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DETERMINAÇÃO DAS CONDIÇÕES ÓTIMAS PARA PRODUÇÃO DE LEVAN DO ISOLADO LOCAL DE *Bacillus subtilis* Cepa AE77

DETERMINATION OF THE OPTIMAL CONDITIONS FOR LEVAN PRODUCTION FROM THE LOCAL ISOLATE OF *Bacillus subtilis* Strain AE77

Bacillus subtilis Strain AE77 تعيين الظروف المثلى لإنتاج الليفان من العزلة المحلية

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RESUMO

Introdução: A levana é um homopolissacarídeo exocelular promissor, seguro, renovável e ecologicamente correto. Recentemente, a levana ganhou crescente interesse de pesquisa devido ao seu impacto significativo nas aplicações da biotecnologia devido à sua biocompatibilidade e capacidade de biodegradabilidade. **Objetivo:** O estudo teve como objetivo determinar algumas das condições culturais ideais para a produção de levana, estudando diversos parâmetros que afetam a produção por isolamento local de *Bacillus subtilis* (B. subtilis, cepa AE77). **Métodos:** A cepa AE77 de B. *subtilis* foi obtida no Departamento de Ciências de Alimentos/Faculdade de Ciências de Engenharia Agronômica como um isolado local, produtor de levana, isolado a partir de doces podres. O meio *Mineral Salts Broth* (MSB) foi aprovado para estudar as condições otimizadas para a produção de levana. Em seguida, a precipitação da levana do meio de fermentação. Posteriormente, a identificação da levana por cromatografia líquida de alta performance (HPLC) estimando o tempo de retenção da levana, comparado-a com o padrão produzido a partir do isolado bacteriano *Erwinia herbicola* (*E.herbicola*). Além das soluções padrão de açúcares (frutose, glicose e sacarose). **Resultados:** Os resultados do estudo comprovaram que o polissacarídeo produzido a partir deste isolado é puro, consiste em frutose e é um homopolissacarídeo pela técnica de HPLC. A melhor produção de levana de *B. subtilis* da cepa AE77 foi com MSB modificado de 20% de sacarose em um período de incubação de 48 horas. Um volume de inóculo de 1×10^8 células/ml a uma concentração de 2%, temperatura de incubação de 35 °C, pH 7,0 e velocidade de agitação de 150 rpm atingiu 4,80 g/100ml. **Conclusões:** HPLC foi usado para separar, identificar e quantificar tanto a cepa AE77 de *B. subtilis* e a levana padrão produzida a partir do isolado bacteriano *E. herbicola*, bem como frutose padrão, glicose e sacarose, respectivamente. Os resultados mostraram que a levana é um homopolissacarídeo constituído por um tipo de monômero (as unidades de açúcar de frutose). As condições para a produção de levana foram determinadas isolando *B. subtilis* da cepa AE77 em meio de sacarose. Os resultados mostraram que o meio ideal para produção continha 20% de sacarose sem qualquer fonte de nitrogênio, pH 7,0 e volume de inóculo $1x10^8$ células/ml a uma concentração de 2% a uma temperatura de 35 °C velocidade de agitação 150 rpm e período de incubação de 48 horas.

Palavras-chave: *Levan, Bacillus subtilis cepa AE77, HPLC, condições ótimas.*

ABSTRACT

Background: Levan is a promising safe, renewable, and environmentally friendly exocellular homopolysaccharides. It has recently gained increasing research interest because of its significant impact on biotechnology applications due to its biocompatibility and ability to biodegradability. **Aim:** The study aimed to determine some of the optimal cultural conditions for levan production by studying many parameters affecting production by local isolation *Bacillus subtilis* (*B. subtilis* Strain AE77). **Methods:** *B. subtilis* Strain AE77 It was obtained from the Department of Food Science/College of Agricultural Engineering Sciences as a local levan-producing isolate isolated from rotten sweets. The Mineral Salts Broth (MSB) medium was approved to study the optimized conditions for levan production. Then precipitation of levan from the fermentation medium. Then the identification of levan with High-Performance Liquid Chromatography (HPLC) technology by estimating the

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retention time of levan, compared with the standard model produced from the bacterial isolate *Erwinia herbicola* (*E.herbicola*). Besides, each of the standard solutions of sugars (fructose, glucose, and sucrose). **Results:** The study results proved that the polysaccharide produced from this isolate is pure and consists of fructose and is a homopolysaccharide with the HPLC technique. The best levan production was from *B. subtilis* Strain AE77 using a modified MSB medium of 20% sucrose in a 48 hours incubation period. An inoculum volume of 1×10^8 cell/ml at a concentration of 2% at an incubation temperature of 35 °C and pH = 7.0 and agitation speed of 150 rpm reached 4.80 g/100ml. **Conclusion:** HPLC was used to separate, identify and quantify both *B.subtilis* Strain AE77 and standard levan produced from the bacterial isolate *E. herbicola*, as well as standard fructose, glucose, and sucrose, respectively. The results showed that levan is a homopolysaccharide consisting of one type of monomer (the fructose sugar units). Conditions for levan production were determined by isolating *B. subtilis* Strain AE77 in a sucrose medium. The results showed that the optimal medium for production contained 20% sucrose without any nitrogen source, pH7.0, and inoculum volume $1x10^8$ cell/ml at a concentration of 2% at a temperature of 35 °C agitation speed 150 rpm, and incubation period of 48 hours.

***Keywords**: Levan, Bacillus subtilis Strain AE77, HPLC, optimum conditions.*

الملخص:
الليفان Levan هو أحد عديدات السكاريد الخارجية Exocellular homopolysaccharides الآمنة والمتجددة والصديقة للبيئة. اكتسب مؤخرًا اهتمامًا بحثيًا متزايدًا بسبب تأثيره الكبير على تطبيقات التكنولوجيا الحيوية نظرًا لتوافقه الحيوي Biocompatibility وقدرته على التحلل البيولوجي Biodegradability. **الهدف:** هدفت الدراسة إلى تحديد بعض الظروف المزرعية المثلى لإنتاج الليفان من خلال دراسة العديد من المعايير المؤثرة في الإنتاج بوساطة العزلة المحلية (*B. subtilis* Strain AE77) *Bacillus subtilis*. **طرائق العمل:** تم الحصول على عزلة *B. subtilis* Strain AE77 من مختبرات قسم علوم الأغذية / كلية علوم الهندسة الزراعية كعزلة محلية منتجة لليفان والمعزولة من الحلويات التالفة rotten sweets, أُعتمد وسط الاملاح المعدنية Mineral Salts Broth (MSB) medium لدراسة الظروف المثلى في إنتاج الليفان, ثم استخلاص الليفان من وسط التخمير, تشخيص الليفان بتقنية الكروموتكرافي السائل عالي الاداء high-performance liquid chromatography (HPLC) من خلال تقدير وقت الاحتجاز للعينة القياسية لليفان المنتج من بكتريا (*E.herbicola*) *Erwinia herbicola* والعينة قيد الدراسة وللمحاليل القياسية للفركتوز والكلوكوز والسكروز على التوالي. **النتائج:** اثبت نتائج الدراسة ان السكر المتعدد المنتج من هذه العزلة نقي ويتكون من وحدات الفركتوز وهو من نوع عديد السكريات المتجانسة homopolysaccharides بتقنية الكروموتكرافي السائل عالي الاداء (HPLC), وقد وجد أن أفضل وسط لإنتاج الليفان من قبل العزلة المحلية *B. subtilis* Strain AE77 هو وسط الأملاح المعدنية السائل (MSB) المحور بنسبة 20% سكروز ومدة حضن 48 ساعة وحجم اللقاح 1 ×10^8 خلية / مللتر بتركيز 2% عند درجة حرارة 35مْ وأس هيدروجيني 7.0 وسرعة تحريك 150 دورة / دقيقة وصل إلى 4.80 غم / 100 مل. **الاستنتاج:** تم استخدام HPLC لفصل وتحديد وقياس كل من *B. subtilis* Strain AE77 والليفان القياسي المنتج من العزلة البكتيرية *E.herbicola* ، وكذلك الفركتوز القياسي والجلوكوز والسكروز على التوالي. أظهرت النتائج أن الليفان هو من نوع السكريات المتعددة المتجانسة homopolysaccharide يتكون من نوع واحد من الوحدات البنائية (monomer) هي وحدات سكر الفركتوز. حددت الظروف لانتاج الليفان من قبل العزلة *B. subtilis* Strain AE77 في وسط السكروز واظهرت النتائج ان الوسط الامثل للانتاج يحوي على 20% من السكروز وبدون اي مصدر نايتروجيني برقم هيدروجيني 7.0 وحجم اللقاح 1× 10^8 خلية / مللتر بتركيز 2% عند درجة حرارة 35مْ وسرعة تحريك 150 دورة / دقيقة ومدة حضن 48 ساعة.

***الكلمات المفتاحية:** الليفان, بكتريا Bacillus subtilis Strain AE77, تقنية الكروموتكرافي السائل عالي الاداء HPLC, الظروف المثلى*

1. INTRODUCTION:

Levan (fructans) with the chemical formula $(C_6H_{10}O_5)_n$ is a linear or branched exogenous sugar whose units are constructed from fructose because of breaking the glycosidic bond of sucrose and one molecule of glucose, either internal or terminal (Joaquim *et al.,*2018, Al-Halbosiy *et al.,*2018).

Levan is produced by a single enzyme or a system of simple enzymes (Han, 1990) which may find in the form of a sticky mucous substance secreted into the fermentation medium (production). Otherwise, in the form of a capsule that surrounds the microorganism microorganisms cell wall is a defensive means to protect the microorganism from extreme conditions. Thus, it strengthens the cellular membranes that protect microbial cells against stress resulting from drought or temperature changes or as a reservoir of energy outside the cell by using carbon as a source of energy (Sukan *et al.,* 2015). Microbial levan has the advantage of having a high degree of polymerization (DP) >100 and a high molecular weight ranging from 1×10^5 to 1×10^8 Dalton, also; having branching regions whose proportions vary according to the strain of the microorganism producing it even within the same type, as it can be observed that it has a branching percentage of 12% in each of *Zymomonas mobilis* (*Z.mobilis*) and some species of *Bacillus*. Whereas the branching percentage of the product from *Serratia levanicum* is less than 6%, while the degree of polymerization of plant levan is DP <100 with molecular weight ranges from 2000 to 33000 Dalton. It has been observed that some chains of Fructooligosaccharides (FOS) have a DP< 9, so the first type is preferred because it is multidisciplinary. In addition to the role played by its structure, especially its branches, in the

biological activities against cancer cells (Ki-Hyo *et al.,* 2006). Finally, Stojković *et al.* (2015) indicated that the degree of branching could range from (2 to 12)%.

2. MATERIALS AND METHODS:

2.1. Materials

2.1.1. Equipment and Apparatuses

Equipment and apparatuses that were used in this experiment were Erlenmeyer conical flasks 250 ml, pH meter Hanna (Italy), Water bath Memmert (Germany), Sensitive balance Sartorius (Germany), Shaker incubator was supplied from Heidolph (Germany), which is used to measure the temperature, as well as to study the effect of agitation speed, high-speed cooling centrifuge Ihanil (Korea) and High-Performance Liquid Chromatography (HPLC) Shimadzu Japanese.

2.1.2. Chemicals

Dipotassium hydrogen phosphate (K_2HPO_4), Potassium dihydrogen phosphate (KH_2PO_4), Sulfate heptahydrate ($MgSO_4.7H_2O$), peptone, yeast extract, and tween (80, 40) were supplied from Sigma-Aldrich(Germany). Nutrient Broth(N.B) was obtained from Himedia (India).

Potassium hydroxide (KOH), Calcium chloride ($CaCl_2$), and Sodium hydroxide (NaOH) were obtained from BHD(England). The other chemical used in these experiments, ethanol, was supplied from Scharlau (Spain).

2.2. Methods

2.2.1. Isolation of B. subtilis Strain AE77

B.subtilis Strain AE77 isolate was obtained from the laboratories of the Department of Food Science/College of Agricultural Engineering as a local isolate of levan genetically diagnosed and registered in GenBank with number MW074171.1 under the name *Bacillus subtilis* subsp.*subtilis* AE77 (Al-Musawi and Al-Shamary, 2021).

2.2.2. Activation of B. subtilis strain AE77 and calculating the number by MacFarland method

The local isolate activated the local isolate *Bacillus subtilis* Strain AE77 using liquid nutrient media N.B and incubated at a temperature of 35 °C for 18 hours. To obtain the approximate number of cells 1×10^8 cell/ml equal to the turbidity of growth of MacFarland standard solution. (Cappuccino and Sherman, 2002).

2.2.3. Levan production medium

This medium was prepared by dissolving KH_2PO_4 0.3g; sucrose 20g;K_2HPO_4 0.3g; $MgSO_4.7H_2O$ 0.05g in 100ml of distilled water by stirring and heating until boiling.

The pH had been adjusted to 7.0± 0.2 by a pH meter. The medium was placed in Erlenmeyer conical flasks 250 ml in volume, and then, these flasks were sterilized by the autoclave at a temperature of 121 °C for 15 min (Shih *et al.,* 2005).

2.2.4. Precipitation of levan

According to Srikanth et al. (2015), Levan production was carried out with some modifications. At the end of the fermentation period, transferring the contents of the production medium to a water bath at a boiling temperature for 30 min to kill the bacterial cells, inhibit the extracellular activity of the enzyme, and left to cool. Further, a centrifuged at 8000 rpm for 30 min to get cell-free supernatant. Thus to eliminate the effectiveness of the extracellular remnants of the enzyme in the supernatant, it was returned to the water bath at boiling for 5 min and left at room temperature for cooling. Later the pH of the supernatant was increased to a range of 9.5 to 10.5 using (1M) KOH. Then chilled ethanol was added at a degree -20 °C and a concentration of 80% at a ratio of 1: 2 (volume of the supernatant: volume of the ethanol).

After adjusting the pH of the supernatant, 1ml of 1% $CaCl_2$ was added to it with continuous stirring for 20 min. Finally, The precipitated levan had been high-speed cooling centrifuge at 13000 rpm for 15 min to obtain levan pellets, and the levan was precipitated again with cold ethanol in a ratio of (1:4 weight levan pellets/volume of the ethanol). Then collected in a glass Petri dish left in the oven at 45 °C till dryness; dried levan was collected by scrubbing and preserved in a clean, screwed glass vial as a dried powder.

2.2.5. Analysis of Levan by HPLC technology

Analysis of Levan from *B. subtilis* Strain AE77 and compared it with the standard levan produced from the bacterial isolate *E.herbicola* and prepared from the German company Sigma-Aldrich by estimating the retention time using Japanese Shimadzu HPLC system model LC-2010 A HT. The instrument operates by using a mobile phase contain Acetonitrile: Distilled water (75:25) (V: V) the flow rate was at 1.5 ml/min, temperature 40 °C and the injection volume 20 µl, while the UV detector was used with a wavelength of 205 nm. The standard sugars used for compared with levan were fructose, glucose, and sucrose.

2.2.6. Optimization of levan production

Experiments to determine the optimal conditions for levan production were performed by the local isolate understudy and twice for each experiment.

2.2.6.1. Effect of the carbon sources (sucrose)

To clarify the effect of different concentrations of sucrose on levan production, different concentrations included (10, 20, 30, 40, and 50)% were added to the production medium mentioned in paragraph (2.2.3), distributed in flasks of 250 ml by 100 ml. the current process aimed to obtain the best production concentration at a pH of 7.0, incubation point of 35 °C, and inoculum size of 2% so that 1ml of it contains 1 x 10^8 cell/ml, the incubation period of 48 hours and a vibrating incubator with a speed of 150 rpm. After the incubation period, After the incubation period expired, the precipitation of the levan process was conducted according to the method of work mentioned in paragraph (2.2.4.).

2.2.6.2. Effect of the initial pH

The production medium was prepared with a pH of (5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) using a solution of (1 M) of HCL or (1 M) of NaOH to adjust the pH. after the incubation period, levan was extracted and its dry weight according to paragraph (2.2.4.).

2.2.6.3. Effect of inoculum volume

The effect of the inoculum volume was studied using different concentrations of *B. subtilis* bacteria that included (1, 2, 4, 6, 8, and 10)% of the inoculum 1×10^8 cell/ml. After the incubation period, levan was extracted, and its dry weight according to paragraph (2.2.4.).

2.2.6.4. Effect of agitation speed

The flasks containing the production medium and inoculated were incubated in the Shaker incubator at different speeds that included (100, 150, 200, 250, and 300) rpm with another sample for comparison, but in a static incubator (static fermentation). Levan was extracted, and its dry weight according to paragraph (2.2.4.).

2.2.6.5. Effect of the temperature

To the effect of temperature on levan production from *B. subtilis* bacteria under study, the flask containing the production medium inoculated with bacteria was incubated at different temperatures including (30, 35, 40, 45, and 50) °C for 48hours. after the incubation period, levan was extracted and its dry weight according to paragraph (2.2.4.).

2.2.6.6. Effect of the incubation time

Various incubation times, including (12,24, 48,72, and 96)hours, were tested for the medium inoculated with *B. subtilis* Strain AE77 to obtain the best fermentation time for the isolate. According to paragraph (2.2.4.), Levan was extracted and its dry weight.

2.2.6.7. Effect of the nitrogen sources

100ml of each Mineral salts broth with 20% sucrose containing two nitrogen sources (yeast extract and peptone) concentrations (0.5,1.0,1.5 and 2.0)% each separately. According to paragraph (2.2.4.), Levan was extracted and its dry weight.

2.2.6.8. Effect of adding tween 80, 40

Tween (80, 40) was used in a mineral salt broth medium as non-ionic surfactants on the permeability of cell membranes to improve the ability of bacteria to produce levan. They were added at concentrations of (0.1,0.3,0.5,0.7, and 1.0)% (v/v) each separately in the production medium. after the incubation period, levan was extracted, and its dry weight according to paragraph (2.2.4.).

2.3. Statistical analysis

Optimization of levan production study results in was analyzed statistically with Graphpad prism version 9 to detect any significant differences in this study at the 1% level ($P<0.01$) (Graphpad Software Inc, Jolla CA).

3. RESULTS AND DISCUSSION:

3.1. RESULTS

3.1.1. identification

HPLC was used to separate, identify, and quantify B.subtilis Strain AE77 and standard levan produced from the bacterial isolate E.herbicola and standard fructose, glucose, and sucrose. The results showed that levan is a homopolysaccharide consisting of one type of monomer (the fructose sugar units) Figure (1, A, B, C).

3.2. Optimization of levan production by *B.subtilis* Strain AE77

3.2.1. Effect of the carbon sources (sucrose)

The dry weight of levan at a concentration of 20% sucrose was 4.80 g/100 ml, noting that there was a decrease in productivity by increasing the amount of sucrose in the medium to reach 1.71 g/100ml when using sucrose at a concentration of 50%, as shown in Figure (2).

3.2.2. Effect of the pH

The study results from Figure (3) showed the effect of the pH of the medium on the production of levan using *B.subtilis* strain AE77. Accordingly, it was observed that the productivity of the levan increased by increasing the pH. The highest amount was obtained at pH 7.0, where the amount of levan produced reached 4.75g/100 ml. then, the amount of levan produced decreased with increasing pH of the medium, reaching (1.99, 0.35, and 0.31) g/100 ml at pH Numbers 8.0, 9.0, and 10.0, respectively.

3.2.3. Effect of inoculum volume

The inoculum volume and concentration are among the factors affecting the production of materials from microorganisms. Figure 4 noted the increase in the amount of levan produced by increasing the inoculum size 1 ml containing 1×10^8 cell/ml. Besides, the highest amount was obtained at a concentration 2%, as the produced amount of levan reached 4.75g/100ml. However, the production decreased by increasing the concentration of the added inoculum, reaching a production of 1.68 gm/100 ml at a concentration of 10%.3.2.4. Effect of the agitation speed

Figure 5 shows the effect of aeration speed on levan production. The first two treatments were carried out in a shaker incubator compared to incubation in a static incubator. The amount of levan produced was 4.90g dry weight /100ml, (96 and 92)% and 0.31g/100ml, respectively, at a speed of 150 rpm, while the amount of levan produced in the static farm was 4.10g dry weight/100ml. Thus, this result gave a positive indication of the effect of stirring speed on enhancing levan production by increasing the transfer of mass in the medium, which leads to an increase in the exposure of the bacterial cell to the basic substrate (sucrose), noting a decrease it noted a decrease in the amount of levan produced, with an increase in the aeration speed and an increase in the biomass compared to the static culture, indicating that the high aeration speed positively affects biomass production and is a negative in the production of levan.

3.2.5. Effect of the temperature

The effect of different temperature ranges on levan production was studied using local isolation is under study. It ranged between (30-50)°C with a difference of 5 degrees of heat from one degree to another. The results are shown in Figure(6) indicate that the optimum temperature for the production of levan was 35 °C, which is almost the same as for previous experiments, as it reached 4.81g/100ml. In contrast, it reached (40, 45, and 50) °C (3.35, 2.84, and 1.70)g/100 ml, respectively, and the degree of 35 °C is the ideal degree to stimulate the effectiveness of cellular enzymes to produce metabolites, including the exopolysaccharides. as well as, their conformity to the degree which was used to isolate it from its natural environment and is the optimum itself used for production in previous experiments.

3.2.6. Effect of the incubation time

The highest yield of levan dry weight was recorded from the most efficient local isolate *B. subtilis* Strain AE77 after 48hours of incubation, as shown in Figure(7). It is the same as previous experiments, reaching 4.84g/100 ml. The efficient production was not observed during the first 12 hours of incubation. Incubation time, while this isolate produced 2.89g/100ml of levan within 24 hours.

3.2.7. Effect of the nitrogen sources

Research has confirmed the importance of the nitrogen source as an influencing factor in bacterial growth and synthesis of many organic compounds, including amino acids and proteins. Besides, the benefit of microorganisms from it depends on the amount of its readiness, which varies according to the source. Furthermore, the difference in the exploitation of nitrogen sources exists even between isolates within the same type (Stanier *et al.,* 1976; Stanbury *et al.,* 1995).

Therefore the effect of organic sources, including (peptone and yeast extract) on levan production was studied using *B.subtilis* isolate. The addition was done separately to the production medium while preserving the inorganic sources originally present in the production medium, including KH_2PO_4, K_2HPO_4, and $MgSO_4.7H_2O$. The results are shown in Figure (8) presented the superiority of the main production medium without any support from organic nitrogen sources; it was 4.81g/100ml, with the note of a decrease in the amount of levan produced when the medium was supplemented with organic sources of nitrogen (peptone and yeast extract, and a decrease in the levan produced by increasing the added concentration of organic sources.

3.2.8. Effect of adding the tween 80,40

It is noticed from Figure(9) that the highest yield of levan was when using the medium mineral salt broth without adding tween 80 or tween 40. At the end of the incubation period, the levan was 4.81g/100ml.

In contrast, the dry weight of levan at concentration was 0.5% of tween 80 and tween 40, excluding the best concentrations for producing levan from *B.subtilis* strain AE77 was 3.87g/100ml and 3.77g/100ml, respectively. A decrease in the amount of levan produced in the highest concentrations of the two compounds.

3.3. DISCUSSIONS

HPLC technical data confirmed that the produced polysaccharide is pure and consists of fructose units, a type of homopolysaccharide, and a type of fructan that belongs to the group of short fructose sugars FOS (Sreenivasan and Kandasamy, 2017).

Benigar *et al.* (2015), who succeeded in extracting levan from isolates *Z.mobilis* and *E. herbicola* and measuring their structures with several techniques, indicated that levan consists of fructose.

While Pei *et al. (*2020) demonstrated that the backbone of levan using HPLC technology consists of 2,6-substituted β-fructoses.

3.3.1. In connection with the discussion of paragraph (3.2.1.).

Shih *et al.* (2005) noted that the amount of levan obtained from the bacterial isolate *Bacillus subtilis* (natto) Takahashi ranged between 4-5 g/100 ml. Also, they indicated the efficiency of *B.subtilis* strains with their high production of levan compared with the productivity rate of other genera due to its possession of the enzyme levansucrase, which is responsible for the production of levan. Ramya *et al.* (2020) explained that sucrose is the only carbon source in catalyzing the enzyme levansucrase, which is catalyzed with sucrose as a basic material in the production medium stimulates cells to convert sucrose into levan Moreover, Dos Santos *et al.* (2013) stated that the initial sucrose percentage in the medium, whenever it is high within the required limits, will increase the percentage of fructose and thus increase the medium. Chidambarama *et al.* (2019) indicated that a gradual decrease in Levan formation in the fermentation medium begins with an increase in sucrose concentration above 30%, which is explained by the phenomenon of the substrate inhibitory effect. The decrease when using high concentrations of sucrose is due to the weak ability of the bacteria under study to withstand the high osmotic pressures resulting from the use of such levels of sugar. Besides, the increase in sucrose concentration is accompanied by a noticeable increase in the rheology of the fermentation medium, which in turn reflects negatively on the production process (Santos *et al.,*2019). therefore, to study the remainder of the other influencing factors in levan production from *B.subtilis* strain AE77, sucrose at a concentration of 20% has been adopted as an upper limit for levan production.

3.3.2. As for paragraph (3.2.2.)

Da Silva (2020) indicated that the pH of the levan production from Bacillus subtilis natto is 7.0, which is the same as what Neeta and Manjusha (2017) indicated when producing levan from the isolate *Bacillus subtilis* (DQ922949), and the role of this pH in catalyzing the enzyme levansucrase. They also indicated the reduction of its effectiveness at pH8.0 due to its participation in removing amino acids from the transfer tRNA, which participates in the protein synthesis process, which leads to the stopping of the protein synthesis process in which it participates. Moreover, Vaidya and Prasad (2012) indicated that the optimum pH for the action of the enzyme levansucrase from *Bacillus subtilis* BB04 is 7.0, its reduction at pH8.0 and reaching zero at pH 9.0. Abou-Taleb *et al.* (2015) also indicated an increase in the amount of levan produced and biomass with an increase in the PH from 5.5 -6.5 and a decrease in the levan at a pH of 8.5. Therefore, a pH of 7.0 was approved in subsequent experiments as optimal for levan production from *B. subtilis* Strain AE77 isolate.

3.3.3. connection with the discussion of paragraph (3.2.3.).

This decrease in production is attributed to an increase in the inoculum concentration, competition for nutrients, and their depletion in a short time, as well as the possibility of producing other compounds that increase the medium alkalinity and thus will limit bacterial growth, which affects the productivity (Yugandhar *et al.,* 2007).

The concentration of the optimal inoculum for levan production varies according to the difference and variation of the strain used even within the same species in its growth rate and the difference in the period of the growth stages of each bacterium in production. The medium components and the surrounding environmental conditions such as temperature and aeration. It should be noted that the study isolation is local and thus differs from others, whether local or global, in terms of its development and production. This study did not agree with the finding of Abou-Taleb *et al.* (2015) and Chidambaram *et al.* (2019) when using a 10% inoculum to produce levan with isolates of *Bacillus lentus* V8 and *Bacillus subtilis*

MTCC 441, respectively. Moreover, Ramya *et al.* (2020) used a 25% inoculum to isolate *Bacillus* sp MTCC 1434. However, Ergene and Avci (2018) found a percentage of 5% as an inoculum for the isolate of *Bacillus* sp. ZBP4 at 35 °C and pH = 7.0 is the best in producing Exopolysaccharides. Based on the data of this experiment, the inoculum size was 2% and the number 1×10^8 cell/ml in the subsequent experiments.

3.3.4. As for the discussion of paragraph

Rahman *et al.* (2005), indicated that oxygen is among the very slow-soluble nutrients in the fermentation medium. Similarly, the aeration and shaking process is important for transporting and penetrating the oxygen in the flask into the medium to help the growth of the biomass needed as the final recipient of electrons in cellular metabolism and demonstrate the effect on the production of levan. The researchers Shih *et al.* (2005), indicated that slight shaking increases the productivity of polysaccharides instead of strong shaking and high aeration working together to inhibit the production of levan. This was confirmed by the study results of reduced production when using a shaking speed of 250-300 rpm. Furthermore, Ishikawa *et al.* (1990) stated that the excess of oxygen leads to a deviation of the metabolic pathway for the production of levan and ethanol to acetaldehyde, which inhibits the production of levan and the growth of *Z.mobilis* cell. Chidambaram *et al.* (2019), when studying the optimum conditions for the production of fibrin from the two isolates *Bacillus subtilis* MTCC 441, found that the agitation speed of 150 rpm was the best speed for the production of levan production. They Indicated the need to pay attention to the ratio between the volume of the production medium and the volume of the flask used in production. Then, the difference in the medium volume inside the flask leads to changes in the shaking intensity and the oxygen supply. Shih *et al.* (2005), stated that in producing Exopolysaccharides from aerobic bacteria, one of their requirements is to provide adequate shaking and oxygenation speeds. Therefore the speed of 150rpm was adopted in the subsequent experiments.

3.3.5. connection with the discussion of paragraph (3.2.5.)

Bersaneti *et al.* (2018) found that the optimum temperature of 35 °C for the production of levan and FOS from *Bacillus subtilis* natto when using levansucrase, while Bansal *et al.* (2019) stated that the rate of optimum production temperature ranges from 25 to 37 °C, levan production may be inhibited at temperatures higher than 47 °C, with some temperature exceptions depending on the type of microorganisms used in the production of levan. Abou-Taleb *et al.* (2015) reported a variation in the researchers' results about the productivity of levan. The reasons for the difference in the bacterial isolates' ability to grow and produce the levansucrase enzyme at certain temperatures when the inoculation temperature is appropriate for the growth of these isolates. It should be noted that local isolates differ in their ability to produce metabolites and the suitable temperatures for their growth. Ergene and Avci (2018) indicated that Exopolysaccharides play an important role in protecting the cell from stress conditions such as temperature, pH, and light intensity. Thus, their production is a direct response to environmental conditions. Therefore, a temperature of 35 °C was adopted to complete the subsequent experiments.

3.3.6. As for the discussion of paragraph (3.2.6.)

Abou-Taleb *et al.* (2015), within 48hours of cuddling, observed a decrease in sucrose levels, matched by an increase in the level of levan within two days. Dos Santos *et al.* (2013) attributed the low levan yield to the increase in the incubation time due to the lack of nutrients and the accumulation of metabolic toxins. The possibility of self-decomposition of the bacterial cells was negatively affected levan production. It was noted in Figure(7) the gradual decrease of levan by increasing the incubation period. Thus this study indicates that the stability of the levan produced takes place in the stationary phase. This is confirmed by Shih *et al.* (2005) indicated that the maximum production of levan occurs in the stationary phase.

3.3.7. As for paragraph (3.2.7.)

Melo *et al.* (2007), proved that levan production was affected by the increase in yeast extract concentration in the production medium. So, the increase in the concentration of (2-10) g/l resulted in a decrease in levan production with increasing the biomass production of the isolate *Z. mobilis* and pointed out that yeast extract improves cellular metabolism, which subsequently leads to enhanced substrate consumption. Although a significant increase in substrate consumption was verified, only a small portion of the consumed sugar was converted to levan. The large amount consumed was directed to biomass production and possibly other by-products of fermentation.

3.3.8. In connection with the discussion of paragraph (3.2.8.)

Researchers Guruviah and Alamu (2013) observed an increase in the amount of levan

produced from *B.subtilis* when it was added to substances tween 40,80, and triton X-100 separately at a concentration of 0.5 g/l. Moreover, these substances are considered growth supports and catalyzing for the levansucrase enzyme. Therefore, their addition led to a decrease in the surface tension between the cell membranes and the surrounding water medium, along with a change in the cellular membrane components from fatty acids, which increases the secretion of the enzyme levansucraseincreases its effectiveness. This is reflected in the increase in the productivity of levan, as Shaheen *et al.* (2017) pointed out the importance of these materials in reducing tension (surface tension). Then, the effect in stimulating the enzyme activity produced from the bacterial isolation KIBGE-IB14 *Zymomonas mobilis* by adding 20,80, and triton X-100 in different concentrations. They also noticed a similarity in the effect of these substances in increasing the effectiveness of the levansucrase enzyme produced from bacteria. However, as the concentration increases at a certain level, the enzymatic activity decreases, Arockiasamy and Banik (2008) state when studying optimal conditions for gellan gam production from *Sphingomonas paucimobilis* ATCC 31461 by using shake flasks when adding the surface-active substances 40,80, and triton X-100, a simultaneous increase between the gellan and the biomass of the cells, where they observed a decrease in production when the concentration increased, They attributed the reason for this to the aggregation of cells together in the form of aggregates, which affects the secretion of polymer from cells leading to decrease the production yield.

4. CONCLUSIONS:

1. HPLC was used to separate, identify and quantify both *B.subtilis* Strain AE77 and standard levan produced from the bacterial isolate *E.herbicola*, as well as standard fructose, glucose, and sucrose, respectively. The results showed that levan is a homopolysaccharide consisting of one type of monomer (the fructose sugar units).

2. Conditions for levan production were determined by isolating *B. subtilis* Strain AE77 in sucrose medium. The results showed that the optimal medium for production contained 20% sucrose without any nitrogen source, pH 7.0, and inoculum volume ($1x10^8$) cell/ml at a concentration of 2% at a temperature of 35 °C, agitation speed 150 rpm/min, and incubation period 48 hours.

3. There are significant differences at the level of $P<0.01$ between all treatments of Optimization of levan production by *B.subtilis* Strain AE77.

5. DECLARATIONS

5.1. Study Limitations

The study is limited to the sample size.

5.2. Acknowledgements

The author is grateful to the Market Research and Consumer Protection Center and who contributed to the accomplishment of the current study.

5.3. Funding source

The authors funded this research.

5.4. Competing Interests

The authors declare that they have no competing interests.

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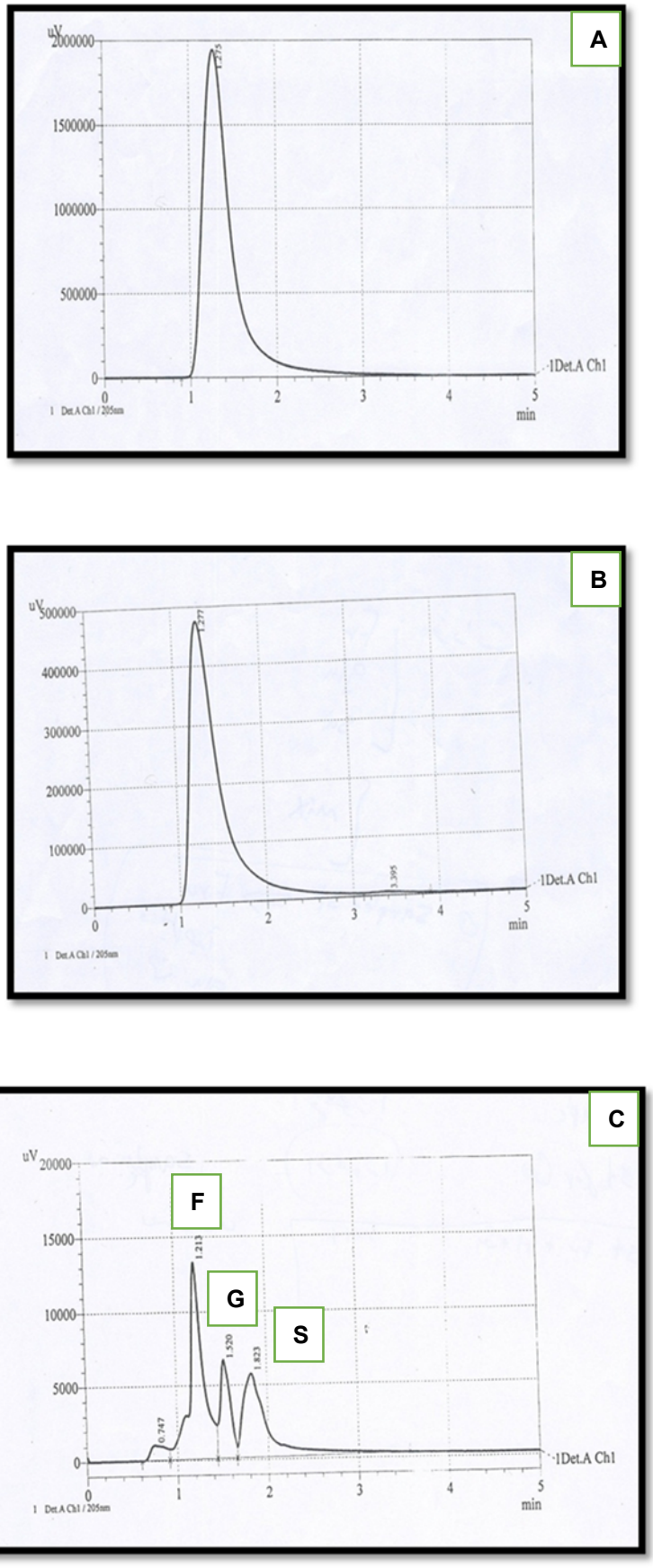

Figure 1: HPLC analysis of levan production, A: standard from *Erwinia herbicola*, B: sample from *B. subtilis* Strain AE77 in MSB, C: standard sugar (F: Fructose, G: Glucose, S: Sucrose)

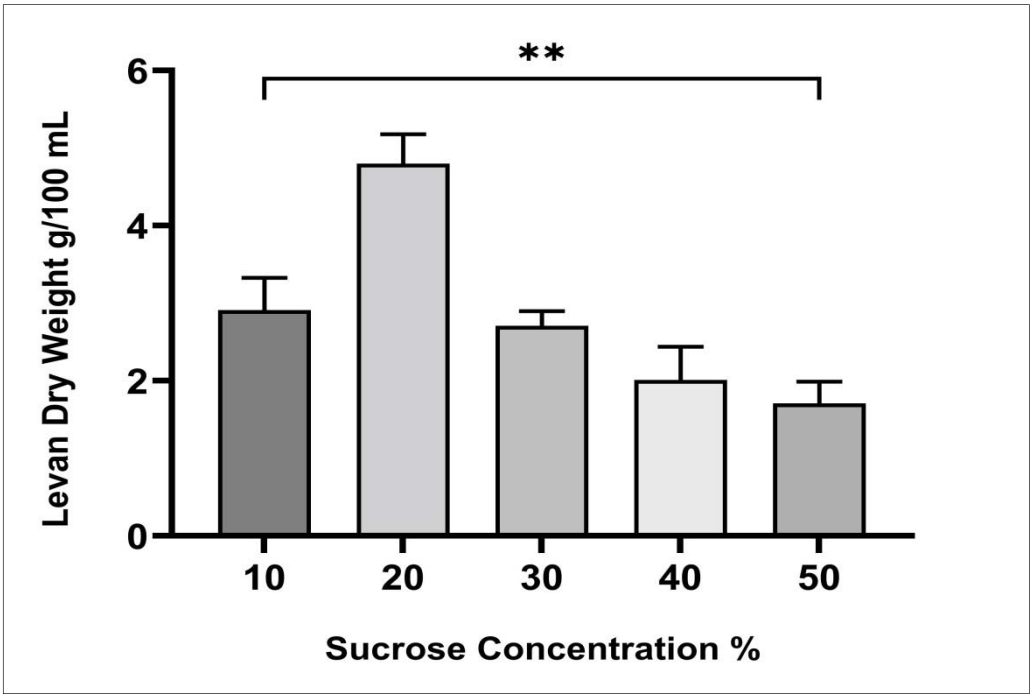

Figure 2: production of levan from *B. subtilis* Strain AE77 in MBS containing different sucrose concentrations incubated at 35 °C for 48hours and pH = 7.0 and inoculum volume 2%, and agitation speed 150 rpm

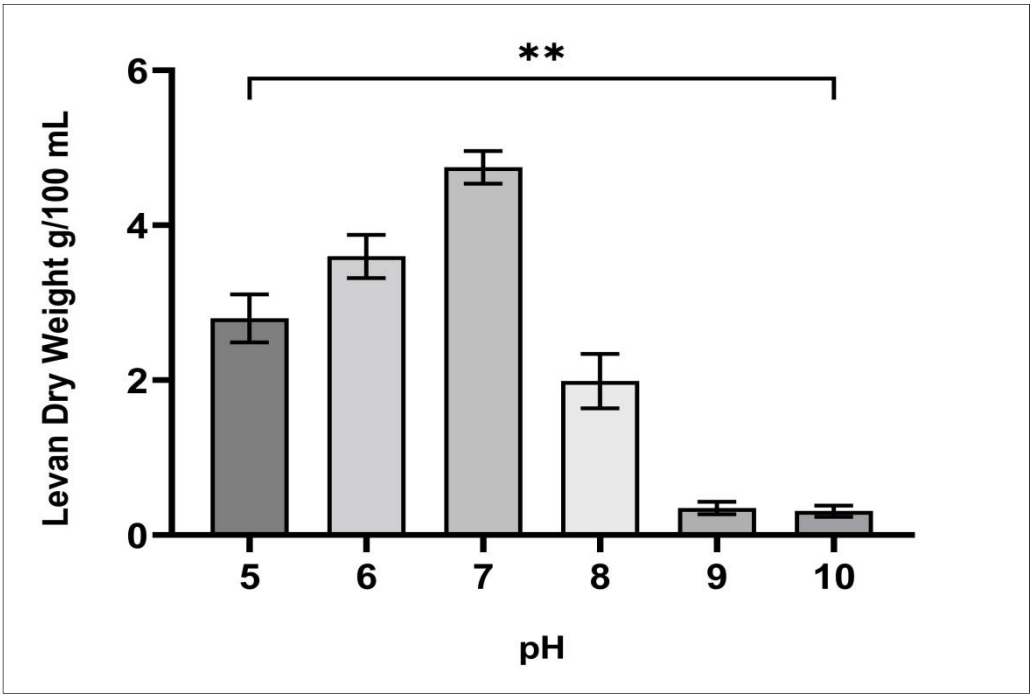

Figure 3: Effect of pH on levan production from *B. subtilis* Strain AE77 in MSB containing 20% sucrose incubated at 35 °C for 48 hours and inoculum volume 2% and agitation speed 150 rpm

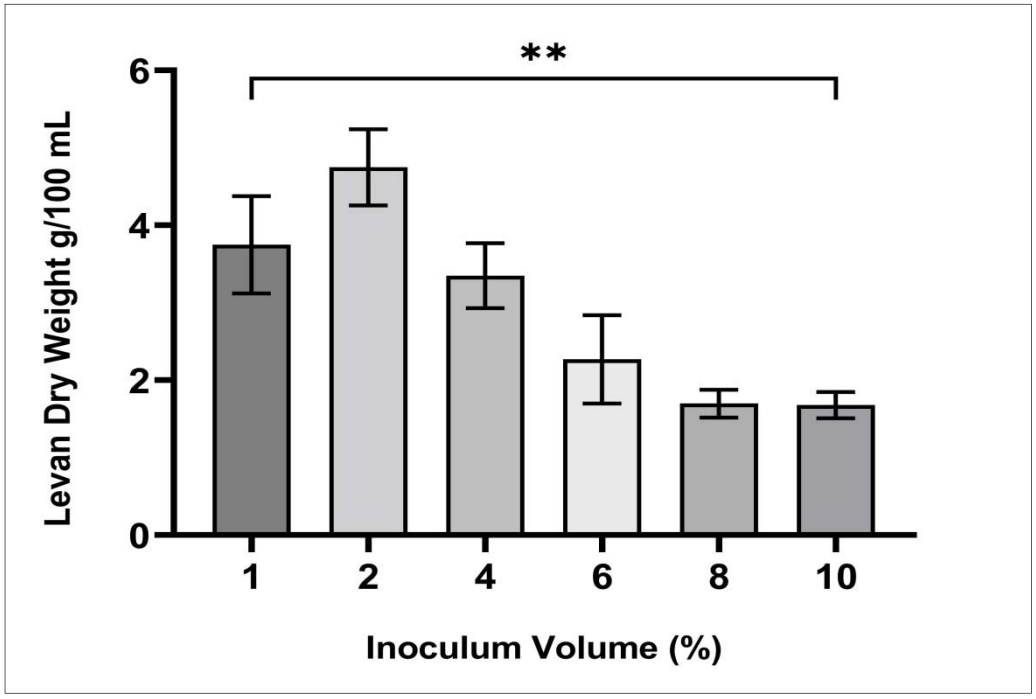

Figure 4: Effect of inoculum volume on levan production from *B. subtilis* Strain AE77, in MSB containing 20% sucrose, incubated at 35 °C for 48 hours and pH = 7.0 and agitation speed 150 rpm

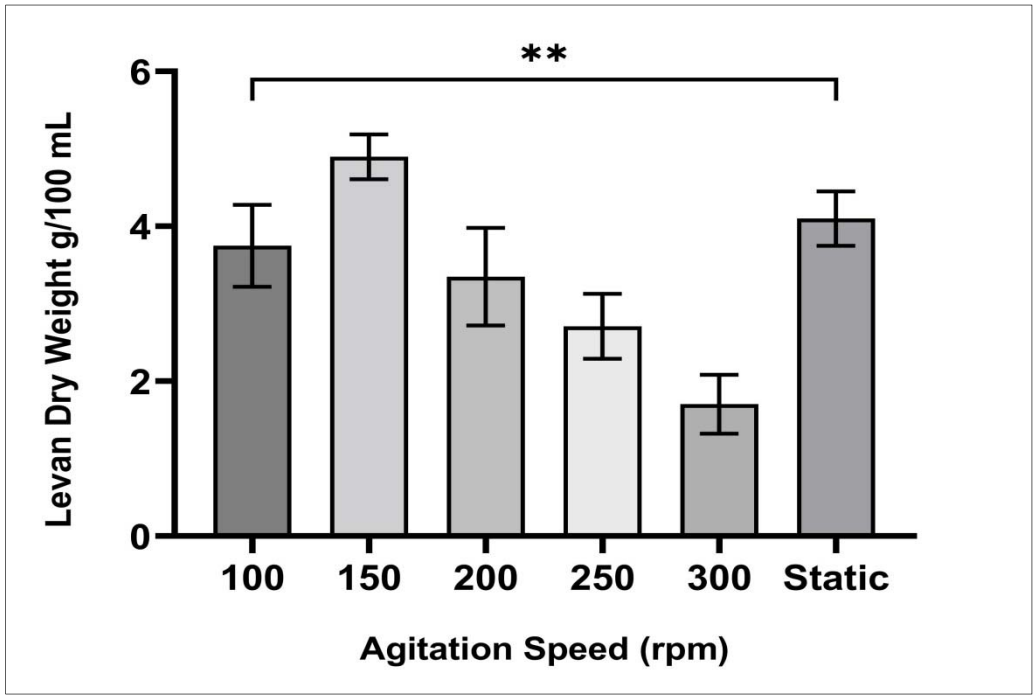

Figure 5: Effect of agitation speed on levan production from *B.subtilis* Strain AE77, in MSB containing 20% sucrose, incubated at 35 °C for 48 hours, pH = 7.0, and inoculum volume 2%

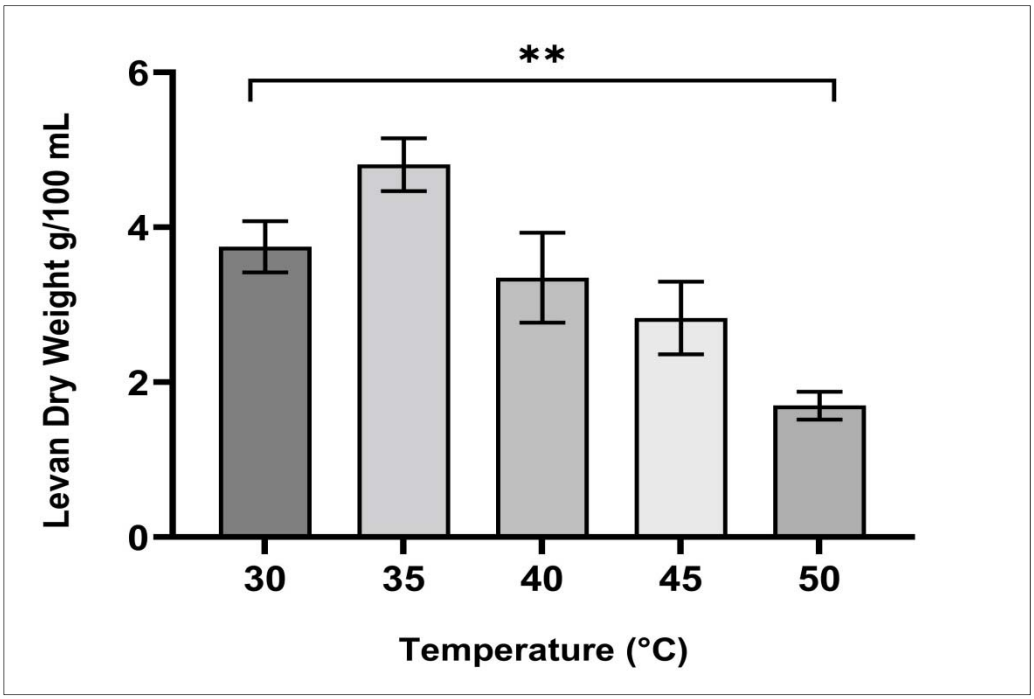

Figure 6: Effect of temperature on levan production from *B. subtilis* Strain AE77, in MSB containing 20% sucrose, incubated at 35 °C for 48 hours, pH 7.0, inoculum volume 2%, and agitation speed 150 rpm

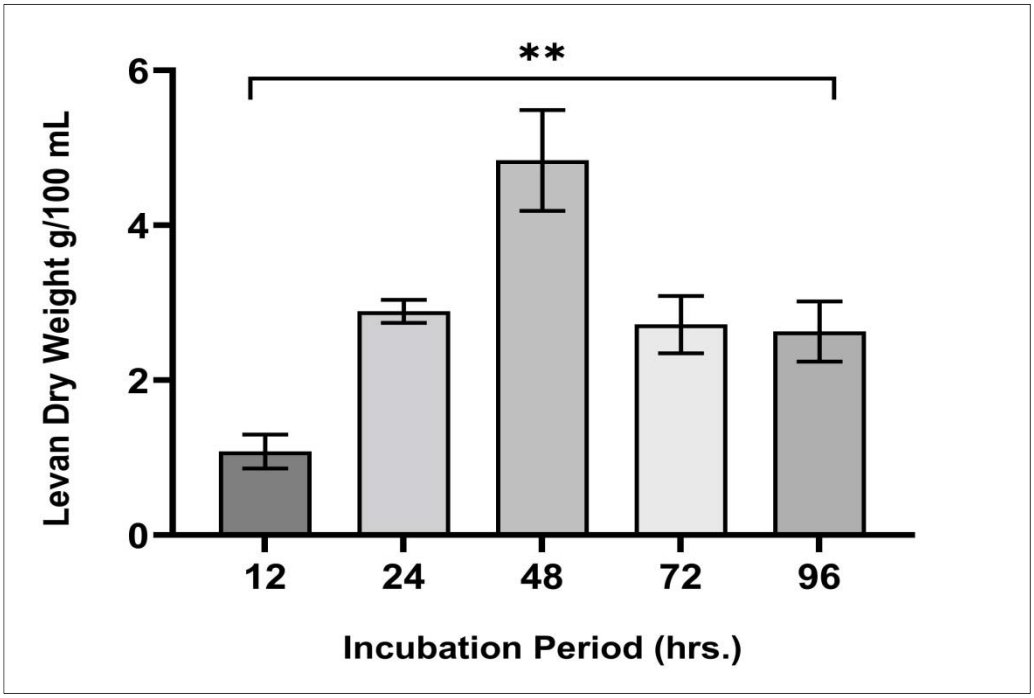

Figure 7: Effect of incubation period on levan production from *B. subtilis* Strain AE77 in MSB containing 20% sucrose incubated at 35 °C for 48 hours, pH 7.0, inoculum size 2%, and agitation speed 150 rpm

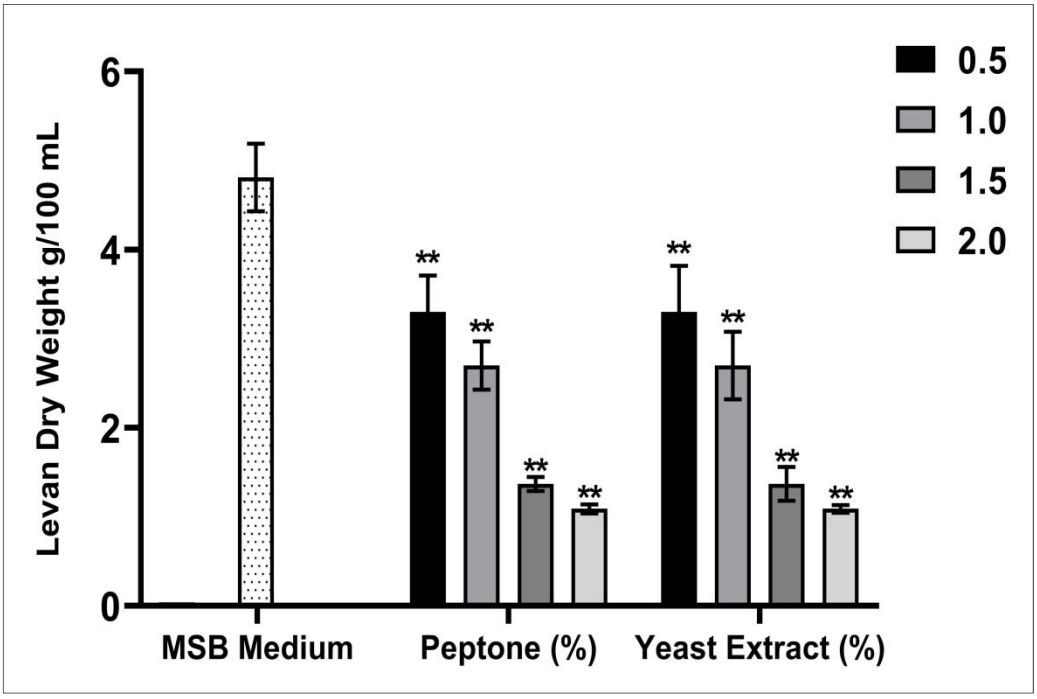

Figure* 8*: levan production from *B.subtilis* Strain AE77 in MSB containing 20% sucrose and containing peptone and yeast extract incubated at 35 °C for 48 hours, pH 7.0, inoculum volume 2%, and agitation speed 150 rpm

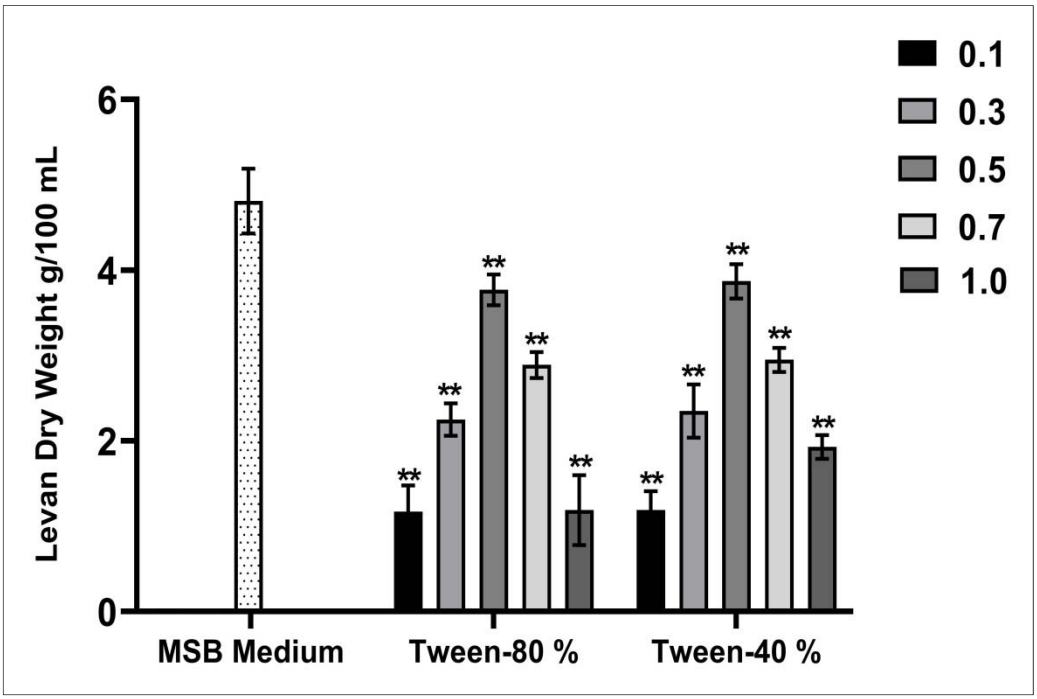

Figure 9: production on levan production from *B. subtilis* Strain AE77 in MSB containing 20% sucrose, and containing tween 80 and tween 40 incubated at 35 °C, for 48 hours, pH 7.0, inoculum volume 2%, and agitation speed 150 rpm