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Isolation and Partial Characterization of Glycolipopeptide Biosurfactant Derived from A Novel *Lactiplantibacillus plantarum* Lbp_WAM

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Abstract: Biosurfactants are amphipathic molecules generated by a variety of microorganisms with different biological functions. In this study, lactic acid bacteria were screened for their emulsification properties. However, the *Lactiplantibacillus plantarum* strain LBpWAM was molecularly identified using 16S rRNA, and its ability to produce surface-active peptides was investigated. The biosurfactant derived from *L. plantarum* LBp_WAM was shown to have the potential to reduce water surface tension from 72 $mN.m^{-1}$ to 32 mN/m within a critical micelle concentration (CMC) of 2.4 $mg.ml^{-1}$. The emulsification index (E_{24}) values were evaluated for sunflower oil (60 ± 3.0%), glycerol (53.9 ± 0.11 %), olive oil (49.0 ± 2.0 %), mineral oil (50.7 ± 0.60 %), hexane (36.03±0.05 %), and kerosene (31 ±0.05 %). The biosurfactant was purified using gel filtration chromatography (GFC), and the molecular weight was determined using the SDS-PAGE method, indicating an approximate molecular weight of 19 kDa. Thin-layer chromatography (TLC) and Fourier transform infrared spectroscopy (FT-IR) were used to determine the molecular structure of the obtained molecule, which was found to be composed of protein, lipid, and polysaccharides. The biosurfactant's antibacterial activity was also examined, as it showed inhibitory effects against different species of Gram-positive and Gram-negative bacteria.

Keywords: Antimicrobial activity, Biosurfactant, FTIR, Glycolipopeptide, *Lactiplantibacillus plantarum*.

Introduction

The genus *Lactobacillus* has long been associated with humans, whether through fermented foods or via the digestive and vaginal ecosystems (Borges *et al.*, 2014; Hill *et al.*, 2018). Since March 2020, there have been more than 260 *Lactobacillus* species with a wide spectrum of physical, ecological, and genotypic features. These species were consequently reclassified into 25 genera (Zheng *et al.*, 2020). *Lactiplantibacillus plantarum* (previously *Lactobacillus plantarum*), is generally described as a Gram-positive heterofermentative microaerophilic bacterium with a rod-like morphology that may be found singly or in short chains (Fidanza *et. al*, 2021). Frequent strains of *L.*

plantarum have been isolated from various ecological niches, such as milk, meat, fish, fruits, vegetables, and so on (Todorov & Franco, 2010). Lactobacilli, mainly *L. plantarum*, are generally known for their capacity to produce a wide range of primary and secondary metabolites, including lactic acid, hydrogen peroxide, bacteriocins, biosurfactants, enzymes and other related compounds (Arena *et al*., 2016; Satpute *et al*., 2016). These metabolites are thought to be a defensive mechanism that bacteria deploy to prevent other microorganisms from colonizing their natural habitat by inhibiting their proliferation and survival (Peterson *et al.*, 2020). In particular, biosurfactants can play a vital role in suppressing pathogen adherence, which is a crucial stage in bacterial growth and some other important activities such as biofilm formation (Abruzzo *et al.*, 2021; Markande *et al*., 2021). Biosurfactants are diverse groups of surfactants and amphiphilic molecules generally produced by living microorganisms, explicitly bacteria, fungi and yeasts (Marchant & Banat, 2012). The synthesis of biosurfactant precursors requires several metabolic pathways depending on what type of the main carbon sources are used in the medium of production (Santos *et al*., 2016).

Nevertheless, microbial surfactants are classified according to the chemical structure and producing microorganisms (Malakar & Deka, 2021). They are categorized into molecules with a low molecular weight that efficiently declines surface and interfacial tension, and polymers of large molecular weight that well assist to stabilize emulsions (Rosenberg & Ron, 2013). Glycolipids, lipopeptides, phospholipids, and glycolipopeptides are the major groups of low molecular weight biosurfactants, whereas molecules with high molecular weight are usually categorized as polymeric biosurfactants. They are synthesized by various microorganisms and are comprised of different groups such as lipoproteins, proteins, polysaccharides, lipopolysaccharides or their complexes (Ron & Rosenberg, 2001; Mulligan *et. al.*, 2019). The increasing concern of chemical surfactants draws more attention to naturally synthesized biosurfactants owing to their low toxicity and biodegradability (Mondal *et. al.*, 2015; Jimoh & Lin, 2019). Consequently, the demand for biosurfactants produced by lactobacilli is becoming ever more interesting for application in industrial processes such as food additives, cosmetics, and pharmaceuticals (Hajfarajollah *et al*., 2018; Adu *et al.*, 2020). Similarly, the body of literature on the characterization of new innovative biosurfactants has grown, although there were few investigations on Lactobacilli glycolipopeptides surfactants (Twigg *et al*., 2021). However, efforts have been made in the current study to evaluate the ability of selected bacterium *L. plantarum* LBp_WAM to produce a surface-active substance, as well as to characterize its functional and antimicrobial properties.

Materials & Methods

Isolation and phenotypic identification of lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) strains were isolated from natural buffalo milk yogurt samples obtained from a local market (Garmat-Ali) located in the north of Basrah city, Iraq. A total of 10 samples refered as (B7, YL1, YL2, CH2, CH3, WAM, MB6, YR1, and YR2) were collected aseptically in sterile containers and rapidly transferred to the laboratory. Precisely, one ml of each sample was enriched in 9 ml of de Man, Rogosa, and Sharpe (MRS) broth then incubated at 37^{o}C for 24 h. The samples were

diluted with phosphate buffer saline (PBS) :(10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl, and pH 7.0), then cultured anaerobically on MRS agar plates and incubated at 37°C for 24 h.

The standard identification procedures based on morphological, cultural and biochemical features were followed in selecting all the probable isolates within the scope of the present study. Gram-stained isolates were chosen according to Gram-positives and cell shape. Further tests of catalase and oxidase have also been conducted. The selected isolates were maintained on MRS agar slants at 4°C. Whereas stock cultures were kept in cryovials with their culture broth containing 30% sterile glycerol at -20°C until use.

Screening for biosurfactant-producing LAB

The procedure described by Mouafo *et al*. (2018) was followed for biosurfactant production with some modifications. The culture medium comprising $g.L^{-1}$: ($NaNO_3$ 2.0, KCl. 0.1, KH_2PO_4, 1.0, K_2HPO_4, $CaCl_2$ 0.01, $MgSO_4 \cdot 7H_2O$ 0.5), peptone: 8, yeast extract 5, and carbon source 9% (w/v) was prepared and supplemented with 1 ml of trace elements solution containing $mg.100\ ml^{-1}$: ($CuSO_4.7H_2O$ 0.5; H_3BO_3 1.0; $MnSO_4.7H2O$ 1.0; and $ZnSO_4$ 0.7). The pH of the medium was adjusted to 6.7 and a standard culture medium of MRS was used as a control.

Afterwards, a sterilized culture medium with a volume of 100 ml was distributed in Erlenmeyer flasks. Each flask was inoculated with 2.5 ml of an overnight culture of LAB then incubated at 37°C in a shaker incubator (Sartorius-Certomat IS, Germany) operating at 150 rpm for 72 h. After incubation, the cultures were centrifuged (Hermle Labortechnik GmbH, Germany) at 10,000 rpm and 4°C for 10 min and the cell-free supernatant (CFS) of each culture was neutralized to pH 6.7 with 0.1N NaOH before being used for subsequent analysis.

Genotypic characterization of biosurfactant-producing LAB

Genomic DNA extraction

A single colony of *L. plantarum* LBp_WAM was cultivated in 5 ml MRS broth and incubated for 24 h at 37°C. Next day, the growing bacteria were harvested by centrifugation at 4000 rpm for 3 min. The precipitated pellets were washed twice with nuclease-free water and centrifuged. Then, the bacterial gDNA was extracted by using a commercial DNA extraction kit (Genaid, Korea). All the extraction steps were applied as recommended by the manufacturer's instructions. The extracted gDNA purity and quantity were detected by using a nanodrop spectrophotometry apparatus (DNA/Protein Analyzer, Quawell, USA).

Gene amplification by using Polymerase Chain Reaction (PCR)

A universal pair of primers (Forward: 5'-AGA GTT TGA TCC TGG CTC AG-3' and Reverse: 5'-TAC GGT TAC CTT GTT ACG ACT-3') described by Fei *et al.* (2014) was used to amplify the 16S rRNA gene by applying the following PCR conditions. Primary denaturation at 95°C for 5 minutes, cycling denaturation at 95°C for 30 sec, annealing 55°C for 30 sec, cycling extension 72°C for 2 min, and final extension 72°C for 10 min. The resulting amplicons were analyzed by running on 1% agarose gel and visualized using a UV transilluminator imaging system.

DNA nucleotides sequencing and analysis

The detected 16S rRNA bands were extracted from the agarose gel and sent to the Macrogen

Company (Seoul, Korea) for nucleotides sequencing. The obtained 16S rRNA sequence was analyzed using NCBI blast and compared to similar sequences from the GenBank database. The top sequence hits were selected and downloaded for further analysis such as nucleotides sequence alignment. Clustal O (https://www.ebi.ac.uk/Tools/msa/clustalo/), an online multiple DNA sequences alignment application, was used to clarify the similarity and differences between the downloaded sequences. Molecular evolutionary relationship and phylogenetic analyses (phylogenetic tree construction) were conducted by applying the trimmed aligned sequences into the MEGA 11 software programme (Tamura *et al*., 2021) using the UPGMA method constructing condition. The alignment sequences were trimmed and uploaded to the MEGA 11 programme. A bootstrap of 1000 replicates was used to associate taxa clustered together. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004). This analysis involved six 16S rRNA nucleotide sequences including four best-hit nucleotide sequences, which were *L. plantarum* strain IMAU98304, *L. plantarum* strain LN-3-1, *L. plantarum* strain 4310 and *L. rhamnosus* strain 6870 with accession numbers (MT473659.1, MN372114.1, MT544853.1 and MH791026.3 respectively). In addition to the novel isolate sequence (*L. plantarum* LBp_WAM OL913130.1) and a hetero bacterial strain, a local isolate *Bacillus cereus* SAMU1, as a reference strain (Al-Hejjaj *et al*., 2020).

Emulsification activity (EA)

The emulsification activity of the produced biosurfactant was determined according to Patel & Desai (1997) with some modifications. Precisely, cell-free supernatant (CFS) with a volume of 0.5 ml was added to a screw-capped tube containing 7.5 ml of Tris-Mg buffer (20 mM Tris HCl (pH 7.0) and 10 mM $MgSO_4$) and 0.1ml of dodecane. The mixture was vortexed at high speed for 2 min then allowed to stand for one hour. Emulsification activity was expressed as the measured optical density (OD) by assessing the absorbance at a wavelength of 540 nm using a UV/Vis spectrophotometer (SP 3000nano, Japan). The test was carried out in triplicates.

Oil-Spreading Method

The oil-spreading method was performed in triplicates as described by Morikawa *et al*. (2000). First, a volume of 50 ml of distilled water was added to a Petri dish of 15 cm in diameter, followed by adding 100 µl of crude oil to the surface of the water. Next, 10 µl of the CFS solution was gently dropped on the oil surface. The formation of a clear zone on the oil surface was observed and measured after 30 sec.

Determination of surface tension (ST) and critical micelle concentration (CMC)

A ring-type tensiometer (Sigma 700, KSV Instruments LTD - Finland) was performed to determine the surface tension of crude biosurfactant at different concentrations (0.0-10 mg. ml^{-1}), using the Du Nouy method at room temperature. Likewise, the CMC values were obtained by measuring the surface tension for various CFS dilutions. Distilled water (72 ± 0.10 $mN.m^{-1}$) was used to verify the measurements before each reading. The CMC was defined as the point where the surface stress increases abruptly.

Emulsification index (E_{24})

The emulsion index was estimated depending on Madhu & Prapulla (2014) with a slight modification. Briefly, a mixture of 1 ml of crude biosurfactant (1mg.ml^{-1}), 4ml of distilled water, and 6ml of (olive oil, sunflower, hexane, kerosene, glycerol, and mineral oil) was vortexed vigorously for 2 min and left to stand for 24 h. Tween 80 (1%) was used as a positive control. The $\%E_{24}$ was calculated by using the following equation

$$E24\% = \frac{\text{The height of the emulsified layer(mm)}}{\text{The total height of the liquid layer(mm)}} \times 100$$

Biosurfactant extraction

A biosurfactant extract has been obtained according to Fracchia *et al.* (2010) with some modifications. Initially, the acid precipitation was carried out by centrifuging 100 ml of an overnight culture at 10.000 rpm and 4°C for 10 min (Hermle Labortechnik GmbH, Germany). The CFS was acidified to pH 2, with 6 N HCl, then maintained at 4°C for 24 h. After centrifugation at 10.000 rpm and 4°C for 15 min, the precipitate was collected and dissolved in 10 ml of distilled water. Next, a solvent extraction step has been executed with a mixture of chloroform/methanol (2:1 v/v) by a rotary evaporator under vacuum conditions. Finally, the extraction solvents were thoroughly evaporated before collecting the crude biosurfactant.

Gel filtration chromatography (GFC)

A concentrated crude biosurfactant (5 ml) has been passed through a Millipore membrane (0.2µm) to eliminate any insoluble substance. This filtered solution was then applied to a Sephadex® G-100 (Pharmacia, Sweden) column (2.6 cm×90 cm) pre-equilibrated and eluted with a phosphate buffer (pH 7.2). Blue dextran (2000 kDa) was used to estimate the void volume of the column by measuring the optical density at 650 nm using a UV/Vis spectrophotometer (SP 3000nano, Japan). Likewise, a flow rate of elution buffer (1 ml.min^{-1}) was maintained and fractions of 3 ml were collected and examined for biosurfactant content by absorption at 280 nm. The purified fractions were lyophilized with a freeze-drier (Crist, Germany) to obtain a pure concentrate of biosurfactant that finally was stored at -20^{o}C until use.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight of the purified biosurfactant was determined by using SDS-PAGE analysis technique as described by Sambrook & Russel (2006). Briefly, 10% polyacrylamide resolving gel consisting of 2.6 ml resolving buffer (1.5M Tris-HCl, 0.4% SDS, pH8.8), 3.3 ml acrylamide stock solution (30% acrylamide, 0.8% Bis-acrylamide), 4 ml distilled water, 100µl ammonium persulphate (APS 10%) and 10µl N,N,N',N'-tetramethylethylenediamine (TEMED) were used. In addition to a stacking gel prepared by mixing 2.5 ml stacking buffer (0.5 M Tris-HCl, 0.4% SDS, pH 6.8), 1.3 ml acrylamide stock solution, 6 ml distilled water, 100 µl APS (10%) and 10 µl TEMED. Gel-purified biosurfactant samples were denatured, as described by (Al-Hejjaj, 2017), in protein loading buffer (40% glycerol, 200 mM Tris-HCl pH 6.8, 400 mM DTT, 0.4% bromophenol blue and 8% SDS). Samples were heated at 95°C for 5 minutes and centrifuged at 13000 rpm for 1 min. The gel was loaded with a 20µl protein sample. To estimate the detected proteins' size, a 5 µl (Pageruler, Thermo Scientific, UK) prestained protein ladder was loaded onto the gel as well. Subsequently, electrophoresis was applied for approximately 2 h at 100 Volts in protein running solution (25mM Tris, 0.1% SDS and 250mM glycine).

Subsequently, gels were stained with Coomassie blue (0.1% Coomassie Blue R-250, 50% methanol, 10% acetic acid) for at least 1 h with gentle shaking. Finally, the stained gel was washed with destaining solution (10% v/v methanol, 10% v/v acetic acid) to remove the blue background of the stained gel and visualise the detecting bands.

Thin-layer chromatography (TLC)

TLC analysis was realized as Lamilla *et al*. (2018) described with slight modifications. A precise volume (5µl) of concentrated crude biosurfactant has been spotted on a TLC plate (HiMedia, India). Chloroform, methanol, and water (60: 20: 1 v/v) were used as a mobile phase. The dried plate was sprayed with Ninhydrin, iodine and sulfuric acid then air-dried and heated for 5 min at 100°C to visualize the fractions of peptides, lipids, and carbohydrates, respectively.

$$\text{Rf} = \frac{\text{Distance travelled by the sample from the origin(cm)}}{\text{Distance travelled by the solvent from the origin(cm)}}$$

Fourier transform infrared spectroscopy (FTIR)

The infrared spectrum was used to determine the chemical structure of the biosurfactant through the identification of functional groups. An aliquot of the crude biosurfactant was deposited in the FTIR instrument (FTIR-8400S, Shimadzu, Japan) expressing the transmittance between the frequencies in the range of 4000 - 500 cm^{-1}.

Antimicrobial activity

The inhibitory activity of the produced biosurfactant was assessed using the agar well diffusion method as described by Al-Seraih *et al*. (2017). For this purpose, clinical bacterial strains were used as target strains including *Enterobacter* sp., *Escherichia coli*, *Staphylococcus aureus, Micrococcus luteus*, *Pseudomonas aeruginosa*, and *Bacillus cereus*, that were kindly provided by the department of biology, college of science at Basrah University. The CFS of *L. plantarum* LBp_WAM was obtained by centrifugation (10.000 rpm for 10 min at 4°C, Hermle Labortechnik GmbH, Germany) of a fresh culture cultivated for overnight in MRS broth at 37°C. Afterwards, wells bored onto the surface of Muller Hinton agar, that pre-inoculated with 1% (10^6 $CFU.ml^{-1}$) of a target strain, were filled with 50µl of a neutralized CFS (pH 6.5). The Petri plates were held for 1 h at 4°C before being incubated for 24 h at 37°C. Antibacterial activity of biosurfactant was evaluated by measuring the diameters of the clear zones surrounding the wells that contain CFS. The zone of inhibition was expressed in millimetres.

Statistical analysis

All measurements were done in triplicate and data presented are mean values ± standard deviation. A one-way analysis of variance (ANOVA) was applied to assess the diffusion diameters of biosurfactant droplets, however, a two-way analysis was performed to analyse the screening of biosurfactant-producing lactic acid bacteria, and emulsification index data. Mean comparisons were carried out using the L.S.D test at a level of significance $p \leq 0.05$. SPSS program ver. 25 was applied to analyse data.

Results & Discussion

Screening and selection of biosurfactant-producing LAB

The potential strains were isolated from different samples of traditional buffalo milk yogurt, based on their ability of oil emulsification and surface tension reduction. The screening of biosurfactant-producing isolates was carried out by measuring the emulsification activity (E.A). Thus, in order to

select the best biosurfactant-producing isolates, different carbon sources (kerosene and sunflower oil) were initially used as shown in fig. (1). All the ten isolates tested in this study displayed significant variations in their capability to emulsify and reduce surface tension ($p \leq 0.05$).

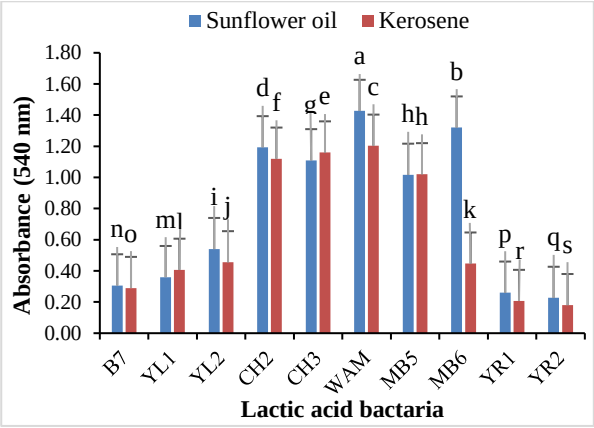

Fig. (1): Screening and Selection of Biosurfactant-Producing lactic acid bacteria. (Results are represented by three replicates). Different letters above bars indicate significant differences (≤ 0.05).

The isolate *L. plantarum* LBp_WAM displayed the most significant emulsifying activity with sunflower oil carbon source compared to the other LAB isolates. Likewise, the oil spreading technique was applied to confirm the presence of surface-active biomolecules. This test revealed the efficacy of *L. plantarum* LBp_WAM strain for biosurfactant production as a clear zone with a diameter approaching 7.6 cm via measuring the diffusion of the biosurfactant drop on a crude oil-water interface (Figs. 2, and 3).

Biosurfactant-producing bacteria are capable to generate surface-active molecules in aqueous media1 in the presence of carbon sources. Carbohydrates, hydrocarbons, and vegetable oils are the most common carbon sources utilized in the production of biosurfactants (Kosaric & Sukan, 2010). The use of various types of oils as carbon sources in the synthesis of biosurfactants has been generally recognized. This is owing to the fact that there are four possible pathways for biosurfactant synthesis: The first hypothesis is that the hydrophobic and hydrophilic moieties are produced by separate metabolic pathways. Second, the carbon source has an impact on the synthesis of both hydrophilic and hydrophobic sides of the biosurfactant. Third, the hydrophilic moiety is initiated de novo, while the carbon source drives the hydrophobic side's synthesis. Lastly, the hydrophobic part is generated de novo, while the hydrophilic end is influenced by the carbon source (Nurfarahin *et al.*, 2018).

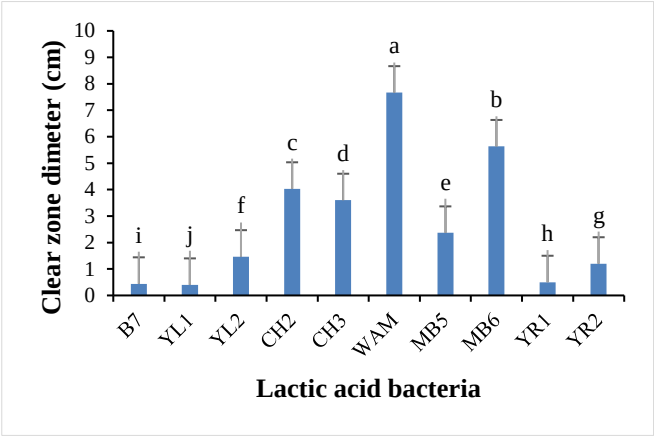

Fig. (2): Diffusion diameters of biosurfactant droplets produced by lactic acid bacteria on the crude oil-water interface. Results are expressed as mean ± standard deviations of values from triplicate experiments. Different letters above bars indicate significant differences ($p \leq 0.05$).

Sunflower oil, which is used in the current study, is an example of carbon sources that could be applied to produce biosurfactants since it contains a mixture of monounsaturated and polyunsaturated fatty acids. As a hydrophobic substrate, it may stimulate the synthesis of biosurfactants which increases its solubility in the aqueous medium. Furthermore, it has been suggested that the high production of biosurfactants by sunflower oil can also be attributed to the high content of linoleic acid (about 60%) in this substrate (Ferraz *et al.*, 2002; Wasoh *et al.*, 2017).

Similarly, hydrocarbons are usually utilized for microbial production of biosurfactants. For instance, Mnif *et al.* (2014) evaluated the ability of *B. subtilis* SPB1, a biosurfactant-producing strain, to consume diesel and kerosene as carbon sources. Their findings showed that adding yeast extract (2%) to the liquid mineral medium, which was supplemented with hydrocarbons as substrates, significantly boosted the microbial growth and improved biosurfactant production. When a hydrocarbon is employed as a carbon source, the cellular metabolic mechanism is mostly directed toward the lipolytic pathway and gluconeogenesis (the synthesis of glucose from various hexose precursors) allowing it to be used for the formation of fatty acids or sugars (Santos *et. al.,* 2016). Hommel (1994) considers two mechanisms of hydrocarbon absorption: (i) interfacial uptake (cells in direct contact with hydrocarbon droplets) and (ii) biosurfactant-mediated hydrocarbon transfer (cell contact with emulsified or solubilized hydrocarbons). Both processes are usually possible, but which one takes the priority depends on the strain itself.

The variations among the selected bacterial isolates in their ability of producing surface-active substances could be belonged to the phenotypic variability and behavioural flexibility in the bacterial cells, which are becoming increasingly acknowledged (Harrison, 2013; Frankel *et al.,* 2014).

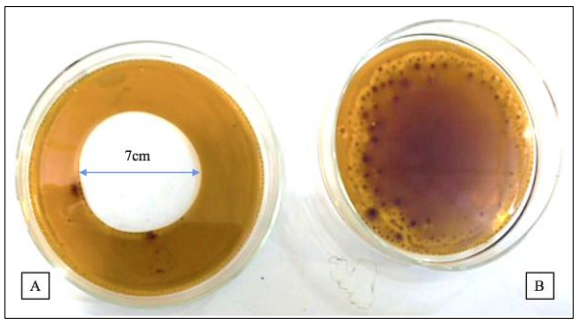

Fig. (3): (A) the diffusion of the biosurfactant drop of *L. plantarum* LBp WAM on the crude oil-water interface. (B): Negative control of non-producing strain.

Genetic variations such as single nucleotide variants (SNV) and larger structural variants are prevalent when natural or engineered strains are re-sequenced. The range of effects that these genetic variants play on individual gene or protein function or expression is enormous (Cardoso *et. al.,* 2015). Nevertheless, in order to develop extrapolations about how a genotype impacts a certain phenotype, it may need to evaluate intermediate phenotypes such as transcript, protein, or metabolite levels for these strains.

Taxonomic affiliation of the biosurfactant producing bacterial strain *L. plantarum* LBp_WAM

The surfactant producing selected strain was initially identified depending on the morphological and biochemical features followed by molecular techniques confirmation. The 16S rRNA gene was amplified using a universal pair of primers in PCR technology. The nucleotide sequence was obtained and analysed before deposing at the GenBank as *L. plantarum* strain LBp_WAM with an accession number of OL913130.1 The resulting bacterial sequence was compared with available sequence data in geneBank followed by constructing the phylogenetic tree using the MEGA 11 genetic analysis programme (Fig. 4).

Surface tension (ST) and critical micelle concentration (CMC)

Lactobacillus species are capable of producing a variety of surface-active metabolites that may efficiently reduce water's surface tension to very low levels, compared to chemically synthesized substances (Satpute *et al.,* 2016).

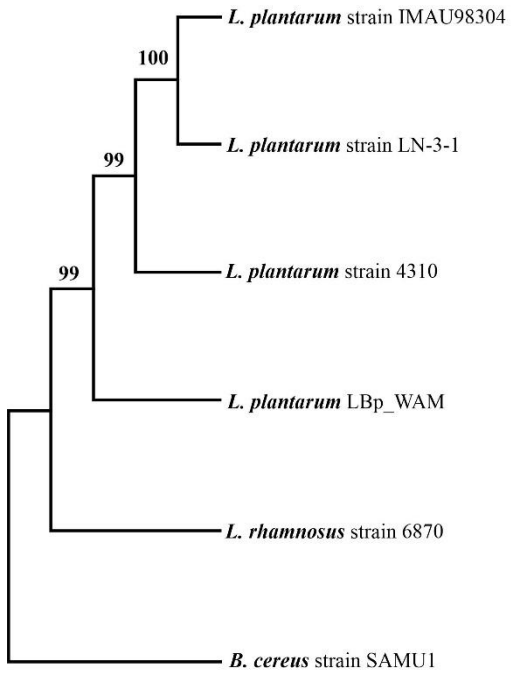

Fig. (4): Phylogenetic evolutionary analysis of *L. plantarum* strain LBp_WAM (LBp_WAM) based on the 16S rRNA nucleotides sequence. The newly identified 16S rRNA sequence of LBp_WAM align with top hits *Lactobacillus* 16S rRNA bacterial sequences obtained from GenBank database using Clustal O. The phylogenetic tree was constructed based on the UPGMA methods and Bootstrap value of 1000 replicates using the MEGA 11 genetic analysis application.

In the current study, the biosurfactant generated by *L. plantarum* LBp_WAM revealed the capacity to reduce the surface tension of water from 72 $mN.m^{-1}$ to 32 $mN.m^{-1}$. At this level, additional biosurfactant concentrations did not show a significant decrease in surface tension (Fig. 5).

Furthermore, the critical micelle concentration (CMC) value of *L. plantarum* LBp_WAM was 2.4 $mg.ml^{-1}$. These values were exceeded those obtained by Mouafo *et al.* (2018), who have demonstrated surface tension values close to (47.5, 41.9, and 44.20 $mN.m^{-1}$) by the *Lactobacillus plantarum* G88, *Lactobacillus delbrueckii* N2, and *Lactobacillus cellobiosus* TM1, respectively. Along the same lines, Madhu & Prapulla (2014) found that the crude surfactant of *Lactobacillus plantarum* CFR 2194 reduced the surface tension of the phosphate-buffered saline solution from 72.2 $mN.m^{-1}$ to 44.3 $mN.m^{-1}$. Evidently, effective surfactants reduce the surface tension of water and air from 72 to 35 $mN.m^{-1}$ and the interfacial tension of water and n-hexadecane from 40 to 1 $mN.m^{-1}$. However, more surfactant monomers when added to the combination, the surface or interfacial tension drops until the surfactant concentration reaches the CMC, at which point there is no further decrease is possible (Soberón-Chávez, 2010; Alwaely *et al*., 2019).

Emulsification index (E_{24}) and emulsification activity

One of the most important properties of a surfactant is the capacity to emulsify liquids. The biosurfactant's emulsification ability was compared to that of nonionic chemical surfactant Tween 80. Thus, fig. (6) depicts the crude biosurfactant and Tween 80 emulsification indexes against various substrates. It was realized that crude biosurfactant and Tween 80 emulsified two immiscible phases in very identical ways. Olive oil, sunflower oil, glycerol, mineral oil, hexane, and kerosene were used to test the crude biosurfactant's emulsification index (1 $mg.\ ml^{-1}$).

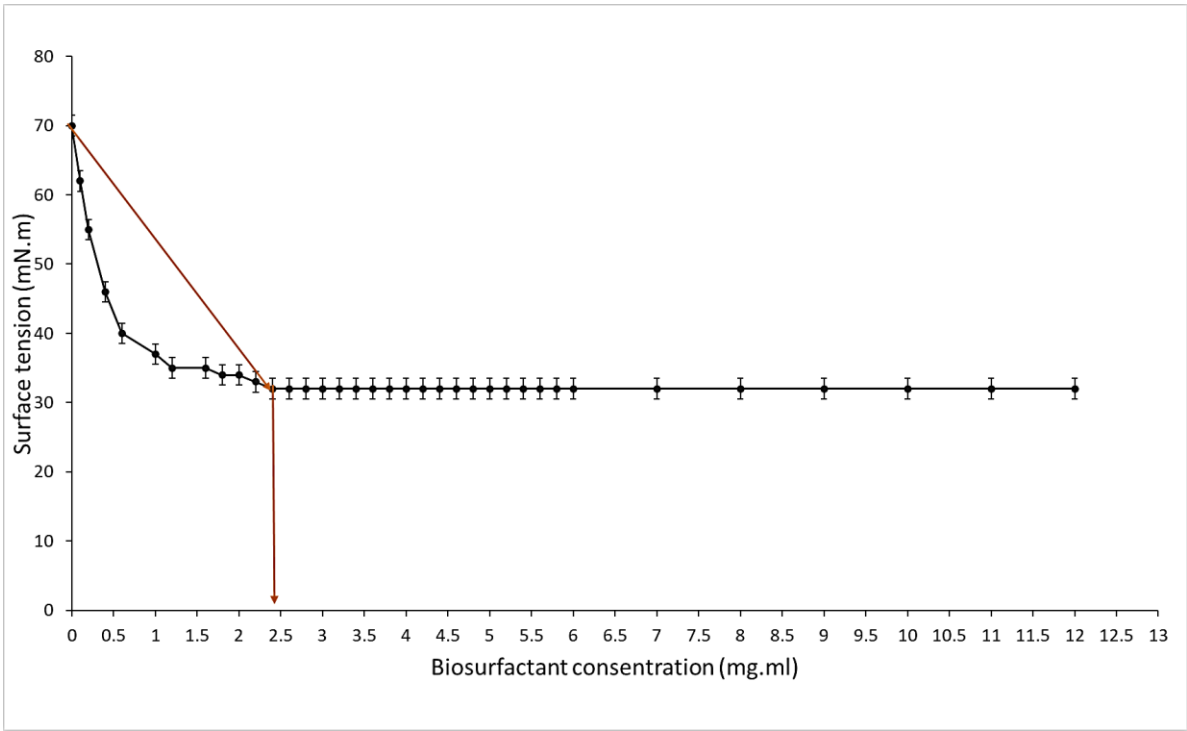

Fig. (5): Surface tension and CMC of the biosurfactant produced by *L. plantarum* LBp_WAM. Results are expressed as mean ± standard deviations of values from triplicate experiments.

The results displayed that the sunflower oil (60% ± 3.0), glycerol (53.9% ± 0.11), and olive oil (49.0% ± 2.0), had significantly higher E_{24} among all emulsions used in this study, whereas mineral oil (50.7% ± 0.60) seemed to show the highest E_{24} value among the hydrocarbons, followed by hexane (36.03% ±0.05), and kerosene (31% ±0.05).

Accordingly, Madhu & Prapulla (2014) were used kerosene, xylene, coconut oil, sunflower oil, hexane, and heptane to assess the emulsification index of the biosurfactant (1 mg. ml^{-1}) derived from *L. plantarum*. For hydrocarbons, their findings indicated that the highest E_{24} value was 38.2% for heptane, followed by 16.22% for xylene, 15.2% for kerosene, and (13.6%) for hexane. However, the E_{24} values were 37.9% and 19.4% for coconut oil and sunflower oil respectively. They also noticed that coconut oil and sunflower oil emulsions were more stable than hydrocarbon emulsions. Equally, Cornea *et al.* (2016) have verified the emulsification index of the biosurfactants synthesized by *Lactobacillus* spp. strains against kerosene, sunflower oil, and olive oil as substrates. They reported that kerosene had the lowest E_{24} value, whereas olive oil had the highest E_{24} value across all strains. Nevertheless, one or more lipophilic and hydrophilic moieties can be found in biosurfactants.

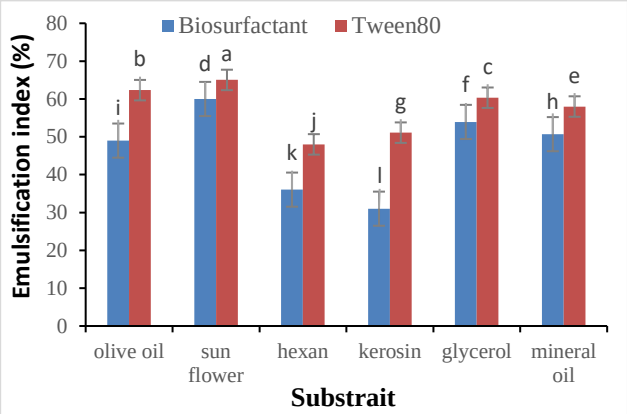

Fig. (6): Emulsification index (E_{24}) of biosurfactant of *L. plantarum* LBp_WAM for different substrates. Results are expressed as mean ± standard deviations of values from triplicate experiments. Different letters above bars indicate significant differences (≤ 0.05).

The lipophilic moiety can be a protein or peptide with a large fraction of hydrophobic side chains, but it is most often the hydrocarbon chain of a fatty acid with 10–18 carbon atoms, however, an ester, a hydroxy, a phosphate or carboxylate group, or carbohydrate can all function as a hydrophilic moiety (Bognolo, 1999).

Purification and characterization of crude Biosurfactan

To recover the crud biosurfactant produced by *L. plantarum* LBp_WAM, the cell-free supernatant was acidified, and followed by a solvent-solvent extraction step. However, the concentrated biosurfactant solution was applied to a Sephadex® G-100 column for further purification. The biosurfactant was eluted at a rate of 1 $ml.min^{-1}$, as it appeared in the fractions that ranged between 81-148 forming a sharp peak (Fig. 7). Thereafter, the gel-purified biosurfactant was submitted to SDS-PAGE in order to estimate its molecular weight. The protein profile showed that it has a molecular mass of approximately 19 KDa (fig. 8). However, the molecular characterization of the biosurfactant on a polyacrylamide gel under denaturation conditions revealed a single protein band, indicating electrophoretic homogeneity. The obtained result is comparable to those previously reported for low molecular weight biosurfactants generated by *Lactobacillus* spp or other microorganisms characterized by SDS-PAGE. Shokouhfard *et al*. (2015) have used the SDS-PAGE technique to examine a freeze-dried biosurfactant produced by Lactobacillus acidophilus ATCC 4356. Only one band with a size of roughly 10 KDa was identified in the protein profile, according to their findings.

Similarly, Satpute *et al.* (2019) have partially described a surface-active molecule derived from *Lactobacillus acidophilus* using SDS-PAGE, which revealed several bands in a molecular range of 14.4 to 60 KDa. Moreover, it was reported that *Streptomyces* sp. DPUA1559 has been developed as a biosurfactant having a chemical composition of 20% proteins, 38% carbohydrates, and 12% lipids with an approximate molecular mass of 14.3 KDa (Santos *et al.*, 2017).

Based on the above studies, it appears that the predicted description of this 19 KDa biosurfactant is either a lipoprotein or a glycolipid, as the majority of low molecular weight biosurfactants often fall into one of these two classes (Rosenberg & Ron, 2013). Furthermore, it was confirmed that low molecular weight biosurfactants with low CMCs efficiently boost the apparent solubility of high molecular weight hydrophobic combinations by integrating hydrocarbons into the hydrophobic parts of micelles (Miller & Zhang, 1997) which approves the elevated solubility and high emulsification rates determined in the current study.

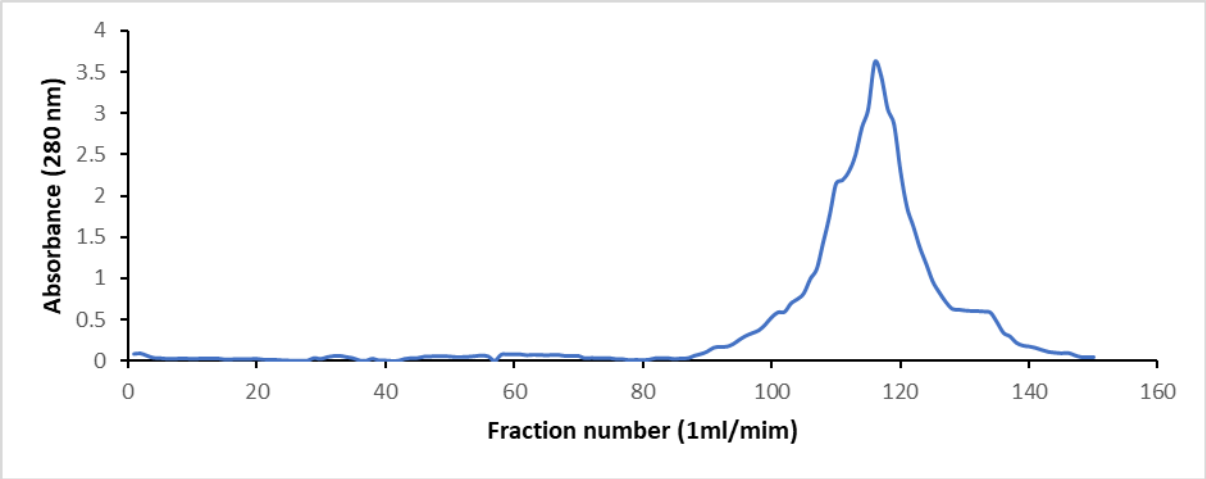

Fig. (7): Sephadex G-100 elution profile of biosurfactant from *L. plantarum* LBp WAM.

Thin-layer chromatography (TLC)

The TLC plates were visualized using UV light (254 nm), and the results suggested subsequently a glycolipoprotein nature of the crude biosurfactant extracted from *L. plantarum* LBp_WAM as it was developed a purple spot with an approximate Rf of 0.57 after treating with the ninhydrin reagent, whereas another Rf value of 0.54 was measured with the iodine vapour confirming the existence of a lipid part stained as a yellow-brown spot. However, the treatment with sulfuric acid indicated the presence of a carbohydrate part as it was presented as a brown spot with a 0.52 Rf value. Furthermore, potassium permanganate was used as a universal stain to visualize the oxidizable functional groups (Fig. 9).

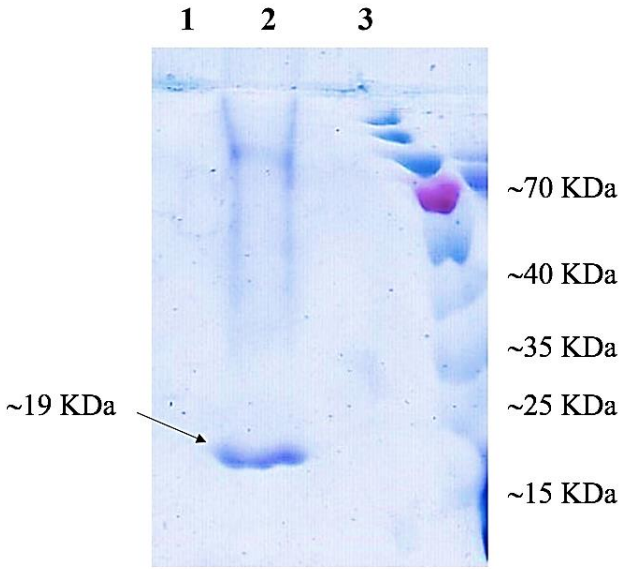

Fig. (8): Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile analysis of *L. plantarum* LBp_WAM. Lane 1: negative control; Lane 2: purified biosurfactant; lane 3: protein ladder (Pagerular 10 to 170 KDa, Thermo Scientific, UK).

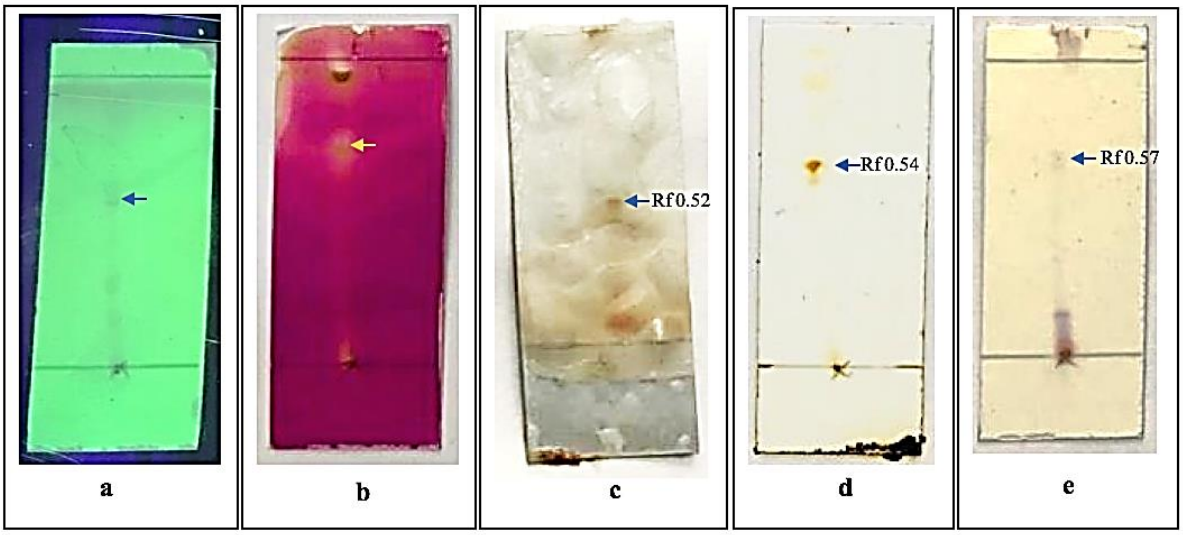

Fig. (9): Thin layer chromactography (TLC) assays of the chemical composition of the crude biosurfactant of *L. plantarum* LBp_WAM. (a) U.V. light, (b) Permanganate, (c) H_2SO_4, (d) Iodine, (d) Ninhydrin.

Several studies have shown that biosurfactants generated by *Lactobacillus* species have varying Rf values depending on the nature of the biomolecules. Abdalsadiq *et al.* (2018) have identified biosurfactants derived from *Lactobacillus acidophilus and Lactobacillus pentosus* as a lipopeptide, and a glycolipid respectively. Proteins were recognized using ninhydrin, which confirmed the presence of amino acids as a purple zone, and lipids were identified using iodine reagent, which revealed the presence of fatty acids as a light brown zone on the TLC plate. A glycolipoprotein derived from *Lactobacillus acidophilus* was described by Satpute *et al.* (2019) showed different Rf values ranged between 0.68, 0.54 and 0.46 for sugars, lipids and protein, respectively. Similarly, a crude glycolipid with polysaccharides and lipid fractions produced by *Lactobacillus helveticus* was characterized by Sharma *et al.* (2014), which exhibited a dark yellow single spot, when stained with iodine vapor, with an Rf value of 0.68 proposing the presence of a glycolipid moiety. The RF values are obviously affected by experimental conditions, substance nature, and generating microorganisms. Following treatment with ninhydrin solution, TLC results for biosurfactants produced by *Ochrobactrum* sp. and *Bacillus* sp. indicated the presence of positive spots, suggesting the lipopeptides nature. However, the protein fragment showed as single spots with Rf values of 0.55 and 0.72 for *Ochrobactrum* and *Bacillus* respectively (Joy *et al.*, 2017).

Structure Analyses of Biosurfactant by FTIR Spectroscopy

The molecular composition of the crude biosurfactant was unveiled by FTIR analysis, which showed the presence of peptide groups resulting from OH and NH stretching by the bands at 3487 cm^{-1}, and 3475 cm^{-1} respectively. Furthermore, the FTIR spectrum revealed the existence of protein-related weak bonds, with C=O bonds at 1743 cm^{-1} (amide I bond), and N-H bonds at 1543 cm^{-1} (amide II bond). The existence of C-H stretching matching with CH_2 and CH_3 groups of aliphatic chains is indicated by absorbance

bands between 2924 cm^{-1} and 2854 cm^{-1}, as well as bands at 1458, and 1381 cm^{-1}. Polysaccharides are recognized by the band at 1091 cm^{-1} and 1168 cm^{-1} (sugars' C-O stretching vibration) (Table 1, and Fig. 10). These findings are comparable with those of previous studies on glycolipoproteins generated from *Lactobacillus* species. Vecino *et al.* (2015) have analyzed the FTIR spectrum of a surface-active glycolipopeptide produced by *L. pentosus.* They demonstrated the presence of C=O bond at 1644 cm^{-1}, and N-H bonds at 1644 $cm^{-1}$and 1544 cm^{-1} bond. The occurrence of C-H stretching corresponding to aliphatic chains (CH_2 and CH_3) is identified by bands between 2961 cm^{-1} and 2856 cm^{-1}, along with bands at 1456, 1403 and 1385 cm^{-1}. Polysaccharides are also indicated by the band at 1089 cm^{-1}.

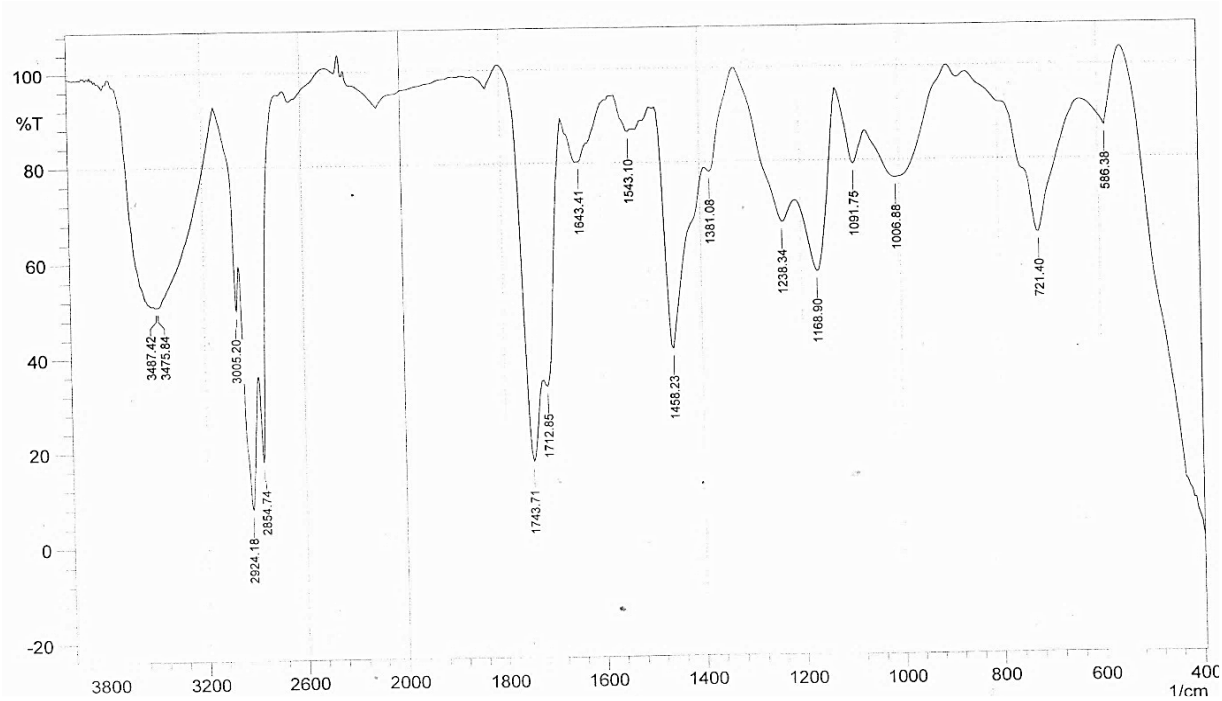

Fig. (10): FTIR spectrum of the biosurfactant obtained from *L. pla-ntarum* strain LBp_WAM.

Table (1): FTIR spectra of functional groups identified in *L. plantarum* LBp WAM biosurfactant.

Range (cm^{1-})	Assignment
12381-1168	C–O sugar stretching
1458-1381	C–H vibrations of groups CH2 e CH3 Groups N–H in proteins
1543	Groups N–H in proteins
1743-1712	C=O stretching of a carbonyl group
2924-2854	CH stretching absorption
3487-3475	OH, and NH stretching

Similarly, the FTIR spectrum of a crude biosurfactant derived from *L. helveticus* revealed polysaccharides, protein, and lipid content. The most significant bands for O–H stretching were observed at 3456 and 3286 cm^{-1}. The C–H stretching vibrations in the transmittance range 2800-2950 cm^{-1} identified the aliphatic chains. Sugars have bands of 1700 cm^{-1} (C=O ester bond) and 1273 cm^{-1} (C–O stretching vibration), whereas glycolipid moieties have bands of 1041 cm^{-1} (polysaccharides), 702 cm^{-1}, and 648 cm^{-1} (for the CH 2 group) (Sharma *et al.,* 2014)

Antimicrobial activity

The antibacterial efficacy of the glycolipopeptide biosurfactant against several Gram-positive and Gram-negative bacteria is summarized in table (2). For Gram positive bacteria, the highest inhibitory effect was shown against *Micrococcus luteus* with an inhibition zone of 16.5 ± 0.707 mm followed by *Staphylococcus aureus* and *Bacillus cereus* as they revealed inhibition diameters of 13.0 ± 0.816 mm and 11.6 ± 0.577 mm respectively. Nevertheless, the Gram-negative target strains including *Pseudomonas aeruginosa*, *E. coli*, and *Klebsiella pneumoniae* showed lower inhibitory values with inhibition diameters of 11.3 ± 1.527, 10.3 ± 1.154, and 8.6 ± 0.577 respectively.

Table (2): Antimicrobial activity of biosurfactant derived from *L. plantarum* LBp_WAM against Gram-positive and Gram-negative bacteria.

Strains	Antimicrobial activity (Mean± standard deviations (mm)
G+	
Staphylococcus aureus	13 ± 0.816
Bacillus cereus	11.6 ± 0.577
Micrococcus luteus	16.5 ± 0.707
G-	
Pseudomonas aeruginosa	11.3 ± 1.527
Escherichia coli	10.3 ± 1.154
Klebsiella pneumoniae	8.6 ± 0.577

According to these observations, the bioactive molecule has been presented to be more efficient against Gram-positive bacteria than Gram-negative bacteria. Similarly, other glycolipoproteins derived from *L. plantarum* 60FHE, *L. paracasei* 75FHE, and *L. paracasei* 77FHE exhibited a wide range of antimicrobial activity against Gram-positive and Gram-negative bacteria but not against fungi and yeasts. The inhibition zone diameters were 20.3± 0.25 mm, 23.0 ± 0.96 mm, and 36.3± 0.12 mm for *Staphylococcus aureus* ATCC29737, *Bacillus subtilis*, and *Micrococcus luteus* ATCC10240 respectively, whereas the antagonistic effect was about 38.0± 0.10, 20.3± 0.81, and 14.0± 0.17 for *Escherichia coli* ATCC10536, *Pseudomonas aeruginosa* ATCC9027, and *Salmonella typhimurium* respectively (Sakr *et al.,* 2021). The anti-staphylococcal activity of glycolipoprotein biosurfactants produced by commercial probiotic strains *Lactobacillus acidophilus* NCFM and *Lacticaseibacillus rhamnosus* GG was evaluated by Nataraj *et al.*

(2021) as they reported inhibitory zones ranged between 11.0±0.0 mm up to 34.0 ± 0.0 mm against *Staphylococcus aureus* ATCC 25923 and MRSA strains depending on the biosurfactants' concentrations used. However, Sriram *et al.* (2011) showed similar results for the antibacterial efficacy of the lipopeptide biosurfactant against Gram-positive and Gram-negative bacteria. *Bacillus megaterium* showed a maximum zone diameter of 18.7 mm, whereas the other Gram-positive bacteria had a diameter of 10 mm. For gram-negative bacteria, a 9 mm diameter halo was measured. Surfactants have been shown to completely defeat pathogenic bacteria. Antimicrobial efficacy has been proven in all main surfactant classes, however not all surfactants exhibit competence, and not all microorganisms are equally vulnerable to their effect. Structural changes in a surfactant type might affect antimicrobial efficiency (Falk, 2019). Biosurfactants have been shown to have antimicrobial activity through a variety of mechanisms, the most common of which include compromising the integrity and permeability of the cell wall or cytoplasmic membrane. More precisely, biosurfactants interact with cell membranes due to their amphiphilic properties and affinity for lipid bilayers, causing cell lysis and metabolite leakage, which leads to cell death (Shu *et al*., 2021). Antimicrobial biosurfactants have been found to be produced by a variety of bacteria, with glycolipids and lipopeptides being the two most common studied families with widespread antimicrobial action (Zhang *et. al.*, 2021). It is important to mention that only a few papers assessed the production and antimicrobial effect of glycoproteins and glycolipopeptides by *Lactobacillus* species (Satpute *et. al.*, 2016).

Conclusion

The current study shows that the identified strain *L. plantarum* LBp_WAM is capable of producing a surface-active biomolecule. The presence of protein, lipid, and carbohydrate fractions of the biosurfactant was confirmed by TLC test and spectral FTIR analyses. SDS-PAGE characterization of the gel-purified molecule revealed a low molecular weight biosurfactant. Along with its significant emulsification activity, this substance may be used as an emulsifying agent for hydrocarbons and oils. Furthermore, the antibacterial activity of this glycolipopeptide against food-borne pathogens was also validated in this work. The results suggested that this biosurfactant might be employed in the medical industry as an alternative antimicrobial agent to combat bacteria that cause diseases and infections, making it a viable alternative to conventional antibiotics.

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Conflict of interest

As for the requirements of the publishing policy, there is no potential conflict of interest for the authors.

Contributions of Authors

A.A.A.: Samples collection, designing and planning the experiments, Revision of Manuscript.

W.A.A.: Samples collection, Methodology, designing and planning the experiments, Revision of Manuscript.

F.H.A.: Samples collection, Revision of Manuscript.

M.Y.A: Methodology, revision of manuscript.
A.K.G: Writing draft manuscript.

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العزل والتوصيف الجزئي للمركب الحيوي الخافض للشد السطحي جلايكولايبوبيتايد المشتق من *Lactiplantibacillus plantarum* العزلة LBp_WAM

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المستخلص: المواد الحيوية الخافضة للشد السطحي هي جزيئات مزدوجة الألفة تنتج بواسطة مجموعة متنوعة من الكائنات الحية الدقيقة للقيام بوظائف بايولوجية مختلفة. في هذه الدراسة تم غربلة بكتيريا حامض اللاكتيك للتعرف على خصائص استحلابها. شخصت العزلة LBp_WAM *Lactiplantibacillus plantarum* جزيئيا باستخدام 16SrRNA وتم التحقق من قدرتها على انتاج الببتيدات النشطة سطحيا. أثبتت النتائج ان المركب الحيوي المنتج من العزلة LBp_WAM *L. plantarum* له القدرة على تقليل التوتر السطحي للماء من 72 ملي نيوتن. م$^{-1}$ إلى 32 ملي نيوتن. م$^{-1}$ عند تركيز مذيلة حرج (CMC) يبلغ 2.4ملغم.مل$^{-1}$. قدرت قيم مؤشر الاستحلاب (E_{24}) لزيت عباد الشمس (60 ± 3.0٪)، الجلسرين (53.9 ± 0.11٪) ، زيت الزيتون (49.0 ± 2.0٪)، الزيوت المعدنية (50.7 ± 0.60٪)، الهكسان (36.03 ± 0.05) ٪)، والكيروسين (31 ± 0.05٪). تمت تنقية المادة الحيوية باستخدام كروماتوجرافيا الترشيح الهلامي (GFC)، وتم تحديد الوزن الجزيئي باستخدام طريقة SDS–PAGE، مما يشير إلى وزن جزيئي تقريبي قدره 19 كيلو دالتون تقريبا. تم استخدام كروماتوغرافيا الطبقة الرقيقة (TLC) والتحليل الطيفي للأشعة تحت الحمراء لتحويل فوريه (FT–IR) لتحديد التركيب الجزيئي للجزيء الذي تم الحصول عليه، والذي وجد أنه يتكون من البروتين والدهون والسكريات. فحص النشاط المضاد للبكتيريا للمركب، حيث أظهر تأثيرات مثبطة ضد طيف واسع من البكتيريا الموجبة والسالبة لصبغة غرام.

الكلمات المفتاحية: نشاط مضاد للميكروبات، المواد الحيوية الخافضة للشد السطحي، FTIR، جلايكولايبوبتايد، *Lactiplantibacillus plantarum*.