FTIR spectroscopy and Optimization of Biosurfactant Production by *Lactobacillus acidophilus* and*Lactobacilluspentosus*

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Abstract: Two *Lactobacillus* strains (*L. acidophilus* and *L. pentosus*) isolated from dairy products, showed good biosurfactant properties. The purpose of this work was to optimize of biosurfactant production by *L. acidophilus* and *L. pentosus* and to characterize thesebiosurfactant by FTIR spectroscopy. Maximumbiosurfactant production was achieved when the two isolates grown at pH 7 and 6, incubated at 37 ºC and 36 ºCfor 24 h, 150 rpm and 2% of NaCl respectively. Types of cooking oil as a carbon source and nitrogen source significantly ($p<0.05$) increased the biosurfactant production. The FTIR analysis showed that biosurfactants from *L. acidophilus* were composed of a mixture of protein and lipid, while biosurfactants from *L. pentosus* were composed of a mixture of lipid and carbohydrate,suggesting that the typical structure of biosurfactant were lipopeptides and glycolipids respectively which are affected by medium composition and bacterial growth. The *L. acidophilus* and *L. pentosus*are a promising biosurfactant producer which reduced surface tension under a wide range of pH, temperatures, agitation, salinities, time of cultivation, carbon and nitrogen sources and therefore, these strains of *Lactobacillus* spp. can be added to food formulations to prevent of pathogenic microorganisms growth also they showed a high emulsification index(EI_{24}) values with cooking oil indicating their potential properties as emulsifying agent and could be further exploited for food and pharmaceutical applications.

Keyword: Biosurfactant,*L. acidophilus*,*L.pentosus*, EmulsificationIndex,Surface tension,lipopeptides, glycolipids.

I. Introduction

Physical parameters, carbon and nitrogen sources affect the production of biosurfactant by microorganisms. The pH, temperature, dissolved oxygen concentration, and degree of aeration influenced cell growth and metabolite accumulation in the culture medium composition [1]. Temperature, aeration and pH were reported to greatly influence the amount and type of biosurfactant being produced [2].However, in some cases, biosurfactant production can be mainly regulated by pH and growth temperature [3].Reference [1]showed that the *L. lactis* subsp. *lactis* CECT-4434 showed higher biosurfactant production after 6h at 30-40°C. This bacteria has optimum growth temperature between 20°C and 45°C and survive at pH 5 or less.The main parameters in biosurfactant production are the carbon source, so the amount and type of them are extensively considered. Several microorganisms can use different types of carbon sources to produce biosurfactants. In general, glucose, sucrose, glycerol, diesel and crude oil have been reported as good sources of carbon for biosurfactant production. However, the use of different substrates influences the biosurfactant structures, and consequently their properties [4]. Studies using cooking oil as carbon source to produce biosurfactants seem to be an interesting and low cost alternative. There are few reports, which

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utilized the vast potential of these cooking oils for biosurfactant production [5]. Nitrogen is an important constituent in the culture medium for biosurfactant production, since it is an essential component of the proteins that play a role in the growth of microorganisms, and therefore, in the production of enzymes required for the fermentation process. Several sources of nitrogen have been studied for the production of biosurfactants, such as urea, peptone, ammonium sulphate, ammonium nitrate, sodium nitrate, meat extract and malt extract. Yeast extract has been widely used for the production of biosurfactant. However, its concentration greatly depends on the nature of the producing-microorganism and the specific culture medium used [2] [6]. The optimum biosurfactant production by *B. amyloliquefaciens* was achieved by adding peptone (4 g /L) as nitrogen source [7]. Reference [8] reported that the best nitrogen source for biosurfactant production by *P. aeruginosa* PBSC1 was sodium nitrate. Effect of different nitrogen sources on biosurfactant production was evaluated by [9] and they reported that peptone was the most important factor for biosurfactant production by *L. paracasei*ssp. *paracasei*A20. Reference[10] reported that the surface tension of culture broth was linearly correlated with the yield of biosurfactant. They showed that sucrose was the best carbon source tested with respect to surfactant yield efficiency, since it had the most significant reduction in surface tension (from 60.7 to 28.5 mN/ m). For nitrogen source utilization, all the nitrates (NaNO3, KNO3, and NH4NO3) contributed to biosurfactant production as indicated by the surface tension of culture broth was below 30 mN m. The purpose of this study was to optimize of biosurfactant production by *L. acidophilus* and *L. pentosus* and to characterize them by FTIR spectroscopy.

II. Materials and methods

1. Culture preparation

The LABs namely, *L. acidophilus* FM1and *L. pentosus* Y1were sub-cultured twice in de Man,Rogosa and Sharpe (MRS) broth (Oxoid CM0361) incubated at 37ºCto activate bacterial growth. A 200 µl of 24h cultured LAB was inoculated to 20 mL of MRS broth and incubated at 37°Cfor 24 h. This 24 h culture was used in the following experiments.

2. Effect of physical parameters, carbon and nitrogen sources on production of biosurfactant by *L. acidophilus and L. pentosus*

Biosurfactant production was optimized using the following parameters namely, pH, temperature, salinity, agitation (rpm), nitrogen sources and different kinds of vegetable oils as carbon sources following the method described by [11]. For all the experiments below, the following standard procedure was used. Ten percent (v/v) of overnight culture in MRS broth from *L. acidophilus* and *L. pentosus*was inoculated into 250 mL conical flask containing 200 mL of MRS broth and incubated at 37°C in an orbital shaker at agitation speed of 120 rpm for 24 h. The negative control in these tests was MRS broth without inoculation.

3.Measurement of parameters

For both physical and nutrient parameters, after 24 h of incubation, the culture was centrifuged at 10000g at 4°C for 15 min. The supernatant was collected and the surface tension was read using a tensiometer (model (KSV-sigma 703D Finland). Results were expressed in dynes/cm. All the experiments were carried out in triplicates and the average values were calculated [12].

3.1 Effect of pH and temperature on biosurfactant production

For determination of optimal pH, ten percent (v/v) of overnight culture in MRS broth from *L. acidophilus* and *L. pentosus*was inoculated in MRS broth at different pH (4, 5, 6, 7, 8 and 9adjusted using 1 N NaOH), then incubated at 37°C on an orbital shaker at 120 rpm. After

optimal pH had been determined, the bacteria were grown in MRS broth at optimized pH and incubated at different temperatures (28, 31, 37 and 40°C) on an orbital shaker at 120 rpm for 24 h. The culture was centrifuged at 10000g at 4°C for 15 min. The cell free supernatant (CFS) was collected and the surface [12]. The pH and temperature of incubation that induced the highest biosurfactant production were demonstrated by showing the lowest surface tension**,** andwas subsequently chosen for variation in agitation and salt concentration.

3.2 Effect of agitation and salt concentration on biosurfactant production

To determine the optimal agitation speed, ten percent (v/v) of overnight culture in MRS broth from *L. acidophilus* and *L. pentosus*was inoculated into 250 mL conical flask containing 200 mL of MRS broth on an orbital shaker and incubated at agitation speed of 50, 120, 150, 200 and 250 rpm for 24 h. The cultures were incubated at 37°C and 31°C and pH 7 and 6 for *L. acidophilus* and 31°C *L. pentosus*, respectively. After optimal agitation speed had been determined, the bacteria were grown in MRS broth containing varied concentrations of NaCl (1, 2, 3, 4 and 5%) at optimized pH, temperature and agitation speed on an orbital shaker for 24h. The culture was centrifuged at 10000 g at 4°C for 15 min. The CFS was collected and the surface tension was evaluated [12]. The agitation speed and the salt concentration that induced the highest biosurfactant production as demonstrated by the lowest surface tension valueswas subsequently chosen for variation in carbon and nitrogen sources.

3.3 Effect of carbon source on biosurfactant production

Ten percent (v/v) of overnight culture in MRS broth from each strain at optimized pH, temperature, agitation speed and salt concentration wasinoculated into fermentation media containing 5% (v/v) of cooking oils (olive oil, sunflower oil, corn oil, soy bean oil and palm oil) as carbon sources and incubated at 37°C *L. acidophilus* and 31°C *L. pentosus* for 24 h in an orbital shaker speed of 150 rpm. The pH of fermentation media was adjusted to 7 for *L. acidophilus* and 6 for*L. pentosus*with adding 2% sodium chloride. The culture was centrifuged at 10000g at 4°C for 15 min. The CFS was collected and the surface tension and the $EI_{24\%}$was evaluated [12]. The carbon source that induced the highest biosurfactant production as demonstrated by the lowest surface tension and higher $EI_{24\%}$valueswas subsequently chosen for variation in nitrogen sources.

3.4 Effect of nitrogen source on biosurfactant production

To determine the best nitrogen source for optimized production of biosurfactant, each time one source of nitrogen (peptone, meat extract and yeast extract) in MRS broth was replaced with the same amount of ammonium sulphate ($(NH_4)2SO_4$). Ten percent (v/v) of overnight culture in MRS broth from each strain was inoculated into fermentation media containing different nitrogen sources and incubated for 24h at 150 rpm at the predetermined optimized pH and temperature (pH 7 and 37°C for *L. acidophilus* and pH 6 and 31°C for *L. pentosus*) with added 2% sodium chloride and appropriate carbon source. The type and amount of nitrogen sources added to the MRS broth is as shown in Table 1. The culture was centrifuged at 10000g at 4°C for 15 min. The CFS was collected and the surface tension was evaluated [12].

Table 1 Type and amount of nitrogen sources (g/L) added to MRS broth

Nitrogen sources	Media 1	Media 2	Media 3	MRS broth
Peptone (g/L)	10	10	-	10
Meat extract (g/L)	8	-	8	8
Yeast extract (g/L)	-	4	4	4
Ammonium sulphate (g/L)	4	8	10	-

3.5 Biomass estimation

Bacterial cell growth was monitored by measuring the dry cell weight. LABs were inoculated in MRS broth and incubated for 24 h at the optimum conditions as previously described in section 3.4.Bacterial cell growth was determined by centrifugation (10000 g for 15 minutes) 20 mL culture broth and the cell pellet was washed with distilled water twice followed drying the cells at 50ºCin an oven until constant weight was attained [13].Dry weight of cell was calculated as = (Mass of the plate after drying with cell) – (Mass of the empty plate).

3.6 Effect of cultivation time on biosurfactant production

LABs were inoculated in MRS broth and incubated for 24 h at the optimum conditions as previously described in section 3.4.At time intervals (24, 48 and 72 h) 50 mL sample were taken and centrifuged at 10000 g for 15 min at 4^{o}C. The CFS was collected and the surface tension and the EI24% was evaluated [12].

4. Extraction of the Crude Biosurfactant Under Optimized Conditions

*L .acidophilus*and *L. pentosus* were grown in medium under optimum conditions, as mentioned before in section 3.4. The cells were eliminated by centrifugation at 10,000 rpm for 15 min at 4ºC. The CFS was found and pH of the CFS was set to 2, using 1 N HCl and kept at 4ºC overnight. Following this biosurfactant was gathered by centrifugation at 12,000 rpm for 15 min at 4ºC. The resulting dry pellet was lyophilised by freeze-drying (Freeze dryer FD-550), stored at −20°C. Extracts were concentrated and kept at -20°C until characterisation [14].

5. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR can be employed to identify the functional groups and chemical bonds in an unknown mixture of crudebiosurfactants. Samples (1-5 mg) were subjected to milling with 80 mg of KBr (potassium bromide) to produce smooth powder, which was then squeezed into a very fine pellet. FT-IR system (Perkin Elmer Spectrum BX) was used to analysis of biosurfactant pellet. The spectral were measured using absorbance [15].

6.Statistical analysis

Results from triplicate experiments are presented as the mean ± standard deviation. ANOVA were performed using SPSS software and significant differences between means were determined by two-way ANOVA and the Tukey test to indicate any significant difference at $p<0.05$ among parameters and the variables.

III.Results and discussion

1. Effect of pH on biosurfactant production

Environmental factors are extremely important in the yield and characteristics of the biosurfactant produced by microorganisms. In order to obtain large quantities of biosurfactant it is necessary to optimize the culture conditions because the production of a biosurfactant is affected by environmental variables such as pH, temperature, aeration and agitation speed and type as well as carbon and nitrogen sources [16].It was observed that the activity of

biosurfactant produced by *L. acidophilus* and *L. pentosus* significantly affected ($p<0.05$) by pH. However, there was no significant difference ($p>0.05$) in surface tension reduction at pH 6, 7 and 8 (Table 2 and Figure 1). At pH 6 and 7 the highest surface tension reduction of 34.2 and 35.4 mN/m was recorded for CFS of *L. acidophilus* and *L. pentosus*, respectively.Similarly, the biosurfactant production by *B. amyloliquefaciens* reached maximum at pH [7].Reference [17] showed that the effect of pH (4 to 8) on biosurfactant produced in the CFS by six bacterial strains (*Acinetobacter* sp., *Pseudomonas* sp., *Bacillus* sp., *Arthrobacter* sp., *Gluconobacter* sp., and *Pseudomonas*sp) and reported that the maximum of surface tension reduction was at pH range from 6.0 (38mN/m) to 7.0 (36mN/m).

Table 2 Effect of pH on surface tension reduction of biosurfactantproduced by*L. acidophilus* and *L. pentosus*

pH	Surface tension reduction	
	L. acidophilus	*L. pentosus*
4	58.0 ± 0.2^{aC}	57.3 ± 0.01^{aB}
5	52.2 ± 0.0^{aB}	55.0 ± 0.0^{aB}
6	36.0 ± 00^{aA}	35.4 ± 0.1^{aA}
7	34.2 ± 0.2^{aA}	37.1 ± 0.1^{aA}
8	37.0 ± 07^{aA}	38.2 ± 0.1^{aA}
9	48.0 ± 0.0^{aB}	53.0 ± 0.2^{aB}

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

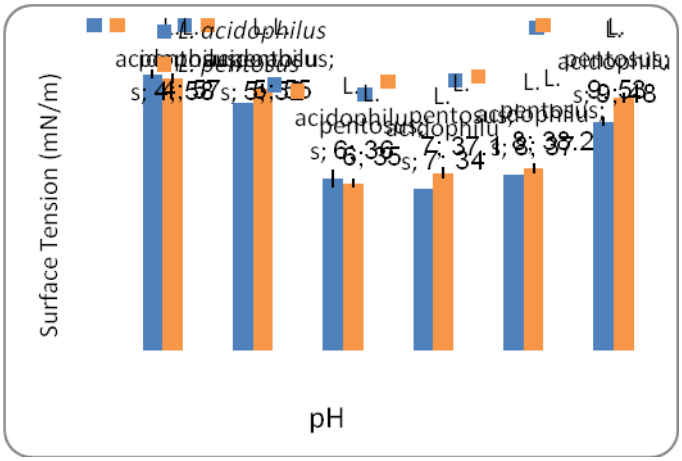

Figure 1: Effect of pH on surface tension reduction of biosurfactant produced by *L. acidophilus* and *L. pentosus*

2. Effect of temperature on biosurfactant production

Biosurfactant production by *L. acidophilus* and *L. pentosus* was achieved at 31 and 37°C (Table 3and Figure 2). Results showed that significantly ($p<0.05$) lowest surface tension reduction of 34.7±00(mN/m) was recorded for *L. acidophilus* cultured at 37ºCwhile 35.0±0.0(mN/m) was recorded for *L. pentosus*at 31^{o}C.Biosurfactant activity was not observed in CFS from cultures incubated at 28 and 40ºCfor both the isolates. The effect of temperature (20-50°C) on biosurfactant produced in the CFS by (*Acinetobacter* sp., *Pseudomonas* sp., *Bacillus* sp., *Arthrobacter* sp., *Gluconobacter* sp., and *Pseudomonas*sp) were investigated by [17] who noted that the best range of temperature for selected strains to produce biosurfactant was between 30 to 40°C surface tension reduction was from 35 to 37 mN/m, respectively.

Table 3 Effect of temperatures on surface tension reduction ofbiosurfactant produced by*L. acidophilus* and *L. pentosus*

Temperatures	Surface tension reduction	
	L. acidophilus	*L. pentosus*
28	42.0±0.0^{aB}	47.3±0.1^{bB}
31	37.2±0.0^{aA}	35.0±0.0^{aA}
37	34.7±00^{aA}	37.2±0.2^{aA}
40	50.4±0.0^{aC}	52.0±0.2^{aC}

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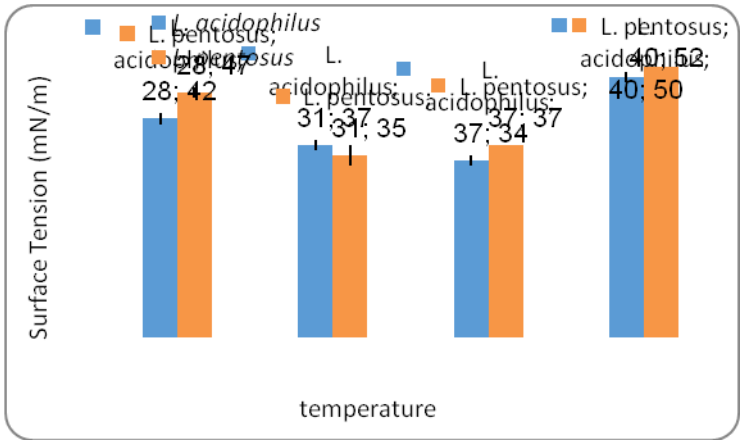

Figure 2: Effect of temperatures on surface tension reduction of biosurfactant produced by *L. acidophilus* and *L. pentosus*

3.Effect of agitation on biosurfactant production

Agitation speed seems to affect biosurfactant production It was observed that significantly ($p<0.05$) low surface reduction was recorded at agitation of 50, 100 and 150 rpm compared to at 200 and 250 rpm for both the LAB isolates incubated at the defined pH and temperature (Table 4and Figure 3). Lowest surface reduction of 31.0±00and 33.2±0.2(mN/m) were recorded at 150 rpm for *L. acidophilus* and *L. pentosus*, respectively. Biosurfactant production were totally inhibited at 200 and 250 rpm, indicating that the biosurfactant produced by *L. acidophilus* and *L. pentosus*were agitation-dependent. Similarly, [9] noted that the highest biosurfactant production by L. paracasei ssp. and paracasei A20, a strain isolated from a Portuguese dairy plant, with a decrease in the surface tension of 6.4mN/m and 22.0mN/m, respectively was at 120 rpm and 37°C. Biosurfactant production in some yeast was stimulatedwhen the agitation and aeration rates were increased [18].

Table 4 Effect of agitation rate on surface tension reductionof biosurfactant produced by*L. acidophilus* and *L. pentosus*

rpm	Surface tension reduction	
	L. acidophilus	*L. pentosus*
50	34.2±0.0^{aA}	37.0±0.1^{aA}
120	34.0±0.0^{aA}	36.3±0.0^{aA}
150	31.0±00^{aA}	33.2±0.2$^{a\ A}$
200	45.4±0.0^{aB}	48.5±0.2^{aB}
250	56.0±00^{aC}	54.2±0.4^{aC}

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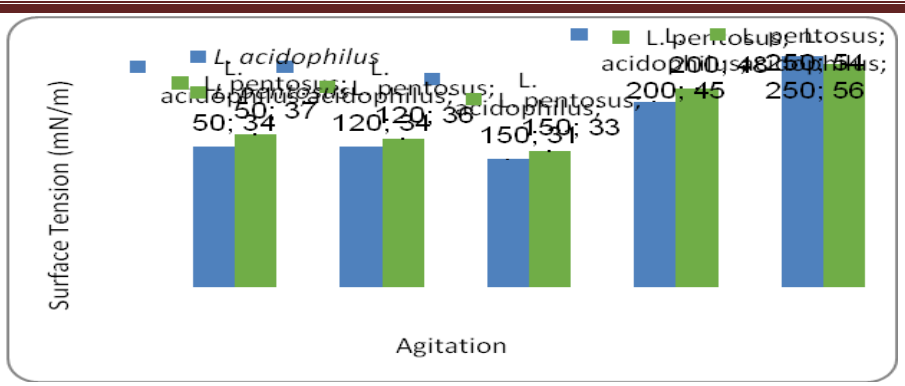

Figure 3: Effect of agitation rate on surface tension reductionof biosurfactant produced by *L. acidophilus* and *L. pentosus*

4. Effect of salt concentration on biosurfactant production

Sodium chloride was reported to affect biosurfactant production and depended on its effect of cellular activity [17]. It was observed that addition of salt to MRS broth at 1, 2 and 3 percent salt (w/v) recorded significantly ($p<0.05$) low surface tension reduction of about 30 and 32 (mN/m).Biosurfactant activity of *L. acidophilus* and *L. pentosus* was not detected at salt concentration greater than 4% (w/v) (Table 5 and Figure 4). Reference [17] reported that the surface tension of CFS of six strains (*Acinetobacter* sp., *Pseudomonas* sp., *Bacillus* sp., *Arthrobacter* sp., *Gluconobacter* sp., and *Pseudomonas* sp.) was reduced at all NaCl concentrations tested (1 to 13% w/v) from 60 to 32 mN/m. Similar observation was reported by [19] who reported that *B. licheniformis* BAS50 produced a lipopeptide surfactant when cultured on a variety of substrate at different salt concentrations and reported that the biosurfactant production was optimal at 5% NaCl. In the presence of 3 to 9% NaCl, micellization could be enhanced and emulsification was maximized.

Table 5 Effect of salt concentration on surface tension reductionof biosurfactant produced by*L. acidophilus* and *L. pentosus*

NaCl (% w/v)	Surface tension reduction	
	L. acidophilus	*L. pentosus*
1	33.2 ± 0.0^{aA}	35.0 ± 0.1^{aAB}
2	31.0 ± 0.0^{aA}	30.2 ± 0.0^{aA}
3	$32.4.0\pm0^{aA}$	32.0 ± 0.2^{aA}
4	38.1 ± 0.0^{aB}	39.2 ± 0.2^{aB}
5	46.0 ± 00^{aC}	47.0 ± 0.4^{aC}

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

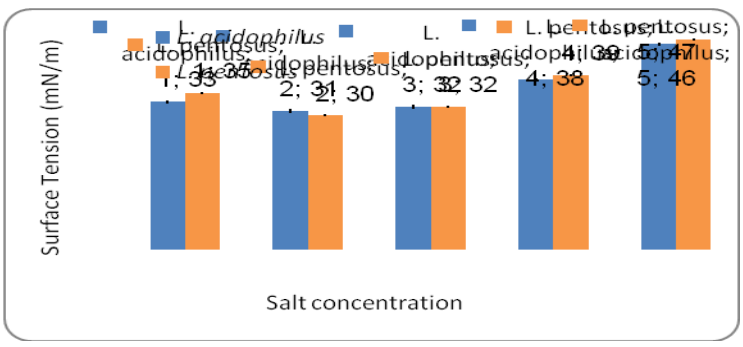

Figure 4: Effect of salt concentration on surface tension reductionof biosurfactant produced by *L. acidophilus* and *L. pentosus*

5.Effect of carbon source on biosurfactant production

The main parameters in biosurfactant production are the carbon sources or nitrogen sources, so the amount and type of them are extensively considered. A variety of materials, such as plant derived oils have been used as substrates for cultivation of microorganisms [4].Interestingly, LAB and oil type showed significant differences ($p<0.05$) in the biosurfactant activity as indicated by the ability of *L. acidophilus* and *L. pentosus*to utilize various types of cooking oil (soy bean, sunflower, palm, corn and olive oil at 5% (v/v) as carbon sources. The surface tension reduction for the CFS of the two LAB was in the range of 20.0±00to 33.0±0.2mN/m (Table 6and Figure 5).

Table 6 Effect of carbon sources added to MRS on surface tension reduction of biosurfactant produced by*L. acidophilus* and *L. pentosus*

Carbon sources	Surface tension reduction	
	L. acidophilus	*L. pentosus*
Soybean	28.0 ± 0.0^{aB}	$30.1.\pm0.1^{aB}$
Sunflower	31.2 ± 0.0^{aB}	$30.2.\pm0.0^{aB}$
Palm	29.0 ± 00^{bB}	21.0 ± 0.0^{aA}
Corn	30.0 ± 0.0^{aB}	33.0 ± 0.2^{aB}
Olive	20.0 ± 00^{aA}	29.0 ± 0.3^{bB}

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

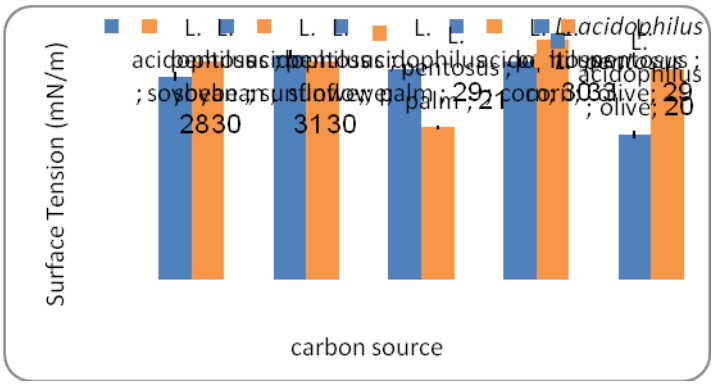

Figure 5: Effect of carbon sources added to MRS on surface tension reduction of biosurfactant produced by *L. acidophilus* and *L. pentosus*

Significant differences ($p<0.05$) were observed for the $EI_{24\%}$ values of CFS from both *L. acidophilus*and *L. pentosus*and were in the range between 81.2±0.2and 100% for all the cooking oil used as carbon source (Table 7 and Figure 6). This result is in agreement with [4]who demonstrated the surfactant activity by *E. dermatitidis* was detected in vegetable oils supplemented group, such as soybean oil, corn oil and palm oil using drop collapse test. Basal culture medium without carbon source (no oil) as well as supplementation with glycerol, glucose or n-hexadecane could not induce the biosurfactant production. The researchers observed that palm oil induced highest surfactant activity (3.9 mm) followed by soybean oil (3.7 mm) and then corn oil (3.6 mm) with significant difference at p-value < 0.05. Similarly, [20] investigated the production of biosurfactant by two *S. marcescens* strains (LB006 and 0710 strains) on minimal culture medium supplemented with vegetable oils (soybean, olive, castor, sunflower, and coconut fat). Sunflower oil gave the best results

because it contains about 60% of linoleic acid which decreased the surface tension to 28.39 suggesting that linoleic acid stimulates the biosurfactant production by the LB006 strain.

Table 7 Effect of carbon sources added to MRS on $EI_{24\%}$ values of biosurfactant produced by*L. acidophilus* and *L. pentosus*

Carbon sources	Emulsification index ($EI_{24\%}$)	
	L. acidophilus	*L. pentosus*
Soybean	91.6 ± 0.0^{abB}	87.0 ± 0.1^{bB}
Sunflower	87.0 ± 0.0^{aC}	83.9 ± 0.0^{abBC}
Palm	88.5 ± 02^{bBC}	99.3 ± 0.0^{aA}
Corn	83.3 ± 0.0^{aD}	81.2 ± 0.2^{aC}
Olive	100.0 ± 00^{aA}	87.0 ± 0.3^{bB}

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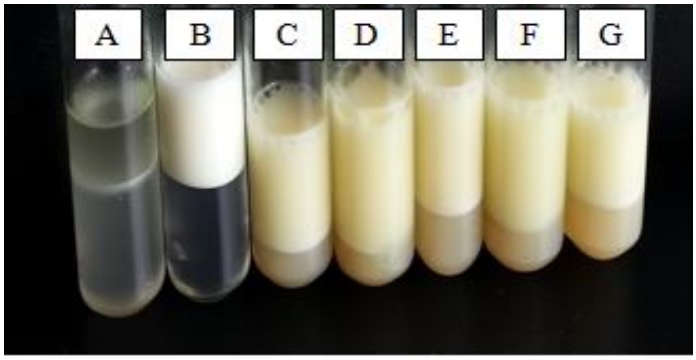

Figure 6: The emulsion layer of biosurfactant mixed with oil at 24h. A. negative control, B. 1%SDS, C. soybean, D. sunflower, E. palm, F. corn, G. olive oil

6.Effect of nitrogen source on biosurfactant production

Nitrogen is one of the main elements in the composition of protein and nucleic acid, and makes up 12–15 % of cell dry weight. It was observed that yeast extract was essential for *Lactobacillus* growth and the minimum biomass (g/L) was achieved when yeast extract was replaced by ammonium. The maximum growth rate was achieved using MRS both containing 4 g/L yeast extract, 8 g/L meat extract and 10 g/L ammonium sulphate (*L. acidophilus*14.4 and *L. pentosus*15.02 g/L). However, peptone was very important for biosurfactant production and there was significant difference ($P<0.05$) between MRS broth containing peptone and MRS broth without peptone for biosurfactant production. The maximum biosurfactant production was achieved using MRS broth containing 10 g/L peptone, 8 g/L meat extract and 4 g/L ammonium which reducing the surface tension to 18.3 and 19.6 mN/m by *L. acidophilus* and *L. pentosus*respectively. In case of MRS broth was supplemented by 10 g/L ammonium, 8 g/L meat extract and 4 g/L yeast extract without peptone, L. acidophilus and L. pentosus were unable to produce biosurfactant (Table 8).

Table 8 Effects of nitrogen sources on surface tension reduction of biosurfactant production by *L. acidophilus* and *L. pentosus*

Nitrogen sources	Surface tension reduction	
	L. acidophilus	*L. pentosus*
10 g/L peptone, 8 g/L meat extract and 4 g/L ammonium sulphate	18.3 ± 0.5^{aA}	19.6 ± 0.0^{aA}
4 g/L yeast extract, 8 g/L meat extract and 10 g/L ammonium sulphate	27.8 ± 0.0^{aB}	29.4 ± 0.9^{aB}
4 g/L yeast extract, 10 g/L pepton and 8 g/L ammonium sulphate	44.9 ± 0.0^{aC}	52.1 ± 0.2^{bC}
MRS broth (4 g/L yeast extract, 10 g/L pepton and 8 g/L meat extract)	20 ± 0.7^{aA}	21 ± 0.0^{aA}

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

Reference [9] reported that the yeast extract is essential component for bacterial growth and peptone is the most important factor for biosurfactant production by LABs. Similarly, [9] observed that yeast extract was found to be an essential component for bacterial growth (2.80 g/L), while peptone was the most important factor for biosurfactant production (24.5 mN/m) by *L. paracasei*ssp. *paracasei* A20. Combination of peptone and meat extract resulted in a higher biosurfactant production (24.5 mN/m) when compared to the standard medium (51 mN/m). He reported that the *L. paracasei*ssp.*paracasei* A20 are a promising biosurfactant producer , [9**]**.Reference [21] explained that using ammonium sulfateas a nitrogen source provided the maximum value of biosurfactant production because this salt is very soluble and is easy utilized as a nitrogen source for cell metabolism and it play significant role in biosynthesis and extracellular secretion (Figure 8).

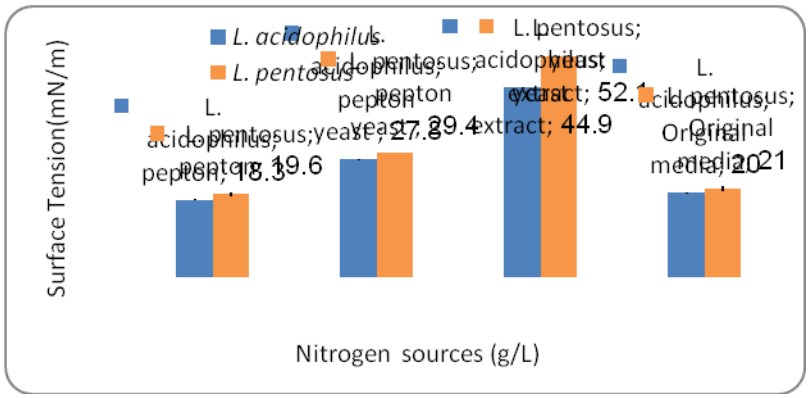

Figure 7: Effects of nitrogen sources on surface tension reduction of biosurfactant production by *L. acidophilus* and *L. pentosus*

7.Effect of cultivation time on biosurfactant production

This study showed that the biosurfactant production by *L. acidophilus* and *L. pentosus* started at 12 h and the best incubation period for its production was 24 h (The lowest surface tension was 18.3 and 19.6 mN/m and the highest $EI_{24\%}$ was 100% by *L. acidophilus* and *L. pentosus* respectively) at which the growth reached approximately its maximum for biomass (*L. acidophilus*3.4 g/l and *L. pentosus* 3.0 g/l) (Figure 8 and 9). There was no significant differences ($P<0.05$) after 24 h between different cultivation time on biosurfactant production and cell growth and their levels were nearly constant (all the incubation time gave approximately same the surface tension (18 and 19 mN/m) and same $EI_{24\%}$ value (100%) by

L. acidophilus and *L. pentosus*respectively. In agreement with this study [21] showed the biosurfactant production by all strains occurred mainly in the first hours (4 h) where cell growth is almost inexistent and the substrate consumption is very low. However, the biosurfactant production continues during all 72 h of fermentation but at a very slow production rate. Maximum bisurfactant production was at 24 h demonstrated by surface tension reduction.

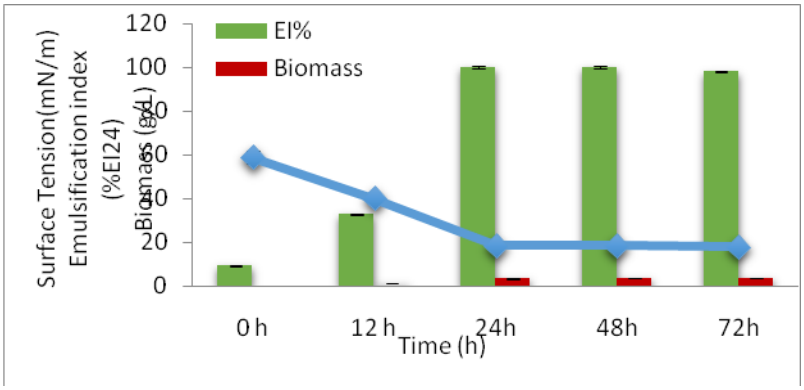

Figure 8: Effects ofincubation time on biomass and biosurfactantproduction by *L. acidophilus*

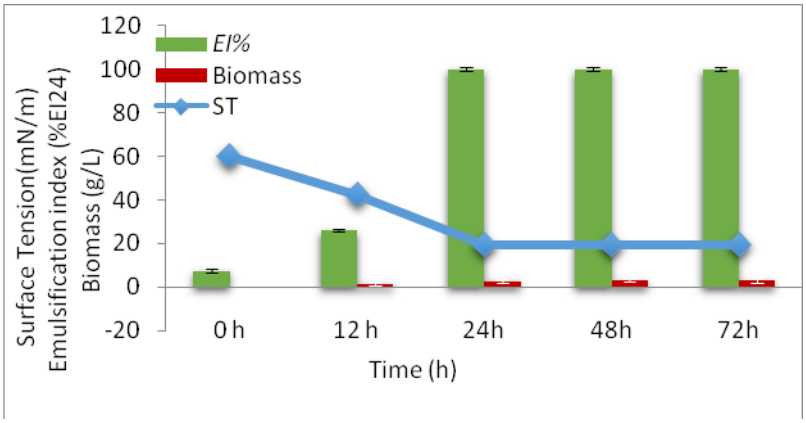

Figure 9: Effects of incubation time on biomass and biosurfactant production by*L. pentosus*

8.FTIRspectroscopy of biosurfactant from *L. acidophilus*

Most biosurfactants consist of two parts (hydrophobic and hydrophilic chain). Results of FTIR showed that biosurfactant from *L. acidophilus* are a lipopeptide compound which contain of a protein and lipid. Results of biosurfactant analysis by FTIR showed groups of peptide as a result of OH stretching (wave numbers 2500 and 2826 cm−1) and NH stretching (wave numbers 3250 and 1630 cm−1). The bands at ν = 2826 and 2500 cm^{-1} suggesting the presence of hydroxyl group (OH) of the carboxylic acids in the chemical structures of the biosurfactant [22]. Similarly, the spectrum of FTIR of the biosurfactant showed the existence of protein with thin bands; C=O bond at1760 and 1700 cm−1 N-H (amide I bond) bonds at 3250 and 1630 cm−1 (amide II bond). The band at ν =1050 cm-1 is related to vibrations of C–O bond. The absorption peak at 638 cm-1 indicates the existence of -CH2 group [13]. Another study reported that the absorbance bands at 1455 and 1400 cm−1denote the existence of stretching of C-H related to groups of CH3 and CH2 of aliphatic chains[23].These results strongly indicate that the crude biosurfactant possesses a peptide-like moiety and aliphatic hydrocarbons (Figure 10). This result is in agreement with other studies on *L. pentosus*[24]

which showed peptide groups that were the result of OH at 3419 and NH at 3290 cm−1 and protein with thin bands; N-H bonds at 1544 cm−1(amide II bond) and C=O bond at 1644 cm−1 (amide I bond). FTIR band between 2856 and 2961 cm−1 as well as bands at 1385, 1403 and 1456cm−1, which indicate the existence of CH stretching in the aliphatic chains related to groups of CH2 and CH3. They reported that the biosurfactant resulting from *L. pentosus*was also a complex compound contain protein, lipid and carbohydrate. Similarly, the strong adsorption peaks present at 2000 cm^{-1} were probably related to stretching of C≡C. Additionally, absorption peak at ν = 1740 cm^{-1}as a result of the carbonyl group stretching (C=O) [25]. Another study reported that *Corynebacterium kutscheri* biosurfactant consisted of carbohydrate, lipid, and protein [26]. Spectrum showed the existence of and CH2-C-H asymmetric vibrations and carboxylic acids, N-H/C-H bonds of protein, which verifying that Corynebacterium *kutscheri* biosurfactant contain alkanes.

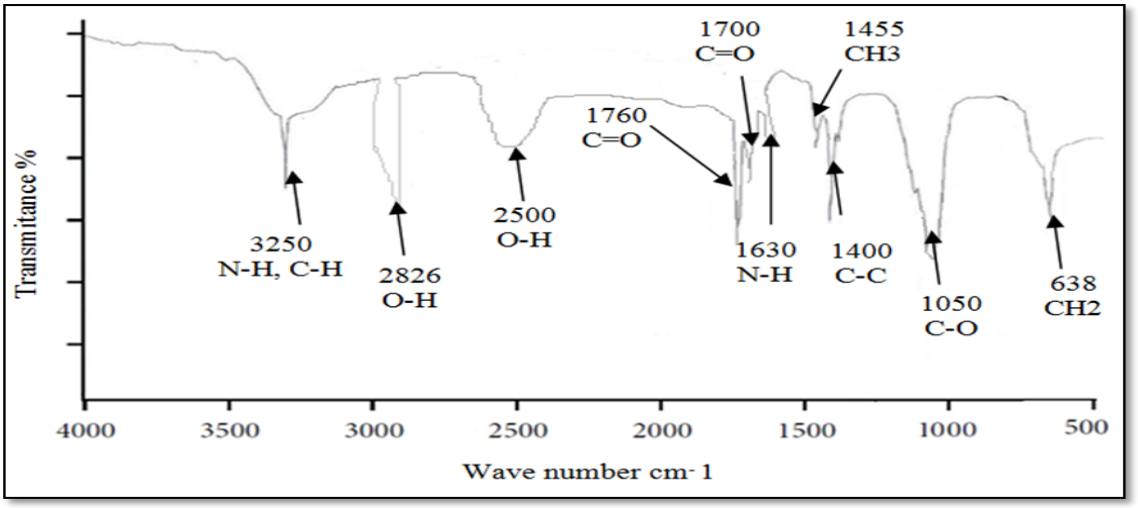

Figure 10: Spectrum of Biosurfactant Isolated from *L. acidophilus*

9.FTIR spectroscopy of biosurfactant from *L. pentosus*

In accordance with FTIR and comparison with the literature, it was found that biosurfactant from *L. pentosus* was a glycolipid compound composed of a mixture of lipid and carbohydrate. The FTIR spectrum showed absorbance peaks at 3,352 hydroxyl group (OH), 3,010, 2910 and 2876 unsaturation carboxylic acids in aliphatic chain (CH2 CH3), 1,740 ester linkage (C=O), 1677 carbonyl groups (C = O), 1450 glycolipid moieties (C-H), 1205 and band of sugar stretching at 1068 produced between groups of hydroxyl and atoms of carbon in the chemical structure (C-O and 730 cm−1 attributed to alkenes group (C-H) (Figure 11).This study in agreement with the studies by[27] of biosurfactant produced by *L. delbrueckii*which classified it as a glycolipid composed of lipid and carbohydrate. They reported the most significant bands for the CH aliphatic stretching at 2854, 2924 and 2962 cm^{-1}, the C = O ester bond at 1793 cm^{-1} and OH groups at 3388 and 3696 cm^{-1} validating the existence of glycolipid. Similarly, [28] reported that *E. faecium* MRTL biosurfactant was biochemically characterised as glycolipid. Analysis of FTIRshowed that most pronounced adsorption bands were C-H stretching bands of CH2 and CH3 groups and C-H stretching bands of CH2 and CH3 groups at 2955, 1, 2855 and 3009 cm^{-1}, C = O stretching of the carbonyl groups at 1674 cm^{-1}, C-O stretching bands; produced between hydroxyl groups and carbon atoms at 1103 cm−1 and CH2 group at 771 cm^{-1} which clearly verified the existence of glycolipid biosurfactant. Another study by [29] on biosurfactant produced by *L. casei* MRTL3 and *L. helveticus*reported that the biosurfactant was characterised as glycolipid composed of lipid and carbohydrate.

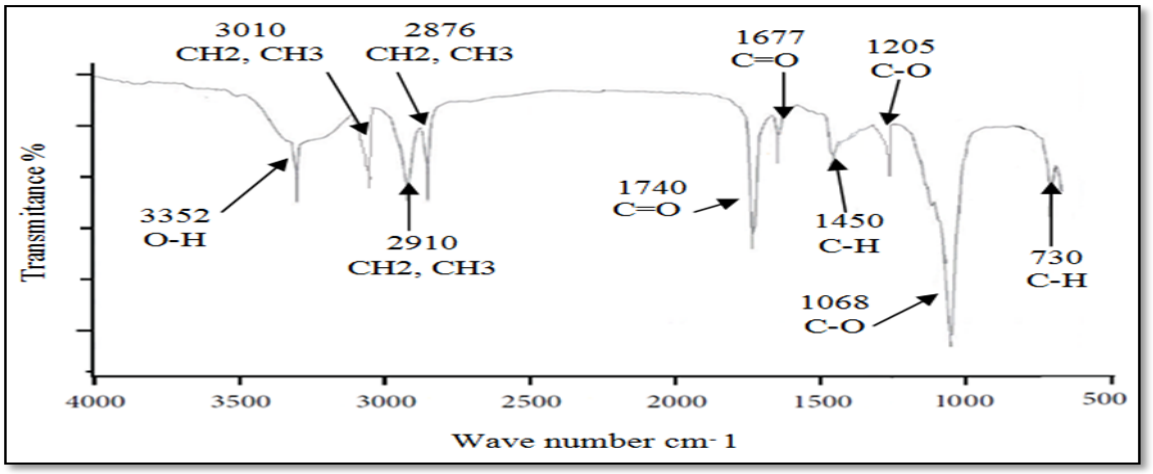

Figure 11: Spectrum of Biosurfactant Isolated from *L. pentosus*

IV. Conclusion

Microorganisims such as *L. acidophilus* and *L. pentosus* require special growth conditions to produce a special type of biosurfactant. The quantity and structure of the biosurfactant produced vary according to the microorganism type and its growth conditions**.** *L. acidophilus* and *L. pentosus*respectively were able to effectively grow on several cooking oils as sole carbon and peptone as nitrogen source at optimum physical parameters with concomitant synthesis of biosurfactants, suggesting their possible exploitation in future biotechnological processes.*L. acidophilus* produced lipopeptide when it was grown with olive oil as a carbon source while *L. pentosus* produced the glycolipid when it was grown with palm oil as a carbon source. *L. acidophilus L. pentosus*are a promising biosurfactant producer and therefore, these strains of *Lactobacillus.*spp. can be added to food formulations to prevent of pathogenicmicroorganisims growth also they showed a high EI_{24} values with cooking oil indicating their potential properties as emulsifying agent.

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References

[1] Cortes-Sanchez, A. J., Sánchez, H.H. & Jaramillo-Flores, M.E. 2013. “Biological activity of glycolipids produced by microorganisms: New trends and possible therapeutic alternatives”. *Microbiol. Res.* Vol. 168: p. 22–32.

[2] Fernandes, E.C.R., Rodrigues, L.R.M. and Pérez, E. J. G. 2013. “Study of Biosurfactant “Cocktails” with Enhanced Properties, (Master Thesis), Minho's University, Portuguese, pp. 3.

[3] Mulligan, C.N., Sharma, S.K., Mudhoo, A & Makhijani, K., Biosurfactants. 2014. Research trends and applications, Green chemistry and biossurfactantresearch. 1–30.

[4] Chiewpattanakul, P., Phonnok, S., Durand, A., Marie, E., Thanomsub, B. W. 2010. Bioproduction and Anticancer Activity of Biosurfactant Produced by the Dematiaceous Fungus Exophiala dermatitidis SK80, Journal of Microbiology and Biotechnology, 20 (12), 1664-1671.

[5] Abouseoud, M., Maachi, R., Amrane, A., Boudergua, S. and Nabi, A. 2008. Evaluation of different carbon and nitrogen sources in production of biosurfactant by Pseudomonas fluorescens. Desalination, 223, 143–151.

[6] Ghribi D . &Ellouze-Chaabouni S. 2001. "Enhancement of Bacillus subtilis Lipopeptide Biosurfactants Production through Optimization of Medium Composition and Adequate Control of Aeration. Enzymes from extremophiles". Curr. Opin. Chem. Biol. Vol. 5: p.144–151.

[7] Zhang, w., Zhang, x and Cui, h. 2015. Isolation, Fermentation Optimization and Performance Studies of a Novel Biosurfactant Producing Strain Bacillus amyloliquefaciens, Chem. Biochem. Eng. Q, 29 (3) 447–456.

[8] Demirjian D.C., Moris-Varas F. & Cassidy C.S. 2001. "Enzymes from extremophiles". Current Opinion in Chemical Biology. Vol. 5. p. 144-151.

[9] Eduardo J., Gudina, J., José A., Teixeira. &Lígia R. Rodrigues. 2011. "Biosurfactant-Producing Lactobacilli: Screening, Production Profiles, and Effect of Medium Composition". Applied and Environmental Soil Science. Vol. 2011: p.1–9.

[10] Liang, X., Shi, R., Radosevich. M., Zhao. F., Zhang. Y., Han. S and Zhang. Y. 2017. Anaerobic lipopeptide biosurfactant production by an engineered bacterial strain for in situ microbial enhanced oil recovery, RSC Advances, 33, 20667–20676.

[11] Wei,Y. H., Lai,C.C and Chang, J.S. 2007. Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactinproductionby Bacillus subtilis ATCC21332, Process Biochem, 42, 40–45.

[12] El-Sersy, N., A. 2012. Plackett-Burman design to optimize biosurfactant production by marine Bacillus subtilis N10. Rom, Biotechnol. Lett, 17 (2), 7049–7064.

[13] Aparna, A., Srinikethan, G. and Hedge, S. 2010. Effect of addition of biosurfactant produced by Pseudomonas sp. on biodegradation of crude oil, 2nd International Conference on Environmental Science and Technology, ISSN: 2229-791X www.researchinbiotechnology.com.

[14] Anandaraj, B., & P., Thivakaran. 2010. "Isolation and production of biosurfactant producing organism from oil spilled soil". Journal of Bioscience Technology. Vol.1 (3): p.120-126.

[15] Mukherjee, S., Das, P. & Sen, R. . .2006. "Towards commercial production of microbial surfactants". Trends Biotechnol. Vol. 24(11): p. 509–515.

[16] Saharan,B.S.,Sahu,R.K. and Sharma,D,. 2011. Areview on biosurfactants: fermentation,current developments and perspectives. Genet. Eng.Biotechnol.J. 29, 1–14.

[17] Dhail, S. 2012. Isolation of potent biosurfactant producing bacteria from oil spilled marine water and marine sediments, African Journal of Biotechnology, 11(103), 16751-16757.

[18] Georgiou, G., S. C. Lin. C. and Sharma, M. M. 1993. Surface-active compounds from microorganisms, Biotechnology, 10(1), 60–65.

[19] Yakimov, M., Timmis, K., Wray, V and Fredrickson, L. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface Bacillus licheniformis BAS50, Appl. Environ. Microbiol, 1706-1713.

[20] Ferraz, C., De Araujo, A.A. and Pastore, G.M. 2002. The influence of vegetable oils on biosurfactant production by Serratia marcescens, ApplBiochemBiotechnol, 98, 841–847.

[21] Rodrigues, L., Teixeira, J., Oliveira, R. & Van der Mei, H. 2005. "Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria". Process Biochemistry. Vol. 41: p.1-10.

[22] Dusane, DH.Matkar, P., Venugopalan, VP., Kumar, AR., &Zinjarde, SS. 2011. "Cross species induction of antimicrobial compounds, biosurfactants and quorum sensing inhibitors in tropical marine epibiotic bacteria by pathogens and biofouling microorganisms". CurrMicrobiol Vol. 62: p. 974–978.

[23] Pornsunthorntawee, O., Wongpanit , P., Chavadej, S., &Rujiravanit, Abe. M. R. 2008. "Structural and physicochemical characterization of crude biosurfactant produced by Pseudomonas aeruginosa SP4 isolated from petroleum-contaminated soil". Bioresource Technol. Vol. 99(6): p. 1589–1595.

[24] Vecino, X., Barbosa-Pereira, L., Devesa-Rey, R., Cruz, JM., &Moldes, AB. 2014. "Optimization of extraction conditions and fatty acid characterization of L. Pentosus cell bound Biosurfactant/Bioemulsifier". J Sci Food Agric. Vol. 95(2): p. 1313–1320.

[25] Chandran, P. & N. Das. 2010. "Biosurfactant production and diesel oil degradation by yeast species trichosporonasahii isolated from petroleum hydrocarbon contaminated soil". International Journal of Engineering Science and Technology. Vol. 2(12): p. 6942-6953.

[26] Thavasi, R., Jayalakshmi, S., Balasubramanian, T., & Banat, I. M. 2007. "Biosurfactant production by Corynebacterium kutscheri from waste motor lubricant oil and peanut oil cake. Lett. Appl. Microbiol". Vol. 45: p. 686–691.

[27] Thavasi R., Jayalakshmi S., Balasubramanian T., & Banat IM. 2008. "Production and characterization of a glycolipid biosurfactant from Bacillus megaterium using economically cheaper sources". World J MicrobiolBiotechnol. Vol. 24(7): p. 917–925.

[28] Sharma, D., Saharan, BS., Chauhan, N., Procha, S. & Lal, S. 2015. "Isolation and functional characterization of novel biosurfactant produced by Enterococcus faecium". Springer Plus. Vol. 4 (1): p. 1-14.

[29] Sharma, D., Saharan, BS. Chauhan, Nv, Bansal, A., &Procha, S. 2014. "Production and structural characterization of Lactobacillus helveticus derived biosurfactant". Sci World J. Vol. 2014: p. 1-9.