Original article

# Partial characterization of levan polymer from *Pseudomonas fluorescens* with significant cytotoxic and antioxidant activity

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ABSTRACT

Microbial levan has great potential as a functional biopolymer in different fields including foods, feeds, cosmetics, and the pharmaceutical and chemical industries. In this study, a good levan producer bacterial strain of *Pseudomonas fluorescens* strain ES, isolated from soil in Egypt in a previous study, was used. Levan production by this strain was optimized using Plackett-Burman experimental design (PBD) to screen the critical factors of several process variables and Centered Central Composite Design (CCD) was applied for further estimation of the relationship between the variables and the response as well as optimization of the levels. Plackett-Burman (P-B) design showed a p-value 0.0144 less than 0.05 indicated the significance of the model. Sucrose, potassium dihydrogen phosphate, yeast extract and pH value showed the most significant effect on levan concentration at the values of 89.17, 65.83, 24.17, and 15.83, respectively. The purified levan polymer was characterized using different Physico-chemical methods such as Fourier Transform Infrared Spectrometer (FTIR), Nuclear magnetic resonance (NMR), and High-Performance Liquid Chromatography (HPLC) to determine the main composition and functional groups in the obtained polymer. HPLC results indicated that the polymer purification increased the percentage of fructose residue from 75 up to 89. Furthermore, $^1$H and $^{13}$C NMR spectroscopy analysis showed great matching between the obtained signal for our polymer with that reported in other people's work. The obtained levan polymer exhibited cytotoxic activity against Human epidermoid Skin carcinoma and Hepatocellular carcinoma with IC50 of 469 and 222.7 μg/ml, respectively. Antioxidant activity was determined using DPPH assay and the percentage of inhibition at 1000 μg/ml was found to be <50 (13.89 ± 1.07) with IC50 of (24.42 ± 0.87).

## 1. Introduction

Exopolysaccharide (EPS) is a high molecular weight polymer comprising various sugar molecules introduced externally into the medium by certain organisms (Rehm, 2009). Recently, some bioactive polysaccharides have been isolated from medicinal plants (Zhang et al., 2020). EPS are also produced by microorganisms (Nouha et al., 2018) such as lactic acid bacteria with unique

physical and chemical applications in the pharmaceutical industry and food (Hooshdar et al., 2020). Also, fungi and algae have been reported to be considered as a faster alternative source of EPS in comparison to chemical and plant-derived sources (Barcelos et al., 2020). Furthermore, these polysaccharides polymers, are classified as environmentally friendly substances, which are also called "the sleeping giant of biotechnology" (Esawy et al., 2012; Freitas et al., 2011).

Levan is counted as one of the most encouraging microbial polymers, it is one of the two main types of fructan polysaccharide (Srikanth et al., 2015), it is a neutral homopolysaccharide consisting of fructofuranosyl residues linked by β-(2,6) as the backbone chain with some β-(2,1) branching points (Venugopal, 2011) which has been gained major research attention for its biomedical and efficient food properties because of characteristics like bioactivity and biodegradability. Microbial levan is produced in the medium by extracellular levansucrase (EC: 2.4.1.10) using sucrose as a substrate. Diversity of bacterial genera such as *Erwinia*, *Bacillus*,

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*Acetobacter*, *Pseudomonas Paenibacillus, Zymomonas, Halomonas,* and, *Streptococcus* are capable of producing levansucrase enzyme (Gojgic-Cvijovic et al., 2019).

Quantitative and qualitative determination of levan can be obtained by using usual methods for carbohydrate polymer estimation but the variations in molecular weight and particle size of levan polymer are known to be mainly due to the microbial source (Öner et al., 2016). Therefore, to explore this polysaccharide's full potential from novel sources, a more detailed analysis of the construction and functional groups is needed (Gojgic-Cvijovic et al., 2019). The production process of levan is influenced by various factors such as carbon and nitrogen source, fermentation period, inoculum size, initial pH (Srikanth et al., 2015). Along with the sources of phosphates, temperature, agitation rate, oxygen content, and chemical composition of the fermentation media all affect levan yields to different extents (Sarilmiser et al., 2015). Experimental design techniques have become an effective alternative method to one-variable-at-a-time (OVAT) for understanding and analyzing interactions between different physiological parameters interrupted within levan production in the cultivation medium (Razack et al., 2013).

Moreover, characterization techniques that determining the physical and chemical structure, morphology, molecular weight, mechanical, thermal properties, and few other chemical properties of levan are important before introducing it in any application (Srikanth et al., 2015). Many techniques have been applied for the characterization of levan polymers such as Fourier Transform Infrared (FT-IR), Nuclear Magnetic analysis (^{1}H and ^{13}C NMR spectroscopy) (Dahech et al., 2012), and other physiochemical techniques. Therefore, the unique hysico-chemical features of levan such as low toxicity, ocular non-irritant, low viscosity, and heat stability made it superior in a lot of commercial industrial along with the medical field as an anti-inflammatory, antioxidant, anti-AIDS anti-carcinogenic and hyperglycemic inhibitory (Srikanth et al., 2015).

It has been reported that *P. fluorescens* isolate was used for fabrication of levan polymer after the formation of the characteristic appearance of levan polymer on screening sucrose medium (Srikanth et al., 2015). This investigation was carried out to statistically optimize the different physiological parameters as well as the cultural condition for levan production by the good levan producer *P. fluorescens* strain ES using Plackett-Burman design followed by Central Composite Design (CCD) as the second step in the optimization process. Characterization of levan polymer was also carried out using different chemical and physical analyses such as Fourier Transform Infrared Spectrometer (FTIR), Nuclear magnetic resonance (NMR) spectroscopy, and High-Performance Liquid Chromatography (HPLC). Finally, the biological activity of levan polymer was demonstrated by studying its cytotoxic and antioxidant activity.

## 2. Materials and methods

### 2.1. Bacterial growth, maintenance and levan screening medium

One levan producing *P. fluoresces* strain ES was kindly provided by Professor Hoda H. El-Hendawy, Botany and Microbiology Department, Faculty of Science, Helwan University, Egypt, and used for production of levan polymer in this study. This strain was routinely grown on nutrient agar slope at 30 °C for 48 h then, kept at 4 °C, and subcultured monthly. Screening for levan production was carried out on sucrose medium, it contained (1 g tryptone, 0.5 g yeast extract, 1.5 g agar, 10 g NaCl, 0.25 g $K_2HPO_4$, 20 g sucrose) (Nasir et al., 2015).

### 2.2. Inoculum preparation and fermentation medium

A batch fermentation system in stationary flask cultures was used for levan production according to Silbir et al. (2014) with slight modification, *P. fluorescens* strain ES was freshly grown in TS broth at 30 °C and 150 rpm for 24 h then inoculated into seed culture containing (Sucrose, 50 g; yeast extract, 7.0 g; $KH_2PO_4$ 2.5 g; $(NH_4)_2SO_4$ 1.6 g; $MgSO_4.7H_2O$ 1.0 g per litre pH 5.0) at the concentration of 5% (v/v) and incubated under the static condition at 28 °C for 24 h. Then, an inoculum at the concentration of 10% was inoculated into a fermentation medium contained (Sucrose, 300 g; yeast extract, 2.5 g; $KH_2PO_4$ 1.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g per litre pH 5.3) in a 250 ml Erlenmeyer flask and further incubated under the static condition at 28 °C for 72 h. The broth was centrifuged at 10,000 rpm at 4 °C for 10 min to eliminate the bacterial cell and coagulated proteins. The supernatant was adjusted to pH 10 by (0.1 M KOH), 3 volumes of pre-chilled 75% ethanol were added to the supernatant to precipitate levan. 1 ml of (1% $CaCl_2$) was added to every 10 ml of the supernatant to enhance levan precipitation. The precipitate was collected by centrifugation under the previously mentioned conditions and was freeze- dried for further analysis.

### 2.3. Quantitative estimation of reducing sugar concentration

Levan powder (0.1 g) was hydrolyzed by 0.1 N HCl 5% (v/v) at 100 °C for 1 h. Then estimated by using the dinitrosalicylic acid method (DNS) (Garriga et al., 2017; Yoshida et al., 1990). Glucose standard solution was prepared in 0.05 M acetate buffer (pH 4.8) over the range of 100–600 (μg/ml). Then 1 ml of 3,5-dinitrosalicylic acid was added to 1 ml of the standard and test sample with appropriate dilution as well as blank test tube with distilled $H_2O$ and mixed well, then placed in a boiling water for 10 min then left to cool at room temperature for 30 min. Then, 10 ml of distilled $H_2O$ was added to each tube and absorbance was measured at 540 nm.

### 2.4. Optimization of the medium components and conditions for levan production

Optimization of levan production was performed statistically in two steps, the first one by using Plackett-Burman design (PBD) and then by using response surface methodology Central Composite Design (CCD). We used statistical methods to avoid time-consuming and to estimate the interactions between the most significant variables (Ananthalakshmy and Gunasekaran, 1999; Moghannem et al., 2017).

### 2.5. Selection of the significant variables by Plackett-Burman (P-B) design

Ten different independent variables were diversified between medium composition and culture conditions and one Dummy factor was tested by the Plackett-Burman test. Each variable was signified at two levels, high and low (−1) as indicated in Table 1. Thirteen trials in Table 2 were obtained and done in duplicates to avoid any error. Plackett-Burman experimental design depends on the first-order model: $Y = \beta 0 + \Sigma \beta_i X_i$

Where, Y is the response of levan concentration (μg/ml) which was calculated by the dinitrosalicylic acid (DNS) method, $\beta_i$ is the linear coefficient, $\beta_0$ is the model intercept and $X_i$ is the level of the independent variable.

**Table 1**
Experimental variables and their levels for Plackett-Burman (PB) design.

| Number | Variable name | Variable abbreviation | Variable unit | Low level | High level |
|---|---|---|---|---|---|
| 1 | Shaking incubation | A | RPM | 0 | 150 |
| 2 | Temperature | B | °C | 25 | 35 |
| 3 | Incubation time | C | Day | 2 | 5 |
| 4 | Sucrose | D | g/l | 200 | 400 |
| 5 | Yeast extract | E | g/l | 1.5 | 3.5 |
| 6 | Ammonium sulfate | F | g/l | 0.5 | 1.5 |
| 7 | Magnesium sulfate | G | g/l | 0.2 | 1 |
| 8 | Potassium dihydrogen phosphate | H | g/l | 0.5 | 1.5 |
| 9 | Peptone | J | g/l | 5 | 15 |
| 10 | pH | K | N | 5 | 7 |
| 11 | Dummy | L | N | −1 | 1 |

N = nothing.

**Table 2**
The trials and the obtained results for Placket- Burman design.

| Trial | A | B | C | D | E | F | G | H | J | K | L | Levan Conc. (µg/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 150 | 25 | 2 | 200 | 3.5 | 0.5 | 1 | 1.5 | 5 | 7 | 1 | 350 |
| 2 | 0 | 25 | 2 | 400 | 1.5 | 1.5 | 1 | 0.5 | 15 | 7 | 1 | 400 |
| 3 | 150 | 25 | 5 | 400 | 3.5 | 0.5 | 0.2 | 0.5 | 15 | 5 | 1 | 210 |
| 4 | 0 | 25 | 2 | 200 | 1.5 | 0.5 | 0.2 | 0.5 | 5 | 5 | −1 | 300 |
| 5 | 150 | 35 | 5 | 200 | 1.5 | 0.5 | 1 | 0.5 | 15 | 7 | −1 | 160 |
| 6 | 0 | 25 | 5 | 200 | 3.5 | 1.5 | 0.2 | 1.5 | 15 | 7 | −1 | 400 |
| 7 | 0 | 35 | 5 | 200 | 3.5 | 1.5 | 1 | 0.5 | 5 | 5 | 1 | 160 |
| 8 | 0 | 35 | 2 | 400 | 3.5 | 0.5 | 1 | 1.5 | 15 | 5 | −1 | 350 |
| 9 | 75 | 30 | 3.5 | 300 | 2.5 | 1 | 0.6 | 1 | 10 | 6 | 0 | 210 |
| 10 | 0 | 35 | 5 | 400 | 1.5 | 0.5 | 0.2 | 1.5 | 5 | 7 | 1 | 600 |
| 11 | 150 | 35 | 2 | 200 | 1.5 | 1.5 | 0.2 | 1.5 | 15 | 5 | 1 | 110 |
| 12 | 150 | 35 | 2 | 400 | 3.5 | 1.5 | 0.2 | 0.5 | 5 | 7 | −1 | 500 |
| 13 | 150 | 25 | 5 | 400 | 1.5 | 1.5 | 1 | 1.5 | 5 | 5 | −1 | 210 |

### 2.6. Optimization of the significant variables by response surface methodology (RSM)

#### 2.6.1. Central composite design (CCD)

The CCD step involved levels optimization and the effect of interaction between the significant variables that have a positive effect with (P-B) design step (El-Naggar and Hamouda, 2016). In this work, the experimental plan consisted of 25 trials as shown in Table 4, and the four independent variables (pH, sucrose, dihydrogen potassium phosphate, and yeast extract) were analyzed at five different levels (−alpha, low, middle, high and +alpha) as represented in Table 3. The levels are a combination between two factorial points (low and high), two axial points (−alpha and +alpha), and a central point (middle). The other factors (shaking incubation, temperature, incubation time, ammonium sulfate, magnesium sulfate, and peptone) were fixed at 0, 25 °C, 2 days, 0.5 g/l, 0.2 g/l, and 5 g/l, respectively. All the experiments were done in duplicate and levan concentration (µg/ml) was considered as a response (Y). The experimental results of CCD were fitted using the following polynomial second-order equation:

$$Y = \beta_0 + \beta_i X_i + \beta_{ii} X_i^2 + \beta_{ij} X_i X_j$$

where Y is the predicted response, βi is the linear coefficient, βii is the quadratic coefficients, βij is the interaction coefficients β0 is

the regression coefficients, and $X_i$ is the coded levels of independent variables.

#### 2.6.2. Central composite design validation

The model was validated to verify its adequacy and accuracy, one of the obtained software solutions in Fig. 1 was performed in triplicates.

### 2.7. Purification of levan

The precipitated levan polymer was dissolved and dialyzed against distilled water for three days minimum for removal of low molecular weight impurities (Gojgic-Cvijovic et al., 2019). Then the purified precipitate of levan polymer was dried in a hot air oven at 60 °C for 4–6 h. The dry weight was estimated gravimetrically (Yoshida et al., 1990).

### 2.8. Levan polymer characterization

#### 2.8.1. Fourier transform infrared spectrometer (FTIR)

IR spectra were analyzed in the National Research Centre, Dokki, Giza, Egypt, by using JASCO FTIR 6100, Japan to obtain information about chemical groups. The obtained polymer was read at a

**Table 3**
Experimental variables and their levels for Central Composite (CC) design.

| No. | Factors | Symbol | Units | Levels | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Low | High | Middle | −alpha* | +alpha* |
| 1 | pH | A | – | 5 | 7 | 6 | 4 | 8 |
| 2 | Sucrose | B | g/l | 200 | 400 | 300 | 100 | 500 |
| 3 | Potassium dihydrogen phosphate | C | g/l | 0.5 | 1.5 | 1 | 0 | 2 |
| 4 | Yeast extract | D | g/l | 1.5 | 3.5 | 2.5 | 0.5 | 4.5 |

*1.68179.

**Table 4**
Trials and results for Central Composite Design.

| Variables &Response Trials | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A- pH | 6 | 7 | 5 | 5 | 4 | 6 | 5 | 5 | 6 | 7 | 6 | 5 | 5 | 6 | 8 | 6 | 7 | 7 | 7 | 5 | 7 | 6 | 7 | 7 | 5 |
| B- Sucrose (g/l) | 100 | 200 | 200 | 400 | 300 | 500 | 200 | 400 | 300 | 200 | 300 | 200 | 200 | 300 | 300 | 300 | 400 | 400 | 400 | 400 | 200 | 300 | 200 | 400 | 400 |
| C- Potassium dihydrogen phosphate (g/l) | 1 | 0.5 | 0.5 | 1.5 | 1 | 1 | 0.5 | 0.5 | 1 | 0.5 | 0 | 1.5 | 1.5 | 1 | 2 | 2 | 0.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1 | 1.5 | 0.5 | 0.5 |
| D-Yeast extract (g/l) | 2.5 | 1.5 | 3.5 | 3.5 | 2.5 | 2.5 | 1.5 | 1.5 | 1.5 | 3.5 | 2.5 | 1.5 | 3.5 | 2.5 | 2.5 | 2.5 | 3.5 | 3.5 | 1.5 | 1.5 | 1.5 | 4.5 | 3.5 | 1.5 | 3.5 |
| Levan concentration (μg/ml) | 360 | 580 | 420 | 600 | 270 | 580 | 590 | 860 | 290 | 210 | 400 | 340 | 460 | 150 | 380 | 350 | 400 | 710 | 300 | 330 | 300 | 350 | 400 | 580 | 280 |



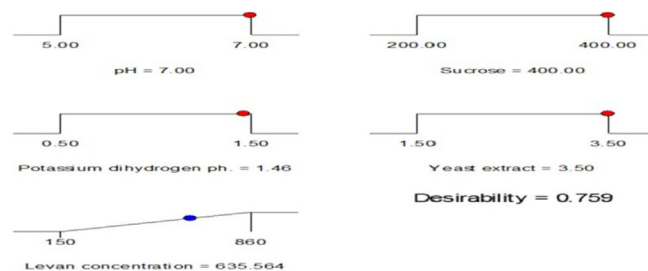**Fig. 1.** The model solution that validated actually.

400–4000 cm$^{-1}$ wavelength range with a resolution of 4 cm$^{-1}$ (Kuppusamy et al., 2015).

### 2.8.2. High-performance liquid chromatography (HPLC)

Levan sample was extracted into DMSO for HPLC analysis according to (AOAC, 1997). Then was filtered through a 0.45 μm cellulose nitrate Millipore membrane filter (Sartorius). Sugar in the filtrate was analyzed in the National Research Centre, Dokki, Giza, Egypt, by using HPLC, Shimadzu Class-VPV 5.03 (Kyoto, Japan) supplied with LC-16ADVP binary pump, refractive index RID-10A Shimadzu detector, DCou-14 A degasser, Shodex PL Hi-Plex Pb column (Sc 1011 No. H706081), and Sc-Lc Shodex as guard column, at 80°C. Separation and quantitation were done on an amino-bonded column with a mobile phase of CH$_3$CN and H$_2$O (80/20 V:V).

### 2.8.3. Nuclear magnetic resonance (NMR) spectroscopy

The purified polymer was characterized by ($^1$H NMR and $^{13}$C NMR spectra) using Bruker Corp., Billerica, MA, USA spectrophotometer 400 and 100 MHz, respectively (NMR Lab., Microanalytical unit (MAU), Faculty of Pharmacy, Cairo University, Egypt). Levan sample was dissolved in D$_2$O (Sigma Aldrich) cold and poured into 5 mm tubes (Abou-taleb et al., 2015; Jathore et al., 2012).

### 2.9. Determination of the biological activity of the bacterial levan

### 2.9.1. Cytotoxic activity (SRB assay)

Cell lines A-431: Human epidermoid skin carcinoma and HepG2: Hepatocellular carcinoma were used. Cells were preserved in DMEM media provided with 100 mg/mL of streptomycin, 100 units/ml of penicillin, and 10% of fetal bovine serum (heat-denaturation) in humidified, 5% (v/v) CO$_2$ atmosphere at 37 °C. The viability of cells was determined by SRB assay. Cell suspension (5x10$^3$ cells) placed in 96-well plates (100 μl), then incubated in complete media for 24 h. Cells were subjected to another liquid of media containing drugs with different concentrations (100 μl). After 72 h of exposure to drug, cell fixation takes place by replacing media with 150 μl of TCA (10%) and incubated at 4 °C for 1 h. The TCA solution was discarded, cells were washed with distilled water 5 times. Aliquots of 70 μl SRB solution (0.4% w/v) were added then incubated at room temperature for 10 min in dark. Plates were washed with 1% acetic acid 3 times and left to air-dry overnight. Then, 150 μl of TRIS (10 mM) was added to dissolve protein-bound SRB stain; absorbance was measured at 540 nm by a BMG LABTECH®- FLUOstar Omega microplate reader (Ortenberg, Germany). (Allam et al., 2018; Skehan et al., 1990).

### 2.9.2. Antioxidant activity (DPPH assay)

Levan sample was prepared in concentrations of 1000 and 100 μg/ml in water to identify a range within which the inhibitory concentration 50 (IC50) lies. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical assay was carried out according to the

method of Boly et al. (2016) Briefly; 100 μl of DPPH reagent freshly prepared in (0.1% methanol) was added to 100 μl of the sample in 96 wells plate (n = 6), then incubated at room temp. in dark for 30 min. Finally, the resulting reduction in DPPH color intensity was determined at 540 nm. Data are represented as means ± . SD by using the following equation:

Percentage inhibition

$= ($Average absorbance of blankaverage absorbance of the test/average absorbance of blank$) \times 100$

The results were documented by microplate reader FLUOstar Omega, and the IC50 value was determined by Graph pad Prism 5® by changing the concentrations to their logarithmic value and selecting non- linear inhibitor regression equation (log inhibitor vs. normalized response – variable slope equation) (Chen et al., 2013).

### 2.10. Statistical analysis

The "Design-Expert" software (version 7.0, State-Ease, Inc., Minneapolis, USA) was applied for the experimental design and data analysis. The data were submitted to determine the analysis of variance, regression coefficients, and the studies of the interaction between the response and the tested variables. Antioxidant activity data were analyzed using Microsoft Excel®.

## 3. Results

### 3.1. *Levan production on sucrose* medium

Formation of the shiny, slimy mucoid appearance indicating the formation of levan polymer on sucrose medium by *P. fluorescens* strain ES (Fig. 2).

### 3.2. Selection of the significant variables by Plackett-Burman (P-B) design

#### 3.2.1. P-B design

In P-B results, levan concentration ranged from 600 μg/ml in run 10–110 μg/ml in run 11. The model p-value was 0.0144 less than 0.05 indicated the significance of the model. Additionally, the variables shaking incubation (A), Incubation time (C), sucrose (D), yeast extract (E), ammonium sulfate (F), magnesium sulfate (G), potassium dihydrogen phosphate (H), peptone (J), pH (K) were significant model terms with values 0.0095, 0.0236, 0.0081, 0.0335, 0.0335, 0.0130, 0.0219, 0.0130 and 0.0059, respectively as presented in Table 5 The obtained results also, indicated that pH (K), sucrose (D), potassium dihydrogen phosphate (H), and yeast extract (E) showed the most significant effect on the levan concentration with values 89.17, 65.83, 24.17, and 15.83, respectively as indicated in the Pareto chart Fig. 3 and Table 5. In contrast, each of shaking incubation (A), Incubation time (C), ammonium sulfate (F), magnesium sulfate (G), and peptone (J) had a low effect on levan concentration. Additionally, the dummy variable (L) involved



**Fig. 2.** Levan production on sucrose medium A: negative levan production strain B: positive levan production by *P. fluorescens* strain ES.

in the design did not have any effect on the produced levan polymer. The model Adeq precision ratio was 176.672 indicate adequate signal and the model may be helpful to navigate the design space because a ratio larger than 4 is suitable.

#### 3.2.2. Central composite design (CCD)

The results of the central composite quadratic model indicated that the predicted levan concentration was achieved by applying the polynomial second-order equation (No. 1). The linear coefficients results are 1.10, −0.14, −0.22, −0.51 for sucrose (B), pH (A), yeast extract (D) and potassium dihydrogen phosphate (C), respectively; the interactive coefficients are 3.21, 0.51, 0.43, 0.42, 0.34 and 0.16 for CD, AB, AC, BD, AD and BC, respectively; the quadratic coefficients are 2.64, 2.09, 1.75 and 1.72 for $B^2$, $C^2$, $A^2$ and $D^2$. R-square value was 0.8026 (80.26%). The model F- value of 2.91 (P-value = 0.04) means the model is significant, there is only a 4.48% chance that a "model F-value" this large could occur due to noise. Values greater than 0.1000 indicated that the model terms are not significant, in this study CD. $B^2$ and $C^2$ are significant model terms as indicated in Table 6.

#### 3.2.3. Final equation in terms of actual factors

**Sqrt(Levan concentration)** =
$+ 156.64425 - 24.31983 *$ pH $- 0.19138 *$ Sucrose$- 40.02026 *$ Potassiumdihydrogenphosphate $- 18.53340 *$ Yeastextract $+,$
$5.06576E - 003 * pH * $ Sucrose $+, 0.86555 * pH *$
Potassiumdihydrogenphosphate $+, 0.33630 * pH *$
Yeastextract $+, 3.29520E - 003 *$ Sucrose $*$
Potassiumdihydrogenphosphate $+ 4.17399E - 003 *$ Sucrose $*$
Yeastextract $+ 6.42574 *$ Potassiumdihydrogenphosphate
Yeastextract $+ 1.74628 * PH_2 + 2.63773E - 004 *$ Sucrose$_2$
$+ 8.37664 *$ Potassiumdihydrogen phosphate$_2 + 1.72283 *$
Yeastextract$_2$      **Equation1**

#### 3.2.4. Interactions between the significant variables

Interactions between the four significant variables were studied through contour and 3D graphs. A significant interaction was noticed between potassium dihydrogen phosphate (C) and yeast extract (D) with a fixed value of pH (A) and sucrose (B) as indicated in Fig. 4. Where, at a constant value of pH and sucrose concentration (7 and 400 g/l, respectively), levan concentration reached 600.891 and 710 μg/ml in the presence of both the lowest and highest levels of (D) and (C), respectively. On the other hand, levan concentration decreased to 333.486 μg/ml in presence of the low level of (D) 1.5 g/l and the high level of (C) 1.5 g/l. Additionally, it was 400 μg/ml with vice versa.

Finally, the model was validated, and the results were 640 μg/ml, it deviated by +0.7% from the predicted value which was 635.564 μg/ml. Also increased by 2 folds when compared with the value obtained from the ordinary conditions 300 μg/ml before the optimization as in Fig. 5.

### 3.3. Levan polymer characterization

#### 3.3.1. Fourier transform infrared spectrometer (FTIR)

The FTIR spectrum analysis Fig. 6 was carried out to detect the functional groups of purified levan produced by *P. fluorescens* strain ES, the characteristic peaks of levan indicated that the observed band at 2930 and 2885 cm$^{-1}$ corresponding to the stretching vibration bands C—H aliphatic symmetric and asymmetric methyl and methylene, respectively, that coupled with the C═O groups.
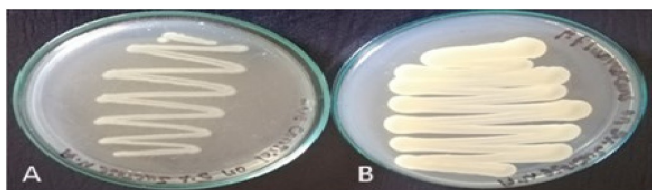
**Table 5**
ANOVA and regression coefficients of the P-B design results.

| Variables | Coefficients | Standard error | Sum of square | P-value |
|---|---|---|---|---|
| A-Shaking incubation | −55.83 | 0.83 | 37408.33 | 0.0095 |
| C- Incubation time | −22.50 | 0.83 | 6075.00 | 0.0236 |
| D- Sucrose | +65.83 | 0.83 | 52008.33 | 0.0081 |
| E- Yeast extract | +15.83 | 0.83 | 3008.33 | 0.0335 |
| F- Ammonium sulfate | −15.83 | 0.83 | 3008.33 | 0.0335 |
| G- Magnesium sulfate | −40.83 | 0.83 | 20008.33 | 0.0130 |
| H-Potassium dihydrogen phosphate | +24.17 | 0.83 | 7008.33 | 0.0219 |
| J- Peptone | −40.83 | 0.83 | 20008.33 | 0.0130 |
| K- pH | +89.17 | 0.83 | 95408.33 | 0.0059 |
| L- Dummy | −7.50 | 0.83 | 675.00 | 0.0704 |
| **Model statistics** | | | | |
| **Model P- value** | Adjusted R-Square | R-Square | Adeq Precision | |
| **0.0144** | 0.9996 | 1.0000 | 176.672 | |
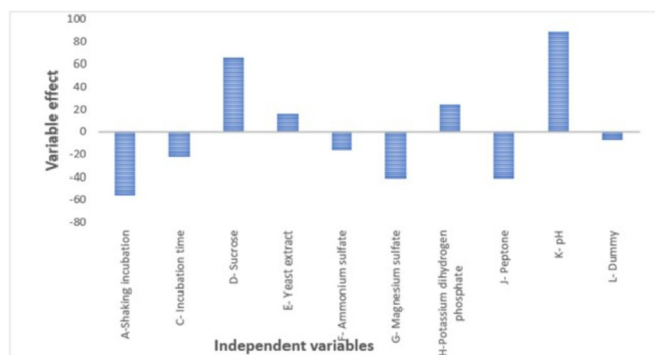


**Fig. 3.** Pareto chart showing the effect of different independent variables on the produced levan concentration.

Besides, there is a shoulder peak at 1745 cm$^{-1}$ corresponding to the carbonyl group. Furthermore, the observed band at 1422 and 1340 cm$^{-1}$ corresponding to the stretching vibration bands of the CH$_3$ functional group. In addition, the substituted aromatic conjugated rings present C—H weak distortion bands raging between 1100 and 1300 cm$^{-1}$. The strong absorption peak at around 1061 cm$^{-1}$ represents the stretching vibration of C—O of intramolecular C—O—C ring ether. In addition to the presence of stretching vibration bands of CH$_2$—OH and CH—OH, which found in cellulose and polysaccharides were observed at 1010 cm$^{-1}$. Also found peak near (844 and 891 cm$^{-1}$), weak absorption peak was found around 870 cm$^{-1}$.

### 3.3.2. High-performance liquid chromatography (HPLC)

HPLC analysis was used to detect the quantity of each component in the produced polymer in comparison with fructose sugar as standard Fig. 7, the results show that the percentage of fructose was increased from 75.8 to 89.008 after levan purification (Table 7), which indicated that the purified levan was mainly composed of fructose subunits.

### 3.3.3. Nuclear magnetic resonance (NMR) spectroscopy

The $^1$H NMR spectrum of the purified levan Fig. 8 revealed that the obtained polymer of *P. fluorescens* was found to contain proton chemical shift signals indicating the existence of sugar protons which was observed at $\delta$ 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 (H-1b), and 3.48 ppm (H-6b).

The $^{13}$C NMR spectrum of the purified levan recorded six-carbon signals at $\delta$ 104.17, 80.26, 76.26, 75.17, 63.36, and 59.87 ppm Fig. 9. The chemical shifts of carbon atoms were represented in Table 8.

### 3.4. Determination of cytotoxic activity (SRB assay)

The cytotoxic effect of levan on A-431: Human epidermoid Skin carcinoma and HepG2: Hepatocellular carcinoma were estimated at 7 concentrations (0.001, 0.01, 0.1, 1, 10, 100, and 1000 μg/ml) for 24 h. Levan showed dose-dependent anticancer activities and IC50 was 469 and 222.7 μg/ml, respectively at 24 h as shown in Table 9 and (Fig. 10).

**Table 6**
Variance analysis and regression statistics of CCD results.

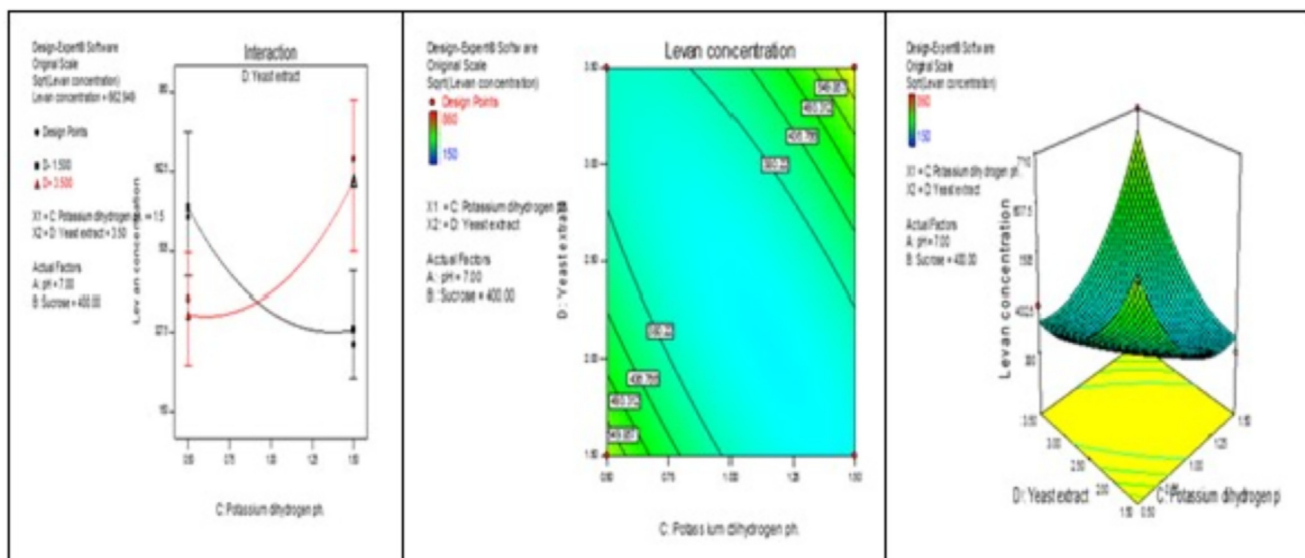| Variables | Coefficients | Standard error | Sum of squares | P-value |
|---|---|---|---|---|
| A-pH | −0.14 | 0.55 | 0.46 | 0.8068 |
| B-Sucrose | 1.10 | 0.55 | 29.09 | 0.0736 |
| C-Potassium dihydrogen ph. | −0.51 | 0.55 | 6.25 | 0.3761 |
| D-Yeast extract | −0.22 | 0.55 | 1.20 | 0.6936 |
| AB | 0.51 | 0.67 | 4.11 | 0.4702 |
| AC | 0.43 | 0.67 | 3.00 | 0.5358 |
| AD | 0.34 | 0.67 | 1.81 | 0.6290 |
| BC | 0.16 | 0.67 | 0.43 | 0.8121 |
| BD | 0.42 | 0.67 | 2.79 | 0.5501 |
| CD | 3.21 | 0.67 | 165.16 | 0.0008 |
| A$^2$ | 1.75 | 0.80 | 34.44 | 0.0548 |
| B$^2$ | 2.64 | 0.80 | 78.58 | 0.0082 |
| C$^2$ | 2.09 | 0.80 | 49.53 | 0.0262 |
| D$^2$ | 1.72 | 0.80 | 33.52 | 0.0576 |
| **Model statistics** | | | | |
| **Model *P*- value** | Adjusted R-Square | R-Square | Sum of squares | |
| **0.0479** | 0.5264 | 0.8026 | 296.35 | |

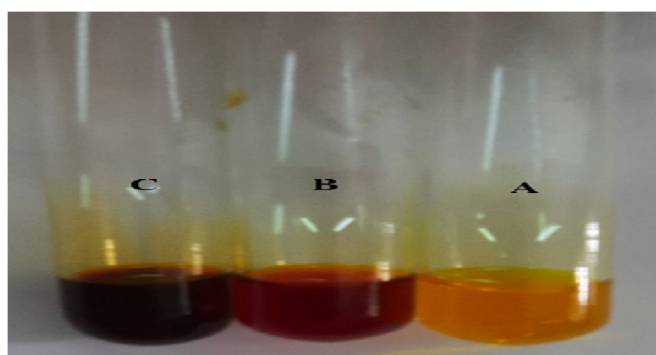**Fig. 4.** 3-D, contour, and interaction graphs of CC results.



**Fig. 5.** Optimization of levan concentration: (A) negative control, (B) the ordinary medium and (C) the optimized medium.

*3.5. Determination of antioxidant activity (DPPH assay)*

The antioxidant activity of the obtained levan polymer was evaluated by using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical assay. The scavenging activity of the levan from *P. fluorescens* strain ES on DPPH radical at 0.1 mg/ml concentration was

13.89 ± 1.07% with IC50 value (inhibitory concentration giving 50 % reduction of the free radical DPPH) of 24.42 ± 0.87 mg/ml as shown in Table 10. Trolox was used as standard.

## 4. Discussion

Carbohydrate polymers continue to spark scientists' interest due to their broad range of possible applications, structural diversity, and complexity (Dumitriu, 2004). Levan is one of the most promising natural homopolysaccharides composed of d-fructose residues (Srikanth et al., 2015), that has enormous potential in a variety of industrial and medical applications so, the present study was based on levan production by *P. fluorescens* strain ES strain was isolated from soil in Egypt, in a previous study (unpublished) and proved to be capable of producing characteristic feature of mucoidal consistency when grown on sucrose medium since levan polymer usually obtained from sucrose-based substrates by different microorganisms (Tomulescu et al., 2016). This characteristic feature of the colony could be attributed to the production of an extracellular levansucrase enzyme which is capable of converting sucrose in the screening medium to exopolysaccharides in the form of β- d fructoside (levan polymer) (Thakham et al., 2020),
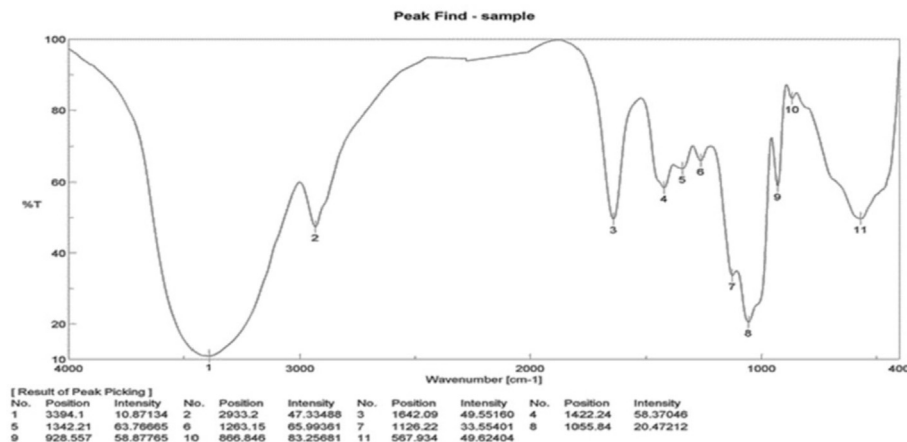


**Fig. 6.** FTIR spectra of levan polymer produced by *P. fluorescens* strain ES.
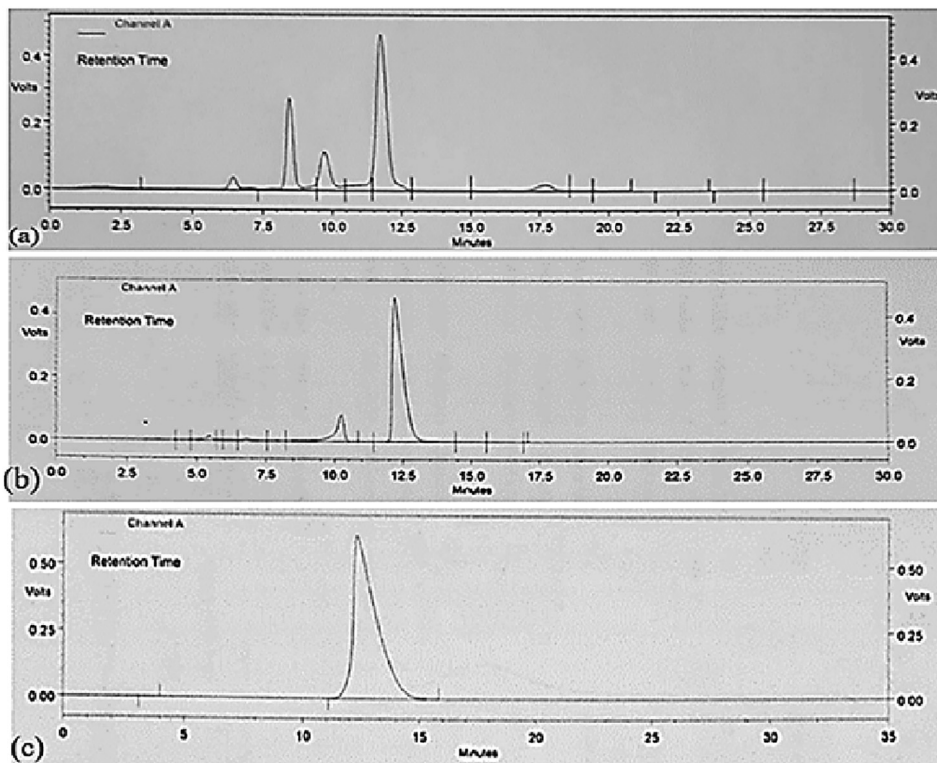
**Fig. 7.** HPLC analysis of levan polymer (a) before purification (b) after purification (c) standard sugar (Fructose).

**Table 7**
HPLC analysis of levan polymer before and after purification.

| Sample | Fructose |
|---|---|
| **Before purification** | 75.8 * |
| **After purification** | 89.008* |

Value is expressed as the relative area percentage.

Levansucrase enzyme biosynthesis have been reported to occur in many gram-negative and gram-positive bacteria (Benkeblia, 2013). Jathore et al. (2012) reported the production of levan by *P. fluo-*

*rescens* bacterium when incubated aerobically for 6 days. In the case of the Egyptian strain, levan was produced was obtained after 72 h incubation in broth medium under a static condition then pre-chilled 75% ethanol was added to precipitate it. However, to meet the requirements for industrial and medical use, large-scale production must be done through optimization of growth condition and composition of the medium. To screen the significant medium components and environmental factors for levan processing, Plackett-Burman design was used since it provides a convenient and consistent method to enhance levan production (Vinusha and Ganduri, 2019).

Response Surface Methodology (RSM) is a useful experimental design technique for selecting optimal conditions in a multivari-
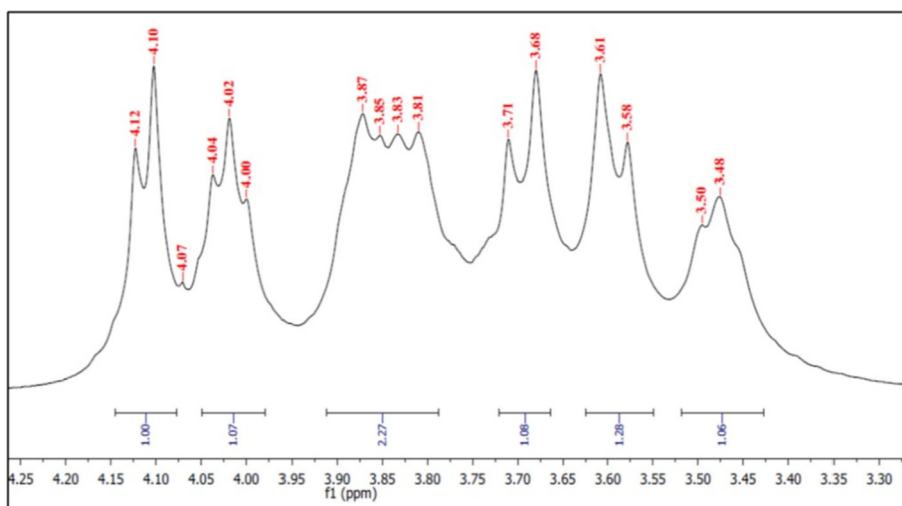


**Fig. 8.** The $^{1}$H NMR spectrum of levan from *P. fluorescens* strain ES. The spectrum was measured at 400 MHz for the sample in $D_2O$.
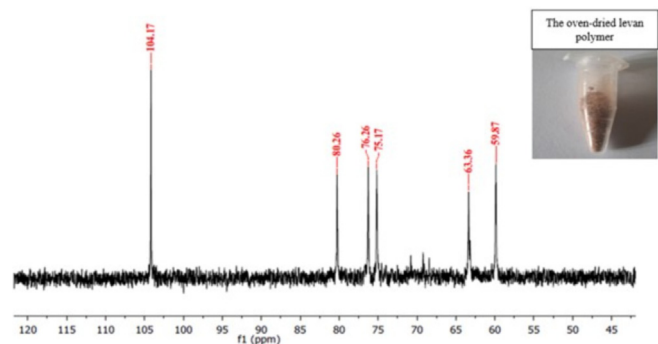
**Fig. 9.** The $^{13}$C NMR spectrum of levan from *P. fluorescens* strain ES. The spectrum was measured at 100 MHz for the sample in $D_2O$.

**Table 8**
Comparison of $^{13}$C NMR results of *P. fluorescens ES* levan with $^{13}$C NMR values of *Bacillus licheniformis* and *Z. mobilis* levan.

| Carbon atom ($^{13}$C NMR) | Chemical shift (ppm) | | |
|---|---|---|---|
| | Standard Levan of *Z. mobilis* (Han and Clarke, 1990) | *B. licheniformis* Levan (Dahech et al., 2012) | *P. fluorescens* ES Levan |
| C-1 | 60.76 | 59.78 | 59.87 |
| C-2 | 104.64 | 104.17 | 104.17 |
| C-3 | 77.68 | 76.17 | 76.26 |
| C-4 | 75.75 | 75.12 | 75.17 |
| C-5 | 80.78 | 80.25 | 80.26 |
| C-6 | 63.95 | 63.95 | 63.36 |

**Table 9**
Cytotoxicity of levan polymer against Cancer cell lines.

| Cancer cell lines | (SRB) IC50 μg/ml |
|---|---|
| A-431: Human epidermoid Skin carcinoma | 469 |
| HepG2: Hepatocellular carcinoma | 222.7 |

able system. (Vinusha and Ganduri, 2019). In this study, the concentration of levan produced by *P. fluorescens* strain under test, was optimized as the response (Y) in each trial of PB and CC trials. Levan concentration was determined spectrophotometrically at 540 nm by DNSA method according to (Yoshida et al., 1990) and (Garriga et al., 2017). The selection of the concentration as a response not the yield (weight) of the levan was due to the final weight in our study was not completely corresponding to the levan product. The p-value of PB and CC models was 0.0144 and 0.04, respectively. Both p-values were less than 0.05 indicated the significance of the two models. These results agreed with Xavier

**Table 10**
Antioxidant scavenging activity of levan polymer.

| Sample | % inhibition at 1000 μg/mL (Mean ± SD)) SD)SD) | IC50 |
|---|---|---|
| **Levan** | <50 (13.89 ± 1.07) | 24.42 ± 0.87 |

and Romana (2017) as they reported that the lower *p-values* indicate the high significance nature of the corresponding coefficients (Xavier and Ramana, 2017). Results of Dos Santos et al. (2013), Moghannem et al. (2017), and Bouallegue et al. (2020) agreed with ours in that sucrose concentration had a positive effect to maximize the production of levan. In contrast, to the result of Jathore et al. (2012) who reported that $KH_2PO_4$ exhibited a very low effect, it was significantly effective in our study. In CCD, R-square value was 0.8026 (80.26%). The values of R2 was larger than 75% indicating the fitness of the model (Xavier and Ramana, 2017). Additionally, as we found, Moghannem et al. (2017) reported that yeast extract was the most favorable nitrogen source. It is likely that, Yeast extract might have a role in providing the culture medium with amino acids and vitamins to sustain bacterial growth (Luthra et al., 2015).

A very critical stage in the sample analysis of the obtained levan polymer after optimization is the physical and chemical characterization that demonstrates the existence of the desired product. On the other hand, characterization methods give the whole picture on morphology, mechanical, molecular structure, molecular mass, thermal, and little other chemical properties (Srikanth et al., 2015). FTIR is an analytical technique that is used to gain an infrared spectrum of absorption or emission of a gas, solid, or liquid substance to distinguish organic and inorganic materials (Leela and Anchana, 2017). Levan could be characterized easily using FTIR spectral analysis which determines the functional group composition of the structure of *P. fluorescens* levan. The obtained results, showed three different large and strong peaks at the Wavenumber of 2930 and 2885 cm$^{-1}$ and particularly at 3394 cm$^{-1}$ corresponding to the stretching vibration bands C—H aliphatic asymmetric and symmetric methyl and methylene, respectively which being conjugated with the C=O groups because of intermolecular hydrogen bonding (Thakham et al., 2020). Moreover, the observed band at the Wavenumber of 1650 cm$^{-1}$ might correspond to the characteristic of the C bond, which could be related to uronic acid groups. There was a strong peak at the Wavenumber of about 1061 cm$^{-1}$ representing the stretching vibration of C—O of intramolecular C—O—C ring ether, that characterize pyranose carbohydrate ring absorption peak. Besides, there are two types of α, and β carbon–glucoside bonds, that may be characterized by FT-IR. The C—H bond α-type has an absorption peak of about 844 cm$^{-1}$, whereas that of the C—H bond β-type is about 891 cm$^{-1}$ (Jang et al., 2001). The weak peak at around 870 cm$^{-1}$ might indicate the
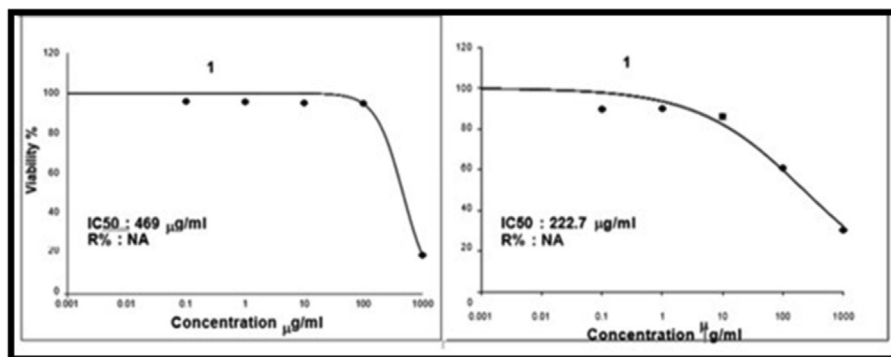


**Fig. 10.** Cytotoxicity of levan polymer against Cancer cell lines showing IC50 μg/ml.

existence of β-glycosidic bond type pyranose in the polysaccharide (Jathore et al., 2012; Yu et al., 2016; Taylan et al., 2019; Thakham et al., 2020).

The HPLC analysis revealed that fructose is the main structure unit of the obtained *P. fluorescens* strain ES levan. Furthermore, its percentage was increased from 75.8 to 89.008 after the purification. Similar finding with HPLC analysis of levan from *Leuconostoc mesenteroides* showed that fructose was the main monosaccharide found in the repeating unit structure (Taylan et al., 2019) that emphasizes the chemical structure of levan of fructose polymer (Tomulescu et al., 2016). Pei et al. (2020) also reported that levan from *Bacillus megaterium* PFY-147 contained a backbone of 2,6-substituted β-fructose by HPLC analysis.

Spectroscopy of $^1$H and $^{13}$C NMR was also commonly used in the characterization of levan polymer since it gives unique features and quite predictable for each compound, especially in small molecules (Srikanth et al., 2015). NMR spectroscopies help in the determination of structural properties and saccharide conformation with its ratio within the mixture. Furthermore, it also gives complete information on the existence of specific "functional groups" in the specimen and differentiates branched and linear levan from a plant (Hammer and Morgenlie, 1990). The obtained signals by $^1$H NMR spectrum of the purified levan from *P. fluorescens* strain ES at the 3.48–4.11 ppm region was associated with the presence of sugar protons. The spectra were almost the same as the previous $^1$H NMR spectra of levan polymer which was mainly a fructan consisting of (2 → 6) fructofuranoside (Matsuzaki et al., 2017) indicating that the obtained polysaccharides were only a levan type as a β-(2 → 6)-linked fructan (Taylan et al., 2019). The $^{13}$C NMR spectrum of *P. fluorescens* strain ES strain polymer also confirmed the structure of levan, the spectrum includes 6 signals, indicating that there was only one main type of monosaccharide (Pei et al., 2020). In an agreement with those of the previously recorded NMR studies, signals in the 60–110 ppm range were indicative of typical polysaccharide spectra. For example, *Streptococcus mutans* strain produced levan type EPS with resonance signals almost identical to the obtained one in this study (Shimamura et al., 1987). Also, the $^{13}$C NMR spectrum of polysaccharides from *Leuconostoc mesenteroides* S81 also confirmed the structure of EPS as a levan-type (Taylan et al., 2019). Moreover, Moussa et al.(2017) compared six resonance signals from *Brachybacterium phenoliresistens* to the resonance signals of the standard levan from *Z. mobilis* (Han and Clarke, 1990) and *B. licheniformis* resonance signals (Dahech et al., 2013).

To investigate the biological activity of the purified *P. fluorescens* strain ES levan, cytotoxicity (SRB assay) was determined against A-431: Human epidermoid Skin carcinoma and HepG2: Hepatocellular carcinoma cell line that showed IC50 > 100 μg/ml, to assess the suitability of the polymer to be efficient in drug delivery applications since scientists are searching for causes that put the body at risk of cancer as well as factors that could protect the body from cancer (Ragab et al., 2019). The results revealed that in the liver HepG2 cells, levan has moderate cytotoxicity with IC50 of 222.7 μg/ml, whereas lower cytotoxic effect indicated by elevated IC50 of 469 μg/ml was recorded against human epidermoid Skin carcinoma. Differences in insensitivities of cell lines towards different anticancer substances could be due to their physiological behavior and structural characteristics (Alqahtani et al., 2020). The previous study recorded the strong anti-cancer activity of levan from *B. subtilis* especially after sulfation, phosphorylation and carboxymethylation were done (Esawy et al., 2013; Ragab et al., 2019). Furthermore, bacterial levan was effective towards stomach cancer where its anticancer activity was increased proportionally to the complexity in the branching of levan structure (Srikanth et al., 2015). Generally, no significant cytotoxicity was recorded at low concentrations but by increasing levan concentration loss in cell viability was observed which indicated that levan anticancer effect was dose dependent.

The purified levan polymer from *P. fluorescens* strain ES also showed a positive antioxidant activity on DPPH radical at 0.1 mg/ml concentration by 13.89 ± 1.07% with IC50 value 24.42 ± 0.87 mg/ml. However anti-oxidant activity through enhancing the activities of both superoxide dismutase (SOD) and catalase (CAT) enzymes was reported (Dahech et al., 2012, 2013). Levan anti-oxidant activity using DPPH free radical was detected in a variety of bacterial species such as *Acetobacter xylinum NCIM 2526* (Rapala et al., 2015). Also, *Leuconostoc mesenteroides S81* exhibit antioxidant capacity with an EC50 value of 1.7 mg/ml (Taylan et al., 2019), and great antioxidants activity was reported for levan produced by *Lactobacillus plantarum* KX041 (Wang et al., 2017). It has been reported that levan changes the activities of plasma antioxidant enzyme, decreases total cholesterol, and other vital substances that could protect against oxidative stress in diabetes and diabetic in adult rats (Dahech et al., 2012, 2013). Therefore, levan is considered one of the best-known exopolysaccharides for its unique anti-tumor and antioxidant activity.

## 5. Conclusion

In this work, we found that *P. fluorescens* strain ES is a good levan producer when grown on sucrose medium. Levan composition was confirmed using different chemical and physical methods such as Fourier Transform Infrared Spectrometer (FTIR), Nuclear Magnetic Resonance (NMR), and High-Performance Liquid Chromatography (HPLC), which all emphasized that it consists mainly of D-fructofuranosyl residues. The cultural conditions were statistically optimized to enhance levan production by the bacterium using P-B and CCD. The purified polymer proved to be an antioxidant and anti-cancer agent. The percent of inhibition as an antioxidant at 1000 μg/ml was <50 (13.89 ± 1.07) with IC50 (24.42 ± 0.87) and the results of the cytotoxicity against human epidermoid Skin carcinoma and hepatocellular carcinoma recorded IC50 469 and 222.7 μg/ml, respectively. This study reports that levan polymer produced by *P. fluorescens* strain ES has a promising antioxidant and cytotoxic activity against different kinds of cancer cells.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Author contributions**

All authors contributed to data analysis, drafting, or revising the manuscript. All authors approved the final manuscript and are accountable for all aspects of the work.

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