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Screening of biosurfactants properties of cell-free supernatants of cultures of Lactobacillus spp. isolated from a local fermented milk (Pendidam) of Ngaoundere (Cameroon).

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ABSTRACT

The main objective of this work was to characterize cell-free supernatants of cultures of *Lactobacillus* spp. isolated from *pendidam* (a locally fermented milk product) in order to establish their biosurfactants properties. Fifteen (15) strains of *Lactobacillus* spp based on their macroscopic, microscopic, cultural and biochemical characteristics were isolated from three samples of *pendidam* collected respectively at Wakwa, Dang and Ngaoundere's market. Among them, eight strains of *Lactobacillus* spp. showed positive results after the biosurfactants production preliminary test. The cell-free supernatants of these eight isolates cultures were screened for biosurfactants properties using three tests: drop-collapse method, surface tension measurements and emulsification activity. The largest droplet collapse (7.30 mm of diameter after drying of the spread out droplet) and the best emulsification ability (56.80%) were showed by the cell-free supernatant of TM1 strain, whereas the best reducing interfacial tension (45.09 mN/m) were showed by the cell-free supernatant of TM2 strain. In addition, the cell-free supernatants exhibited a strong antibacterial activity against *Salmonella* spp, *E. coli*, *E. faecalis* and *B. cereus*, which wasn't maintained after washing and resuspending the cells in phosphate-buffer saline (PBS). This latest results mean that the activity of *Lactobacillus* spp. is attributed to biosurfactants as excreted factors and not as cell surface components. The inhibitory effect of neutralised cell-free supernatants of the eight isolates, their thermal sensibility and the stability of their activities after treatment with different concentration of NaCl were also studied. The results obtained did not showed any significant loss ($P<0.05$) of emulsification activity and surface activity of the extracts. This is an indication that the *Lactobacillus* spp. strains secreted biosurfactants during their growth on low cost fermentative medium like *pendidam*. This work shows the potential application of autochthonous *Lactobacillus* spp. and their related substances, as an alternative solution to reduce losses of fresh milk in particular, to improve foods preservation against spoilage microorganisms in general.

Keywords: Antibacterial activity, biosurfactant, cell-free supernatant**,** emulsification, interfacial tension, *Lactobacillus* spp., *pendidam.*

1. INTRODUCTION

Milk is a product nearest to the concept of "complete feeding stuff" [1]. It is a food whose nutritional interest is undeniable for young person in growth and for adult, for whom it constitutes a good source of energy, proteins, minerals and vitamins, but also, for the pregnant and nursing woman for its content of essential amino acids [2]. In Cameroon, milk represents a source of income for breeders in general and particularly those of the far North. A study carried out on the dairy economy in the Department of Mbéré (Adamaoua's region, Cameroon), shows that the annual income generated by the dairy activity in a family breeding of bovines is evaluated at 152 000 FCFA [3]. But, the consumption of milk and the dairy products in Cameroon is considered on average at 24 liters/inhabitant/year, satisfied at the present time mainly by imported milks. However, Cameroon has a strong potential for milk production which could provide 25 liters/inhabitant/year [4]. In the same way, a study realized by the ACDIC in 2006 showed that more than 50% of the production is consumed by the breeders and their families and that, the breeders meet many difficulties in particular a weak dairy production of the local cows due to the low genetic potential of the dairy races which are breeding, with the lack of medical follow-up of the herds, the lack of equipment and provisioning of intrants and those of structural order. Thus, let us cited the scarcity or the absence of small units of transformation in the zones of production, the lack of infrastructures for collecting milk which reduces the outlets of the producers, the bad condition of the roads, the lack of specialized structures of formation in the dairy transformation unit, the insufficiency of materials and suitable equipment or the problems of supply in drinkable water [4]. The conjunction of these structural problems involves the deterioration of fresh milk before its arrival to points of transformation and consumption. In fact, milk samples that did not referee standards for

consumption are destroyed or rejected, which leads to post-production losses. An alternative for the valorization of the dairy sector would be by using fermented milks to produce bioconservators usable in food industry to preserve perishable foods. Thus, the use of some micro-organisms which could produce molecules able to inhibit the activity of spoilage micro-organisms by using whey as substrate of fermentation have been studied [5]. Among these micro-organisms, we can cite *Lactobacillus* spp. which can produce a variety of metabolites having antimicrobial properties as hydrogen peroxide, lactic acid, bacteriocins and biosurfactants [6]. Among these molecules, biosurfactants take an important place especially because they represent a field to be explored in food industries. Biosurfactants are amphiphilic compounds produced by diverse microorganisms such as bacteria, fungi and yeasts, at the microbial cell surface or excreted extracellularly [7,8,9]. The amphiphilic molecules contain hydrophobic and hydrophilic moieties. The hydrophilic groups are either ionic or non-ionic and consist of mono-, di-, or polysaccharides, carboxylic acids, amino acids, or peptides. The hydrophobic moieties are usually saturated, unsaturated or hydroxylated fatty acids [7]. Recently, much attention has been directed towards biosurfactants owing to their different advantages such as, lower toxicity [10,11] higher biodegradability, better environmental compatibility, higher foaming, high selectivity, specific activity at extreme temperatures, pH and salinity [12,13] and their ability to be synthesized from renewable feed stocks [7]. The surface activity properties make surfactants one of the most important and versatile class of chemical products, used on a variety of applications in household, industry and agriculture [14]. In addition, biosurfactants were recorded to have variable degrees of antimicrobial activity [15].

The originality of this study is to produce biosurfactants from *pendidam* (as substrate of lactic acid bacteria growth) which is a fermented milk produced in the city of Ngaoundere (Adamaoua's region, Cameroon), work that has not been done until now. The aim of this work is to characterize biosurfactants like substances from cell-free supernatants of cultures of *Lactobacillus* spp. isolated from *pendidam*. This study follows upon work of Mahbou [16] who have evoked the presence of inhibiting thermostable substances in *pendidam* which are active at acid pH.

2. MATERIALS AND METHODS

2.1. Origins of *pendidam* and test microorganisms

2.1.1. *Pendidam:* *Pendidam* is a fermentative acid milk manufactured by skimming, heating and fermenting *biradam* (fresh milk not skimmed and not fermented). Three samples of *pendidam* were collected in sterile tubes from local producers at Wakwa (Ngaoundere), from Ndang (Ngaoundere) and from Ngaoundere city market (Mbé and Meiganga origins), Cameroon). The samples were stored in a cooler during the transport. Samples which were not analysed immediately were kept in the refrigerator at 4ºC.

2.1.2. Test microorganisms: The test microorganisms were bacterial strains *Escherichia coli*, *Bacillus cereus*, *Enterococcus faecalis* and *Salmonella* spp. provided by the Microbiology and Biotechnology Laboratory of the National Advanced School of Agro-Industrial Sciences of the University of Ngaoundere (Cameroon).

2.2. Isolation of *Lactobacillus* spp. from *pendidam*

Lactobacillus spp. strains were isolated from *pendidam* sample after serial dilution (10^{-1} to 10^{-7}) of 1 mL of sample in 9 mL of physiological saline. 0.1 ml aliquots of the appropriate dilutions were surface-plated in triplicate on MRS agar [17], incubated anaerobically at 37ºC for 24 - 48 hours for isolation of *Lactobacillus* strains. Five colonies were randomly picked from MRS agar plates and purified by repeated plating. Purity was checked by streaking on MRS agar. The pure isolates were cultivated in MRS broth at 37ºC for 18 hrs and used in the experiment. Each of the isolates were inoculated in three slants without reloading the platinum handle (inoculation needle) in 3 tubes containing inclined MRS agar and incubated at 37°C for 48 hours then kept at 4°C in the refrigerator for subsequent use if need be.

2.3. Partial identification of *Lactobacillus* spp. isolated from *pendidam*

The partial identification of *Lactobacillus* spp. isolated from *pendidam* was based on some macroscopic, microscopic, cultural and biochemical characteristics. In this assay, small white colonies, pasty, Gram-positive, catalase-negative, mobility-negative, mannitol positive, peroxidase-negative and oxydase-negative isolates from MRS agar were partially assigned to a genus on basis of some key characteristics and tests [18]. The isolates were screened for growth at different temperature (25, 37, 45°C) in 5 ml of MRS broth by visual turbidity after 72 hours incubation. The ability of the isolates *Lactobacillus* spp. strains to tolerate salt were examined in 5 ml MRS broth containing 0%, 2.5% and 6.5% (w/v) of NaCl dispensed in test tubes with incubation period of 4 days at 37ºC by visual turbidity if growth occurs , or otherwise. Homo-hetero fermentation tests were done by using Sperber and Swans' test [19]. Positive growth in acetate agar were used to distinguish the Lactobacilli from others genus of lactic acid bacteria. Each colony suspicious to be a *Lactobacillus* was purified twice by streaking on

MRS agar (incubation in aerobiosis 48 hours at 37°C). Pure cultures resulting from these streaking are carried out and store at -20°C in a MRS medium containing glycerol for later analyses.

2.4. Test of biosurfactants production by *Lactobacillus* strains and cell-free supernatants and bottoms preparations

2.4.1. Preparation of pendidam used as growth medium of strains: *Pendidam* was prepared by using the method described by Rodrigues [5] as follows: after adjusting the pH to 4.5 with 5N HCl, *pendidam* sample was heated at 121°C for 15 min to denature the proteins. The precipitates were removed by centrifugation at 4 °C and 6500×g for 20min. The supernatants were adjusted to pH 6.7, sterilized at 121 °C for 15 min and used as culture media.

2.4.2. Preliminary test of the ability of Lactobacillus strains to produce biosurfactants: *Lactobacillus* spp. isolated from *pendidam* were inoculated on MRS agar medium and colonies were covered by refined palm oil « AZUR ». The apparition of a microemulsion and a clear zone around covered colonies after incubation at 37°C for 24 hours is an indication of biosurfactants production [20,21].

2.4.3. Cell-free supernatants and bottoms preparations: 600 ml of growth medium (*pendidam*) were introduced in flasks, inoculated with 15 ml of an overnight subculture of *Lactobacillus* spp., incubated for 48 hrs at 37°C and 120 rpm as previously described by Gudina [22]. For cell-free supernatants preparation, cells were harvested after 48 hours culture by centrifugation (6500×g, 20min, 4°C), washed twice in demineralised water, and resuspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). For bottoms preparation, the bacteria contained in the bottoms of the above supernatants were left at room temperature for 2 hrs with gentle stirring for biosurfactant release, as previously described [23,24,25] and subsequently removed by centrifugation. Cell-free supernatants and bottoms obtained were kept for further test.

2.5. Partial characterization of cell-free supernatants and bottoms

2.5.1. Drop collapse test: In order to test whether the biosurfactants like substances contained in the cell-free supernatants or the bottoms were able to decrease the surface tension between water and hydrophobic surfaces, the ability to collapse a droplet of water was tested as follows: 25 µL of extracted cell-free supernatant or the bottom was pipetted as a droplet onto a surface coating with oil (refined palm oil); the flattening of the droplet and the spreading of the droplet on the coating surface was followed over seconds or minutes. The droplet was allowed to dry and the diameter of the dried droplet was recorded [26,27].

2.5.2. Determination of emulsion index (E24): This method was described by Abouseouda [9]. Equal volumes of cell-free supernatant or bottom and refined palm oil were vigorously mixed with a vortex for 2 min, and allowed 24 hours to settle. The emulsion index which later called E24 was calculated by the following equation:

$$E24 = \frac{\text{Height of an emulsion layer}}{\text{Total height}} \times 100$$

2.5.3. Determination of surface tension: The surface activity of cell-free supernatant or bottom produced by the bacterial strains was determined by measuring the surface tension of the samples with the ring method. The surface tension of PBS extract containing the biosurfactants from *Lactobacillus spp* was measured using a KRUSS K6 Tensiometer equipped with a 1.9 cm Du Noüy platinum ring. To increase the accuracy an average of triplicates was used for this study [28].

2.5.4. Antibacterial assay: Antibacterial activity of cell-free supernatant and bottom was evaluated on strains of *Escherichia coli*, *Bacillus cereus*, *Enterococcus faecalis* and *Salmonella spp*. using agar well diffusion method [29].

Each pathogens strain were inoculated into 10 mL of TSB broth and incubated at 37°C for 24 hours. Serial dilutions (10^{-1} to 10^{-6}) were done and 0.1 mL of each aliquot was inoculated on their specific medium for counting. A 0.1 ml of inoculums (inoculums size was $12x10^4$ cfu/ml for *E. coli*, $32x10^5$ cfu/ml for *B. cereus*, $60x10^5$ cfu/ml for *Salmonella spp*, $10x10^5$ cfu/ml for *E. faecalis*) was inoculated into the Muller Hinton agar + Tween 80 (1g/l), well stirred and poured into Petri dishes. 20 µL of cell-free supernatant neutralised with 0.1N NaOH to pH 7.0 was poured in individual wells punched in these agar plates. All the plates were observed for zone of inhibition around the wells after incubation period at 37±0.1°C for 18-24±2 hours microbial growth was determined by measuring the diameter of zone of inhibition. Controls were maintained in which sterile distilled water were used instead of the test substances. The experiment was done three times and the mean values are presented.

2.5.5. Thermal sensitivity of cell-free supernatants: Thermal sensitivity were determined by heating 5 ml aliquots of cell-free supernatant at 25, 50, 75 and 100ºC for 15 min prior to evaluation of surface activity and emulsification ability in a water bath as described previously. The results were

recorded and analysed statistically using STATGRAPHICS Plus5.0 and MS-Excel. An untreated cell-free supernatant were used as control.

2.5.6. Stability of cell-free supernatants at different concentration of NaCl: The effect of addition of different concentration of NaCl on the activity of the biosurfactants like substances contained in cell-free supernatant was investigated. These components were re-dissolved after purification with distilled water containing the specific concentration of NaCl (5–20%, w/v). The surface tension and E24 values of each treatment were performed as described above [9]. An untreated cell-free supernatant were used as control.

3. RESULTS AND DISCUSSION

3.1. Isolation, partial identification of *Lactobacillus* spp. from *pendidam* and their ability to produce biosurfactant

After inoculation of appropriate dilutions of different samples of *pendidam* on MRS agar media, a total of 15 different *Lactobacillus spp*. were isolated. For a preliminary identification of the lactic acid bacteria, we based ourselves on the studies of the macroscopic aspect (shape and color of colonies) microscopic aspect (characteristic forms of the microbial cells, their arrangement, the presence or not of spore and their coloring after Gram staining test), mobility test, catalase test, their fermentative type [30] and others tests such as oxidase test, degradation of mannitol, growth in acetate agar, growth in medium containing various concentrations of NaCl (0, 2.5 and 6.5% (w/v)) and at various temperature (25, 37 and 45°C). The results of these tests (Table 1) showed that all the fifteen strains isolated from *pendidam* were rod shape, grouped in pairs or chains, catalase negative, peroxidase negative, oxidase negative, motility negative, mannitol positive, non spore-forming, white creams color colonies and Gram positive. All of them were able to growth on acetate agar. These results obtained are in agreement with those of Mahbou [16] who isolated lactic acid bacteria from *pendidam* and identified most of them as belonging to *Lactobacillus* genus. The potential of milk as lactic acid bacteria substrate had been demonstrated in an earlier study on fermented milk in Burkina Faso which reported that from 100 strains isolated, 98 were lactic acid bacteria [31]. Finally, the results obtained from the present study were similar to those obtained by Ninane [32], Fella [33] and Tabak [34], who showed that *Lactobacilli* strain grown on solid medium MRS gives white creams color colonies which were microscopically rod-shaped Gram positive bacteria, catalase negative, oxidase negative, motility negative, grouped in pairs or chains. Among the fifteen *Lactobacillus* strains isolated, only eight showed positive results after the biosurfactants production preliminary test (Table 1).

Table 1: Some morphological, physiological and biochemical characteristics of the *Lactobacillus* strains isolated from *pendidam* and their ability to produce biosurfactants

Origin of pendidam sample	Strains	Colour of colonies	Surface	Shape	Gram stain	Catalase test	Type of fermentation	NaCl tolerance			Growth temperature			Mobility	Mannitol test	Growth on acetate agar	Oxydase test	Genus	Biosurfactant production
								0%	2.5%	6.5%	25°C	37°C	45°C						
Mbé	A4	white	smooth	rod	+	-	homo	+	+	+	+	+	+	-	+	+	-	*Lactobacillus*	+
Wakwa	A41	white	smooth	rod	+	-	hétéro	+	+	+	-	+	+	-	+	+	-	*Lactobacillus*	-
Dang	S1	white cream	rough	rod	+	-	hétéro	+	+	+	+	+	+	-	+	+	-	*Lactobacillus*	-
Meiganga	BS2	white	smooth	rod	+	-	homo	+	+	+	+	+	-	-	+	+	-	*Lactobacillus*	-
Dang	N2	white	smooth	rod	+	-	homo	+	+	+	+	+	+	-	+	+	-	*Lactobacillus*	+
Mbé	S2	white	smooth	rod	+	-	hétéro	+	+	+	+	+	-	-	+	+	-	*Lactobacillus*	-
Dang	TM1	white	smooth	rod	+	-	homo	+	+	+	+	+	+	-	+	+	-	*Lactobacillus*	+
Wakwa	N1	white	rough	rod	+	-	hétéro	+	+	+	-	+	+	-	+	+	-	*Lactobacillus*	-
Mbé	BS1	white	rough	rod	+	-	hétéro	+	+	+	-	+	+	-	+	+	-	*Lactobacillus*	-
Wakwa	TM2	white cream	smooth	rod	+	-	homo	+	+	+	+	+	+	-	+	+	-	*Lactobacillus*	+
Mbé	HE1	white cream	rough	rod	+	-	hétéro	+	+	+	-	+	+	-	+	+	-	*Lactobacillus*	+
Mbé	FE1	white cream	smooth	rod	+	-	hétéro	+	+	+	-	+	-	-	+	+	-	*Lactobacillus*	-
Mbé	G11	white	smooth	rod	+	-	homo	+	+	+	+	+	+	-	+	+	-	*Lactobacillus*	+
Meiganga	M11	white	smooth	rod	+	-	homo	+	+	+	+	+		-	+	+	-	*Lactobacillus*	+
Wakwa	M41	white cream	smooth	rod	+	-	homo	+	+	+	+	+	+	-	+	+	-	*Lactobacillus*	+

+ = positive ; - = negative ; homo = homofermentative ; hetero = heterofermentative.

3.2. Screening of production of biosurfactants like substances contained in cell-free supernatants or bottoms and their partial characterization

The screening of biosurfactant-producing microorganisms is generally carried out using monitoring parameters that estimate surface activity, such as surface tension, capacity of collapse droplet, ability to emulsify oils, foam stability [35]. In the present study, these parameters were evaluated as potential predictors of surfactant-producing bacteria.

3.2.1. Drop collapse assay: The drop collapse method depends on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface. There is a direct relationship between the diameter of the sample and concentration of the biosurfactant and in contrast, the drop lacking biosurfactant remains beaded due to the hydrophobicity of the oil surface that cause aggregation of droplets [26,36] but this method is not sensitive in detecting low levels of biosurfactant production. Results of Table 2 showed that 8 bacterial isolates (TM1, TM2, N2, A4, M11, HE1, G11 and M41) out of 15 isolates of the total biosurfactant producing bacteria screened positive result in drop collapse assay with diameter of droplets more than 3.1 mm. In this assay, no activity was detected for distilled water as predicted. The biosurfactant droplets diameters do result in a collapsed droplet and this indicates their effects on reduction of surface tension. The biosurfactant activity showed that we are in presence of a real surfactant preparation, since the force or interfacial tension between the drop containing the surfactant and the refined palm oil surface was reduced and resulted in the spread of the droplet. Rodrigues [24], Kuiper [37] and Walencka [38] also showed that surface tension was reduced by biosurfactants. To verify whether biosurfactants are attached to the bacterial cells or excreted in the cell-free supernatant, the drop collapse test was performed on both the cells and the cell-free supernatant. The significant difference ($P<0.05$) between the diameter of dried droplet from cell-free supernatant and bottom indicates that the surface actives compounds were excreted.

Table 2: Diameter of dried droplet obtained from drop collapse assay.

Diameter of dried droplet (mm)			
	strains	**cell-free supernatant**	**bottom**
Inoculum	TM1	7.30±0.50	4.36±0.10
	TM2	6.15±0.25	3.90±0.05
	A4	4.10±1.80	3.10±0.20
	HE1	4.40±0.10	3.98±0.32
	N2	5.90±0.51	3.13±0.00
	M11	3.60±0.70	3.45±0.50
	G11	3.80±0.07	3.64±0.08
	M41	6.10±0.40	3.74±0.02
Control	SDS1%	8.20±0.90	
	H_2O	3.09±0.02	

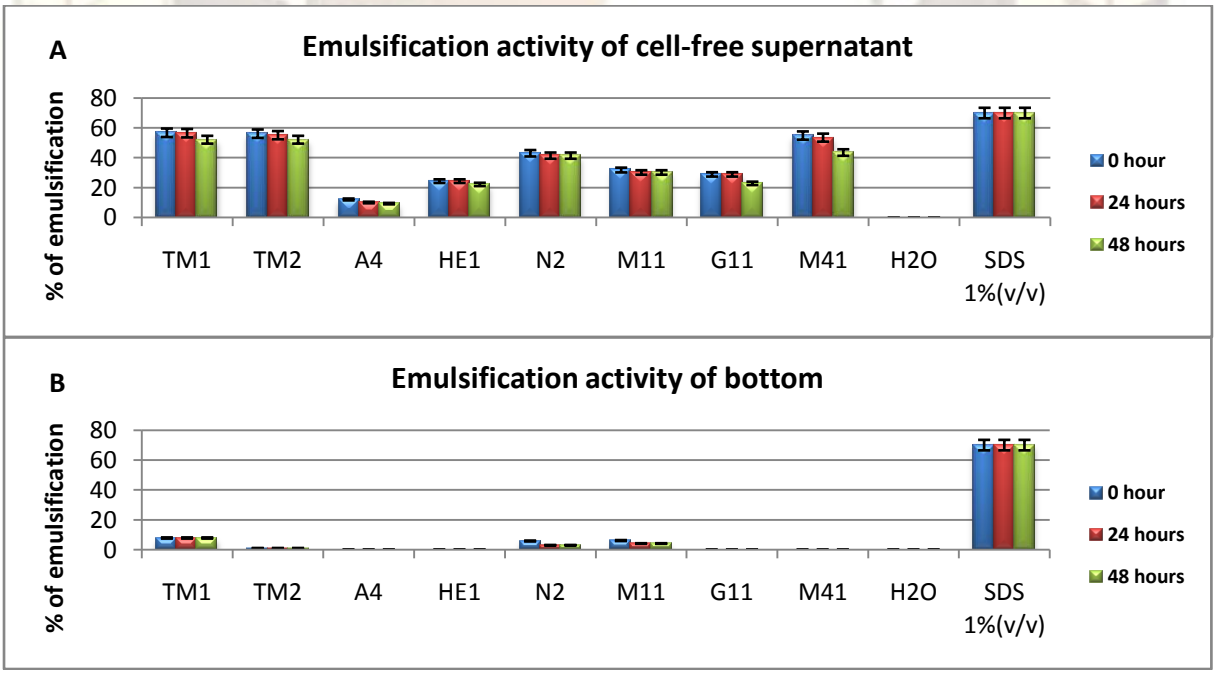

Fig. 1: Emulsification activity of cell-free supernatants (A) and bottoms (B) of *Lactobacillus spp* strains cultures.

3.2.2. Determination of emulsification: Emulsification activity test was performed according to Abouseouda [9]. Results obtained were compared with that of distilled water as negative control and 1% solution of SDS (a common chemical bioemulsifier) as positive control. An approach for screening potential biosurfactant-producing bacteria in this study is the estimation of the emulsification activity (E24).

All the eight strains of *Lactobacillus spp*. which were positive to drop collapse assay, have showed a positive result in emulsification test. The highest potential of emulsifying activity (% E24) of 56.80 was obtained from the fermentation broth of the TM1 isolate (Fig. 1). The E24 value 56.80% obtained in this study for TM1 was comparable to the value of 54.4% obtained by Siripun [39] and means that TM1 isolated from *pendidam* was a good biosurfactant producing strain. Most data published in the literature reported that bacteria with high potential of emulsifying activity are promising microbial candidates for biosurfactant production [15,40]. In that case, we can conclude that our four strains TM1, TM2, N2 and M41 with their high E24 value are promising *Lactobacillus* spp. strains for biosurfactant production. A criterion cited for emulsion stabilizing capacity is the ability to maintain at least 50% of original emulsion volume 24 hours after formation [41]. The study of the stability of emulsion showed that biosurfactants like substances produced by TM1 and TM2 strains were more stable after 48 hours with E24 higher than 50%. However, the significant difference ($P<0.05$) between emulsification activity of cell-free supernatant and bottom mean that, these active compounds are excreted during fermentation by *Lactobacillus spp*. strains.

3.2.3. Determination of surface tension: The effectiveness of a surfactant is determined by its ability to reduce the surface and interfacial tension. For instance, a good surfactant can reduce the surface tension of water from 73.2 to 35.0 mN/m, and the interfacial tension between water and hexadecane from 40.0 to 1.0mN/m [42]. Table 3 compiles the surface activities of SDS, distilled water and several biosurfactants isolated from supernatant and bottom of different *Lactobacillus* strains. The compounds present in cell-free supernatant can reduce the interfacial tension of water from 54.60 to 45.09 mN/m (TM2) and those in bottom after extraction (as described by Velraeds [23] reduced surface tension of water to values around 55.43 to 54.33 mN/m (A4). The interfacial tension obtained for TM2 (45.09mN/m), N2 (45.98mN/m) and M41 (45.74mN/m) were nearly to the value obtained by Ilse [43] who showed that *Lactobacillus sp*. CV8LAC produced biosurfactants which lower the interfacial tension of distilled water from 70.92mN/m to a value more or less 40 mN/m.

According to Table 3, a decrease in surface tension of the culture broth was observed for all eight strains of *Lactobacillus spp*. after 24 hours of culture, although those reductions varied markedly from 1.45 mN/m (A4) to 9.51mN/m (TM2). Therefore, our results are in agreement with those obtained for biosurfactants isolated from other lactobacilli by Gudina [44] who observed that the reduction of surface tension varied from 1.4 to 6.4 mN/m with modified MRS broth where glucose was replaced by lactose. From results showed in Table 3, among those eight strains, TM1, TM2, N2 and M41 exhibited the highest reduction in surface tension (<48 mN/m) and can be classified as best biosurfactants strains producers isolated from *pendidam*. From the small reductions detected in the surface tension of the bacterial cell-surface present in bottom (ranging from 0.17 mN/m (G11) to 1.10 mN/m (A4)) during the fermentation, it can be concluded that surface active compounds were excreted by these strains. Results obtain from drop collapse assay and emulsification test also confirmed the hypothesis that biosurfactants were not attached to the bacterial cell. Ilse [43] also found that biosurfactants were excreted by *Lactobacillus sp*. CV8LAC. On the other hand, Velraeds [23] and Rodrigues [25] have found that biosurfactants remained attached to bacterial cell. This means that, the way that *Lactobacillus spp*. produced biosurfactant varied according the producer strain. The reduction of surface tension obtained by TM1, TM2, N2 and M41 were higher than those obtained by Amaral [45], who found that the *Yarrowia lipolytica* biosurfactant has the ability to reduce surface tension of water up to 50 mN/m. According to Gerson and Kosaric [46], the strain of *L. helveticus*, produced surface-active compounds lowering the surface tension of water down to 39 mNm^{-1}. Similar result were observed by Velraeds [6,23] with strains of *Lactobacillus* such as *L. acidophilus*, *L. casei* subsp. Rhamnosus, and *L. fermentum*. Ours result is not in accordance with their values. It could be because of the fact that, in their studies they have used MRS medium supplemented with glucose. Compared to ours results, Rodrigues [47] also found high decrease in surface tension with biosurfactants obtained from *Streptococcus thermophilus* A and *Lactococcus lactis* 53 probably because they have used whey supplemented by peptone and yeast extract as culture medium. These differences could provide to the fact that in our study, only fermented milk (*pendidam*) without any supplementation have been used.

Table 3: Surface tension of cell-free supernatants and bottoms of cultures of *Lactobacillus* spp. strains isolated from *pendidam.*

Strains	**Surface tension (mN/m)**			
	cell-free supernatant		**bottom**	
	to	**24 hours**	**to**	**2 hours**
TM1	54.93±2.00	47.77±3.05	54.07±3.96	53.78±2.05
TM2	54.60±3.51	45.09±1.45	54.28±5.49	53.99±1.00
A4	53.98±4.15	51.53±1.75	55.43±7.37	54.33±1.08
N2	54.48±3.77	45.98±2.05	53.45±2.12	52.45±6.05
HE1	54.18±1.96	51.66±1.45	51.96±1.63	51.18±7.09
M11	53.71±6.05	50.31±4.00	53.81±4.69	52.98±3.56
M41	55.45±1.88	45.74±2.85	53.69±3.66	52.79±5.16
G11	55.61±1.00	53.68±1.23	54.17±1.45	54.00±3.75
H_2O (control)	66.73±2.04			
SDS (control)	29.32±1.05			

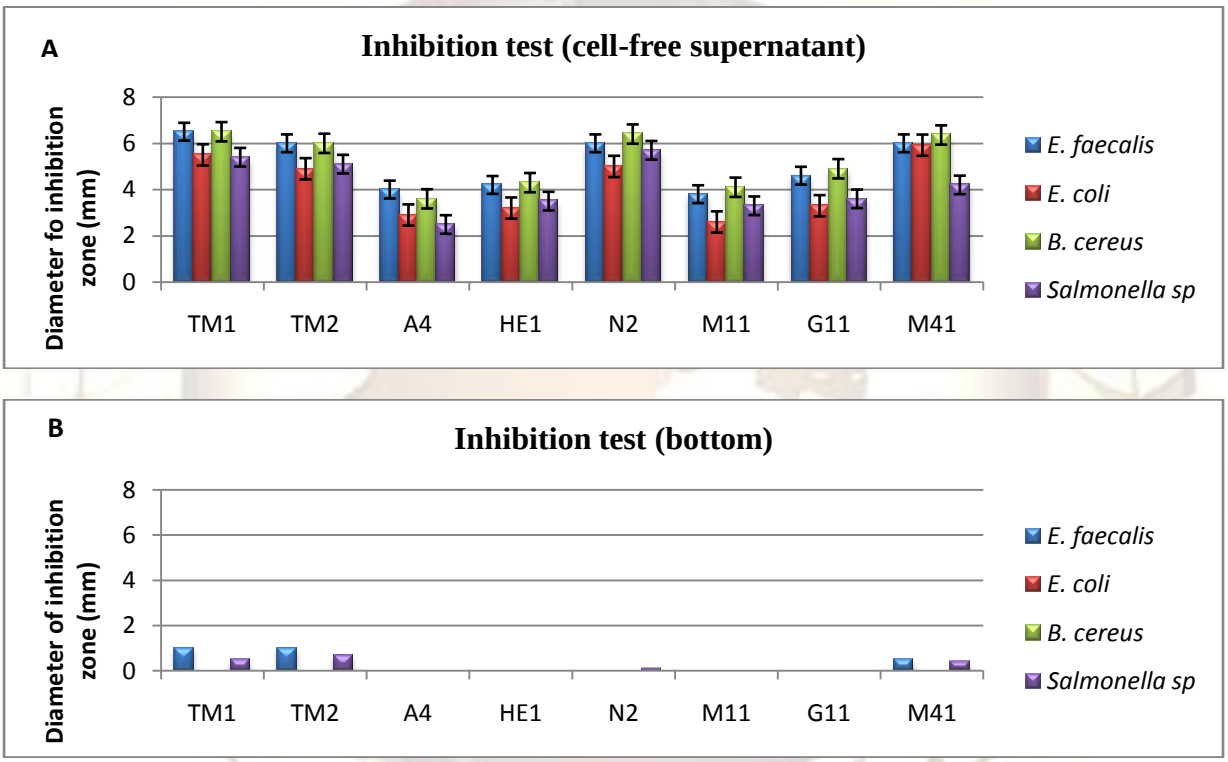

Fig. 2: Antibacterial properties of related substances of cell-free supernatants (A) and bottoms (B) on pathogenic bacteria.

3.2.4. Antibacterial assay: Antibacterial activity of the cell-free supernatant and the bottom of *Lactobacillus* spp. isolated from *pendidam* showed a broad spectrum of activity against the pathogenic microbes tested.

Fig. 2 shows the inhibitory activity of the eight *Lactobacillus spp*. strains (screened positive at the test for biosurfactant production) against *Escherichia coli*, *Bacillus cereus*, *Enterococcus faecalis* and *Salmonella spp*. For these bacterial strains, inhibition by some strains of *Lactobacillus spp.* isolated from *pendidam* were statistically different ($P<0.05$). The cell-free supernatant of strain TM1 showed the best antibacterial activity against *E. faecalis*, *Salmonella spp.* and a similar activity of strains TM2 and N2 against *B. cereus* while the supernatant of strain M41 showed the best antibacterial activity against *E. coli*.

3.3. Additive and partial characterization of biosurfactants like substances contained in the cell-free supernatants

The applicability of biosurfactants in several fields depends on their stability at different temperatures and pH values. In order to characterize these secretions, the emulsification index and surface activity of the cell-free supernatant of different strains were determined.

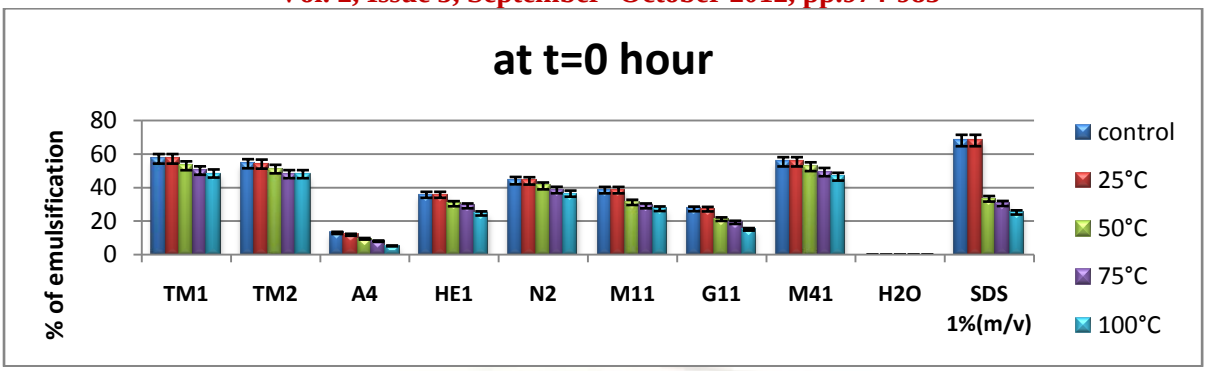

Fig. 3: Thermal sensitivity at 0 hr of related substances of cell-free supernatants of cultures of *Lactobacillus spp*. isolated from *pendidam.*

3.3.1. Emulsification index: Fig. 3 shows the values of E24 obtained after heating cell-free supernatant at 25, 50, 75 and 100°C for 15 minutes of. As can be seen in this figure, emulsification activity showed a few decrease after heating treatment at 50, 75 or 100°C, but those difference remains not significative at ($P<0.05$) and the E24 values do not showed significant difference after incubation at 37°C for 24 and 48 hours. These results are similar to those obtained by Gudina [22] who showed that the biosurfactant remained stable after incubation for 120 hours to temperatures from 25 to 60°C, with practically no loss of activity. Desai and Banat [7] also observed that heat treatment (autoclaving at 120°C for 15 min) on some biosurfactants caused no appreciable changes in their surface and emulsifying activities. Therefore, it can be concluded that this biosurfactant maintains its emulsifications activities unaffected in the range of temperatures between 25 and 100 °C after incubation times of at least 48 hours.

The stability of biosurfactant was tested over a wide range of temperature. The biosurfactant produced by *Lactobacillus spp*. strains was shown to be thermostable (Fig. 3). Heating the supernatant to 100°C caused no significant effect ($P<0.05$) on the biosurfactant performance. The emulsification activities were quite stable at the temperatures used with a minimal variation of E24 (6.23%) for TM2 and maximal variation (11.15%) for G11. Abouseouda [9] have also found that biosurfactant produced by *Pseudomonas fluorescens* was thermostable and the emulsification activity was quite stable with a variation of E24 value of 8%.

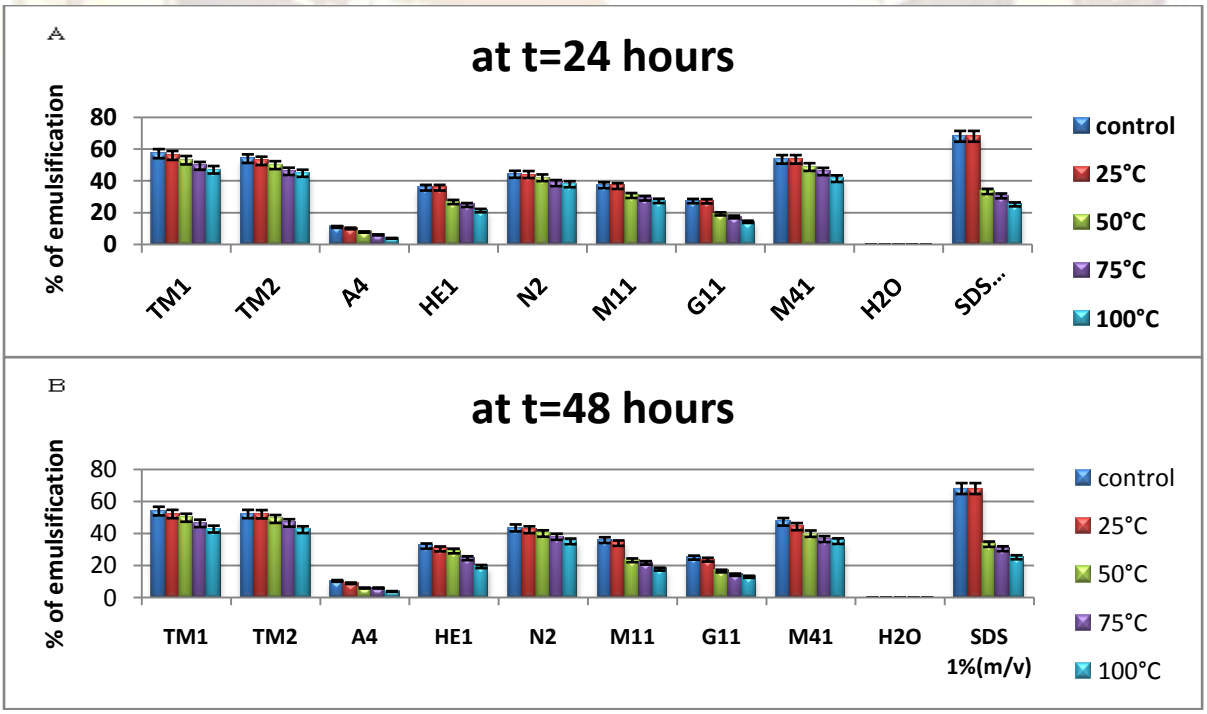

Fig. 4: Stability to heat treatment of related substances of cell-free supernatants after 24 hours (A) and 48 hours (B).

From Fig. 3 we can observe that SDS exhibits a significant loss ($P<0.05$) of emulsification activity beginning at 50°C. Similar result were observed by Kim [48] who showed that synthetic surfactants such as SDS which exhibits a significant loss ($P<0.05$) of emulsification activity beginning at 70°C.

Fig. 4 shows the stability of those emulsions after 24 and 48 hours. No significant ($P<0.05$) variation of E24 observed means that biosurfactants activities were stable according to Batista [41].

3.3.2. Surface activity: The results obtained after heating cell-free supernatant at 25, 50, 75 and 100°C for 15 minutes (Table 4) showed that the variation of interfacial tension were not significant ($P<0.05$). The minimal value of surface tension variation was obtained by TM1 (3.75mN/m) and the maximal value by G11 (7.87mN/m). These results were similar to those obtained by Abouseouda [9] who showed that the surface activity of biosurfactant produced by *Pseudomonas fluorescens* was thermostable. Similarly, Gudina [22] have showed that surface activity of biosurfactant produced by *Lactobacillus paracasei* remains unaltered or does not result in a significant loss of activity after heating treatment (25, 37 and 60°C for 120 hours). In fact, from this Table 4, TM1, TM2, N2 and M41 were biosurfactant strains producer which showed the better reduction in surface tension and high stability to heat treatment as compared to others strains.

3.3.3. Stability of related substances of cell-free supernatant at different concentration of NaCl: Results in Fig. 5 show that treatment with NaCl at concentration ranging from 5 to 20% (w/v) were not significantly ($P<0.05$) affected the emulsification activities of cell-free supernatant. Similar results were also obtained by Abouseouda [9] with biosurfactant produced by *Pseudomonas fluorescens*. On the other hand, SDS showed a significant decrease in emulsification activities after treatment with NaCl 10%.

Table 4: Surface tension of related substances of cell-free supernatants after heat treatment.

Strains	Surface tension (mN/m)				
	to	25°C	50°C	75°C	100°C
TM1	47.67±2.75	47.84±2.85	48.47±3.13	49.78±4.83	50.96±2.70
TM2	45.34±2.85	45.12±3.71	46.28±4.39	48.29±5.00	49.09±3.18
A4	52.03±2.05	52.53±3.82	55.43±7.37	56.33±3.48	58.05±2.35
N2	45.56±2.65	46.02±2.61	46.95±2.12	48.26±2.47	49.80±4.83
HE1	51.73±2.66	52.65±2.45	54.16±1.63	55.18±7.09	57.22±3.39
M11	50.49±3.44	53.31±3.07	53.81±4.69	55.98±2.76	57.00±1.45
M41	45.28±3.37	45.94±2.67	46.69±3.96	48.79±5.12	49.40±3.75
G11	53.09±4.26	55.68±3.63	57.17±1.46	58.00±3.75	60.96±2.25
H_2O (control -)	65.02±3.74				
SDS (control +)	29.69±4.35				

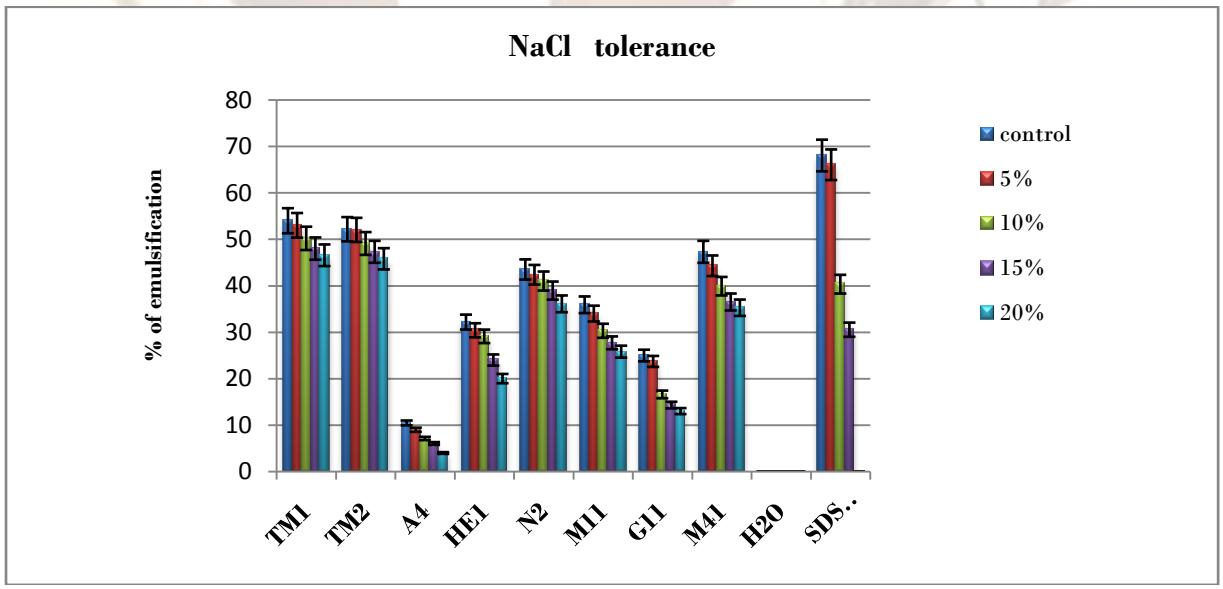

Fig. 5: Effect of NaCl on emulsification ability of related substances of cell-free supernatants.

Table 5: Effect of sodium chloride on interfacial tension of biosurfactants like substances produced by *Lactobacillus spp*. isolated from *pendidam*.

Strains	Surface tension (mN/m)				
	to	**5%**	**10%**	**15%**	**20%**
TM1	46.87±2.72	47.04±2.85	47.97±2.66	49.02±1.83	50.16±2.32
TM2	44.98±2.10	45.10±3.25	46.73±2.56	48.09±3.40	49.15±5.08
A4	53.53±4.15	54.03±3.62	56.13±6.01	57.63±4.68	59.09±2.85
N2	45.56±2.65	46.02±2.61	46.95±2.12	48.26±2.47	49.80±4.83
HE1	51.25±2.33	52.86±3.11	55.06±2.33	55.78±4.09	57.82±4.39
M11	50.12±3.44	52.93±2.05	54.21±3.74	56.08±2.29	57.63±3.05
M41	45.06±3.37	45.24±4.60	46.12±3.09	48.23±2.25	49.15±4.05
G11	53.02±4.31	55.30±1.65	57.70±2.36	58.00±3.12	60.13±4.01
H_2O (control -)	65.72±2.45				
SDS (control +)	31.08±2.96				

After treatment with NaCl 5 to 20% (w/v), the interfacial tension of the cell-free supernatant was carried out and the different values of interfacial tension are showed in Table 5. From this table we recorded that, variations of interfacial tension were nearly constant. Abouseouda [9] also recorded that, sodium chloride addition has no significant effect ($P<0.05$) on surface tension of *P. fluorescens* biosurfactant and only little changes were observed in either parameters with addition of up to 20% w/v sodium chloride.

Strain TM1 presented the minimal variation of interfacial tension (3.29mN/m) and M11 the maximal variation (7.51mN/m). The interfacial tension of strains TM1, TM2, N2 and M41 were significant different ($P<0.05$) to those of others *Lactobacillus spp*. strains.

4. CONCLUSION

In the present study, fifteen strains of lactic acid bacteria isolated from *pendidam* were classified according to their genus level to be *Lactobacillus*. From these fifteen strains only eight were screened positive to biosurfactants production. It has been found that, these strains produced the latest substances as excreted active compounds. The emulsification activity, the surface activity, the antimicrobial activity and the stability to heat treatment and salt concentration of substances contained in the cell-free supernatant of *Lactobacillus*'s cultures revealed some of their biosurfactants properties. From the results obtained, *pendidam* with its natural *Lactobacillus spp*. and their ability to secrete actives substances like biosurfactants can be used as an alternative source of antibacterial products. Further studies needed to be initiated to identify completely these *Lactobacillus spp*., to determine the potential applications of biosurfactants produced in food industries and to optimize *pendidam* as a low-cost fermentative medium for biosurfactants production.

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