Nat. Volatiles & Essent. Oils, 2021; 8(4): 5788-5808

NVEO
Natural Volatiles & Essential Oils

Purification And Characterization Of Levan From Lactobacillus Gasseri And Its Effect Against Pseudomonas Aeruginosa.

Shaimaa M.S. Zainulabdeen[1]

Jehan Abdul Sattar Salmanand KhawlahJebur Khalaf Department of Biology, College of Science, Mustansiriyah University, Baghdad-Iraq Shimaa.majeed@mu.edu.iq

Abstract

Purification and characterization of levan from Lactobacillus gasseri, as well as detection of its antivirulence factors against haemolysin production, pyocyanin production, biofilm formation, and swarming of clinical isolates of Pseudomonas aeruginosa from wounds and burns, are the goals of this study. Thin layer chromatography (TLC),1H Nuclear Magnetic Resonance (HMNR), Fourier transform infrared spectroscopy (FTIR)andScanning Electron Microscopy (SEM), were used for levan characterization. Characterizationproved that pure levan is a homopolysaccharide containing solely fructose, at bond area in 868 cm^{-1} which is typical of carbohydrates with nanosphered structure (24-49) nm. The anti-bacterial activity of purified levanhas been evaluated using the Minimum Inhibitory Concentration (MIC) technique against P.aeruginosa isolates, with concentrations that rising from 1.56 to 400 mg/ml; for all isolates, the MIC was 200 mg/ml. Furthermore, the impactof levan on P.aeruginosa virulence factors was investigated. P.aeruginosa hemolysin production has been reduced after treatment with levan, with inhibition 34.39 %. Pyocyanin production was reduced by levan, with concentrations ranging from 2.30-2.68 µg/ml in comparison to3.84-4.56 µg/ml for the control. Biofilm development was prevented by purified levan at various incubation times (24, 48, and 72 hrs).After 72 hours, the highest level of biofilm suppression had been detected. (86.03%). Also, after 24 h, the greatest inhibition of swarming motility was recoded as 77.27%. Inconclusion, levan purified and characterized from local L. gasserisupressed the develpment and virulent determinants of P. aeruginosa isolated from wounds and burns.

Keywords: L.gasseri, Levan, Anti-bacterial, Anti-virulence

1. Introduction

Levan is the typical form of fructans, in which the fructose chain consists of fructosyl bonds β-(2, 1) and β-(2, 6) respectively, and certain levans derived from microorganisms may have branched structures, fructans are usually produced by organisms as a means of storing energy (Liu et al ., 2017; Ni et al .,2018; Xu et al., 2019).Microorganisms, however, tend not only to make levan as an energy resource (Liu et al., 2017) but also as an essential defense structural component (Benigaret al., 2014).Levan is part of a larger family of polymers known as fructans, which are used as a source of prebiotics and are commercially relevant, a group of enzymes

called levansucrases, which use sucrose as a substrate, catalyzes levan synthesis (Hill et al., 2017; Tezgelet al., 2020). It is a polymer of fructose that a wide variety of microorganisms synthesize from sucrose (Srikanth et al., 2015), its extracellular polysaccharide, non-toxic, biologically active (Khudairet al., 2018). Bacterial levan also has molecular weights above 500,000 Da and is usually branched (Öneret al., 2016).In an aqueous solution, the macromolecular or rather hydrocolloid properties of levan depend primarily on its molecular weight and can be additionally affected by its degree of branching and polydispersity (Hundschellet al., 2020).

P. aeruginosa is a significant human pathogen that causes serious infections (Ali et al., 2017). Furthermore, it is a common multidrug-resistant gram-ve bacterium which tends to cause pneumonia in hospitalized patients and is a common cause of nosocomial infections across the world(Asuphonet al., 2016). P. aeruginosa is a bacterial pathogen related to higher morbidity, mortality, and worse value of living in a number of human illnesses, involving burns, ulcers, and lung damage(Sweereet al., 2020).In wound infection, it's one of the most common pathogens, it's also renowned for forming difficult to remove antimicrobial-resistant biofilms, as well as chronically wounds, consistent infection, severe inflammatory processes, and a significantly delayed healing process are a tremendous burden on patients and healthcare systems around the world (Serraet al., 2015 and Vanderwoudeet al., 2020)

2. Materials and Methods

2.1. Microorganism

2.1.1. Lactobacillus gasseri isolate

Lactobacillusgasserihad been obtained of the vaginal swabs of healthy womenand cultivated anaerobically inMRS medium at 37°C for (24-48) hours. Cultural, cellular, biochemical, and molecular assays, as well as the Vitek 2 system, have been used to identify the isolate.

2.1.2. Pseudomonas aeruginosa

Clinical P. aeruginosa isolatesfrom burns and wounds. The Vitek 2 method, furthermore cultural, microscopic, and biochemical tests, were used to test the isolates.All isolates' virulence factors (hemolysin, pyocyanin, swarming, and biofilm formation) were assessed, the four with the greatest virulence factors were chosen for further step.

2.2. Levan Production

This procedure has been carried out according to Adnan (2018), in which 250ml flasks containing 100ml of levan production medium were inoculated with 4% of selected Lactobacillus spp. suspension containing (9×10^8cfu/ml) (equal to 0.5 ml of McFarland standard absorbance at a wavelength of 600 nm about 0.134) andhad been incubated at 30°C for 24 h.

2.3. Levan's Precipitation

After 24 hours of incubation, cultures had been centrifuged at 10,000 rpmfor 10 min, by mixing two volumes of cold absolute ethanol to the superfluous,levan has been precipitated. The levanwas measured as dry weight.

2.4. Levan Purification fromL.gasseri

To thoroughly eliminate low molecular weight impurities, the precipitated levan polymer had been resuspended in deionized water and dialyzed (cut-off 14 kDa) against running water for at least two days, levan was solubilized in water, the non-solubilized materials have been separated from the supernatant by centrifugation at 10000 rpm/10 min, and 2 volumes of cold absolute ethanol were added to get the material that precipitated in 2 volumes of 50% ethanol, repeat this step with 2 volumes of 75% ethanol, after centrifugation at 10000 rpm for 10 min the separated levan was dissolved in water and dialyzed (cut-off 14 kDa) (Haddaret al., 2021). To determine levan concentration, the O.D. was measured at 400 nm,theequation below has been used (González-Garcinuñoet al., 2017):

" y = 0.1645x −0.035"

Where y has been represented the abs. at 400 nm and x the levan conc. in mg/ml.

2.5. Levan's characteristics Purified from the L.gasseri

2.5.1. Thin Layer Chromatography (TLC)

Using a silica gel covered plate, 0.01 gm of levan purified from L. gasseri had been hydrolyzed in 5% HCl (v/v) and boiled in a water bath at 100oC for an hour. Equivalent weights (0.01gm) of glucose, sucrose, and fructose had been dissolved in 1 ml of 1% ethanol (as standard).Then, capillary tubes were used to spot 10 µl of hydrolyzed levan and various sugar suspensions at identical intervals around 2 cm from the lowest edge of the TLC plate,the plate was moved to a closed jar containing a mobile phase made up of acetontrile, ethyl acetate, propanol, ethanol, and water in a ratio of (8:2:5:2:10) and allowed to diffuse through the silica gel plate, after a duration of time, when the diffusion had reached around 15 cm, the plate was removed from the jar and allowed to dry at room temperature.The dry plate was sprayed with specific reagent composed of ethanol solution containing 0.3% (w/v) α-naphthol and 5% (v/v) H_2SO_4 and put in oven for 5-10 min at 105 ºC. Levan components appeared as dark colored spots (Chung, 2006), The distances of the spots wave been measured, The relative flow (Rf) was estimated according to the instructions given by Radhi et al. (2013):

$$R_f = \frac{\text{Distance moved by substance}}{\text{Distance moved by the solvent front}}$$

2.5.2. Fourier Transform Infrared Spectroscopy (FTIR) analysis

Fourier transform infrared spectroscopy (FTIR)analysishad been performed to determine the functional group deposition of the structure of levan.TheFTIR spectra were recorded in transmittance mode from 4000 to 400cm^{-1}at the Department of Chemistry, college of Science, MustansiriyahUniversity, Iraq.

2.5.3.1 H Nuclear Magnetic Resonance (HNMR) Spectroscopy

Purified levanhad been characterized by using HNMR spectroscopy, by sending the powder of levan to Tehran, Iran**.**

2.5.4. Field Emission Scanning Electron Microscopy (FE-SEM)

By transporting the powder of levan to Tehran, Iran, Field Emission Scanning Electron Microscopy (FE-SEM) has used to characterize purified levan.

2.5.5. Melting Point

In the Chemistry Department/College of Sciences /MustansiriyahUniversity/Iraq, a melting point assessment was done utilizing (Melting Point, Digital, Advanced, SMP30). Load a capillary tube whichclosed at one end with levan to a depth of 2-3 mm, subsequently positioned it in a melting point apparatus and heat it.Thetemperature at which levan becomes completely liquefied was recorded.

2.5.6. Water solubility index

The WSI of levanhas been estimated according to Domżał-Kędziaet al. (2019)technique. To achieve a homogeneous suspension, 200, 150, 100, and 50mg of the sample were dissolved in 5 ml Milli-Q water and agitated for 40 minutes in a water bath at 40 °C. The sample was then subjected to centrifugation at 4000g/10min, the supernatant had been placed on a Petri plate and dried at 105°C/4 hours to produce a dry weight. Finally, the WSI was calculated based on the following equation:

$$WSI\ (\%) = \frac{dry\ weight\ of\ solids\ in\ supernatant}{weight\ of\ dry\ sample} \times 100\%$$

2.5.7. Water-holding capacity

The WHC of pure levanhad been calculated using the Doma-Kdziaet al. (2019) technique.The sample was dissolved in ten ml of Milli-Q water and had been kept at 40°C/10 min. After that, the sample has been centrifuged for 30 min/at 14,000g before the supernatant has been thrown.The pellet was placed on pre-weighed filter paper to eliminate the water. The weight of the precipitated sample has recorded.The proportion of WHC was determined by the equation:

$$WHC\ (\%) = \frac{Total\ sample\ weight\ after\ water\ absorption}{Total\ dry\ sample\ weight} \times 100\%$$

2.6. Anti-bacterial effect of purified levan from L.gasseri against P. aeruginosa isolates

Anti-bacterialactivity of purified levan from a chosen Lactobacillus spp. isolate against P.aeruginosa was evaluated using the microdilution technique in a 96 well flat-bottom microplate titer based on MIC values. In sterilized distilled water, a stock solution of pure levan was diluted to quantities ranging from (1.56-400) mg/ml. In Column 1, 100µl of the levan was mixed with 100µl of Muller Hinton Broth (MHB) and in each well, 50µl of MHB broth was distributed, whereas columns 2-10 only received 50µl of MHB broth. Column 11 held 100µl of diluted standardized inoculum, whereas Column 12 had 100 µl of medium broth. After that, the standardized P.aeruginosa(equivalent to 0.5 McFarland standard) had been diluted in MHB broth by 1:100, and 50µl of the adjusted bacterial suspension was then added to all wells containing levan and to the growth control wells. Incubation of microplates for 24 h /37 °C. Finally, 0.015 percent resazurin dye was applied to all wells (30 µl each well) and incubated for 4 hours to observe color change.The MIC value had been calculated using the column with no resazurin (blue) color change during incubation. Inoculation of the suspension of wells greater than the MIC value on Muller Hinton agar has been used to demonstrate the minimum bactericidal concentration (MBC).When there was no colony growth on the agar plate, the MBC had been recorded(Elshikhet al., 2016).

2.7. Impact of levan purified from L. gasserion Virulence Factors of P.aeruginosa"

2.7.1. Inhibition of Biofilm Formation

Purified levan's antibiofilm action against P.aeruginosa isolates had been measured using a 96-well flat bottom microtiter plate according to the technique published by Salman and Kareem (2021), P.aeruginosa isolates were cultivated at 37°C for (24,48,72) hours with and without of biopolymer levan at sub-MIC concentrations, In each well containing 80µl of sterilized Brain Heart Infusion broth with 2% sucrose and mixing with 100 µl of levan, 20 µl of P.aeruginosa suspension was introduced compared to 0.5 MacFarland,whereas the control contained just 180µl and 20µl of P.aeruginosa suspension, after incubation, the medium was taken from the wells and washed three times with sterile PBS to remove the unattached P.aeruginosa cells and left to dry for 15 minutes at room temperature, the wells were then filled with 200µl of crystal violet (0.1%) and allowed to sit for 20 minutes.To remove unbound dye, the stained wells were washed three times with PBS (PH 7.2) and allowed to dry at room temperature for 15 minutes, finally, 200µl of 95% ethanol was poured to each well, and the optical density was measured using an Eliza reader at 630 nm. The equation was used to determine the percentage of biofilm production inhibition indicated by Namasivayamet al. (2012).

$$\% \text{ Inhibition of biofilm formation} = \frac{\text{OD control} - \text{OD treatment}}{\text{OD control}} \text{ x } 100$$

2.7.2. Hemolysin ProductionInhibition

Lee et al.(2014) used a procedure to evaluate the suppression of hemolysin production.P.aeruginosaisolates have been cultivated at sub-MIC levels with and withoutlevan, in comparison to 0.5 McFarland, 20µl of bacterial suspension had been inoculated to the treatment tube(980µl Nutrient broth + 1000µl of levan), while control tube only (1980µl of Nutrient broth + 20µl ofP.aeruginosa),incubated at 37˚C for 24 h, then at room temperature, 1ml of culture medium (treatment and control) were centrifuged at 7400 x g/10 minutes.Bacterial pellet has been rinsed twice in PBS before being re-suspended.One ml of cell suspensions were added to equal volumes of 8% suspension of RBC and incubated at 37°C/1hr, centrifugation has been used for 10 min at 12,000 rpm, the (O.D) was measured for each control and treatment at 571 nm, then the percentage of hemolysis production was estimated according to Hertleet al. (1999) by this equation:

$$\textbf{Hemolysis}\% = \frac{A_{571}(\text{sample with hemolysin}) - A_{571}(\text{control without hemolysin})}{A_{571}(\text{total lysis caused by Triton X100}) - A_{571}(\text{control without hemolysin})} \times 100$$

2.7.3. Inhibition of Pyocyanin Production

2.7.3.1. Qualtitative assay:

The impact of pure levan on the production of pyocyanin by P.aeruginosa isolates had been identified.Overnight culture of P.aeruginosa isolates had beendiluted1:100 in nutrient broth aftergrowth in nutrient broth and then treated with sub MIC concentration of levan, (1:1) (levan: culture), control included culture broth only . Culture supernatants have been collected after 24 hours of growth at 37°C by centrifugation at 6000rpm/15min, the colour change has been noticed and photographed (Lee et al., 2014).

2.7.3.2. Quantitative assay:

Using the pyocyanin quantitative assay described by Essar et al. (1990), the impact of pure levan on the production of pyocyanin by P.aeruginosa isolates had been evaluated.P.aeruginosa supernatant had been produced by centrifugation at 1000rpm/10min of P.aeruginosa cultured in pepton broth (P.broth) with levan (sub MIC) at 37°C for 24 hours.As a control, the supernatant of bacterial isolates grown on P.broth alone, without levan, was utilized,4.5 ml chloroform was used to extract 7.5 ml of supernatant, which was then agitated by vortex for 10 seconds until the bottom layer became blue.The two volumes of blue layer was then extracted with one volume of (0.2N) HCl and stirred well for 10 seconds to provide a pink to deep red solution.At 520nm, the absorbance of an acidic solution was measured. The concentrations were determined

using the following equation, which is expressed in micrograms of pyocyanin produced per ml of culture supernatant, and the findings were compared between the control and levan treatments.

O.D520 ×17.072= Conc. of Pyocyanin (µg/ml)

The proportion inhibition of pyocyanin production has been determined using the equation below:

$$\%\ \textbf{Inhibition of pyocyanin production} = \frac{\text{conc.of pyocyanin}\left(\mu g/ml\right)\text{control} - \text{conc.of pyocyanin}\left(\mu g/ml\right)\text{treatment}}{\text{conc.of pyocyanin}\left(\mu g/ml\right)\text{control}} \times 100$$

2.7.4. Inhibition of swarming motility

Pseudomonas aeruginosa isolates were inoculated on swarming plates (2g agar and 3.2g nutrient broth in 400ml of distilled water. After autoclaving, 10%D-glucose was added to a final concentration of 0.5%) supplied with purified levan and incubated for 24 h at 37°C (26). The capability to swarm with and withoutlevanhad been determined by the distance of swarming from the central inoculation site, the inhibition of swarming was determined using the equation below:

$$\textbf{\% Inhibition ofswarming} = \frac{\text{distance of swarming(mm)for control} - \text{distance of swarming (mm)for levan treatment}}{\text{distance of swarming(mm)for control}} \times 100$$

3. Results

3.1. Purification of L.gasseri-produced levan

It was decided to purify the levan produced by L. gasseri. At each phase of the purification process, the concentration of levan was calculated. The concentration of levan at each step of purification, with concentrations of (2.0365, 6.4742, 8.6018, 9.8845)mg/ml before precipitation, after precipitation, after dialysis, and after the final purification (Figure 1), respectively.

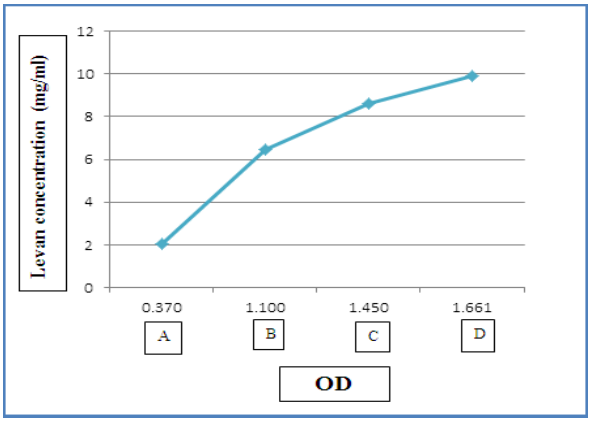

Figure1. The concentration of levanpurified from L. gasseri,A: before precipitation, B: after precipitation, C: after dialysis, D: after last purification.

3.2. Characterization of Purified Levan

3.2.1. Thin Layer Chromatography (TLC)

TLC was used to identify the monosaccharide components of the Levan contents purified by L. gasseri. Fructose Rf value was same or very near to that of acid hydrolyzed levan.Sucrose, fructose, and glucose exhibited Rfs of 0.40, 0.66, and 0.60, respectively, whilst Levan had an Rf of 0.66.This meant that the purified levan from L.gasseri was fructose-based (Figure 2).

Levan Sucrose Glucose Fructose

Figure 2. Thin Layer Chromatographyanalysis of purified levan from L.gasseri, Lane L: Levan; Lane 1: sucrose; Lane 2: Glucose; Lane 3: Fructose

3.2.2. Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups of pure levan from L.gasseri were detected by Fourier Transform Infrared spectroscopy (FTIR) spectrum analysis. The polysaccharide peaks on the purified levan revealed that it was of the polysaccharide type.The hydroxyl (O-H) stretching vibration is represented by the band in the 3275.24 cm^{-1} range, whereas the (C–H) stretching vibration is represented by the band in the 2935.76 cm^{-1} range.C=O stretching is represented by the band area of 1635.69 cm^{-1}, The band areas of 1415.80 cm^{-1}-118.75cm^{-1} reflect C-H plan deformation and aromatic skeleton stretching, respectively, 868.00cm^{-1} has a typical binding area for carbohydrates (identification of polysaccharides)(Figure 3).

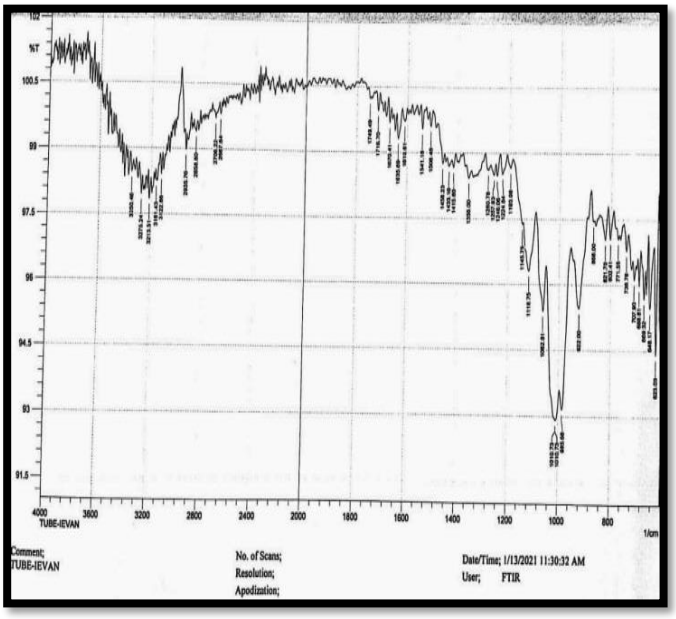

Figure 3. FTIR spectra of purified levanfromL. gasseri

3.2.3. Analysis of 1HNMR

1H spectroscopy adds to our understanding of the levan structure. In the 1H NMR spectra, the proton chemical shift signal corresponding to fructose as a monomer of levanhas been also detected (Figure 4,5).This signal was found at 4.68 ppm (OHg), 4.75 ppm (OHe), 4.62 ppm (OHf), 3.79 ppm (H1c), 3.99 ppm (H1h), 3.63 ppm (2Ha), 3.71 ppm (H1b), and 3.65 ppm (2Hd).

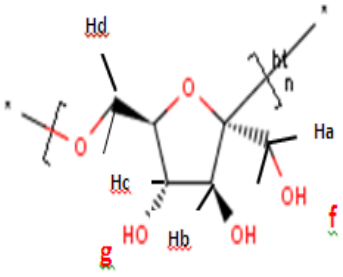

Figure 4. In the current study, a putative structure of pure levan from L.gasserihad provided based on the functional groups of the FTIR results.

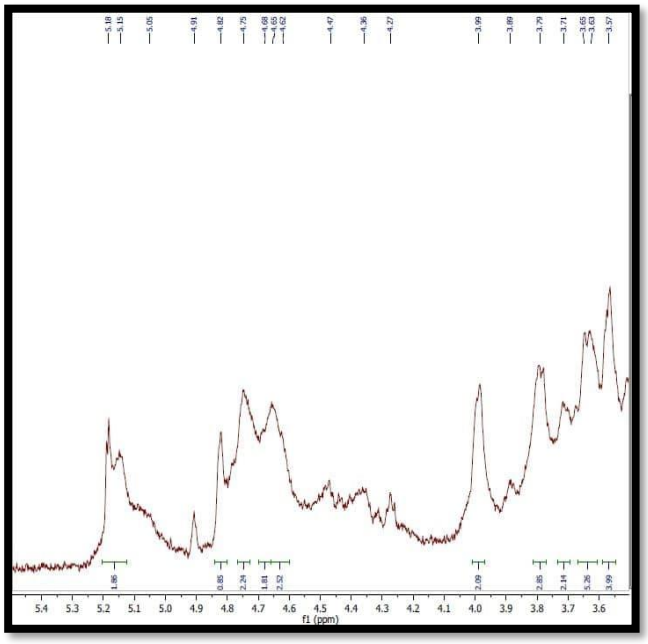

Fig 5. 1H Nuclear magnetic resonance (HNMR) of levanpurified fromL.gasseri

3.2.4. Field Emission Scanning Electron Microscopy (FE-SEM)

Images in Figure 6(a) showed thick, high density and compact fragments of the biopolymer, with different shapes and sizes. These fragments arranged randomly over each other and appeared as multi-layers. However, a vary large-scale image of this biopolymer (Figure 6b) showed that these layers consisted of many clusters of highly organized spherical-nanostructures, with different dimensions ranged from ~24 - 49 nm. These nanospheres have large surface area and low volume, which increased their potential for different applications such as industry, pharmacy and medicine.

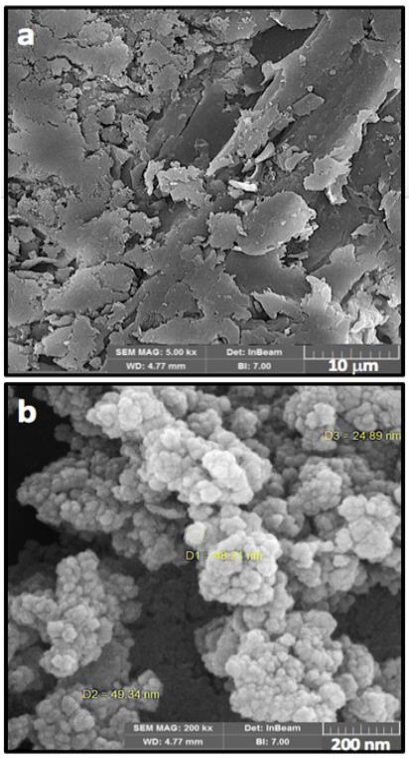

Figure 6.FE-SEM images of the biopolymerlevan purified from L.gasseri.a)shapeand topography of the surface at a scale bar of 10 um, where lots of fragments arranged as multilayers. b)highmagnification image of the biopolymer shows clusters of very organisednanoshepherd structures (~24-49 nm) oriented over each other.

3.2.5. Melting Point

The temperature at which the chemical completely melted had been determined using melting point analysis.It can also offer information on the sample's purity. According to findings, the purified levan from L.gasserihas been completely melted at 236 C^{o}.

3.2.6. Water solubility index and water-holding capacity

The water solubility index has been used to determine a substance's degree of solubility in water (WSI).The water holding capacity (WHC) of a substance is the amount of water it can hold. The results are expressed in terms of how many times the weight of the levan powder could be held in water. For all samples containing pure levan, the WSI and WHC (Table 1) were evaluated. The greatest WSI percentage was 90% at concentration

100mg/5ml, while the lowest WSI was 79% at concentration 200mg/ml, and the highest WHC value was 252 at concentration 50mg/5ml, while the lowest was 150 at concentration 200mg/5ml.

Table 1. Percentage of water solubility index(WSI) and Water holding capacity (WHC).

Levan (mg/5ml)	WSI%	WHC%
200	79	150
150	86.67	196.67
100	**90**	224
50	88	**252**

3.2. Anti-bacterial effect of Purified Levan against P.aeruginosa Isolates

Purified levan from L.gasserihad been used to determine the MIC of levan at concentrations ranged from (400-1.56)mg/ml against P.aeruginosa isolates. As compared to the control, Levan's MIC against all 19 P.aeruginosa isolates evaluated and had been 200mg/ml, with P.aeruginosa(w3), P.aeruginosa(b9), P.aeruginosa(w11), and P.aeruginosa(b14) being the four isolates chosen for subsequent studies based on their virulence factors (Table 2).

Table2. Levan's Minimum Inhibitory Concentration (MIC) against P.aeruginosa isolates.

Bacterial isolates	Levan Concentration (mg/ml)									
	400	200	100	50	25	12.5	6.25	3.12	1.56	Control
P. aeruginosa (w3)	-	-MIC	+	+	+	+	+	+	+	+
P. aeruginosa (b9)	-	-MIC	+	+	+	+	+	+	+	+
P. aeruginosa(w11)	-	-MIC	+	+	+	+	+	+	+	+
P. aeruginosa (b14)	-	-MIC	+	+	+	+	+	+	+	+

"-" no growth of bacteria; "+" growth of bacteria; "Control" Muller Hinton broth only; "b" isolate from burns; "w" isolated from wounds; " MIC " Minimum Inhibitory Concentration

3.4. Impact of purified Levan on virulence factors of P.aeruginosa isolates

3.4.1. Inhibition of Biofilm Formation

The formation of biofilms in P.aeruginosaisolated from burns and wounds was suppressed by purified levan from L. gasseri.Levan inhibited biofilm formation in all P.aeruginosa isolates from burns and wounds, according to the findings, in comparison to the control, biofilm formation in P.aeruginosa isolates had been reduced at different incubation periods (24,48,72hrs).P.aeruginosa (w11) isolated from wounds had a high inhibition percentage of 72.46 percent for biofilm development after 72 hours, while P.aeruginosa (w3) isolated from wounds had a low inhibition percentage of 27.16 percent at 24 hours (Table 3).

Table 3: Purified levan from L. gasseri inhibits biofilm formation by a percentage in different incubation times.

Bacterial isolates	Biofilm inhibition by Levan(100 mg/ml)		
	Incubation period		
	24h	48 h	72h
P.aeruginosa(w3)	27.16	41.79	48.68
P.aeruginosa(b9)	51.95	68.43	86.03
P.aeruginosa(w11)	28.92	69.55	72.46
P.aeruginosa(b14)	33.65	62.30	67.56

3.5.2. Inhibition of Hemolysin Production

It was discovered that levan purified from L.gasseri inhibited the production of hemolysin by P.aeruginosa isolated from burns and wounds. In this study, Levan had an influence on the production of hemolysin in P.aeruginosa isolates, hemolysin production was reduced in the tested P.aeruginosa isolates, compared to the control percentage (91.22%) of hemolysis, P.aeruginosa(w3) isolated from wounds inhibited hemolysis with a high proportion of 67.75%, while P.aeruginosa (b14) isolated from burns had a low inhibition percentage of 59.19% of hemolysis (Table 4).

Table (4): Percentage of hemolysiswith and without purified levan from L.gasseri.

Bacterial isolates	% Of hemolysis without levan	%Of hemolysis with levan

P. aeruginosa (w3)	91.22	67.75
P. aeruginosa (b9)	87.59	62.98
P. aeruginosa(w11)	90.65	63.93
P. aeruginosa(b14)	90.17	59.16

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3.5.3. Pyocyanin Production Inhibition

Purified levan from L. gasserihas shown to decrease the production of pyocyanin in all P.aeruginosa isolates tested in this research. In comparison to control, after being treated with pure levan, the concentration of pyocyanin produced by P.aeruginosa isolates has been reduced (sub MIC) (Fig7).P.aeruginosa isolated from burns and wounds had inhibition percentages of 41.23% and 40.10%, respectively, whereas P.aeruginosa isolated from wounds had inhibition percentages of 39.19% and 43.84% (Table 5).

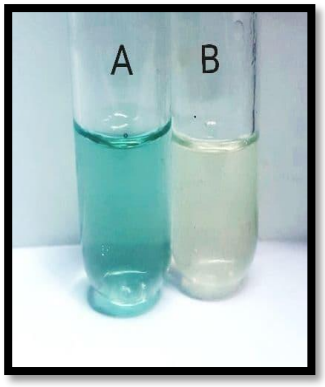

Fig 7.P.aeruginosa pyocyanin production inhibitionby purified levan from L.gasseriA- control without purified levan, B- after treatment with purified.

Table 5. Inhibition pyocyanin production byPurified Levan from L. gasseri.

Bacterial isolates	**Pyocyanin concentration(µg/ml)**		
	Without levan Control	With levan Treatment	% Reduction of pyocyanin con.
P.aeruginosa(w3)	3.93	2.39	39.19
P.aeruginosa(b9)	4.56	2.68	41.23
P.aeruginosa(w11)	4.22	2.37	43.84

P.aeruginosa(b14)	3.84	2.30	40.10

3.5.4. Inhibition of swarmingmotility

Swarmingmotility is necessary for biofilm formation and antibiotic resistance, so it is considered a virulence factor (Ugurluet al., 2016).All four tested were showeddecreasedin theirswarming after grown in the present of levan. For P.aeruginosa isolated from burns, the inhibition percentage had been 75% and 77.27%, respectively, whereas for P.aeruginosa isolated from wounds, it was 53.33%and 70.58% (Figure8).

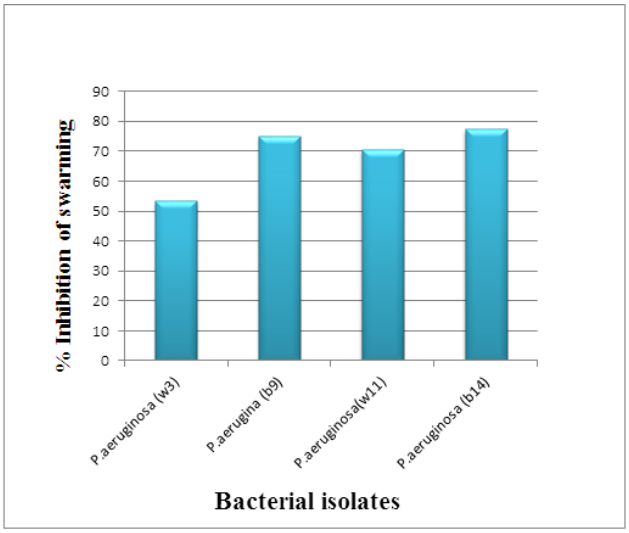

Fig 8. Inhibition of swarming by purified levanfrom L.gasseri.

4. Discussion

Levan is a polysaccharide made up of (β2→6)-linked fructofuranosyl residues that has branched through (β2→1) linkage. It's a water-soluble polysaccharide that bacteria may make by using levansucrase and sucrose as fructose donors in a transfructosylation process (Ni et al., 2018; Haddaret al.,2021).To analyze the functional groups and components of pure levan, in this research levan had been isolated and characterized from the local probiotic bacteria L.gasseri.Furthermore, from sucrose medium, L.gasseri generated levan comprised entirely of fructose, indicating that the Lactobacillus isolate contained levansucrase.FTIR and HNMR analysis revealed that the fructan is a levan composed of consecutive β-(2,6) fructopyranose units.Salman et al. (2019) revealed that the Rf value of acid-hydrolyzed levan from Leuconostocmesenteroideswas similar to that of fructose, and When thin-layer chromatography(TLC)had been used to examine levan, it revealed that it was entirely made up of fructose, similarly, Al-Qaysiet al. (2021) reported that levan produced by P. agglomerans ZMR7 and exposed to acid hydrolysis and TLC is fully made up of fructose.

Salman et al. (2019) revealed that O-H vibration stretching occurred at a wavelength of about 3271.26cm^{-1}, whereas C-H stretching showed at a wavelength of around 2932.40cm^{-1},according to the FTIR spectra study of levanpurified from L.mesenteroides, C=O stretching is characterized by a peak at 1634.83cm^{-1}.FurthermoreGamal et al. (2020) employed FTIR to analyze levan produced by Enterococcus faecalis and revealed that the absorptions at 1070.390 and 1271.82 cm^{-1} had been corresponded to pyranose sugars, and Koranyet al. (2021) indicated that FTIR analysis confirmed the existence of stretching vibration bands of CH_2-OH and CH-OH in the levan purified from Pseudomonas fluorescens, at 1010 cm^{-1}, which was detected in polysaccharides, furthermore had been found peak near (844 and 891 cm^{-1}).

According to Koranyet al. (2021) study, determined the 1HNMR spectra of P. fluorescens revealed proton chemical shift signals at 4.11 ppm (H-3), 4.02 ppm (H-4), 3.86 ppm (H-5), 3.82 ppm (H-6a), 3.69 ppm (H-1a), 3.59 ppm (H1b), and 3.48 ppm (H-6b), indicating the presence of sugar protons at δ4.11 ppm (H-3), 4.02 pp.FurthermoreAğçeliet al. (2020) performed using 1 HNMR analysis for levan from P.mandelii. The protons in the 1 HNMR spectrum are between 3.2 and 4.2 ppm, suggesting that the levan type is present, with the fructofuranoside linkage (2→6), in addition to using Scanning Electron Microscopy (SEM) analysis to observe the microstructure of the levan from P.mandeliithat showed an irregular structure of levan.

Pei et al. (2020) observed a compact structure of levan constituted of nonuniform block sizes after SEM analysis, and demonstrated that it also have high WHC (231.29 ± 3.76%) and WSI (97.34 ± 1.72%)

The antibacterial and antivirulence activities of levan purified from L.gasseri against P.aeruginosa isolated from burns and wounds were determined in this research, including biofilm formation, pyocyanin production, swarming motility, and hemolysin, and it may inhibit the generation of virulence factors mediated by quorum sensing, according to our findings.

On microbial pathogens, the levan has been discovered to exhibit antimicrobial and antibiofilm properties (Ağçeliet al., 2020), it displays significant inhibitory effect against the New Castle disease virus in general, and in particular, suggesting a prospective therapeutic use (Gamal et al., 2020).

The polysaccharide polymer's inhibitory impact might have been caused by changes in fluidity, which could also enhance membrane permeability (Wu et al., 2016). Metabolic dysfunction, energy production inhibition, and cell death had been occurred from the decrease of cell membrane permeability(Zou et al., 2015). Entrapment of cation or nutrients, which can impair nutritional bioavailability, is another explanation for the inhibitory influence of many polysaccharides (Jun et al., 2018).

The pure levan from Enterococcus faeciumdemonstrated promising antibiofilm action against Listeria monocytogenes, S. enterica, S. typhi, and Vibrio anguillarum(Tilwani,et al., 2021).L.coryniformis EPS inhibited the development of Bacillus cereus and S. typhimurium biofilms by around 80% and 40%, respectively (Xu et al., 2020).

Song et al. (2020) investigated the influence of pure EPS from L. plantarum12 on S. flexneribiofilm formation and growth, furthermore the primary active component's effects on S. flexneri biofilm development.AlsoLiu et al. (2017)reported thatthe pure exopolysaccharide from L. plantarum inhibited harmful bacteria such as P. aeruginosa, E. coli, S. typhimurium, and S. aureus from forming biofilms.

Lactobacillus polysaccharide acts as a signal, causing many genes involved in biofilm formation to be down-regulated(Rendueleset al., 2013). P.aeruginosa lectin-dependent adhesion or sugar-binding proteins on the bacterial membrane, along with fimbriae and pili tipped adhesins, may indeed be inhibited by the polysaccharides (Zinger-Yosovich and Gilboa-Garber, 2009).

In this study, Levan purified from L.gasserihas shown to exhibit antibiofilm activity against P. aeruginosa isolates.Many Candida albicans virulence factors were suppressed by the pure levan produced by L. mesenteroides, according what had reported by Salman and Khudair (2019).Salman and Kareem (2021) also had been observed that the biopolymer dextran purified from L. gasseri prevented the formation of biofilms and the production of virulence factors by P. aeruginosa.

5. Conclusion

In conclusion, P.aeruginosa isolated from wounds and burns had been inhibited by pure levan from local L. gasseri, as well as certain virulence factors. Also it can self-assemble into nanoparticles in water, making it an important encapsulation agent and can be used in the pharmaceutical industry.

Acknowledgments

The authors would like to express their gratitude toMustansiriyah University in Iraq(www.uom ustansiriyah.edu.iq) because of assistance with this work.

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