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RESEARCH ARTICLE

The Role of Levan Produced from *Pseudomonas fluorescens* as Antipathogenic Substances and Phagocytic Enhancer against Some of Bacterial Isolates

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Abstract

Levan is a natural substance that produced from many organisms, levan recognized as a homopolysaccharide with multi-functional features and a wide range of potential industrial applications. *Pseudomonas fluorescens* had the capacity to produce levan in the total absence of air which exhibited broad antibacterial spectra against foodborne pathogenic bacteria. The results showed that three isolates of bacterial isolates were classified as a member of *Pseudomonas fluorescens* with antibacterial activity when tested against various pathogenic bacteria; levan has antibacterial activity against *Staphylococcus. aureus, E.coli* and *Listeria* but it had no activity against *Salmonella typhi*, the results showed that levan increased phagocytic activity, The phagocytic index against *S.aureus* increased in the presence of levan in compared with control (without levan). The phagocytic index was (65%) when used levan in concentration (200µg/ml|) while the phagocytic index was (40%) in control (without levan).

Keywords: *Levan, Antimicrobial activity, FT-IR, Pathogenic bacteria, Phagocytic index.*

Introduction

Levan is a homopolysaccharide which is composed of D-fructofuranosyl residues joined by 2, 6 with multiple branches by 2, 1 linkages. Microbial levan is extracellularly produced from sucrose-based substrates by levansucrase that catalyses levan biosynthesis by transferring fructose on sucrose donor to acceptor molecule. Levan can be produced either from plants or microorganisms. Plant levan also called as phelins, have much lower molecular weight than bacterial levan [1]. It is recognized that levan is a homopolysaccharide with multi-functional features and a wide range of potential industrial applications. Many research works attribute levan a variety of potential applications in diverse fields like: medical, chemical, pharmaceutical, cosmetics and food industries [2].

Pseudomonas fluorescens is an obligate aerobe and Gram negative bacterium that can use oxygen as well as nitrate as a hydrogen acceptor. This nitrate-reducing strain can produce levan in the total absence of air [3].The antibacterial activity of levan compounds, including high-molecular weight levan, low-molecular-weight levan and difructosedianhydride IV (DFA IV) was studied by [4]. The levan exhibited broad antibacterial spectra against foodborne pathogenic bacteria. The strongest in vitro inhibitory effect was observed with low-molecular-weight levan and followed by the other tested levan compounds [4]. It was assumed that levan may have two different modes of inhibitory action: induction of osmotic stress and/or reduction in water activity and competitive interference with bacterial absorption of an essential nutrient (s) [5]. Phagocytosis is the primary function of macrophages, which leads to enhance a diverse range of antimicrobial and cytotoxic responses including generation of respiratory bust, secretion of inflammatory mediators and antigen presentation.

The effects of levan and Di-D-fructose-2, 6':6, 2'-dianhydride (DFA-IV) DFA-IV on phagocytosis were studied by [6, 7] when RAW264.7 cells treated with various concentrations of DFA-IV or levan in the same concentration range (1～100 µg/mL) for

24 h, phagocytic activity was significantly increased but levan had no effects on phagocytic activity of macrophages at low concentrations (1 µg/mL) [8].The objective of this study was to investigate the antibacterial activity and phagocytic activity of levan that produced from local isolate of *Pseudomonas fluorescens*.

Materials and Methods

Collection of Samples

Thirty two of different food samples were collected from different local markets in Baghdad governorate in sterile container and transported to the laboratory until using.

Isolation and Identification of Bacteria

For food Samples, suspension was prepared by adding 1g (dry weight) of each food sample in 10ml of sterile distilled water, and mixed well. Flask containing 100ml of selective liquid mineral salt medium, consisted of (1gm KH_2 PO_4)1gmK_2PO_4, 1gm NH_4No_3, 1gm $(NH_4)_2So_4$, 0.2gm $MgSo_4.7H_2O$, 0.5gm NaCl and 0.5gm $FeSo_4.7H_2O$), all these components were dissolved in 900ml of distilled water and 1 ml from trace element solution consisted of (0.23gm $Znso_4.7H_2O$, 0.18gm $MnSo_4.5H_2O$, 0.1gm $CnSO_4.5H_2O$) all these components were dissolved in 100ml of distilled water was added [9].

The culture was incubated in a shaker incubator at 120 rpm at 35°C for seven days, then in order to gel separated colonies, a loop full of the broth culture mentioned above was transferred to inoculate the nutrient agar (dispensed in sterile petri dishes) using ABC streaking method, then incubated at 35°C for seven days. The process was repeated several times to get pure culture [10]. The isolated bacteria were purified by inoculating then on plates containing nutrients medium. The bacteria were purified by repeated inoculation.

After ensuring purity, the cultures were sub-cultured on nutrient slants and allowed to grow for a period of 24 hrs and subsequently stored at 4°C as stock cultures were transferred to fresh nutrient slants at regular intervals of 3 months. *Pseudomanas* isolates were identified by morphological features, microscopic examination and biochemical test.

Detection of Levan Produced Bacterial Isolates

The isolated bacterial colonies on Nutrient agar plates were transferred to YPS agar plates (1% yeast extract, 2% peptone and 5% sucrose), and then incubated at 30°C for 24hrs. Bacterial colonies with glutinous were selected and incubated in YPS broth medium and incubated for 48 hrs at 30°C.

Isolation and Purification of Levan Produced from Bacterial Isolates

For the preparation of levan from bacterial isolates, cells with glutinous appearance were taken and grown in the YPS broth media incubated for 48 hrs at 30°C, after incubation period, the culture fluids were collected by centrifugation. Three volumes of cold ethanol were added to culture supernatant, and the mixtures were mixed by vortex for 1 min and then centrifuged 8,000 rpm for 10 min.

The precipitated materials were re suspended in water and mixed again with three volumes of cold ethanol. Samples were collected by centrifugation, and the procedure was repeated. The amount of levan was determined by measuring the final dry weight of the pellets after centrifugation [11].

Analysis of Levan by Fourier Transform Infrared Spectroscopy (FT-IR)

To complete the diagnosis of levan, Fourier Transform Infrared (FTIR) spectroscopy was carried out to identify the functional groups, Each 1 mg dried sample was mixed with 200 mg of KBr (Spectranal) and pressed under vacuum to form thin tablet. The tablet was immediately analyzed with a spectrophotometer [12].

Determination the Role of Levan as Antipathogenic Substances [13]

The antipathogenic activity of levan (10mg/ml) was examined against some of gram positive and gram negative bacterial isolates by agar well diffusion method as following:

- Gram positive bacteria (*Staphylococcus aureus* and *Listeria spp*.) and gram negative bacteria (*Salmonella typhi*, *E. coli*) were inoculated in test tube containing 5 ml of nutrient broth and incubated at 37° C for 18 hrs.

- Decimal dilutions were prepared from both gram positive and gram negative bacterial isolates to obtain dilution similar to the turbidity of Mcfarland tube.
- About 200µl of bacterial dilutions were spread on Muller- Hinton agar plates by using sterilized swabs.
- Two wells were done with sterilized cork borer on Muller- Hinton agar plates.
- One of the wells was filled with 200µl of levan product and the other well was filled with200µl of sterilized D.W. as control.
- The plates were kept in refrigerator for 10 min. for diffusion of compounds.
- The plates were incubated at right side at 37^0C for 18 hrs.
- The inhibition zones around the wells were measured in (mm).

Determination the Activity of Levan as Antiphagocytic Enhancer against *Staphylococcus aureus*

Preparation of *Staphylococcus aureus* Suspension

Brain heart infusion (BHA) agar plate was prepared and inoculated with *Staphylococcus aureus*, incubated at 37^0C for 18 hrs, after incubation period 50 ml of Brain Heart Infusion broth (BHI) was inoculated with a single colony of *Staphylococcus aureus*, incubated at 37^0C for 24hrs. Bacterial cells were collected by centrifugation at a 3000 rpm for 10 min., washed twice with D.W., cell were harvested and suspended in D.W. [14].

Preparation of Levan Suspension

Levan was dissolved in normal saline to obtain concentration (250µg / ml**).**

Blood Sample Collection

Sterilized single using syringe was used to obtain blood from the vein; blood was collected in anticoagulant tube, this blood was used to calculate the percentage of phagocytic cells.

Calculation the Phagocytosis index

Phagocytosis index was calculated against *Staphylococcus aureus* before and after supplement blood sample with levan suspension according to Furth *et al* [15]. As in the following:

- A volume of 250 µl of blood was mixed with 250 µl (1×10^8 cell/ ml) of *Staphylococcus aureus* suspension before and after the additional of levan suspension to blood sample.
- The mixture was incubated for 30 minutes in 37^0C, and mixed every 5minutes.
- Smear of mixture was done by putting one drop of mixture on a glass slide by Pasture pipette, left the slide to dry.
- One drop of absolute methanol was added to fix the mixture.
- One drop of Leishman stain was added, left for 15minutes, after that, washed the slide with D.W, left it to dry, and examined under the microscope.
- Phagocytosis index was calculated by counting 200 phagocytic and non-phagocytic cells.

$$\text{Phagocytosis Index} = \frac{\text{Number of phagocytic cells}}{\text{200 Phagocytic and non-phagocytic cells}} \times 100$$

Statistical Analysis

Using analysis of variance, F-test, t-test. In complete randomized design to explain the differences between parameter means using least significant differences(LSD)at $P\leq 0.05$,and expressed that as (mean ±SEM).

Result and Discussion

Isolation and Identification of Pseudomonas

Three isolates of bacteria were obtained from

Thirty two samples of food, after culturing in liquid mineral salt selective medium. The growth characteristics of this isolated on *Pseudomonas* selective medium indicated that this isolates were classified as a member of genus *Pseudomonas* [9].

When these isolates were further identified by morphological and biochemical tests, the

result showed that they were identified as strains of *Pseudomonas fluorescens* (Table 1) [16].

Table 1: Tests for identification of *Pseudomonas fluorescens*

Test	Result	Test	Result
Indole test	-	Pigment production	+
Acid production	+	Utilization of tryptophan	v
Gas form Nitrate	v	Oxidase	+
Growth at 42^{0}C	-	Arginine dihydrolase	+
Motility test	+	Pectolytic activity	v
O/F mannitol	v	Denitrification	+
O/F maltose	v	Gelatine hydrolysis	+

Note: (-) mean Negative, (+) mean Positive, (v) mean Variable

Detection of Levan Produced *Pseudomonas fluorescens* Isolates

The colonies with the glutinous appearance on production medium agar (YPS) were harvested for further screening of levan production in YPS broth medium. It was found that two *Pseudomonas fluorescens* isolates had the highest glutinous appearance on solid medium, there were selected and underwent further screening in YPS broth medium. The highest levan dry weight was (6.85 mg/100ml), it was extracted from the more glutinous isolate.

Isolation and Purification of Levan produced from *Pseudomonas fluorescens*

Levan was extracted from the production medium (YPS broth) with three volumes of 95% cold ethanol; the amount of levan was established by measuring the final dry weight of the precipitate after centrifugation. Levan appeared as off white powder and the dry weight was 6.85mg/100 ml. Ethanol decreased the solubility of polysaccharide thus clearing their separations from the production media [17]. Ethanol used several times in the processing of extraction of levan from the production medium for more purification of product [18].

Analysis of Levan by Fourier Transform Infrared Spectroscopy (FT-IR)

The functional groups of this biopolymer was determined by FTIR Spectroscopy which it was (C-O, C-H, O-H and C=O). C-H and O-H group ending in (1223.85cm^{-1} and 1450.03 cm^{-1}), C-H stretching in (2819.73cm^{-1}and 3000.01 cm^{-1}), O-H stretching in (3390.63cm^{-1}and 3461.99 cm^{-1}), C=O and C-O stretching group in (1649.02 cm^{-1}and 1114.78 cm^{-1}) respectively Figure (1).

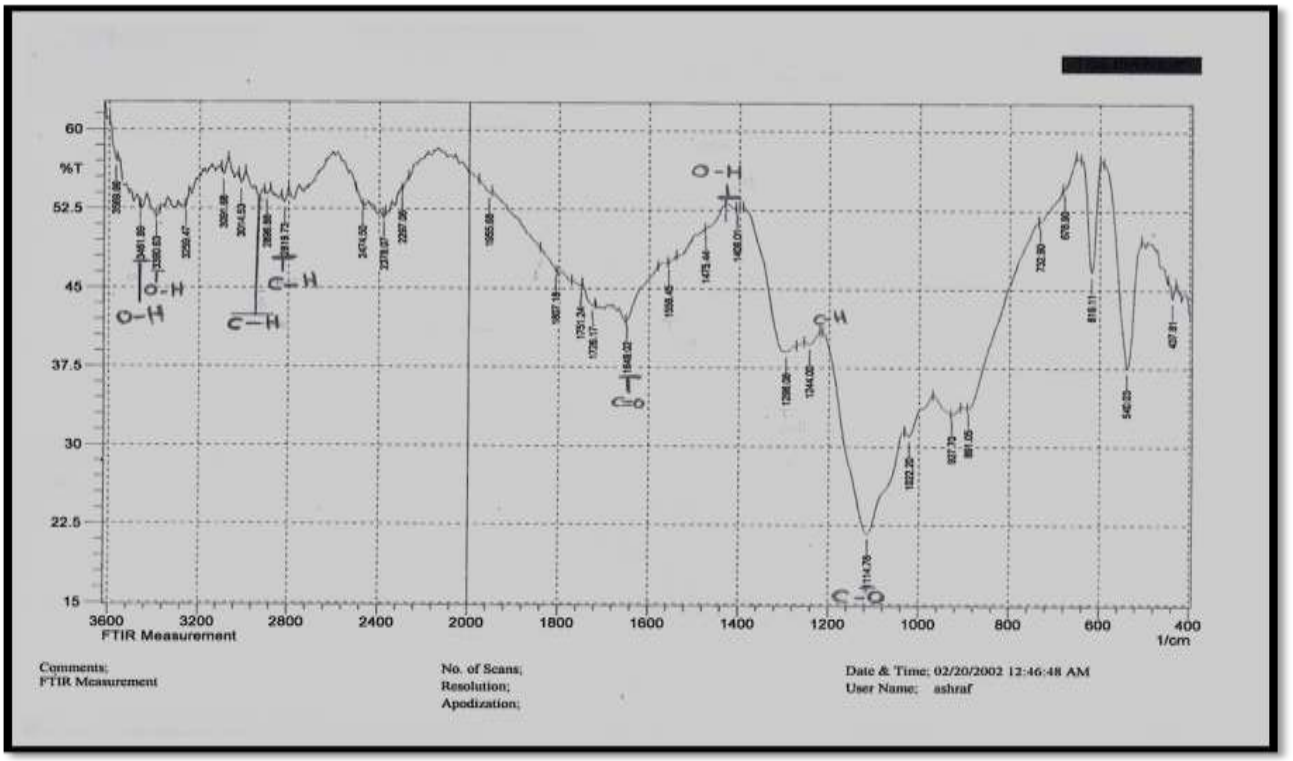

Figure 1: FT-IR analysis of levan produced from *Pseudomonas fluorescens*

The absent of functional groups present in nucleic acid, protein and lipid structure from the levan structure evidence that the basic units of levan was carbohydrate [19].

Antibacterial Activity of *Pseudomonas Fluorescens* Levan against Some Pathogenic Bacterial Species

The antibacterial activity of Levan produced from *Pseudomonas fluorescens* was tested against various pathogenic bacteria frequently involved in food borne diseases worldwide using agar well diffusion method. As shown in Figure (2). levan has antibacterial activity against *Staphylococcus. Aureus, E.coli* and *Listeria* but it had no activity against *Salmonella typhi*. The diameters of inhibition zones were 37mm against *S.aureus*, 28.75mm against *E.coli* and 21.25mm against *Listeria* as shown in Table (2) and Figure (3).There are significant differences between different pathogenic bacteria and *S.aureus* was the most sensitive bacterial isolates and *Listeria* spp. the least sensitive while *Salmonella typhi* not affected completely.

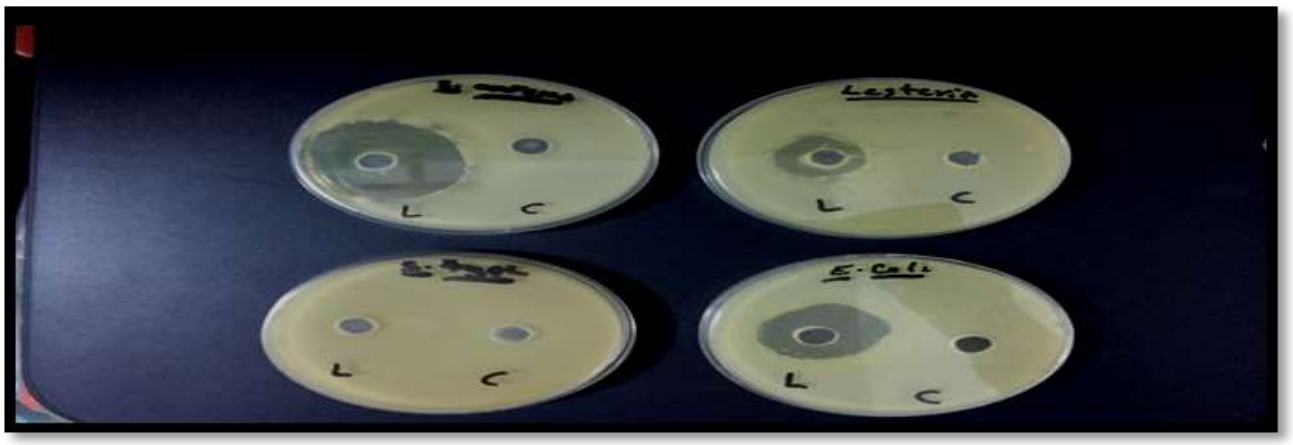

Figure 2: Antibacterial activity of levan against some food borne bacterial pathogens

Table 2: Antibacterial activity of levan against some bacterial pathogenic species

Bacteria	Mean	±	SEM
S.aureus	37.00 a	±	1.78
E.coli	28.75 b	±	1.49
Listeria spp	21.25 c	±	2.50
LSD (P ≤ 0.05)		**6.30**	

Small letter s indicate to comparison in column, similar letters are non-significantly differences between means at (p≤ 0.05), Using (LSD test)

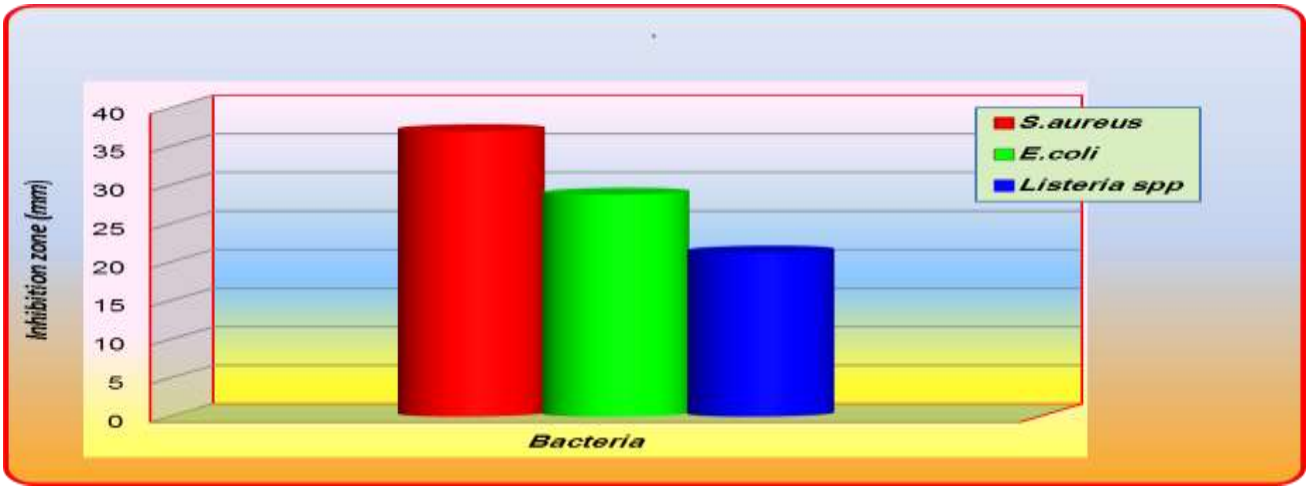

Figure 3: Antibacterial activity of levan against selected pathogenic bacteria

Levan have antibacterial compounds, including high-molecular weight levan, low-molecular-weight levan and difructosedianhydride IV (DFA IV), levan exhibited broad antibacterial spectra against foodborne pathogenic bacteria. The strongest in vitro inhibitory effect was observed with low-molecular-weight levan and followed by the other tested levan compounds. The inhibitory action may be due to induction of osmotic stress and/or reduction in water activity and competitive interference with bacterial absorption of an essential nutrient (s). (4)

Effect of levan on Phagocytic Activity

Human blood cells were treated with levan in order to enhance their phagocytic activity. Phagocytic index against *S.aureus* increased in the presence of levan in compared with control (without levan). The experimental result showed that the phagocytic index was (65%) when used levan in concentration (200µg/ml|) while the phagocytic index was (40%) in control (without levan), this results indicated that levan increases the phagocytosis of phagocytic cells and this may be due to levan reacted with an intense polymorphonuclear (PMN) infiltration followed by accumulation of vacuolated macrophages, levan was found to induce morphological [21] and functional changes in macrophages. It has been further shown that levan is an immunological active agent; it could change the immune reaction of the host [22]. High-mol-wt levan injected locally inhibits the growth of Lewis lung carcinoma in C57BL mice [5]. levan has significantly greater effects on tumoricidal activity than Di-D-fructose-2,6,6,2-dianhydride (DFA-IV) at low concentrations (1µg/ml) [23].

Conclusion

This study investigation the antibacterial activity and phagocytic activity of levan that produced from local isolate of *Pseudomonas fluorescens*. Levan showed antibacterial activity against *Staphylococcus. Aureus, E.coli* and *Listeria* but it had no activity against *Salmonella typhi* also levan enhance phagocytic activity of Human blood cells. Phagocytic index against *S.aureus* increased in the presence of levan in compared with control (without levan).

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