Lactic Acid Fermentation of a Diluted Molasses Medium by Two Strains of *Lactococcus lactis* ssp. Immobilized on Pouzzolane and Bone Bovine

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Summary

In this work, tow supports (pouzzolane and bone bovine) were investigated for accelerate lactic acid fermentation by two strains of *Lactococcus lactis* at pH uncontrolled. The fermentation medium employed consisted of diluted sugar-cane molasses at 50% with supplementation at 25% of Whey. Two strains of *Lc. lactis* subsp. *lactis* isolated respectively from Cow's Milk and cane sugar Molasses were used in this study. Immobilized-cell batch with recycling system was compared with free-cell batch system. In the immobilized-cell batch with recycling fermentations, the initial rate of lactic acid production increased roughly twice especially with organic support during 144 h compared to free-cell batch.

Key words: *Lactococcus lactis*, immobilized-cell with recycling, Lactic acid, pouzzolane, bone bovine.

1. Introduction

Agri-food wastes show a promising alternative with their rich carbohydrate content, relatively stable composition and availability. Therefore, they represent an appealing waste valorization target for an eventual lactic acid. Compared to chemical synthesis, microbial fermentation is a better alternative because it leads to the production of optically pure lactic acid and offers advantages in the utilization of renewable carbohydrates [1]. Molasses is a byproduct of the sugar industry readily available at relatively low cost. It contains water, approximately 50% sugars (sucrose, glucose, fructose, raffinose), nitrogen compounds, organic acids, amino acids, heavy metals, etc [2]. Moreover, molasses and corn steep liquor are prominent culture media in fermentative processes due to the high content of sugars and nitrogen, respectively [3]. In sugar mills, *Lactococcus lactis* is involved in sucrose inversion, due to their potential for producing lactic acid. *Lactococcus* has a homo-fermentative metabolism and produce exclusively L (+) lactic acid [4]. Production of metabolites of *L. lactis* is carried out in batch, fed-batch and continuous fermentations [5, 6, 7, 8]. In these processes, the microorganism can be used either in a free form or immobilized [9].Various immobilization procedures such as covalent coupling, adsorption onto solid inert carriers, and entrapment in semi-permeable inert supports such as hydrogels, fibers and membranes were used. Supports such as Karrageenan gels, calcium alginate, ion exchange resins, vermiculite and y-alumina have been used for cell immobilization [10].

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The industry meat is a food industry generates co-products often valued power animal byproducts including reprocessing becomes more binding. Bones consist of organic (30%) and inorganic compounds (70%). Mineral parts of bones provide their stiffness and proper mechanical properties [11]. Compact bone has a porosity of 5% - 10%, while the porosity of a trabecular bone is 75% - 95%. However, as bone is a dynamic porous structure, its porosity may change [12]. Pouzzolane is defined as a siliceous or siliceous and aluminous material, which chemically reacts with calcium hydroxide at ordinary temperatures to form compounds possessing cementitious properties. Most natural pouzzolane contain substantial amounts of constituents other than silica, such as alumina and iron oxide, which will react with calcium hydroxide and alkalies (sodium and potassium) to form complex compounds [13]. Pouzzolane inorganic support was highly porous, mechanically stable and biologically inert [14]. This study illustrates the possibility of increase of cane sugar molasses supplemented at whey of high rates of lactic acid by immobilized Lactococci on pouzzolane and bovine bone for use as food bio-preservatives.

2. Materials and Methods

2.1. Microorganisms and culture conditions

Lactic acid-producing strains (***L**acto**c**occus lactis* subsp. ***l**actis* isolated from **C**ow's **M**ilk ***(Lc.l$_{CM}$), L**acto**c**occus lactis* subsp. ***l**actis* isolated from cane sugar ***Mo**lasses (**Lc.l$_{ML}$***) were used throughout this study. Microorganisms were maintained on M17 (Difco Laboratories, Sparks, MD) agar plates at 4°C and sub-cultured every 3 weeks. Cells for inoculation of the production medium at a level of 10% (v/v) were obtained from cultures grown on M17 broth (pH 6.5) at 30°C for 24 h.

2.2. Clarification of cane sugar molasses

Molasses (third throw obtained from Sugar Mills, Ain Defla, Algeria) used in the experimental work was initially homogenized. Suitable dilution of molasses was done as required by the experiments. Clarification of cane sugar molasses using sulfuric acid treatment was done according to the method of Ayilvahanan et al. [15]. The pH (Microprocessor pH-meter) of the molasses (total sugar: 31%) was adjusted to 3.0 by adding 0.1 N sulphuric acid. Then molasses were allowed to stand for one hour and thirty minutes and after they were centrifuged (Centrifuge of Hettich EBA III Type) at 3000 rpm for 15 min. The supernatant was collected and diluted with distilled water to obtain 50% of initial sugar concentration and then a supplementation with 25% of whey (acid whey from the Regional Office of Milk sis Aribbs, Ain Defla, Algeria) partially deproteinized [16] was carried. The pH of the diluted sugar-cane molasses at 50% with supplementation at 25% of whey partially deproteinized medium (MSW) was adjusted to 5.6 with 10 M NaOH prior to sterilization. The basal fermentation was used for following fermentation media (g/l) in flasks which were inoculated with 1% (v/v) of inoculums ($60 \pm 5 \times 10^6$ UFC/ml for ***Lc.l$_{CM}$*** and $1 \pm 0.05 \times 10^7$ UFC/ml for ***Lc.l$_{ML}$***. Fermentations were carried out with initial solution (31% of total sugar) and its dilution. The following cultures with immobilized-cell batch with recycling and free-cell batch was carried out at 30°C under shaking conditions at 100 rpm (Agitator of Kika Laboratechik brand) during 144 h [17].

2.3. Inorganic and organic supports

Pouzzolane and bovine bone were used as naturals, solids and respectively inorganic and organic supports.

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2.3.1. Pouzzolane treatment

Pouzzolane from the carrier of Beni Saf, Tlemcen (Algeria) was used and which was washed with tap water for remove impurities. The support was prepared by high temperature before its use. Granular pouzzolane of 06 mm diameter was obtained by size reduction of the rocks sieving. Particles are *60±5%* of porosity and *1.029±0.001* of density.

2.3.2. Bone bovine (BB) treatment

Both cancellous and cortical portions of cow bone (bones collected from university canteens) were used after their freezing (Freezer of Electrostar brand). Soft tissues such as muscles and tendons were removed from the frozen bone. The bones were then cut into small pieces with a diameter less than 2 cm. These pieces were treated by boiling in water for 10 h for deproteinization before their immersion in a 1% sodium hydroxide plus 1% hydrogen peroxide solution for 1h. They were then washed under flowing water and drying in oven brand MEMMERT at 80 °C during 1h. The pieces were shaped into small blocks with 06 mm of diameter and sterilized in autoclave Raypa brand [18]. Particles are *75±5%* of porosity and *1.01±0.005* of density.

2.4. Experimental device

The reactor is a 1.5 l fermentor with 1l working liquid volume (80 mm in diameter and 200 mm in length). Compounds-liquid elimination device are provided at the top of the reactor (Fig. 1). A flow distribution system is composed of sailcloth sis at the bottom of the reactor whose its role is to prevent the filtering material from being carried away. The media inoculated by strains is periodically re-injected in the reactor by a circulation pump collecting the liquid from top through the distributor; this action is necessary in order to collect the off bio-particles and attached biomass. Temperature is maintained at 30°C by a water jacket. The inlet flow from 40 to 50 ml/h during three days was determined for a best microbial fixation on 100 g of granular support. When immobilization was complete, the support was washed twice with 250 ml of substrates with pH initial of *5.6*, then the reactor was conducted with the uncontrolled pH, the inlet flow of 90 ml/h, the dilution rate of 0.36 ± 0.01 h^{-1}, the residence times of *3h 46 min*. The contact time was *33 ± 0.1 min* for pouzzolane and *43 ± 0.3 min* for bovine bone.

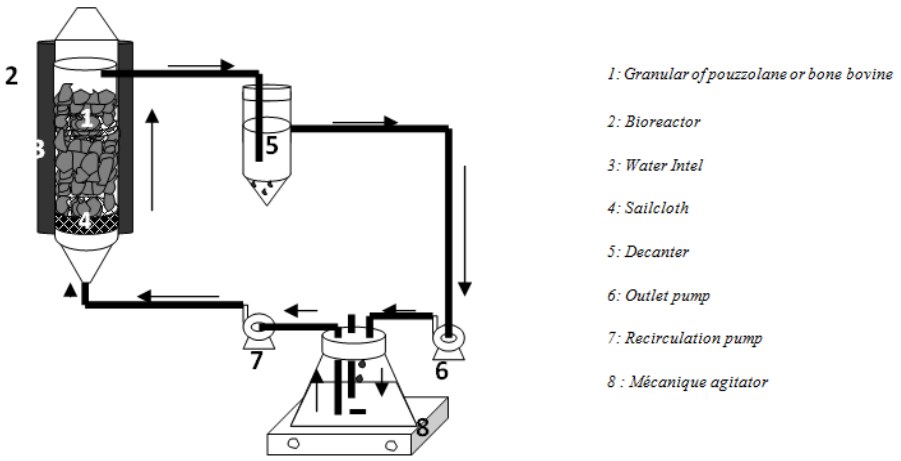

Fig. 1. Schematic diagram of biofilm reactor

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2.5. Analytical methods

Cell growth was monitored by a measurement of optical density (OD) (Spectrophotometer UV OPTIZEN VIEU 2000). *Cell dry weight* was calculated from the optical density using calibration curve for the strain. *Lactococcus* strains were enumerated by the simpler pour plate technique on M17 media. Dry weight, ashes and minerals elements of cane sugar *Molasses treated* were calculated according to the method of Ranganna [19]. *Lactic acid measuring* was carried out by Determination of titrable acidity [20]. *Reducing sugar* was estimated by 3,5-dinitrosalicyclic acid (DNS) method of Miller [21].

2.6. Statistical design

All experiments were replicated three times. Standard deviations were calculated and included in the graphical representation of the data.

3. Results

Repeated-batch fermentation of cane sugar **M**olasses **T**reated and **D**iluted at 50% with **S**upplementation at 25% of **W**hey (**MSW**) wastes to lactic acid was performed using immobilized two strains of *Lactococcus lactis* ssp under anaerobic condition for 144 h and in pH-uncontrolled. Pouzzolane and bone bovine (BB) were used as the immobilization solids porous supports and cell released, total sugar consumption and lactic acid production were explored and were compared with free-cell batch cultures.

The results noted on whey and molasses treated and autoclaved objects of this work show respectively that the density was *0.2±0.03* and *1±0.02*; dry matter was *05±0.1* and *32±0.5*%; proteins were *07±0.1* and *1±0.2*%; total sugar was *55±1* and *31±2*% and mineral mater was *0.6±0.05* and *1.6±0.01*%.

The results indicate that for a homofermentative process, pH 5.6 was found to be optimal. Cell samples from the exponential growth phase ($93 \pm 2\times10^{10}$ *CFU/ml* for $Lc.l_{CM}$ of 24 h and $95 \pm 5\times10^{10}$ *CFU/ml* for $Lc.l_{ML}$ of 18 h) of free-cell batch cultures at pH-uncontrolled were used to estimate biomass immobilized on pouzzolane and bone bovine by dry weight. For free-cell batch fermentations, the substrates uptake and growth kinetics of $Lc.l_{CM}$ and $Lc.l_{ML}$ were compared Elliker broth (data not shown). The immobilized biomass grew continuously during the successive batch cultures and highly adhesion was obtained after 72 h for both strains of Lactococci. During repeated batch cultures, immobilizations of lactococci by adsorption on porous supports for lactic acid fermentation were presented for *45 ± 0.02 g* of granular of each support as following (Fig. 2):

• ***On pouzzolane***, an important biomass concentration of $Lc.l_{CM}$ and $Lc.l_{ML}$ respectively was *0.0986 ± 0.0003 g* and *0.1560 ± 0.0005 g* in **M**olasses **T**reated and **D**iluted at 50% with **S**upplementation at 25% of **W**hey (**MSW**);

• ***On bone bovine***, an important biomass concentration of $Lc.l_{CM}$ and $Lc.l_{ML}$ respectively was *0.1215 ± 0.0001 g* and *0.1499 ± 0.0002 g* in MSW.

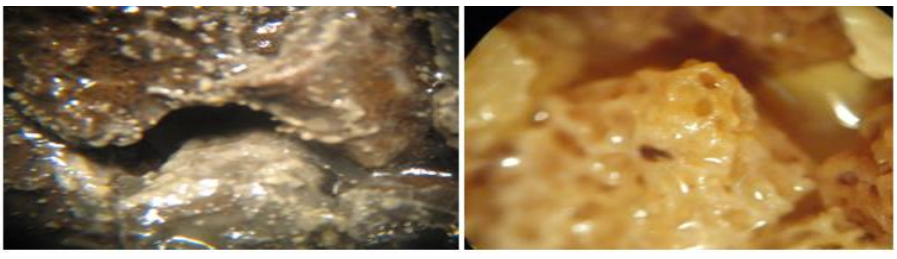

Fig. 2. Binocular observation of biofilm of 24 hours on pouzzolane (a) and on bone bovine (b) (Binocular loupe ZEISS brand)

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Compared with others supports, results of adhesion show that there seems to be an affinity between lactococci and pouzzolane and bone bovine. However, there were better supports for immobilization of the microorganisms.

The overall productivity of the recycle system was higher under all conditions studied in comparison with the batch process using free cells. Enhancement in productivity in the recycle batch reactor was also accompanied by an increase in density of suspended cells. Cell released count was become weak after 24 h and the culture entered stationary phase (Fig. 3).

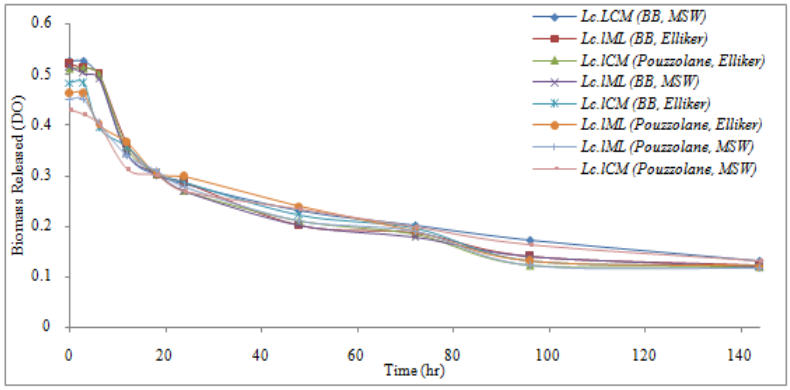

Fig. 3. Bacteria released kinetic in Repeated Batch Immobilized Cells (RBIC) culture on Pouzzolane and Bovine Bone (BB) in MSW (**M**olasses **T**reated and **D**iluted at 50% with **S**upplementation at 25% of **W**hey) at 30°C, the inlet flow of 90 ml/hr, and the dilution rate of 0.36 hr^{-1}

At the end of the sixth consecutive culture, which lasted *144 h*, high total lactic acid content in the medium MSW of *15.8 g/l et 13.6 g/l* respectively with $Lc.l_{ML}$ and $Lc.l_{CM}$ on bovine bone was obtained (Fig. 4), which corresponded to an lactic acid volumetric productivity of *0.105 ± 0.001* and *0.09 ± 0.005 g/l/h* compared with free-cell batch cultures of *0.0041 ± 0.005* and *0.0039 ± 0.005 g/l/h*. At *144 h*, rates of carbon source in the medium, total sugar, was consumed and ranged from *89 ± 1.5* and *90 ± 0.6%* for MSW which initially content *155 ± 0.5 g/l*. On the contrary in Elliker broths containing 14 ± 1.5 g/l assimilation rates vary between *82 ± 0.5 %* and *84 ± 0.3%* for *144 h* (Fig. 5).

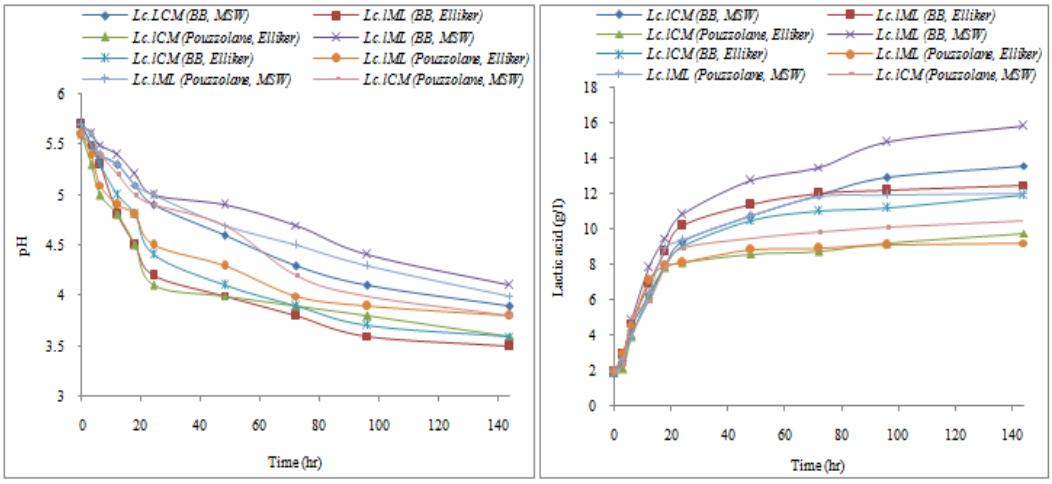

Fig. 4. pH evolution and lactic acid production kinetic in Repeated Batch Immobilized Cells (RBIC) culture on Pouzzolane and Bovine Bone (BB) in MSW (**M**olasses **T**reated and **D**iluted at 50% with **S**upplementation at 25% of **W**hey) at 30°C, the inlet flow of 90 ml/h, and the dilution rate of 0.36 hr^{-1}

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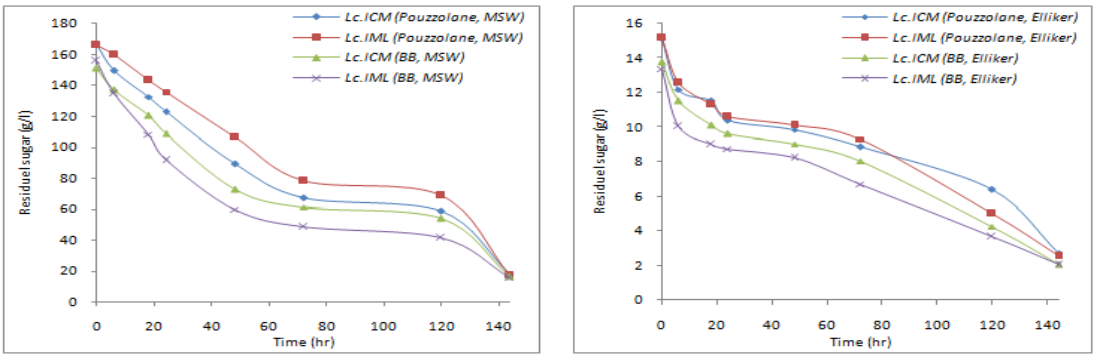

Fig. 5. Total sugar consumed kinetic in Repeated Batch Immobilized Cells (RBIC) culture on Pouzzolane and Bovine Bone (BB) in MSW (**M**olasses **T**reated and **D**iluted at 50% with **S**upplementation at 25% of **W**hey) at 30°C, the inlet flow of 90 ml/hr, and the dilution rate of 0.36 hr^{-1}.

Maximum lactic acid production was also obtained with strains immobilized on supports in MSW compared with free-cell batch cultures (Table 1) at initial pH of *5.6 ± 0.2* (Fig. 4) and temperature of *30 ± 0.5°C*.

Table 1. Maximum lactic acid productions in Repeated Batch Immobilized Cells (RBIC) culture on Pouzzolane and Bovine Bone (BB) in MSW compared with free cells batch.

System	Support	Media	Strain	Lactic Acid (g/l)	Yield (g/g)	Productivity (g/l/hr)	Residue sugar (g/l)
Immobilized cells	*Pouzzolane*	Elliker	$Lc.l_{CM}$	9.76 ± 0.01	0.65±0.003	0.070±0.000	02.6±0.06
			$Lc.l_{ML}$	9.24 ± 0.05	0.62±0.000	0.064±0.000	02.5±0.01
		MWS	$Lc.l_{CM}$	10.5 ± 0.03	0.07±0.000	0.073±0.000	18.0±0.04
			$Lc.l_{ML}$	12.0 ± 0.05	0.08±0.000	0.083±0.000	16.6±0.01
	Bovine bone	Elliker	$Lc.l_{CM}$	11.9 ± 0.05	0.79±0.001	0.083±0.000	2.00±0.05
			$Lc.l_{ML}$	12.5 ± 0.02	0.82±0.020	0.085±0.002	2.00±0.03
		MWS	$Lc.l_{CM}$	13.6 ± 0.04	0.09±0.000	0.094±0.000	16.1±0.05
			$Lc.l_{ML}$	15.8 ± 0.02	0.10±0.000	0.110±0.000	15.3±0.05
Free cells batch		Elliker	$Lc.l_{CM}$	6.65 ± 0.05	0.44±0.000	0.046±0.000	03.8±0.02
			$Lc.l_{ML}$	6.75 ± 0.05	0.50±0.000	0.047±0.000	02.4±0.03
		MWS	$Lc.l_{CM}$	6.69 ± 0.01	0.04±0.000	0.046±0.000	29.0±0.06
			$Lc.l_{ML}$	6.89 ± 0.03	0.05±0.000	0.048±0.000	24.0±0.01

MWS: cane sugar **M**olasses **T**reated and **D**iluted at 50% with **S**upplementation at 25% of **W**hey, $Lc.l_{CM}$: *Lactococcus lactis* subsp. *lactis* isolated from **C**ow's **M**ilk, $Lc.l_{MC}$: *Lactococcus lactis* subsp. *lactis* isolated from cane sugar *Molasses*

The lactic acid volumetric productivity of repeated immobilized cell batch cultures is approximately *2.5 ± 0.5* fold higher than for free-cell batch cultures.

4. Discussion

The lactic acid biotechnological production depends, among other factors, on: the type of microorganism used the immobilization or recirculation of these microorganisms, pH, temperature, carbon source, nitrogen source, type of fermentation, and formation of byproducts. The bacterial type fermentation is characterized for being fast and metabolizing completely the economic substrates, with minimum additions of nitrogen compounds, and growth at lower pH values, show scant production of biomass, and a negligible amount of byproducts [22].

From molasses combined with the manufacture of microorganisms, alcohol, citric acid, itaconic acid, the butanol-acetone, 2,3-butanediol, dextran and other like shoe polishes, rat poisons, fly killers, adhesives; as a fuel and in road paving materials [23] are product.

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Lactic acid fermentations from industrial waste particularly cane sugar molasses with supplementation at 25% of whey were performed on a laboratory-scale bioreactor to investigate the possibility of those raw materials as a sole nutrient source. The ratio C/N of substrate was *0.5*. This result indicates that Syrup of cane sugar Molasses was medium for propagation of lactic acid bacteria to produce lactic acid and other metabolites [24, 3]. Hydrolysed whey protein constituted a richer source of nitrogen compared to yeast extract and whey permeate, which is produced in large amounts and contains high concentrations of lactose and minerals is a good base medium for lactic acid bacteria culture [25].

Lactococcus lactis was chosen as the base strains because it is recognized as being responsible for the lactic acid production and it is one of the dominant lactic acid bacteria [26]. Lactococci may be immobilized by attachment to surfaces, chain formation and by trapping in the extracellular matrix. Use of semi-liquid medium to simulate immobilized growth revealed characteristic properties of non-planctonic lactococcal cultures, which are: limited distribution in the medium, slow growth, a mixture of the stationary and exponentially growing cells, and genetic instability of the culture and cell wall anchored proteins play a positive role in *L. lactis* adhesion [27].

In all the cases, the growth was followed by a decrease in pH due to the production of acid as evidenced by earlier researchers [16, 28]. It has been shown that immobilized strains on pouzzolane and bone bovine with *Lactococcus lactis* ssp. could increase process productivity in the fermentation system with repeated-batch in MWS. In this media, lactic acid volumetric productivity was high compared with that for immobilized cells on pouzzolane in cane sugar molasses treated and diluted without any supplementation cited in the work by Meziane et al. [29].

In this study, data showed that Cell immobilization in organic support as bovine bone or beef bone was increased product concentration as well as production rates than biofilm on inorganic support as pouzzolane. In addition to the porosity of a bone, the level of its mineralization has frequently been shown to have a considerable effect on its mechanical properties.

Conclusion

Immobilized cell technology is a method of immobilization simple, fast, efficient, powerful and inexpensive. In addition, cell immobilization reduces the risk of contamination. There is any negative effect on cell physiology. Our study showed that cane sugar molasses treated and diluted at 50% supplemented with whey is a suitable fermentation medium for lactococci production especially *Lc. lactis* ssp. *lactis* isolated from vegetable matrix [30].

In addition, the results show the quality of bone than the pouzzolane as solid supports for the immobilization of microorganisms and especially lactic acid bacteria. The results encourage the continuation of this work for increase knowledge and improve these systems immobilized microorganisms in mixed culture on solid supports in order to enhance and improve the molasses to other ingredients.

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