

## study of the Optimal Conditions of Levan Production from a Local Isolate of *Bacillus subtilis* subsp. *subtilis* w36

Wafaa H. Khassaf<sup>1\*</sup>, Alaa K. Niamah<sup>2</sup>& Alaa J. A. Al-Manhel<sup>2</sup>

<sup>1</sup>Date Palm Research Centre, University of Basrah , Iraq

<sup>2</sup>Department of Food Science, College of Agriculture, University of Basrah, Iraq

\*Corresponding author E-mail: [wafaakhissaf@gmail.com](mailto:wafaakhissaf@gmail.com)

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**Abstract:** The present study was aimed to test optimum conditions for the levan production from local isolation *Bacillus subtilis* subsp. *subtilis* w36, which was isolated from Basrah city soil by using production medium containing (40 g of sucrose as carbon source 10g pepton, 1g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1g KH<sub>2</sub>PO<sub>4</sub>, 1g MgSO<sub>4</sub>.7H<sub>2</sub>O). The amount of levan produced was 4.9 g.ml<sup>-1</sup>. Molasses and date juice were used as substitutes for sucrose in growth media and substitute percentages were (25%, 50%, 75% and 100%). The results showed the highest production of levan was recorded when sucrose was substituted with 25% molasses, which was 5.2 g.ml<sup>-1</sup>. Therefore, this medium was used to study optimum conditions included incubation period, inoculation volume, temperature, initial pH. The highest production of levan was 6.8 g .100 ml<sup>-1</sup> within 40 hours' incubation, 1 ml inoculum volume, pH 6.5 and 32 °C.

**Keywords:** Bacteria, *Bacillus subtilis*, Molasses, Levan, Production, Optimal conditions.

### Introduction

Several microbial polysaccharides commercially in use in food manufacturer are bacterial cellulose, mannan, bacterial alginates, xanthan gum, gellan gum, levan, and hyaluronic acid (Al-Manhel & Niamah, 2017). Levan is an extracellular polysaccharide (EPS), and it is a natural mono-fructose sugar and its biologically active (Tomulescu *et al.*, 2016). Levan produced naturally from a wide range of microorganisms, especially bacteria, and some plant species. Its microbial source is better economically, artificially and applied aspects, in addition, bacteria produce levan in larger amounts of plants. Bacterial levans

have a higher molecular weight (2-100×10<sup>6</sup>) that produced from plants (200-33000 Dalton), (Srikanth *et al.*, 2015 a). Levan sugar is composed of units of fructose that are bound together by β-glycoside bonds (2, 6) and beta-branch (1, 2). The presence of sucrose in the medium in which bacteria grow stimulates the production of levan, where this enzyme analyzes the sucrose and converts fructose to levan sugar, this process is called “transfructosylation” (Srikanth *et al.*, 2015b). Levan is an exopolysaccharide that synthesized from sucrose by levansucrase enzyme (EC 2.4.1.10), also called 6-fructosyltransferase. It is an enzyme extracellular produced (Gu *et al.*, 2017). Levan has many applications in food,

industrial, technological and health fields, which use as a prebiotic compound in fraction food (Franken *et al.*, 2013), Levan also has healthy properties as it is anti-cancer and antidiabetic, as well as anti-oxidant and anti-inflammatory activities, In cosmetic processes and other therapeutic materials (Gu *et al.*, 2017). Bersaneti *et al.* (2018) reported that levan could be used as a blood plasma substitute and lower cholesterol in the body. In food, it used as a stabilizer, thickener, fat substitute and color and flavor vehicle (Dos Santos *et al.*, 2013). Tomulescu *et al.* (2016) mentioned that levan used as industrial gums (gum Arabic) and fillers (Abou-Taleb *et al.*, 2014), fat substitutes (Santos *et al.*, 2014), sweeteners (Dvya & Sugumaran, 2015). Additionally, levan was used in the manufacture of biodegradable plastic that used in food packaging (Chen *et al.*, 2014), and it used in the manufacture of lozenges Flavor Bearer and Packaging Factors (Divya & Sugumaran, 2015).

The production of levan is influenced not only by the carbon source, but also by other fermentation conditions such as temperature, pH, type of fermentation, cell growth rate and oxygen content in the fermentation medium (Ernandes & Garcia-Cruz, 2005). *Bacillus* is one of the most important bacteria used in the production of microbial levan.

Different species of *Bacillus* genus were used to produce levan, such as *B. licheniformis* (Dahech *et al.*, 2014), *B. subtilis* NATTO (Gu *et al.*, 2017), and *B. subtilis* B58 (Hamid *et al.*, 2018). Due to the importance of levan and its wide applications in food and its role in the chemical and pharmaceutical industries, many studies were performed to improve the conditions of levan production, many attempts have been made to find a sucrose substitute as carbon sources, which

were cheap and available locally. Gojgic-Cvijovic *et al.* (2019) found that the molecular weight of levan produced from molasses was  $5.82 \times 10^6$  Dalton and the viscosity was  $0.24 \text{ dL} \cdot \text{g}^{-1}$ , levan produced from molasses were not significantly different from the levan produced from sucrose  $200 \text{ g} \cdot \text{L}^{-1}$ .

This study was carried out to improve the production of levan by using a substitute productive medium from agricultural residues, and from a local isolate of *Bacillus subtilis* subsp. *subtilis* w36.

## Materials & Methods

### Materials

Nutrient agar medium, nutrient broth medium (Hi-media, India), Ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ ), potassium phosphate  $\text{KH}_2\text{PO}_4$ , HCl (England, PDH) sodium hydroxide NaOH, magnesium sulfate  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , (Germany, Merck), Peptone (USA, Difco), Ethanol (Spain, Scharlau).

### Bacteria used in the production of levan

*Bacillus subtilis* subsp. *subtilis* w36 isolated from soil and characterized in a previously study, nutrient broth medium (Hi-media, India) was used to activate the bacterial isolates in all tests.

### Methods

#### The fermentation process and the medium of levan production

The fermentation process was carried out in 250 ml flasks was contained 100 ml of fermentation medium, which consist of 40 g sucrose, 10 g peptone, 1 g  $(\text{NH}_4)_2\text{SO}_4$ , 1 g  $\text{KH}_2\text{PO}_4$  and 1 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , adjusted pH to 6.8, and was sterilized on  $121^\circ\text{C}$  for 15 min. The flask was inoculated with 1 ml inoculum and incubated at  $32^\circ\text{C}$  for 60 hours (Meng & Fütterer, 2003).

### **Extraction and purification of levan**

At the end of fermentation, the medium containing the levan was heated at 100 °C for 30 min, then cooled and centrifuged for 30 min at 5000 rpm. 100 ml of the supernatant was taken and heated for 5 minutes, then cooled at room temperature. Then pH value adjusted to 10 by using 1N KOH. Levan was precipitated by adding 2 volumes of 80% cold ethanol (V:V), and then added 1% CaCl<sub>2</sub> with constant stirring for 20 minutes. The resulting pellets were washed with cold ethanol of 80% concentration at 1.5 (V:V), then dried and weighed (Jathore *et al.*, 2012).

### **Study of the optimum conditions of levan production**

#### **Effect of carbon source on levan production**

Two local substitutes were used as a carbon sources in media production, sugar cane molasses (obtained from Maysan sugar production factory) and date juice extracted from date palm fruits (Zahdi is a local class) prepared in laboratory. According to Al-Sahlany & Al-Asady (2015), the percentages of substitutes in the medium were (25%, 50%, 75% and 100%) of carbon source. The total sugar in molasses was 65.5% and date juice was 38.5% that were determined by Lane and Eynon method described by Dalaly & Al-Hakim (1987). Molass was purified by activated charcoal (3% W: V), and then centrifuged at 5000 rpm for 15 min., then the supernatant was used in fermentation process (Gojic-Cvijovic *et al.*, 2019). Substitute percentages of molasses and date juice were added to 100 ml of production medium and pH was adjusted to 6.8, after that, incubated at 32 °C for 60 hours (Meng & Fütterer, 2003). used an inoculum volume of 1 ml. 100 ml<sup>-1</sup> (129× 10<sup>9</sup>) colony forming units.ml<sup>-1</sup>.

#### **Effect of different incubation periods on levan production**

Many incubation periods were tested for levan production from the substitute media. The incubation periods were 20, 40, 60, 80 and 100 hours, by using 1 ml of inoculum volume of old isolate, other conditions of medium were an initial pH 6.8 and 32 °C, then the produced levan amount was calculated.

#### **Effect of inoculum volume on levan production**

Different inoculum volumes of a local isolate were used for levan production from the substitute media. The inoculum volumes were (0.5, 1, 2 and 3) ml for each 100 ml culture media, initial pH was 6.8. The cultures media were incubated at 32 °C for the optimum incubation period. Additionally, the number of bacteria was calculated in 1 ml of inoculum volume was (129× 10<sup>9</sup>) CFU.ml<sup>-1</sup>.

#### **Effect of incubation temperature on levan production**

Different temperature degrees (25, 32, 37, 45 and 50)°C were examined for the substitute media of levan production by local isolate, initial pH of media was 6.8, other optimum conditions were used as the conditions of the levan production.

#### **Effect of initial pH on levan production**

To evaluate optimum pH for levan production from substitute media by local isolate, pH of production media was adjusted to 4, 4.5, 5, 5.5, 6, 6.5, 6.8, 7.5 and 8 by using HCl (0.2 M) or NaOH (0.1 M). The optimal conditions were obtained from previous steps were used.

#### **Statistical Analysis**

The mean of the three values (n=3) for all sample tests was calculated. One-way ANOVA table was applied to analyze the

results by SPSS (version 18.0). The level of Significance was 0.05 to compare among the data means using Least Significant Difference (LSD). The diagram of this study was drawn by used Microsoft Excel (version 2013). Results were presented as means  $\pm$  standard deviations ( $\pm$ SD).

## Results & Discussion

### Production of levan from a local isolation of *Bacillus subtilis* subsp. *subtilis* w36

The results of this study showed that, levan production from production media (40% sucrose) by local isolation of *B. subtilis* subsp. *subtilis* w36 was 4.9 g.100 ml<sup>-1</sup>. *B. subtilis* was characterized by its high production of levan compared to that of another species of bacteria. The result of this study was consistent with Shih *et al.* (2005) and Hamid *et al.* (2018). The amount of levan sugar produced from microorganisms depended on microbe used and the amount and type of sugar present in the production media.

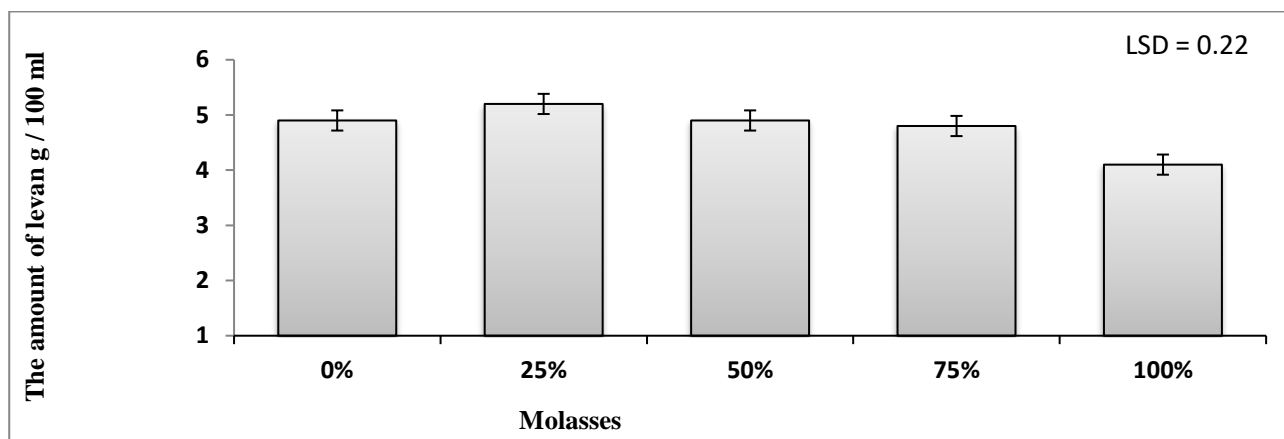
### Effect of using different percentages of molasses and date juice as a carbon source

The results presented in fig. (1) showed that levan production in the medium contains molass (65.5% sucrose) was slightly higher compared with standard production medium (4% sucrose). Statistical analysis showed that, the sample of 25% molass was significantly higher compared with other samples at 0.05 significant level, when sucrose substituted with molasses at 25% led to the significant increase of levan production compared with other substitute percentages, which was 5.2 g.100 ml<sup>-1</sup>, while the levan production was 4.9, 4.8 and 4.1 for substitute percentages 50, 75 and 100 respectively. The lowest levan production was found when sucrose was

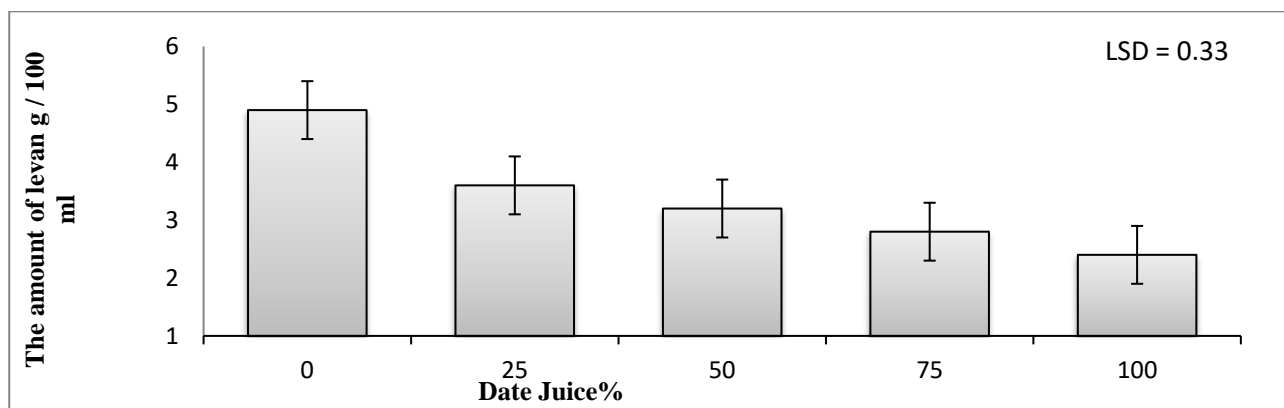
substituted completely with molasses, the lowest levan production was found when sucrose was substituted completely, (Duplicate sentence) and this might be attributed to containing molasses high levels of total sugars, which inhibited bacteria. Previous studies indicated, that sucrose acted as a substrate in the production media induced the enzyme Levansucrase that led to increase in levan production in cells.

Increasing sucrose concentration in media led to increasing fructose concentration, subsequently increase the levan level (Dos Santos *et al.*, 2013). The result of this study was in agreed with the results of Gojgic-Cvijovic *et al.* (2019), when they used molasses (62.6% sucrose) as a carbon substitute for sucrose, using *B. licheniformis* NS032, which found that the growth of this bacteria was very fast when molasses medium was used and the highest production of levan was 5.32 g. 100 ml<sup>-1</sup>. While the results of Silbir *et al.* (2014) when molasses media was used as a carbon source in production media for the production of levan as the estimated amount of levan produced was about 30 g.l<sup>-1</sup>

Data juices presented in fig. (2) revealed the levan production on the media containing date juice as a substitute for sucrose at different percentages, levan production was decreased with the increase of substitute proportion. Date juice contains a high level of glucose and fiber compared with molasses, and that may explain the decrease of levan production, which is affected by the sugar type. The results of this study agreed with the results of Moosavi-Nasab *et al.* (2010) found that 1.048 g of levan.100ml<sup>-1</sup> of date juice compared with 48.9 g . L<sup>-1</sup> levan production from culture media content sucrose 20% when fermentation conditions were 48 hours and pH 6.0.



**Fig. (1): Effect of using different percentages molasses as carbon source substitute for sucrose (25, 50, 75 and 100%).**



**Fig. (2): Effect of Using Different percentages of Date Juice as a carbon source substitute for sucrose (25, 50, 75 and 100%).**

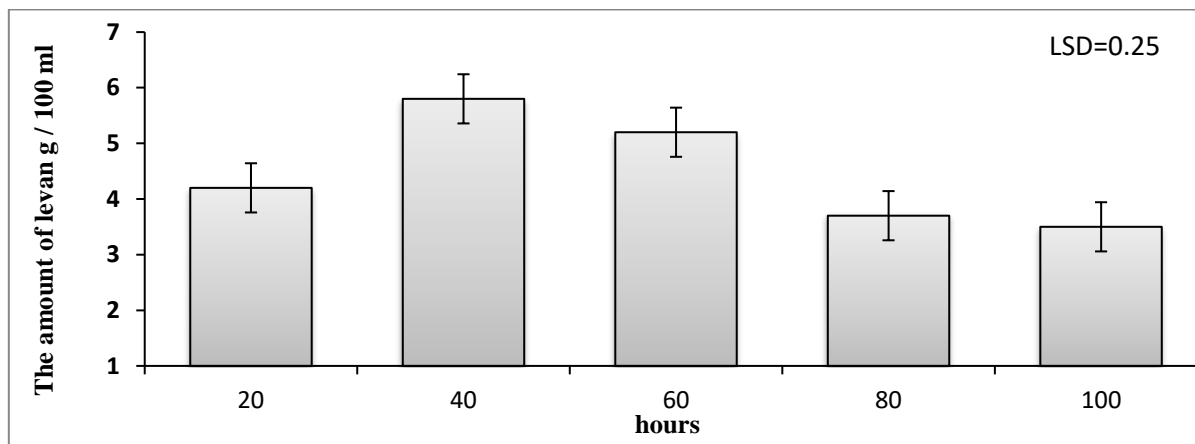
### The periods of fermentation

Fig. (3) shows the periods of fermentation time for levan production, statistical analysis indicates that, incubation time for 40 hours led to a significant increase of levan production compared with other incubation periods, it was  $5.5 \text{ g} \cdot 100 \text{ ml}^{-1}$ . In addition, results showed, that the reduction of levan production was decreased with the increase of incubation period. The decrease in productivity with an increase of the incubation time might be have resulted from changes in the production media as low of nutrients, and increased toxins and the possibility of autolysis of bacterial cells that negatively affected he production of levan.

The results of the current study were similar to the results of Dos Santos *et al.* (2013), which found that the higher levan production was achieved after 38 hours of incubation, and, reported that levan production correlated with fermentation time and sucrose level in the production media. Meanwhile, the results were reversed when the levan level was increased, and the production of levan decreased due to the increase in the viscosity of the medium, which resulted from the presence of levan, which led to the imbalance of osmotic pressure and death of bacterial cells. Gojgic-Cvijovic *et al.* (2019) reported that the highest

production of levan was achieved at fermentation period less than 48 hours when molasses used as a carbon sources in production media, which reached 5.32 g.100

ml<sup>-1</sup>. While Shih *et al.* (2005) found that, *B. subtilis* (Natto) Takahashi produced higher levan production at 21 hours as a fermentation period, which was 4-5 g.100 ml<sup>-1</sup>.

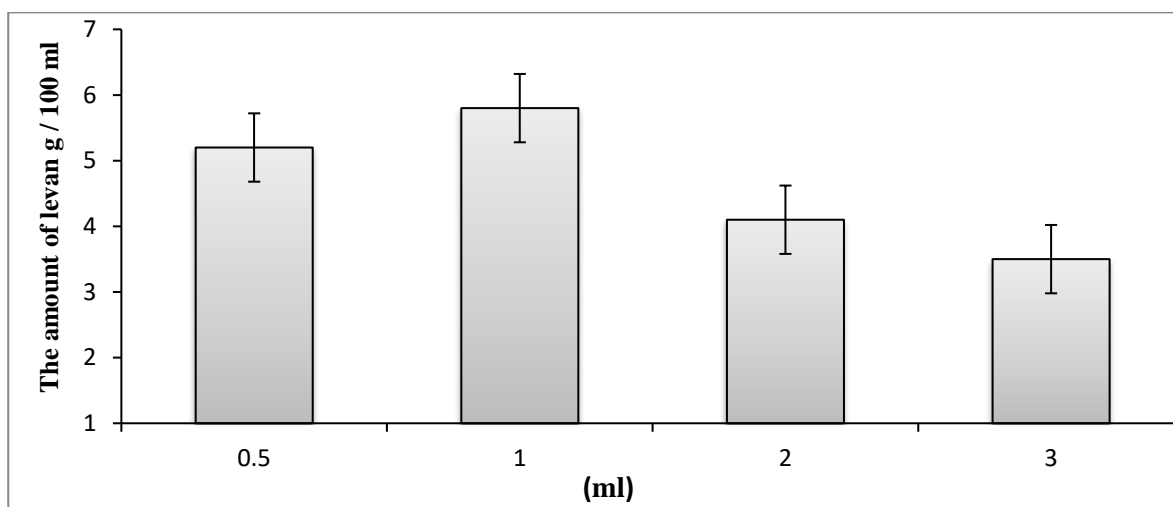


**Fig. (3): Effect of different fermentation periods for levan production from molass media by local isolate.**

#### The inoculum volume

The effect of different inoculum volumes on the levan production was also investigated and presented in fig. (4), the obtained results showed a reduction in levan production with an increase of inoculum volume. The highest levan amount was 6.2 g.100 ml<sup>-1</sup>, which produced when an inoculum volume of 1 ml. 100 ml<sup>-1</sup> (129× 10<sup>9</sup>) colony forms units.ml<sup>-1</sup> was used. The reduction in levan production

might be due to the increasing growth of cells in the production media, which led to the utilization of nutrients and oxygen in a short time and thus a rapid utilization of sugars in the media and reduced production. These results were in agreement with results of Küçükaşık *et al.* (2011) they used 1% inoculum for levan production by *Halomonas* sp. from molasses media. However, the study



**Fig. (4). The inoculum volumes effect on levan production from molass media by local isolate.**

disagree with what some researchers found when 5% of inoculum volume for the production of levan was used. (Dahech *et al.*, 2014). This might be due to the different isolates in their growth rate and the different times of growth phases for each bacterium and the fact that the isolation used in the study is local and may differ from universal isolates and strains in growth and production.

### The fermentation temperature

Fig. (5) shows the efficiency of local bacterial isolation in its ability to produce levan during incubation at different temperatures. The results showed the maximum amount of levan was produced after 40 hours of incubation at 32 °C it was 6.2%, this might be due to the activity of levansucrase to converted sucrose into levan, while the productivity was declined when the temperature reached to 50

°C. The effect of temperature and its suitability for bacterial growth might explain the difference of bacterial isolates in the production of levan. The increase in productivity by increasing or decreasing the temperature was somewhat consistent with the previous research. Dos Santos *et al.* (2013) verified that 35 °C (5.025 g.100 ml<sup>-1</sup>) was the optimum condition for levan production from *B. licheniformis* ANT 179. While Devi & Alamu (2013) found that, the best temperature for levan production was 37 °C, it was (3.3 %) after 60 hours of incubation. The levan increasing productivity was due to the temperature that was appropriate for bacterial growth and production. Their enzymes that produced their metabolites and because they are local isolates might differ from other universal strains in growth and production.

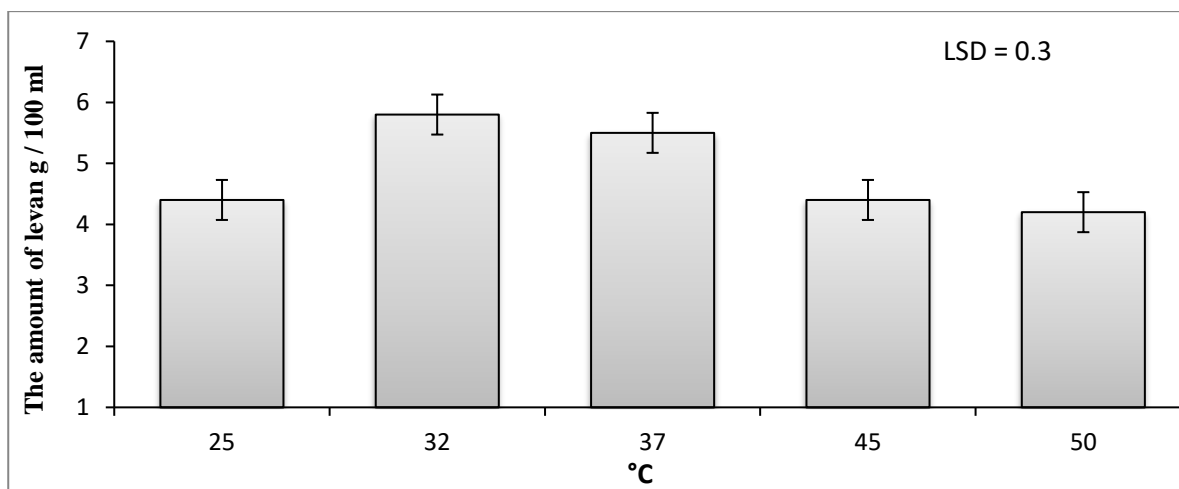


Fig. (5): Effect of incubation at different temperatures on the productivity of levan from a local isolation.

### Initial pH

Fig. (6) shows the effect of the initial pH of the production media on the production of levan by a local isolate of *B. subtilis* subsp. *subtilis* w36. It was noticed from the results that, the productivity of levan increased significantly with increasing pH to 6.5, it was

6.8 g.100 ml<sup>-1</sup>. Meanwhile, levan production was decreased when the pH of the media above 7. The pH of production media considered as an important factor to controlling of metabolic substances included enzymes (levansucrase which responsible for

levan formation), and the optimum pH of *Bacillus* was 6.5 (Abou-Taleb *et al.*, 2015). Belghith *et al.* (2012) concluded that The optimal pH of the levansucrase was 6.5 ,this might be explain increase of levan production. The results of this study were agreed with results of Hamid *et al.* (2018), they reported

the optimum pH of levan production by *B. subtilis* B58 ranged from 6.2-6.5, on the other hand, these results was disagreement with Gojgic-Cvijovic *et al.* (2019) who found that the production of levan by *B. licheniformis* NS032 at an initial pH was 7.2.

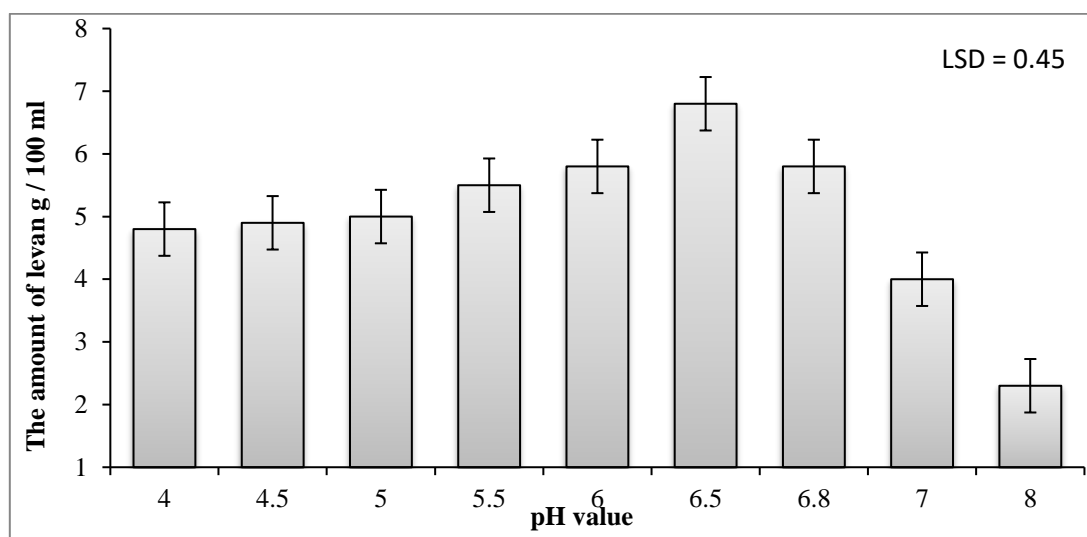


Fig. (6): Effect of pH values on levan production from molass media by local isolate.

## Conclusion

Levan characteristic properties in many fields, such as food, medicine, cosmetics, we have been shown to improve and increase levan production. In that connection, we conducted the present study. Local isolation has proved that *B. subtilis* subsp. *subtilis* w36 was effective in the production of levan at 4.9 g. 100 ml<sup>-1</sup>. The high cost of levan production led researchers to try to reduce the cost of production using some agricultural wastes instead of sucrose such as molasses, which was used in this study, and the results showed that the substitute percentages of 25% of molasses gave the highest production. The optimum conditions of levan production were 40 hours fermentation, 1 ml inoculum volume, initial pH of the medium was 6.5 and the incubation temperature was 32 °C. It

produced 6.8 g of levan from 100 ml of media culture.

## Conflicts of Interest

The authors have declared that there were no conflicts of interest regarding the publication of this paper.

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