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CHARACTERIZATION OF THE PHYSICOCHEMICAL PROPERTIES OF THE BIOSURFACTANT PRODUCED BY L. ACIDOPHILUS AND L. PENTOSUS.

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Absract:-
Biosurfactants or surface-active compounds are biodegradable, non-toxic and eco-friendly compounds released by microorganisms. Biosurfactants are amphiphilic compounds cause surface tension reduction both aqueous solutions and hydrocarbon mixtures. The main purpose of this work was to characterize biosurfactant produced by Lactobacillus strains. Identification using 16s rDNA identified the isolates as L. acidophilus for Fm1 and L. pentosus for Y1. Effect different parameters (temperature, PH and Salinity) were studied to evaluate the stability of biosurfactant after treatment. In addition, critical micelle concentration of biosurfactant, emulsification index and viscosity reduction of palm and engine oils have been studied. The results revealed that, the biosurfactant from L. acidophilus and L. pentosus maintains its emulsifications activities unaffected in the wide range of parameter's study except slightly decreasing in emulsification index values at salinity 15%. The maximum reduction in surface tension was 18.05 mN/m with minimum concentration of critical micelle concentration of 7.5 mg/ml and high decrease of palm and engine oil viscosity of 110.1 and 165.3% respectively. This study concluded that, the emulsification activity, the surface activity and the stability to heat treatment, different PH and salinity of biosurfactant of Lactobacillus strains revealed the application of the biosurfactant in food, pharmaceutical, cosmetics industries and oil recovery.

Keyword: - *Biosurfactant, Emulsification index, Surface tension, critical micelle concentration, L. acidophilus, L. pentosus*

INTRODUCTION

The biosurfactants have several advantages over chemical surfactants including lower toxicity and higher Biodegradability, better environmental compatibility, high selectivity and effectiveness at extreme temperatures, salinities or pH [1]. The disadvantages of the microbial surfactant or biosurfactant, comparing with synthetic surfactant are low yield and high production cost [2]. So, the method to improve the biosurfactant production with low cost is of interest.

Conventional methods include acid precipitation, solvent extraction, centrifugation and ammonium sulfate precipitation, recovery. In recent years, a few unconventional recovery methods have also been described, such as foam fractionation, ultrafiltration and ion exchanged chromatography. Often, a single technique is not enough for biosurfactants recovery and purification. For instance, extraction of low molecular weight biosurfactants normally involves an initial precipitation step, followed by extraction with different organic solvents according to the hydrophobicity and hydrophilic-lipophilic balance value of the compounds. On the other hand, high molecular weight biosurfactants, normally they are extracted by ammonium sulfate precipitation and then purified by dialysis [3]. The stability of biosurfactants to extreme conditions of pH, temperature and salinity make them desirable molecules for applications where these conditions prevail. Several studies showed that many biosurfactants are not affected by extreme environmental conditions. The lichenysin produced by *B. licheniformis* JF-2 is an example of a biosurfactant with good stability, not being affected by temperature up to 50C, pH 4.5 – 9.0, and by NaCl concentrations up to 50 g/l [4]. Therefore, the purpose of this study was to isolate the biosurfactant produced by *L. acidophilus* and *L. Pentosus* and to investigate their physical properties.

Materials and methods

Identification of LAB isolates

The LAB isolates that show biosurfactant activity in all the tests were identified by API 50 CH (API system, BioMérieux, France). The isolates were tested for catalase and Gram stain. Overnight cultures of selected LAB isolates were grown in MRS plates (Oxoid) at 37°C for 24 h. The pure colonies were suspended in API 50 CHL medium (API system, BioMérieux, France) [5]. The suspension was transferred into each of the 50 wells of the API 50 CH strips. All wells were overlaid with paraffin oil to make it anaerobic. Strips were incubated at 30°C as recommended by the manufacturer. Changes in colour from wells were noticed after 24 and 48 h. The result was analyzed with API WEB (BioMerieux). Promising LAB isolates were further identified by 16 s rDNA using primer 16S forward: (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3) (Magnusson et al., 2003). Primer was synthesized at 1st Base, Malaysia. The settings of PCR were as follows: initial at 95 °C for 2 min, denaturation at 92°C for 45 s, annealing at 54°C for 1 min and extension at 72°C for 1 min, with 35 cycles for each steps.

Biosurfactant stability

In order to evaluate the stability of bio surfactant, the effects of temperature, pH and NaCl on the activity of the biosurfactant in the optimized conditions were assessed.

Culture preparation

Two LABs were previously isolated from different sources of fermented milk and showed biosurfactants and antimicrobial activities against pathogenic bacteria were sub-cultured twice in MRS broth incubated at 37°C to activate bacterial growth. A 200 µl of 24h cultured LAB was inoculated to 20 mL of MRS broth and incubated at 37°C for 24 h. This 24 h culture was used in the following experiments.

Surface tension measurements

Surface tension measurement: Culture samples were centrifuged (Jouan Br4i, France) at 12,500 rpm for 15 minutes to remove cells and the resultant CFS was submitted to surface activity measurements. Surface tension was measured by using a du Nouy ring-type tensiometer model (KSV-sigma 703D Finland) [6]. The measurement of the ST of each sample was conducted three times along with a control (water, MRS and 1% SDS). The presence of biosurfactants in the solution was confirmed based on a decrease in the value of surface tension of the isolated sample against the control sample.

Emulsification index

The emulsification index (*%EI24*) was determined according to [7]. The same volume of supernatant and palm oil in a ratio of 1:1 were mixed in a glass test tube (125 mm × 15 mm). Then, the mixture was vortexed for 2 min and left to stand for 24 h. The emulsification index (EI_{24}) was calculated by dividing the total height of the emulsion by the total height of the aqueous layer and then multiplying by 100. The results were compared with 1% sodium dodecyl sulfate (1%SDS) as positive control and distilled water and MRS as negative control.

Effect of temperature

Ten percent (v/v) of 24h culture of *L .acidophilus* and *L. pentosus* were inoculated in 100 mL of MRS and incubated in an orbital shaker for 24h at 150 rpm, pH 7 at the temperature 37°C for *L. acidophilus* and 31°C for *L. pentosus*.. The culture was centrifuged at 10000g at 4°C for 15 min. The CFS was collected and heated. The temperature was maintained within the range of 25100 Cº, then autoclaved at 121 Cº for 15 min and cooled to room temperature (25, 30, 50, 70, 100 and 121 Cº). Thermal stability of the biosurfactant was determined by measuring and the surface tension reduction and the $EI_{24\%}$ value. The 1 % SDS was used as the positive control in triplicates and the average values were calculated [8].

Effect of pH
Ten percent (v/v) of overnight culture in 100 mL MRS broth from each strain was inoculated into fermentation media and incubated for 24h at 150 rpm, pH 7 at the temperature 37°C for *L. acidophilus* and 31°C for *L. pentosus*. The culture was centrifuged at 10000g at 4°C for 15 min. The CFS was collected and adjusted to a range of pH from 4 to 11 using 1 N HCl to determine the effect of pH on the surface tension reduction and the *EI24%* value and the results was comparing with % SDS [8].

Effect of salinity
Ten percent (v/v) of overnight culture in 100 mL MRS broth from each strain was inoculated into fermentation media and incubated for 24h at 150 rpm, pH 7 at the temperature 37°C for *L. acidophilus* and 31°C for *L. pentosus*. The culture was centrifuged at 10000g at 4°C for 15 min. The effect of the addition different concentrations of NaCl (1-10 up to 15 %) on the stability of the biosurfactant was determined by measuring the surface tension reduction and the $EI_{24\%}$ value. Each experiment was conducted using three replicate and comparing with 1 % SDS [8].

Physical properties of biosurfactant produced by *L. acidophilus* and *L. pentosus*
The biosurfactant was extracted from *L. acidophilus* and *L. pentosus* culture to evaluate their Physical properties. The acid precipitation for biosurfactants which become insoluble at low pH values were used to extract the biosurfactants from the culture medium.

Acid precipitation
Biosurfactant extraction as described by [9]. Ten percent (v/v) of overnight culture in 100 mL MRS broth from each strain was inoculated into fermentation media and incubated for 24h at 150 rpm, pH 7 at the temperature 37°C for *L. acidophilus* and 31°C for *L. pentosus*. The bacterial cells were removed by centrifugation 10000 rpm for 15 min at 4Cº. The CFS was taken and pH of the CFS was adjusted to 2, using 1 N HCl and kept at 4ºC overnight. Then biosurfactant was collected by centrifugation 12000 rpm for 15 min at 4Cº. The resulting dry pellet was lyophilized by freeze-drying (Freeze dryer FD-550), stored at −20°C and extracted twice with chloroform: methanol (2:1) in a separatory funnel at 28 °C. The biosurfactant was concentrated using a rotary evaporator and the white sediment was collected and weighted. The dry weight of the biosurfactant was measured in a reweighed sterile petri dish. After drying, the dish was weighed. The dry weight of the biosurfactant was calculated by the following formula: Dry weight of biosurfactant = (Mass of the plate after drying with biosurfactant) – (Mass of the empty plate).

Critical micelle concentration (CMC)
The CMC was evaluated as described by [10]. An extracted biosurfactant solution was prepared in sterile distilled water (pH 7) at different concentrations (0.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 17.5mg/mL). Twenty milliliters of biosurfactant solution were used for each measurement. Surface tension measurements were carried out with tensiometer at 25°C. All the experiments were carried out in triplicates and the average values were calculated [10].

Determination of viscosity reduction
Viscosity reduction ability of two oils (palm oil and engine oil) by biosurfactant was tested. 10 mL of biosurfactant (1 to 4 mg/mL) was used to emulsify 10 mL of the two oils. Viscosity was recorded by Ostwald's standard Viscometer at room temperature (28°C). Unemulsified oils were used as a control [11].

Statistical Analysis
Results are presented as the mean ± standard deviation and all measurements were done in triplicate. Statistically significant differences of the conditions tested in the different assays were evaluated by a two-way ANOVA ($P < 0.05$) applying the Tukey test. Statistical analyses were performed using SPSS software and the significant difference was considered if $P < 0.05$.

RESULTS AND DISCUSSION

Identification of LAB Isolates
The two LABs isolates that showed biosurfactant activity were identified by phenotypic and genotypic identification

Phenotypic Identification
Results from API 50 CH test kits and API web identified the two LAB isolates (Fm1 and Y1) as *L. acidophilus and L. pentousus* with similarity 99.2 and 82.9%, respectively.

Genotypic Identification
Genotype identification of DNA using universal primer showed clear bands of isolates (Figure 1) with approximate molecular weight 1500 bp and similarity 99.9% for (LAB-Fm1) *L. acidophilus* and 100% for (LAB-Y1) *L. pentosus*. The sequences of these isolates were determined and deposited in the Gene Bank database under accession number GU138532.1 and GU451063.1, respectively.

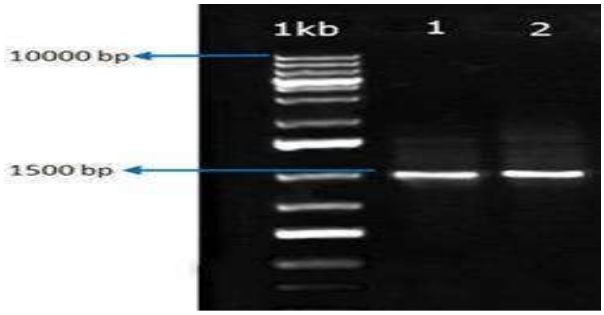

Figure 1: The DNA bands of LABs on the 1.5 % agarose gel using primers 16S.S, Lane 1: Fm1 and 2. Y.

Biosurfactant Stability

Effect of Temperature

The applicability of biosurfactants in several fields greatly depends on their stability at different temperatures, salinities and pH values. Several studies showed that many biosurfactants are not affected by extreme environmental conditions. The stability of the biosurfactant was tested over a wide range of temperature from 25Cº to 100 Cº comparison with 1 % SDS which showed a significant increase ($P<0.05$) in the surface tension and a significant loss ($P<0.05$) of EI24. The biosurfactant produced by L. acidophilus and L. pentosus was shown to be thermo stable. Heating of the biosurfactant to 121 Cº caused no significant effect ($P<0.05$) on the biosurfactant performance when surface tension was measured (18-21 and 19.6-23.4 mN/m by L. acidophilus and L. pentosus respectively as compared with 1 % SDS (32-48 mN/m). The results obtained by [12] concerning heat treatments at 25, 50, 75 and 100ºC for 15 min, show that temperature didn't have any significant effect ($p<0.05$) on the emulsifying and surface activities of biosurfactants extracted from *Lactobacillus* stains. The EI_{24} was stable at the temperature used (EI_{24} = 100 %) in comparison with the synthetic surfactant 1 % SDS, which exhibited a significant loss ($P<0.05$) of EI_{24} beginning at 70 Cº (EI_{24}= 43.2 %). The surface tension activity and EI_{24} were quite stable at these temperatures. Therefore, it can be concluded that this maintains its surface properties unaffected in the range of temperature between 25 and 121 Cº (Figure 2, 3 and 4). These results were in accordance with those reported by [13] who showed that heat treatment of 120°C during 15 min didn't caused any appreciate changes on the emulsifying properties of biosurfactants. But, it differed for results obtained by [14] who showed that biosurfactants produced by *Lactobacillus* spp. were stable ($P<0.05$) only at heat treatment of 25, 37 and 60°C.

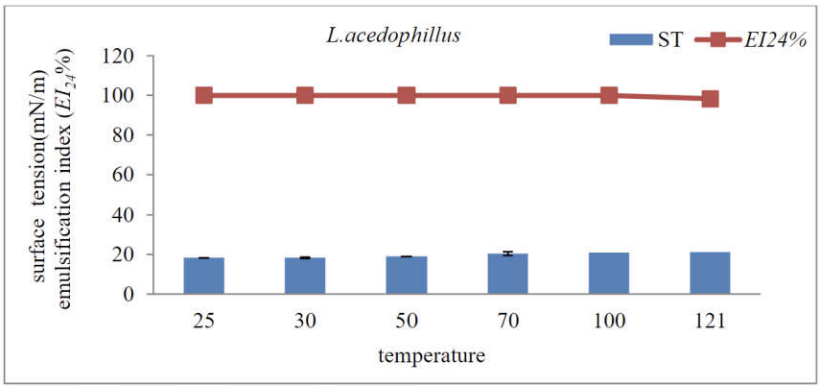

Figure 2: Effect of temperature on *L. acidophilus* biosurfactant stability

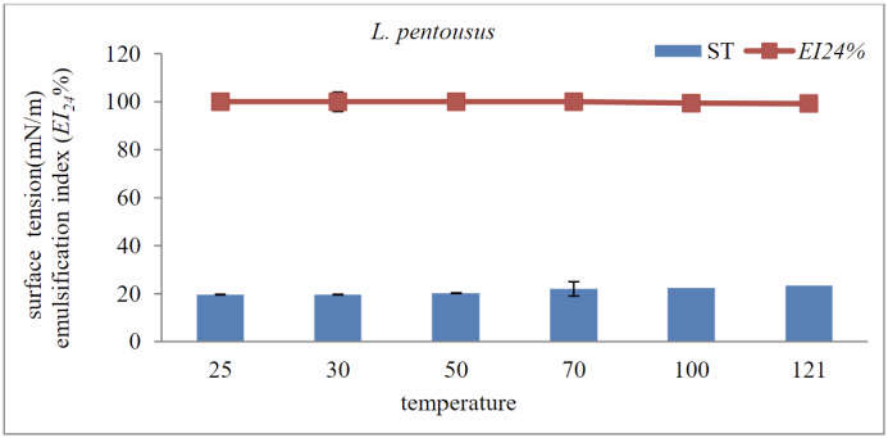

Figure 3: Effect of temperature on *L. pentosus* biosurfactant stability

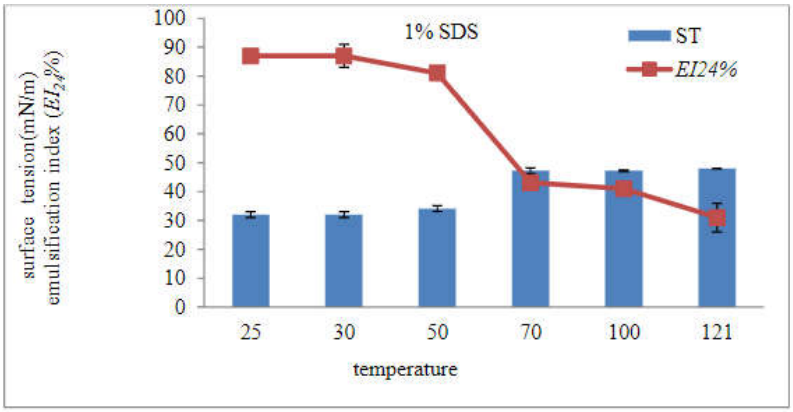

Figure 4: Effect of Temperature on 1 % SDS Stability

Effect of pH

The biosurfactant produced by *L. acidophilus* and *L. pentosus* showed a high stability not being affected by pH up to 11, except slightly decrease in the surface activity at pH 4. The maximum reduction in surface tension (18.6 and 19.7 mN/m) and the higher EI_{24} (100 %) were obtained at pH 7. In comparison with the synthetic surfactant 1 % SDS, there was quite increase in the surface tension from 31 to 48 mN/m and a significant loss ($P<0.05$) of EI_{24} started from pH 7 (87 %) and decrease gradually in both sides (acidic and alkaline) until 20 and 23% at pH 4 and 11%, respectively. The surface tension and emulsification activity of biosurfactant remaining stable from pH 5 to 11 (Figure 5, 6 and 7). This study concluded that emulsifying and surface activities of biosurfactants produced by *L. acidophilus* and *L. pentosus* were stable ($P<0.05$) at acidic, neutral and alkaline pH (5-11). This could be due to the better stability of fatty acid surfactant micelles in the presence of NaOH and the precipitation of secondary metabolites at higher pH values [15]. These results are in accordance with those reported by [16] who showed that strains of *Lactobacillus* with produced biosurfactants stables at pH values of 6 to 12. In the same way, [12] reported pH values studied didn't have a significant effect ($p<0.05$) on the surface activity and EI_{24} of biosurfactants extracted from *Lactobacillus* stains. These results also in agreement with the study by [17] who analyzed the sensitivity of the biosurfactants produced *by L. paracasei sbsp. paracasei* A20 to different values of pH. The surface activity of the crude biosurfactants remained relatively stable between pH 6.0 and 10.0, with a higher stability at alkaline conditions. Similarly, the *L. agilis* CCUG31450 biosurfactants were more stable in the same pH range, although some instability was found for pH 2.0 and 13.0 at which surface tension values was about five units higher than at pH 7.0. The instability of the biosurfactants produced by some *lactobacilli* mainly in acidic conditions has been previously described by other researchers, and has been related to the presence of negative charged groups at the polar ends of the molecules which are protonated under those conditions [17].

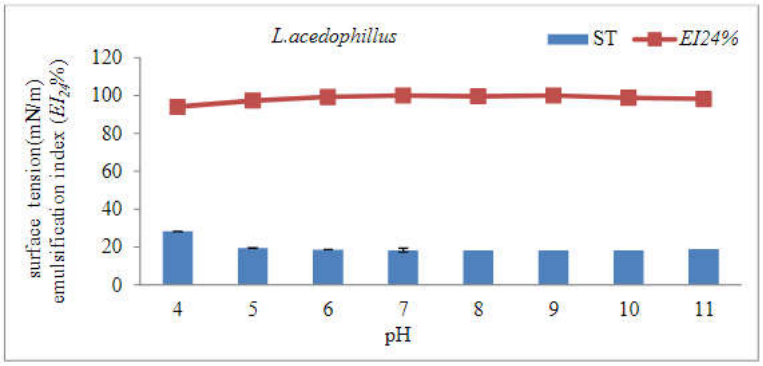

Figure 5: Effect of pH on L. acidophilus biosurfactant stability

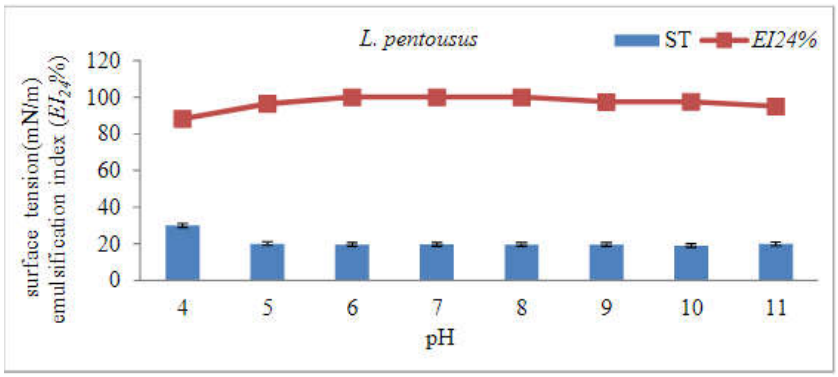

Figure 6: Effect of pH on *L. pentosus* biosurfactant stability

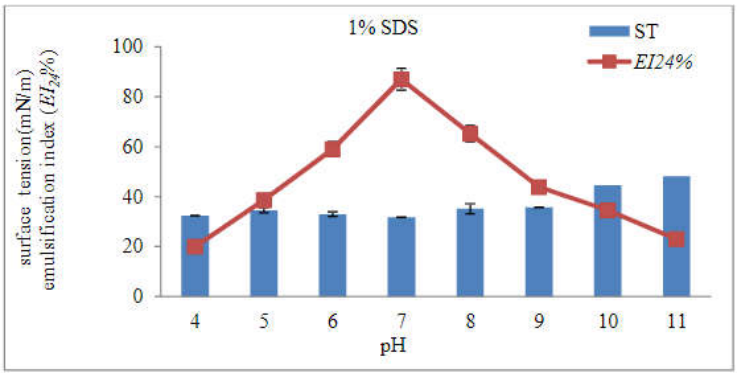

Figure 7: Effect of pH on 1 % SDS Stability

Effect of Salinity

This work showed that there was no significant effect (P<0.05) of NaCl at all concentrations tested on the surface tension reduction and the EI_{24} of biosurfactants except the increase in surface tension from 19 to 41 mN/m and the decrease in the EI_{24} from 92 to 53 % with an increased concentration of NaCl up to 15 % (w/v). The lowest surface tension about 18 and 19 mN/m and the highest EI_{24} about100 and 95 % at 1-8 % NaCl, by *L. acidophilus* and *L. pentosus* respectively. In compare with 1 % SDS, there was a significant increase (P<0.05) in the surface tension at concentration 1 to 10 % from 32 to 70 mN/m and a significant loss (P<0.05) of EI_{24} from 86.7 to 7 % at the same pH range (Figure 8, 9 and 10). These results were in agreement with those reported by [16]*)* which showed that treatment with NaCl at concentrations of 5, 10 and 15 % didn't significantly (P<0.05) affected the surface tension reduction and the *EI24* of the biosurfactants. This stability of biosurfactants activities at NaCl concentration below 15 % could be due to the presence of phosphates groups in the biosurfactants which can prevent the relegate of proteins. Another study by [18] of biosurfactants stability extracted from *Lactobacillus* strains *at* different NaCl concentrations of 5, 10, 15 and 20% have not involved a significant variation (p<0.05) of the surface tension reduction and the EI_{24}. In the same way, [19] reported that LAB produced lipopeptids biosurfactants associated with phosphate groups in the biosurfactants which can prevent the relegate of proteins. Study by [20] reported that the surface activity of the biosurfactants produced by some strains of *B. subtilis* remained stable even after treatment with NaCl at 7%**.** Similarly, [21] investigated the effect of different NaCl concentrations on biosurfactant activity produced by *Lactobacillus* isolates. He found that surface tension did not suffer any pronounced change with increasing NaCl concentrations, except a very small variation from 43.4 mN/m to 46.3 mN/m at higher concentration of NaCl tested. On the other hand, the emulsifying activity is highly affected by salinity, when a NaCl concentration higher than 50 g/l leads to an abrupt loss of emulsifying activity.

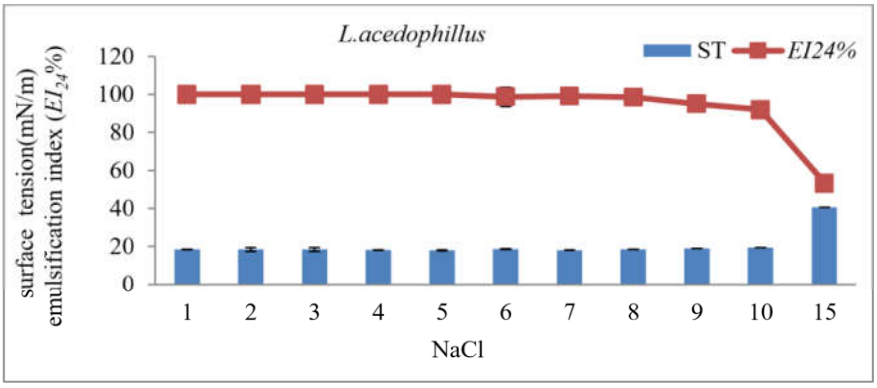

Figure 8: Effect of salinity on *L. acidophilus* biosurfactant stability

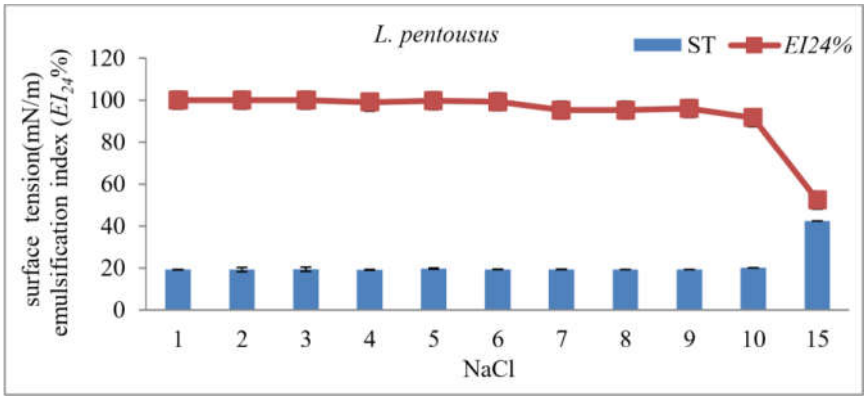

Figure 9: Effect of salinity on *L. pentosus* biosurfactant stability

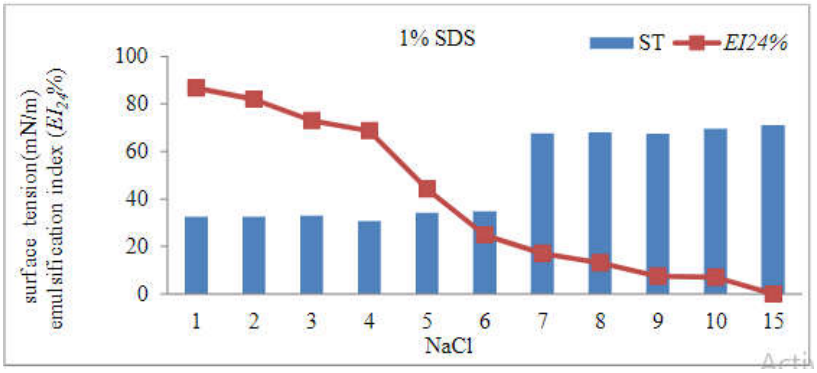

Figure 10: Effect of Salinity on 1 % SDS Stability

Physical properties of biosurfactant produced by *l. acidophilus* and *l. pentosus*

In order to determine the activity of biosurfactant produced by *L. acidophilus* and *L. pentosus*, the biosurfactant was extracted by acid precipitation.

Extraction of the Crude Biosurfactant

Result of acid precipitation (1.93 and 1.86 g/L from *L. acidophilus* and *L. pentosus* respectively) was higher than those obtained by [22] by the *L. lactis* and *S. thermophilus* A (1.45 g/L). The amount of biosurfactant in the this study was also higher than those obtained by [12] which showed the masses of biosurfactants produced by the *Lactobacillus* strains were varying from 0.710 to 1.20 g/L. This difference could be explained by the fact that in the present study MRS was used and supplemented with yeast extract and peptone which according to [14] were essential component for bacterial growth and the peptone is the most important factor for biosurfactant production by *Lactobacillus* spp. Moreover, the total amount of biosurfactants being recovered at the end of fermentation is highly influenced by the recovery technique used.

CMC of Crude Biosurfactant

The CMC defined as the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. The biosurfactant showed the lowest surface tension at 18.05 and 19.50 mN/m with CMC approximately 7.5 and 10 mg/ml by *L. acidophilus* and *L. pentosus* respectively. There was no significant reduction ($P<0.05$) in the surface tension at concentrations above 7.5 mg/mL, meaning that this concentration could be the CMC of biosurfactants produced by *L. acidophilus*. Similar phenomena were observed at 10 mg/mL for *L. pentosus* (Table 1). These results were higher than those reported by [14] with *L. paracasei:* CMC at 2.5 mg/mL or by [23] with *Lactobacillus* sp. CV8LAC: CMC at 0.106 mg/mL. However, they were lower than those obtained by [16] with *Lactobacillus* stains: CMC at 15 mg/mL and 20 mg/mL or by [19] with *S. thermophilus* A: CMC at 20 mg/mL.

Table1: CMC of Lactobacillus biosurfactant

Isolates	ST (mN/m)	CMC (mg/ml)
L. acidophilus	18.0±0.0[a]	7.5±0.2[a]
L. pentosus	19.5±0.4[a]	10.1±0.1[a]

Different letters in the same column represents significant differences at $p<0.05$

Emulsification Index of Crude biosurfactant (EI_{24})

A criterion cited for emulsion stabilizing capacity is the ability to maintain at least 50% of original emulsion volume 24 hours after formation [24]. It was observed that the biosurfactant from *L. acidophilus* and *L. Pentosus* were able to maintain the emulsion stability after 24 h with $\%EI_{24}$ of 100 and 99% for palm oil and 83 and 78% for engine oil respectively (Figure 11).

These EI_{24} values were higher than the value of 54.4% reported by [25] evaluated on lubricant oil using *P. fluorescens.* An EI_{24} values of 56.80 for palm oil was obtained from *Lactobacillus* spp TM1 isolated from fermented milk in Cameroon [12]. Reference [18] evaluated potential biosurfactant properties of *Lactobacillus* spp. on palm oil and reported EI_{24} of 61.11%. Most data published in the literature reported that bacteria with high potential of emulsifying activity of 50 to 60% are promising microbial candidates for biosurfactant production [26].

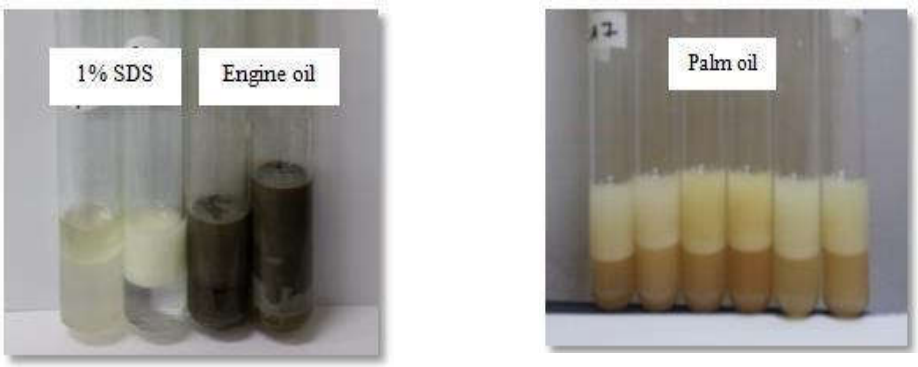

Figure 11: The emulsion layer of biosurfactant mixed with oil at 24h

Determination of Viscosity Reduction

Viscosity is oil's resistance to flow. Due to high viscosity of crude oil, it resists to flow and becomes very difficult for transport. Heavy crude oil contributes significant contents of nitrogen, oxygen, sulphur compounds and heavy metal contaminants. Such viscosity of heavy oils is reduced by using surfactants to increase mobility and ease of transportation [27]. Microorganisms produce surfactants that can reduce oil/water surface tension and cause emulsification. Reference [28] isolated a neutral lipid biosurfactant from the anaerobe *C. pasteurianum*, and anaerobic biosurfactants produced from *Bacillus* and other *Clostridium* spp. have the ability to reduce the viscosity of a heavy oil by as much as 95%. The biosurfactant from

L. acedophillu caused reduction in viscosity of palm oil and engine oil from 402 to 110.1cP and from 736.0 to 165.3 cP while the biosurfactant from *L. pentosus* caused reduction of the viscosity from 402 to 123 cP and from 736.0 to 170.0 cP respectively (Table 2). In comparison with the viscosity of the control (402.0 and 736.0 cP), there was a significant reduction ($P<0.05$) in the viscosity of palm and engine oil respectively caused by biosurfactant. The reduction in the viscosity of engine oil was higher than observed by [29] who found that *Cunninghamella echinulata* biosurfactant, caused decrease in the viscosity of engine oil from 736.6 to 179.0 cP while it caused increase in the viscosity of palm oil from 403.0 to 536.3 cP. They reported that two mechanisms that increase and decrease the viscosity using hydrophobic substrates and the new biosurfactant is a candidate for mediated enhanced oil recovery [30].

Table 2: Reduction of oil viscosity caused by biosurfactant

	Isolates	
Viscosity	*L. acidophilus*	*L. pentosus*
palm oil (cP)	402.0 ± 0.5^{bA}	402.0 ± 0.4^{bA}
reduction (cP)	110.1 ± 0.0^{aA}	123.0 ± 0.1^{aB}
engine oil (cP)	736.0 ± 0.7^{bA}	736.0 ± 0.8^{bA}
reduction (cP)	165.3 ± 0.2^{aA}	170.0 ± 0.0^{aA}

Different letters in the same column (lower case) and in the same row (upper case) represents significant differences at $p<0.05$

Conclusion

The LABs biosurfactant was stable at different temperatures, salinities and pH values. This study reported that biosurfactant are not affected by extreme environmental conditions and it has the ability to reduce the viscosity of oils (palm oil and engine oil) which facilitates the mobility of oils and ease of transportation.

Acknowledgments

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