620 nm the DO of the bacterial suspension must be between 0.320 and 0.400), and it was then inoculated in tubes containing 10 ml of culture medium (MRS liquid for the *Lactobacillus fermentum*, M17 Liquid for the *Lactococcus diacetylactis* and *Streptococcus thermophilus* [1], and this by incubation in a Bain Marie respectively to 45, 30 and 37°C for 24 hours. Mix 10 ml of each young culture to 100 ml of an appropriate culture medium, and after the homogenization, we distribute the mixture in sterilized tubes (10 ml/tube), then we add to each tube 1 ml of sterilized distilled water, incubate under agitation, the tubes for 24 hours and read the DO every 2 hours.

**Determination of the growth of bacteria in suspension with the clay:**

Repeat the process through replacing the distilled water by 1 ml of suspension sterile clay in the tubes, then read it each 2 hours the DO at 620 nm, before and after centrifugation at 4000 rpm for 15 min, but the supernatant and pellet were washed several times in sterilized distilled water to eliminate all bacteria not laid down on the clay. We have also observed that the culture media and the suspension of the clay particles obtained by sedimentation, are transparent to light, corresponding to a DO virtually zero to 620 nm.

**RESULTS AND DISCUSSION**

**Morphological features of the strains:**

The macroscopic and microscopic characterizations serve basically for the identification of genus and species type in some cases. In a solid media cultures of M17 and MRS, the lactic strains have given sometimes lenticular and circulars colonies of small sizes of approximately 1 mm diameter, whitish or yellowish slightly, to regular rim and smooth. In MRS liquid environment, they can grow better in depth, where the pressure of oxygen is low. respecting the microscopic observation, we have remarked that the lactic strains are Gram+, they presented themselves in a form of cocci and bacilli, arranged to be in pairs are in bead chains more or less long, like the Table 2.
Table 2: Macroscopic and microscopic aspects of the retained strains.

<table>
<thead>
<tr>
<th>Code</th>
<th>Aspect of the colony</th>
<th>Gram</th>
<th>Morphology, cellular arrangement</th>
<th>Catalase</th>
<th>Fermentative Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>White lenticular</td>
<td>+</td>
<td>small rod sticks isolated or in short chains</td>
<td>-</td>
<td>Hétéro</td>
</tr>
<tr>
<td>S2</td>
<td>Lenticular creams</td>
<td>+</td>
<td>cocci, diplo, bead chains</td>
<td>-</td>
<td>Homo</td>
</tr>
<tr>
<td>S3</td>
<td>Lenticular creams</td>
<td>+</td>
<td>cocci, diplo, bead chains</td>
<td>-</td>
<td>Homo</td>
</tr>
</tbody>
</table>

Hétér = Hétérofermentation, Homo = Homofermentation

By comparing the results obtained for morphological and biochemical characteristics of our lactic strains with those described by some authors such as Stiles et Holzapfel [22]; Badis et al. [1]; Badis et al [2]; Behira et al. [3]; Zineddine et al. [23]; Guessas et al. [9], Chahrour et al. [7]; Moulay et al. [18], Senouci et al. [21] we have been able to re-identify our bacterial strains, as shown in the Table 3.

Table 3: Different strains of the bacteria studied.

<table>
<thead>
<tr>
<th>Code</th>
<th>bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Lactobacillus fermentum</td>
</tr>
<tr>
<td>S2</td>
<td>Lactococcus lactis subsp. lactis biovar. diacetylactis</td>
</tr>
<tr>
<td>S3</td>
<td>Streptococcus thermophilus</td>
</tr>
</tbody>
</table>

Kinetics of growth of bacteria in the culture medium with or without clay:

Growth of cells in the absence of clay:

The kinetics of growth of the studied strains in a suitable culture medium in the absence of clay particles, are represented in Figure 1.

![Fig. 1: Growth kinetic of strains in the absence of clay.](image)

Our results shows that the strain S1 start the grow after 6th hour, whereas strains S2 and S3, reach their stationary growth phases at 6th and 4th hour respectively.

Kinetics of growth of cells in the presence of clay:

In the presence of clay particles in suspension in the culture medium, some strains studied, their growth rates are changed, as shown in Figure 2. Virtually no changes have been observed concerning the Strains S1 and S3, but there was a clear change in the number increase of cells for strain S2, from an initial number of 40x10^6 cfu/ml to 85x10^7 cfu/ml after 24 hours of incubation. Probably the clay particles in suspension have played a catalytic role in this change.

![Fig. 2: Growth kinetics of strains in the presence of clay.](image)

After centrifugation, the number of cells remaining in the supernatant of cultures in the presence of clay are shown in Figure 3. The reading of the DO to 620 nm after 4 hours of incubation, indicates that the number of
cells decreased in the supernatant for the three strains S1, S2 and S3 respectively to $10^7$, $12 \times 10^7$ and $20 \times 10^6$ cfu/ml. It has been found that these numbers are virtually low for all strains, so they probably indicated a mounting and/or a precipitation with clay.

Fig. 3: Growth kinetic of the strains in the supernatant culture.

However, in the pellet washed several times with sterile distilled water, increases in the number of bacterial cells immobilized on the clay particles are shown in Figure 4, indicating that after 4 hours of incubation, the clay was better able to fix the cells S2 and S3 strains, respectively up to $72 \times 10^7$ and $70 \times 10^7$ cfu/ml, whereas for strain S1, the clay particles have fixed that $20 \times 10^6$ cfu/ml.

Fig. 4: Growth kinetic of the strains in the pellet.

Depending on Dommergues and Mangenot says (1970) the clay particles in suspension have negative charges, but at the ends of their tax slips appear positive charges. Therefore it is possible that in addition to the wetting properties of cell walls, and the accession between microbial cells of the studied strains and the clay particles, are due to electrostatic forces between bacterial walls loaded negatively and the edges of the slips.

The bacterial adhesion on a surface can be described as a process in two steps: According to Bulard et al. [5]; Bulad [6] the first step is physical, instant, and reversible: is the stage of attachment of bacteria on the surface where the microorganisms will be able to accede to the surface by physicochemical connections (depending on van der Waals, it is electrostatic interactions, or hydrophobic, when interactions are governed by the entropy) and by interactions related to the Brownian motion (movements incessant and random in solution). The second step is related to the physiology of bacteria (requiring connections created between the bacteria and the surface during the step of attachment), from the point of view of chemical and cellular properties, it becomes irreversible. It is the stage of adaptation of the bacterium to the surface. The bacterial adhesion is influenced by many other factors. Among those, the nature of the surface from the point of view acido-basic, possible presence of loads, that if they exist, they can play an important role in relation to ordinary forces, which are more low. Another factor to take into account is the roughness of the surface: the irregularities of a surface, the presence of holes and the increase of the porosity, this three factors can increase the number of bacteria adhering to the surface.

Conclusion:

In this work we have carried out a re-identification of three different bacterial strains and we have studied the possibility of Immobilizing on clay particles in suspension to make a complex bio sorbent. So the results obtained, allow us to conclude that:
• the purification of the clay of Maghnia by sedimentation, has led to the recovery of a suspension of clay particles with a size less than 2 μm and with a concentration of 0.58 g/L;
• the macroscopic, microscopic and biochemical characterizations, have led to the re-identification of a studied isolated of lactic acid bacteria;
• the clay particles immobilize better the Lactococcus diacetylactis and Streptococcus thermophilus up to respectively 72x10^7 and 70x10^6 cfu/ml, and this after 4 hours of incubation, whereas the Lactobacillus fermentum are with only 40x10^6 cfu/ml. We even have observed a strong increase in the growth of Lactococcus diacetylactis in the presence of clay particles of Maghnia in suspension in the culture medium up to 85x10^7 cfu/ml after 16 hours of incubation indicating a catalytic role of the clay.

REFERENCES


