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Article

Detection And Environmental Optimization For Levan Produced By *Lactobacillus* spp

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ABSTRACT

Levan is a naturally occurring fructan, the homopolymer of fructose synthesized as an exopolysaccharide (EPS) in the extracellular matrix of bacteria from various genera. Many lactic acid bacteria produce levans. In this study, 30 isolates of *Lactobacillus* spp. have been collected from several hospitals located in Baghdad city. These isolates were 21 *Lactobacillus* spp. isolates from the vagina of healthy women (16 *Lactobacillus plantarum*,5 *Lactobacillus acidophilus*) and 9 isolates (*Lactobacillus plantarum*) for infant stool samples. All isolates were tested for levan production using mucoidy and spectrophotometric methods. The optimum conditions for levan production were studied, including temperature, incubation time, pH, inoculum size, sucrose concentration, Nitrogen source, and culture medium. The optimum conditions for production were at 37 ºC for 24 h at pH 7 with 4 % inoculum size and 40 g/ 100ml sucrose concentration with the best nitrogen source pepton, and the best culture medium for levan production was date medium.

Keywords: Levan homopolymer, *Lactobacillus* spp., screening, optimum conditions

INTRODUCTION

The genus Lactobacillus is taxonomically complex and is composed of over 170 species. However, they are part of the normal human gastrointestinal and vaginal flora [1]. The Lactobacillus genus comprises rod-shaped, non-sporeforming, non-pigmented, catalase-negative and microaerophilic to strictly anaerobic bacteria, widely used in fermented foods. LAB cultures grow optimal in the temperature range of 30°C to 40°C, with an optimum pH range between 4.5-6.5 [2,3]. The Lactic acid bacteria, especially bacteria of Lactobacillus genera, show a promising future in their use as probiotics [4]. Exopolysaccharides (EPSs) are biological polymers secreted by microorganisms, including Lactic acid bacteria (LAB), to cope with harsh environmental conditions [5]. Levan is counted as one of the most encouraging microbial polymers. It is one of the two main types of fructan polysaccharide [6]. Levan is a homo-exopolysaccharide (homopolymer) of fructose composed predominantly of β-(2, 6) fructofuranosyl linkages in the backbone with occasional

β-(2, 1) linkages in the branch chains with varied applications. The transglycosylation activity of levansucrase synthesizes microbial levan (sucrose: 2–6-β-D-fructan 6-β-Dfructosyltransferase, E.C.2.4.1.10) in the presence of sucrose. Levansucrase, belonging to the glycoside hydrolase family 68 (GH68), Due to its well water solubility, high molecular mass and low viscosity, microbial levan can be used as an emulsifying, stabilizing, thickening and encapsulating agent in the food industry [7]. Many reports demonstrated that levan is used in the medicinal and pharmaceutical industries as an antibacterial, antiviral, antiparasitic, antitumor, antioxidant, antiobesity, hypolipidemic, antidiabetic, immunostimulant and cosmeceutical agent (8). Furthermore, owing to its moisturizing properties low cell cytotoxicity, promoting mammalian cell proliferation and anti-inflammatory effect [7]. Various bacterial genera such as Zymomonas, Pseudomonas, Corynebacterium, Leuconostoc, Bacillus, Lactobacillus, Streptococcus, Gluconoacetobacter, Aerobacter, Brachybacterium, and Enterococcus and a few plant species are known to produce levan [8].

MATERIALS AND METHODS

Collection of bacterial isolates

Thirty clinical gram-positive bacterial isolates were collected from different hospitals in Baghdad city. These bacterial isolates obtained from different clinical specimens include the vagina of healthy women and infant stool samples. After collection, all bacterial isolates were identified depending on cultural characteristics, microscopical examination, biochemical tests and VITEK 2 compact system.

Levan production

Mucoidy method

The levan production medium (1 g trypton, 0.5 g yeast extract, 3 g agar, 0.25 g K2Hpo4, 3 g NaCl, 40 g sucrose, in 100 ml DW pH adjusted to 7.2) was inoculated after sterilization with 24 h old culture of Lactobacillus spp. isolates and incubated at 37 ° C for 24 h, the slimy mucoid appearance of isolates was recorded as levan producer [9].

Spectrophotometric method

The levan of selected Lactobacillus spp. isolates, which were recorded as producer isolates, were estimated by the spectrophotometric method. Levan production medium without using agar - agar was inoculated by Lactobacillus spp. suspension containing (9×10^8 cfu /ml) (compared to 0.5 ml McFarland standard absorbance at a wavelength of 600nm about 0.134) with inoculum size 2 % and incubated at 37 ° C for 24 h. After incubation, the culture medium was centrifuged at 10000 rpm for 10 min, the biomass was removed, and the supernatant was used to estimate levan concentration. The OD was measured at 400 nm with the spectrophotometer. The equation determined the levan concentration in the culture medium: $y = 0.1645x - 0.035$, where y is the absorbance at 400 nm, and x is the levan concentration expressed in mg / mL [10].

Determination of The Optimal Conditions For Levan Production

Effect of temperature

The selected isolate was inoculated to a levan production medium and incubated at different temperatures (25, 30, 35, 37, and 40°C). After incubation, the levan concentration and levan production yield were estimated for each temperature.

Effect of pH

The selected isolate was inoculated to a levan production medium and incubated at different values of pH range (4, 5, 6, 6.5,7, 8, and 9). After the incubation period, the levan concentration and levan production yield were estimated for each pH.

Effect of incubation time

The selected isolate was inoculated to a levan production medium and incubated at different times of incubation (24, 48 and 72hrs). After the incubation period, the levan concentration and levan production yield were estimated for each incubation time.

Effect of inoculum size

The selected isolate was inoculated to a levan production medium and incubated at different inoculum sizes of (1-10 %). After the incubation period, the levan concentration and levan production yield were estimated for each inoculum size.

Effect of sucrose concentration

The selected isolate was inoculated to a levan production medium and incubated at various sucrose concentrations (10, 20, 30, 40, 50) g /100 ml. After the incubation period, the levan concentration and levan production yield were estimated for each sucrose concentration.

Effect of nitrogen sources

The selected isolate was inoculated to a levan production medium and incubated at various nitrogen sources that were carried out by using organic and inorganic nitrogen sources, including (yeast extract, beef extract, tryptone, ammonium chloride and potassium nitrate, pepton, silver nitrite). These compounds were tested at 0.25 g/100 ml. After incubation, the levan concentration and levan production yield were estimated for each nitrogen source.

Effect of carbon sources

The selected isolate was inoculated to a levan production medium and incubated at different extracts such as orange, banana, date, fig, grape, tomato and beet at 10 % as carbon sources for levan production. After incubation, the levan concentration and levan production yield were estimated for each carbon source.

RESULTS

Collection of bacterial isolates

The results showed that most collected isolates of Lactobacillus spp. were obtained from the vagina of healthy women with (21) isolates and infant stool with (9) of Lactobacillus spp. isolates, respectively, as displayed in Figure 1.

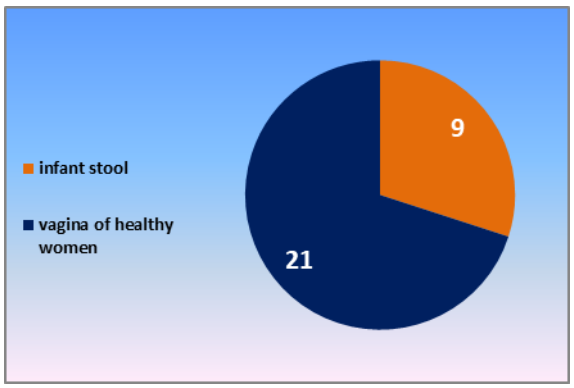

Figure1. Numbers of the collected *Lactobacillus* spp. isolates depending on the source of isolation.

On the other hand, it is obvious from the results shown in Table 1 that most Lactobacillus spp. isolates belonged to the Lactobacillus plantarum, which

included 16 isolates from the vagina of healthy women and 9 isolates from infant stool. In contrast, 5 Lactobacillus acidophilus isolates from the vagina of healthy women.

Bacterial isolates	**Source of isolation**	
	Vagina of healthy w	**Infant stoo**
Lactobacillus planta	**16**	**9**
Lactobacillus acidop	**5**	**-**

Table 1. Distribution of *Lactobacillus* isolates according to source of isolations.

Screening of levan-producing isolates

The ability of Lactobacillus spp. isolates for levan production were examined by using mucoidy and spectrophotometric methods:

Mucoidy method

All 30 isolates of Lactobacillus spp. were tested for levan production. The detection and screening of levan production were recorded according to slimy mucoid colonies on the surface of the levan screening medium. Results showed in Table 2 that only 14 isolates produced slimy mucoid colonies, 2 isolates from 14 isolates produced strong slimy mucoid colonies, and 5 isolates from 14 isolates produced moderate slimy mucoid colonies.

3.2.2. Spectrophotometric method

After selecting the Lactobacillus plantarum isolates that gave high productivity of levan (mucoidy), levan concentration was determined by using the spectrophotometric method; results showed that the bacterial isolate that gave a high concentration of levan was Lactobacillus plantarum V11 with concentration about 30.52 mg /ml, while the other isolates range from (4.72- 28.02) mg/ml Figure 2. V isolates from the vagina of healthy women; S isolates from infant stool.

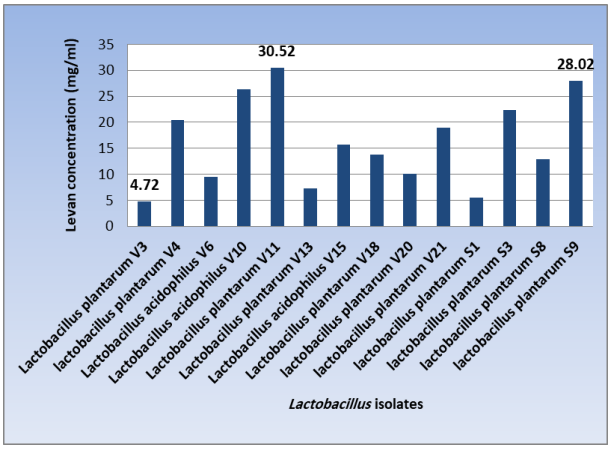

Figure 2. Concentrations of levan produced by *Lactobacillus plantarum*

Bacterial isolates	Viscosity and Mucoidy
Lactobacillus acidophilus V1	**-**
Lactobacillus plantarum V2	**-**
Lactobacillus plantarum V3	+
Lactobacillus plantarum V4	++
Lactobacillus plantarum V5	**-**
Lactobacillus acidophilus V6	+
Lactobacillus plantarum V7	**-**
Lactobacillus acidophilus V8	**-**
Lactobacillus plantarum V9	**-**
Lactobacillus acidophilus V10	++
Lactobacillus plantarum V11	+++
Lactobacillus plantarum V12	**-**
Lactobacillus plantarum V13	+
Lactobacillus plantarum V14	**-**
Lactobacillus acidophilus V15	++
Lactobacillus plantarum V16	**-**
Lactobacillus plantarum V17	**-**
Lactobacillus plantarum V18	+
Lactobacillus plantarum V19	**-**
Lactobacillus plantarum V20	+
Lactobacillus plantarum V 21	++
Lactobacillus plantarum S1	+
Lactobacillus plantarum S2	**-**
Lactobacillus plantarum S3	++
Lactobacillus plantarum S4	**-**
Lactobacillus plantarum S5	**-**
Lactobacillus plantarum S6	**-**
Lactobacillus plantarum S7	**-**
Lactobacillus plantarum S8	+
Lactobacillus plantarum S9	+++

(+++): high production of levan, (++): moderate production of levan, (+): low production of levan, (_): no production of levan.

Table 2. Screening For Levan Production by *Lactobacillus* spp.

Optimization of growth conditions for levan production

Effect of incubation temperature

Different incubation temperatures (25, 30, 35, 37, 40 ºC) were tested to determine the optimum for levan production by Lactobacillus plantarum V11. Maximum production of levan was reached at 37ºC. The levan production yield was 7.7 % at this temperature. However, other temperatures decreased the levan production yield, as shown in Figure 3.

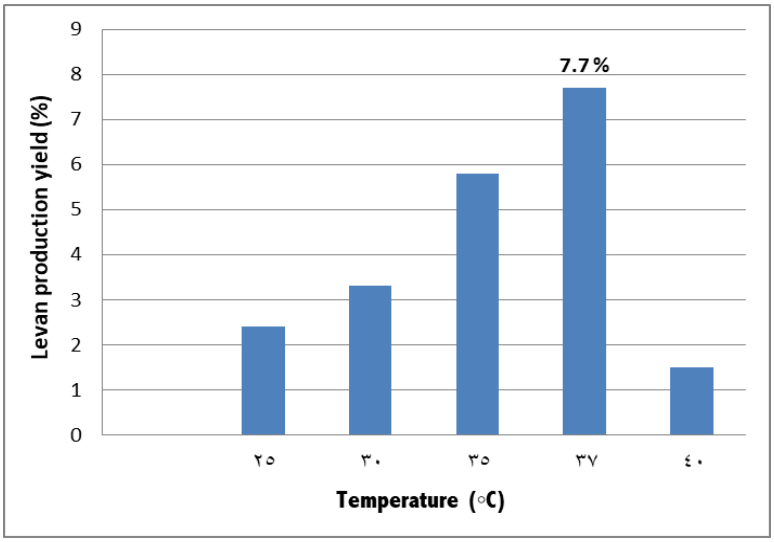

Figure3. Effect of incubation temperature on levan production

Effect of PH

The productivity of levan was variable at different pHs. It was observed that the optimal pH for levan production by Lactobacillus plantarum V11 was 7 with a levan production yield of 7.8 % than other pH values that led to a reduction in the levan production yield to (2.2, 2.6, 4.8, 7.3, 1.8, 1.1 %) when the pH of the medium was (4,5,6,6.5,8,9), respectively as shown in figure 4.

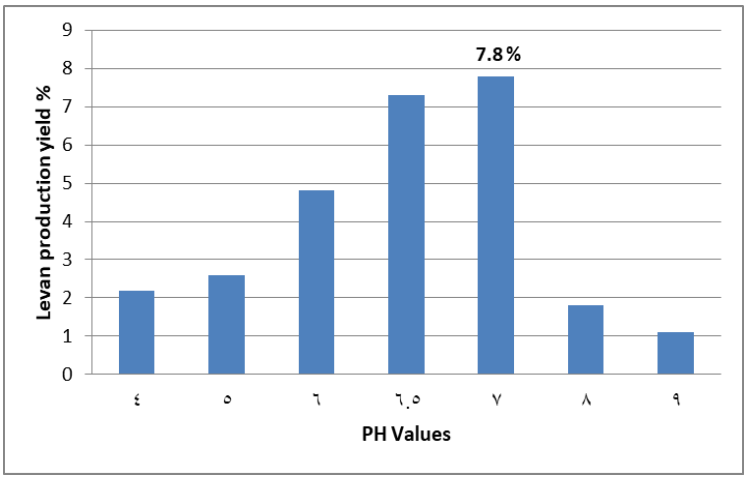

Figure 4. Levan production by *Lactobacillus plantarum V11* at different pHs

Effect of different incubation time

The results revealed that Lactobacillus plantarum V11 after 24 hrs. of incubation gave 7.9 % levan production yield, and the yield decreased with increasing the incubation time and reached (6.3 %) after 48 hrs. Of incubation. After 72h, the levan production yield declined (Figure 5).

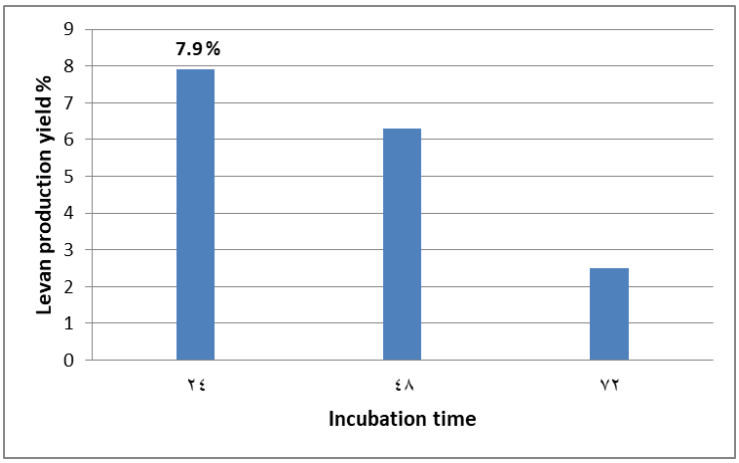

Figure 5. Levan production by *Lactobacillus plantarum V11* at different incubation times

Effect of inoculum size

The Effect of inoculum size on levan production was studied. Lactobacillus plantarum V11 was incubated with various inoculum sizes (1-10) %. Results showed that the best inoculum size for levan production was 4%, with a levan production yield of 8 % Figure 6.

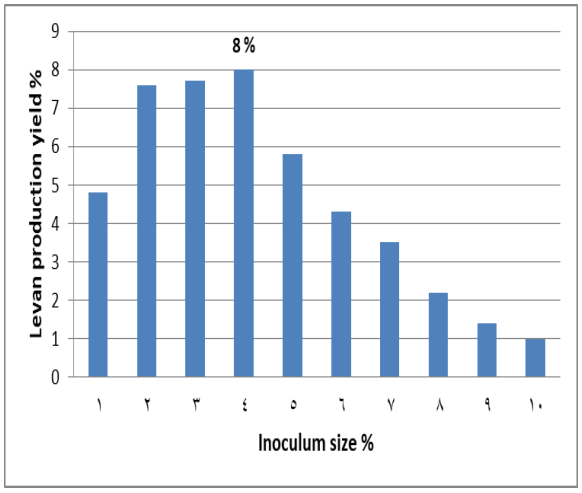

Figure 6. Effect of inoculum size on the levan production by *Lactobacillus plantarum* V11

Effect of sucrose concentration

After optimum inoculum size selection, bacterial isolate Lactobacillus plantarum V11 was inoculated at various sucrose concentrations (10, 20, 30, 40, 50) %. Results showed that the best sucrose concentration for levan production was at 40%, with a levan production yield 8.1 %, as shown in Figure 7.

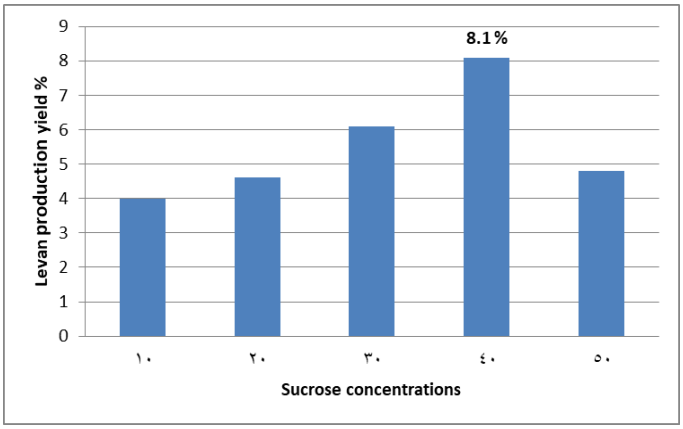

Figure 7. Effect of sucrose concentration on the levan production by *Lactobacillus plantarum* V1

Effect of nitrogen sources

A levan production medium was used to detect the Effect of different nitrogen sources on levan production. The results showed that peptone proved to be the best for levan production from Lactobacillus plantarum V11 With a yield of 8.3 %, followed by potassium nitrate, tryptone and yeast extract, which was the second nitrogen sources suitable for levan production with a yield 8.1 %, 7.9 % respectively. In contrast, the other nitrogen sources represented the poor nitrogen sources for levan production, as shown in Figure 8. Nitrogen source is a major nutrient after carbon essential for growing microorganisms in larger amounts.

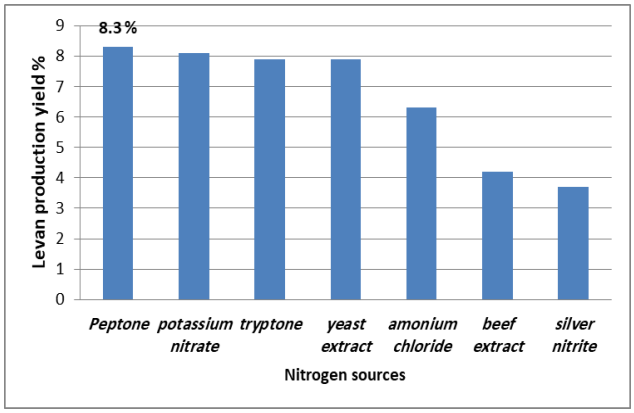

Figure 8. Levan production by *Lactobacillus plantarum* V11 at different nitrogen sources.

Peptone concentration was the factor exerting the strongest Effect on EPS production [16].

Effect of carbon sources

Six different natural carbon sources were used for determining the optimal carbon source for levan production by Lactobacillus plantarum V11. The result showed that a levan production medium supplemented with plant extracts stimulates levan production at different levels. The date extract was the best carbon source and gave a higher levan production yield of 9.3 %, and beet extracts with a yield 9.1 %, While the remaining extracts gave a levan production yield between (8.6-8.8%). as shown in Figure 9.

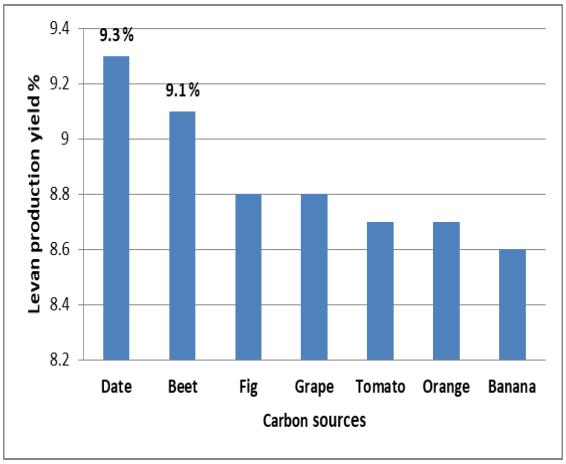

Figure 9 . Levan production by *Lactobacillus plantarum* V11 at different carbon sources.

DISCUSSION

Polysaccharides are a main component in forming the extracellular biofilm matrix. They play an important role in protecting bacteria from adverse environmental factors and in the attachment of microbial cells to solid surfaces [11]. The screening methods to evaluate the microbial ability to produce EPS are based on the cultivation of the LAB in a medium enriched with different sugars (glucose, fructose, sucrose, galactose, or lactose). The easiest way to assess EPS production is to visually observe the phenotypic characteristics of the colonies: slimy or ropy phenotypes. Mucilaginous colonies characterize the slimy phenotype, while the ropy phenotype is characterized by forming long filaments when an inoculation loop is lifted from the colony surface or cell pellet. For a more objective test, ruthenium red staining is used in milk agar plates, producing red colonies of non-ropy EPS producers, while ropy colonies remain white [12]. Detection of mucous colonies has been used as a phenotypical test to select EPS-producing bacteria. The ability of the LAB to produce EPS was analyzed in MRS solid media containing either sucrose or glucose [13].

The optimum temperature required for the levan production ranges from 25°C to 37°C. Temperature above the range may decrease levan production, and production is inhibited at temperatures above 47 °C [14].

Microbial growth depends on the pH of the media. The difference in the pH will alter the growth of microorganisms and, therefore, Affect the levan production. The amount of levan decreased when pH was maintained above 7 and inhibited below pH 4. The maximum amount is observed at pH 6-7. Therefore, it is considered the optimum pH for levan production [14].

After 24 hours of incubation, the highest levan production was obtained at 37°C. [20] . The best levan production from B. licheniformis was when the culture medium containing sucrose was incubated for 24 hrs [9,15].

The optimal inoculum size for exopolysaccharide production by Agaricus blazei was an inoculum size of 3%. Successfully extracted polysaccharides from Cordyceps militaris at inoculum size 4 %. Showed that an increase in the amounts of inoculum possibly had no positive effect on the yield of exopolysaccharides and also reported that the most suitable inoculum size for exopolysaccharide production from Xanthomonas axonopodis was 5% [9].

Sucrose concentration has been identified as the most effective factor in controlling the molecular weight of the levan. Levan production increased notably from 3.48 to 7.97 g/l when sucrose concentration was increased from (50–300) g/l. Z. mobilis and B. subtilis NATTO revealed that the increase in levan production is proportional to the sucrose concentration in the levan production medium [16].

Nitrogen is an essential part of protein, nucleotides, enzymes and a cofactor that plays a vital role in the metabolism [17].

EPS production showed that the amount of EPS produced by plantarum in the dates medium was high. Fructans are fructose polymers produced by several plants and microorganisms, differentiated based on fructose linkage position. Levan is one of the fructooligosaccharide in their studies used date extract for levan production. Microorganisms utilize carbon sources for their growth and in the production of levan. Carbon source is an important factor impacting the quality and quantity of levan production. Many carbon sources are used for levan production, such as sucrose, glucose, fructose, galactose, maltose, mannose and mannitol [14,19,20].

CONCLUSIONS

Lactobacillus plantarum, isolated from the vagina of healthy women, was a better producer of levan. This research was to detect the levan production yield in *Lactobacillus* spp. besides screening and optimization of production conditions of levans.

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References

1. Ellie .J.; Goldstein .C.; Tyrrell . KL and Citron . DM. *Lactobacillus* Species: Taxonomic Complexity and Controversial Susceptibilities, *Clinical Infectious Diseases* **2015**, *60(2):* S98–S107.
2. Huang C.H.; Li SW; Huang L and Watanabe K . Identification and Classification for the Lactobacillus casei Group. Front. *Microbio*l **2018** ,*9*:1974.
3. Yang, E.; Fan, L.; Yan, J. et al. Influence of culture media, pH and temperature on growth and bacteriocin production of bacteriocinogenic lactic acid bacteria. *AMB Expr* **2018**, *8*, 10 .
4. Jaiswal . M. ; Sharma . R.; Subramani. S.; Mishra . V,Sharma. J.; Parthasarathy. P; Bisen.P.S and Shailendra Raghuwanshi. S.. Role of Lactobacilli as Probiotics in Human Health Benefits: Current Status and Future Prospects. *IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)* **2020**, *6(5):* 19-24 .
5. Nguyen .P.T.; Nguyen .T.T.; Bui .D.C.; Hong .P.T; Hoang .Q.K and Nguyen. HT..Exopolysaccharide production by lactic acid bacteria: the manipulation of environmental stresses for industrial applications. *AIMS Microbiol* **2020** *6(4):*451-469.
6. Korany . SH; El-Hendawy . HH; Sonbol .H and Hamad.M. A.Partial characterization of levan polymer from Pseudomonas fluorescens with significant cytotoxic and antioxidant activity, *Saudi Journal of Biological Sciences* **2021** *28(11):* 6679-6689.
7. Han . J.; Feng .H.; Wang . X.; Liu .Z and Wu. Z. Levan from Leuconostoc citreum BD1707: production optimization and changes in molecular weight distribution during cultivation. *BMC Biotechnology* **2021**, *21*:14.

8. Al-Qaysi. S.A.S.; Al-Haideri .H.; Al-Shimmary .S.M.; Abdulhameed .J.M.; Alajrawy O.I.; Al-Halbosiy. M.M.;Moussa. T.A.A and Farahat .M.G. "Bioactive Levan-Type Exopolysaccharide Produced by *Pantoea agglomerans* ZMR7: Characterization and Optimization for Enhanced Production. *J Microbiol Biotechnol* **2021** ,*28;31(5):*696-704.
9. Khudair. A . Y. ; Salman. J. A and Ajah. HA..Production of Levan from Locally *Leuconostoc mesensteroides* isolates. *J. Pharm. Sci. & Res.* **2018**,*10(12*): 3372-3378.
10. Abou-Taleb.; Khadiga and Abdel-Monem.; Mohamed & Hesham.; Mohamed & Draz.; Amal.. Production, Purification and Characterization of Levan Polymer from *Bacillus lentus* **2015**, *8 (5):* 22-32.
11. Jurášková, D.; Ribeiro.; S.C.;Silva.; CCG Exopolysaccharides Produced by Lactic Acid Bacteria: From Biosynthesis to Health-Promoting Properties. *Foods* **2022**, *11, (156).*
12. Prete, R.; Alam, M.K.; Perpetuini, G.; Perla, C.; Pittia, P.; Corsetti. A. Lactic acid bacteria exopolysaccharides producers: Asustainable tool for functional foods. *Foods* **2021**,*10*, 1653.
13. Zarour, K.; Vieco, N.; Pérez-Ramos, A.; Nácher-Vázquez, M.; Mohedano, M.L.; López, P. Chapter 4—Food ingredients synthesized by lactic acid bacteria. In Microbial Production of Food Ingredients and Additives; Handbook of Food Bioengineering; Holban, A.M., Grumezescu, A.M., Eds.; Academic Press: Cambridge, MA, USA, **2017**, pp. 89–124.
14. Bansal, Anu .; Khushboo and Karnwal, Arun. Effects of abiotic factors on production of Levan by microorganisms: A review. *International Journal of Botany Studies* **2019**, *4; (2):* 01-06.
15. Mamay , Wahyuningrum . D and Hertadi .R. Isolation and characterization of levan from moderate halophilic bacteria *Bacillus licheniformis* BK AG21. *Procedia Chemistry* **2015**, *16* ,292 – 298 .
16. Moussa, .T. ; Al-qaysi, .S. ; Alrawi, .Z and Basheer .K. S. Microbial Levan from Brachybacterium phenoliresistens : Characterization and enhancement of production. *Process Biochemistry* **2017**, *57*: 9-15.
17. Suribabu, K., and Hemalatha, K.P.J. Thermal Optimization of α-amylase Production in Brevibacillus sp. *Int. J. Curr. Microbiol.App.Sci* **2016** ,*5(3):* 909-16.
18. Chambi .D.; Romero-Soto .L.; Villca .R.; Orozco-Gutiérrez .F.; Vega-Baudrit .J.; Quillaguamán .J.; Hatti-Kaul .R.; Martín .C and Carrasco .C.Exopolysaccharides Production by Cultivating a Bacterial Isolate from the Hypersaline Environment of Salar de Uyuni (Bolivia) in Pretreatment Liquids of Steam-Exploded Quinoa Stalks and Enzymatic Hydrolysates of Curupaú Sawdust. *Fermentation*. **2021**, *7(1):*33.
19. Zubaidah..E. ; Suryawira.Y.M and Saparianti. E.Comparative Study Production of Exopolysaccharide (EPS) by Lactic Acid Bacteria (L. casei and L. plantarum) in Different Media (Dates and Mulberry juice). *Agroindustrial Journal* **2014**, *3 (1):* 107-111 107.
20. Sarilmiser . H. K.; Ates. O.; Ozdemir. G.; Arga. K. Yand Oner. ET Effective stimulating factors for microbial levan production by Halomonas smyrnensis AAD6T .*Journal of Bioscience and Bioengineering* **2015**, *119, (I 4):* 455-463.

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Jaber, S.; Muslim, S.; Fadel, A. Detection And Environmental Optimization For Levan Produced By *Lactobacillus spp*. Revis Bionatura 2023;8 (2) 62. http://dx.doi.org/10.21931/RB/CSS/2023.08.02.29