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Article

# Screening And Ecological Optimization For Levansucrase Produced By *Lactobacillus* spp

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## ABSTRACT

In this study, 25 isolates of *Lactobacillus* spp. have been collected from several hospitals in Baghdad city. These isolates were 17*Lactobacillus* spp. isolates from the vagina of healthy women as 12 *Lactobacillus plantarum*,5 *Lactobacillus acidophilus* and 8 isolates of *Lactobacillus plantarum* were isolated from feces of healthy infants; The screening for determining levansucrase production was performed by mucoidy and spectrophotometric methods The levansucrase activity measured when this isolate was cultivated under the optimal conditions; only 14 isolates could produce levansucrase revealed specific activity ranged from 9.41 to 28.12 U/mg and the maximum specific activity was for *Lactobacillus plantarum*V11, which was selected as best producer isolates. The levansucrase activity reached its maximum level when this isolate was cultivated under the optimal conditions, which consisted of using a levansucrase production medium incubated at 37°C for 24 hours at pH 7 with 4 % inoculum size and 40 g/100ml sucrose concentration with the best nitrogen source was pepton and the best carbon source was dated.

**Keywords:** *Lactobacillus* spp., levansucrase, production, optimum conditions

## INTRODUCTION

The vaginal microflora was Gram-positive rods. These bacillus have been identified as *Lactobacillus* spp. Lactobacilli, are the predominant microorganisms of the vaginal macrobiotics. Lactobacilli are facultatively anaerobic, non-spore-forming, catalase-negative, rod-shaped lactic acid bacteria. Numerous strains of the genus *Lactobacillus* are used as probiotics. *Lactobacillus* plays a main role in preserving a healthy genital tract by avoiding the colonization of pathogenic bacteria. In healthy women, the vaginal microflora is ordered by *Lactobacillus* species at a level of $10^7$ -$10^8$ CFU g⁄l of fluid, which significantly affects the microflora of the ecosystem[1]. Most *Lactobacillus* species are non-pathogenic tenants of the animal and human intestine and support the intestinal microbiota. Studies on the gut microbiome show that *Lactobacillus* is an invariable content of the intestinal microbiota [2]. Lactobacilli are also generally associated with infantile intestinal microbiota and play important roles in nutrition, metabolism, immunity, and defense against pathogens [3]. The *Lactobacillus spe-*

*cies* most frequently isolated and detected in infant feces were *L. brevis, L. fermentum, L. reuteri, L. rhamnosus, and L. plantarum* [4]. Levansucrase (LS, EC 2.4.1.10, sucrose: 2,6-b-D-fructan 6-b-D-fructosyltransferase) [5]. In bacteria, The gene encoding levansucrase is widely distributed in both gram-positive and gram-negative bacteria [6]. Levansucrase belongs to the family of fructosyltransferases and catalyzes three individual reactions depending on the corresponding fructosyl acceptor: polymerization (the growing fructan chain as an acceptor), transfructosylation (monosaccharides, disaccharides, or oligosaccharides as acceptors), and hydrolysis (water as an acceptor). The synthesis of levan and levan-like fructooligosaccharides uses sucrose as a substrate in a transfuctosylation reaction catalyzed by levansucrase, releasing glucose as a byproduct [7]. Levansucrase shows three activities: transfructosylation (using monosaccharides, disaccharides or oligosaccharides as acceptors), hydrolysis (using water as an acceptor) and polymerization (using the growing fructans as an acceptor), releasing fructose, glucose, and synthesizing fructooligosaccharides and levan. These molecules show relevant applications in the food, pharmaceutical and cosmetic industries [8].

## MATERIALS AND METHODS

### *Collection of bacterial isolates*

Twenty-five clinical of gram-positive Lactobacillus spp. isolates were collected from different hospitals in Baghdad city. These Lactobacillus spp. isolates obtained from different clinical specimens include: vaginal of healthy women and feces of healthy infants samples. After collection, all Lactobacillus spp. isolates were identified depending on cultural characteristics, microscopical examination, biochemical tests and VITEK 2 compact system.

### *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 D.W pH adjust to 7.2) was inoculated after sterilization with 24 h old culture of Lactobacillus spp. isolates and incubated at 37 $C^o$ for 24 h , the slimy mucoid appearance of isolates was recorded as levan producer [9], and levansucrase activity was estimated by levan forming.

### *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. a suspension containing ($9 \times 10^8$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 $^oC$ 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 levansucrase activity [10].

### *Levansucrase activity assay*

Levansucrase activity was checked by a spectrophotometer. It was measured by reacting with sucrose as a substrate to the product of levan and glucose by modification of the method described by [11]. 0.5 ml of crude extract was incubated for 30 min at 37°C with (0.5) ml of (1 gm of sucrose as substrate dissolved in 50 ml of 0.1M sodium acetate buffer at pH =5.0. The enzyme reaction was stopped by putting the mixture in a boiling water bath for 15 min. After that, 1 ml of 3,5-Dinitrosalicyclic acid (DNS) reagent was added to the mixture, and the final volume was 2 ml. Then, we measured the absorbency at 540 nm by

spectrophotometer. The blank was prepared by adding 0.5 ml of D.W to 0.5 ml of (1 gm of sucrose dissolved in 50 ml of 0.1M sodium acetate buffer at pH =5.0).

Levansucrase activity was calculated from the following formula: Enzyme activity (U/ml) = µg of liberated glucose / V x T, where µg of liberated glucose can be taken from the standard curve, V is the volume of enzyme sample, and T is the hydrolysis time. One unit of levansucrase activity is the enzyme releasing 1µmol of glucose per minute under specific conditions [12].

*Determination of protein concentration*

Protein concentration was measured by the method of Bradfored, (1976) with Bovine serum albumin (BSA) [13].

*Determination of the optimal conditions for levansucrase production*

Effect of temperature

The selected isolate was inoculated to a levan production medium, and the optimum temperature was determined by incubating the culture medium at different temperatures (25, 30, 35, 37, and 40°C). After incubation for 24 h, the culture was centrifuged, and levansucrase activity protein concentration was measured.

Effect of pH

The selected isolate was inoculated to a levan production medium to determine the optimum PH and the culture medium was adjusted to different value ranges (4, 5, 6, 6.5,7, 8, and 9). After incubation at 37 °C for 24 h, the culture was centrifuged, and levansucrase activity protein concentration was measured.

2.6.3. Effect of incubation period

The selected isolate was inoculated to a levan production medium, and the effect of the incubation period was studied for different periods of incubation (24, 48 and 72hrs.). After incubation, the culture was centrifuged, and levansucrase activity and protein concentration were measured.

Effect of inoculum size

The selected isolate was inoculated to a levan production medium. The best inoculum size was achieved by inoculating the production medium individually with an inoculum size of (1-10 %). After incubation at 37 º C for 24 h, the culture was centrifuged, and levansucrase activity protein concentration was measured.

Effect of sucrose concentration

The selected isolate was inoculated to a levan production medium. To determine the best sucrose concentration, the culture medium was inoculated at various sucrose concentrations (10, 20, 30, 40, 50 g/100 ml). After incubation at 37 °C for 24 h, the culture was centrifuged, and levansucrase activity and protein concentration were measured.

Effect of nitrogen sources

The selected isolate was inoculated to a levan production medium that contained different organic and inorganic nitrogen sources, including 0.25 g /100ml (yeast extract, beef extract, tryptone, ammonium chloride and potassium nitrate, pepton, silver nitrite ). After incubation at 37 °C for 24 h**,** the culture was centrifuged, and levansucrase activity and protein concentration were measured.

Effect of carbon sources

The selected isolate was inoculated to a levan production medium and incubated at different plant extracts such as orange, banana, date, fig, grape, tomato and beet at 10% as carbon sources. After incubation at 37 º C for 24 h, the culture was centrifuged, and levansucrase activity and protein concentration were measured.

## RESULTS

*Collection of bacterial isolates*

The results showed that most collected isolates of Lactobacillus spp. were obtained from vaginal of healthy women with [17] isolates and feces of healthy infants with (8) of Lactobacillus spp.

isolates, respectively, as displayed in Figure 1.

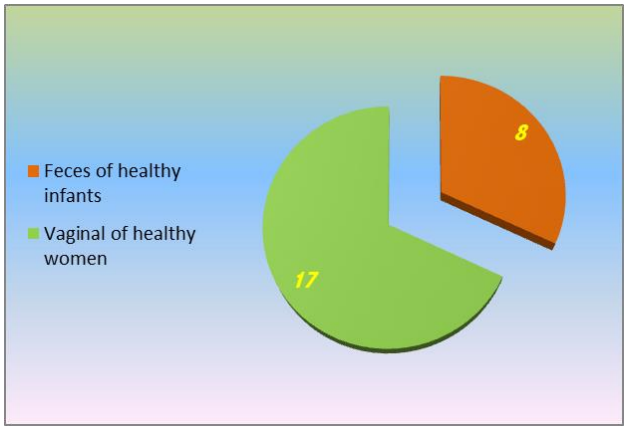


**Figure 1. Numbers of the collected *Lactobacillus* spp. isolates depending on the source of isolation.**

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 12 isolates from the vaginal of healthy women and 8 isolates from infant feces. In contrast, 5 Lactobacillus acidophilus isolates from the vaginal of healthy women.

| Bacterial isolates | Source of isolation | |
|---|---|---|
| | Vagina of healthy w | Infant stoo |
| ***Lactobacillus planta*** | **12** | **8** |
| ***Lactobacillus acidop*** | **5** | **-** |

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

*Screening of levansucrase-producing isolates*

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

*Mucoidy method*

All 25 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 were slimy mucoid colonies, 2 isolates from 14 isolates produced strong slimy mucoid colonies, and 5 isolates from 14 isolates produced moderate slimy mucoid colonies.

| 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 V9* | - |
| *Lactobacillus acidophilus V10* | ++ |
| *Lactobacillus plantarum V11* | +++ |
| *Lactobacillus plantarum V12* | - |
| *Lactobacillus plantarum V13* | + |
| *Lactobacillus plantarum V14* | - |
| *Lactobacillus acidophilus V15* | ++ |
| *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 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 of mucoid method for levan production**

*Spectrophotometric method*

After selecting the Lactobacillus plantarum isolates that gave high mucoidy, results showed that the Lactobacillus plantarum V11, with maximum levansucrase activity, equals 11.53 U/ml and 28.12 U/mg of specific activity. These 14 isolates revealed different levels of levansucrase activity with specific activities ranging from 9.41 to 27.23 U/mg, as shown in Figure 2. V isolates from the healthy vagina; S isolates from infant stool.

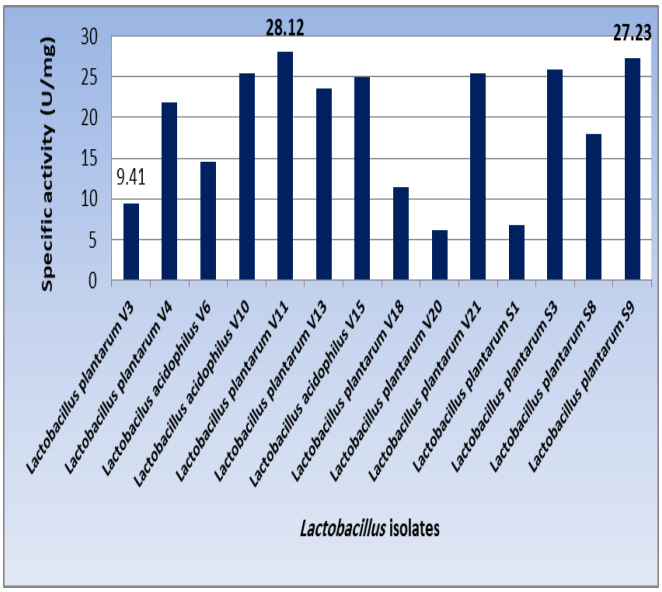


**Figure 2. Specific activities for *Lactobacillus* spp. in spectrophotometric method.**

### *3.5. Optimization of growth conditions for levansucrase production*

#### 3.5.1. Effect of incubation temperature

Different incubation temperatures (25, 30, 35, 37, 40 ºC) were tested to determine the optimum one for levansucrase activity by Lactobacillus plantarum V11. Maximum activity of levansucrase was reached at 37ºC. The specific activity 28.39 U/mg was reached at this temperature. However, other temperatures led to a decrease in levansucrase activity, as shown in Figure 3.

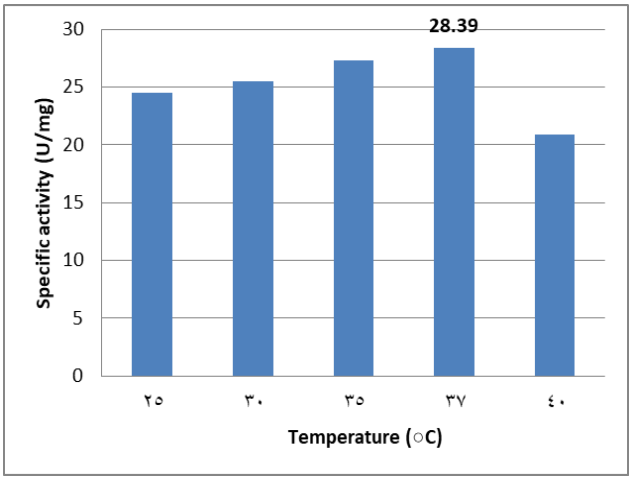


**Figure3. Effect of incubation temperature on levansucrase production.**

#### Effect of PH

The specific activity of levansucrase was variable at different pHs. It was observed that the optimal pH for levansucrase activity by Lactobacillus plantarum V11 was 7 with specific activity 28.57 U/mg than other pH values that led to reducing the levansucrase specific activity, as shown in figure 4.

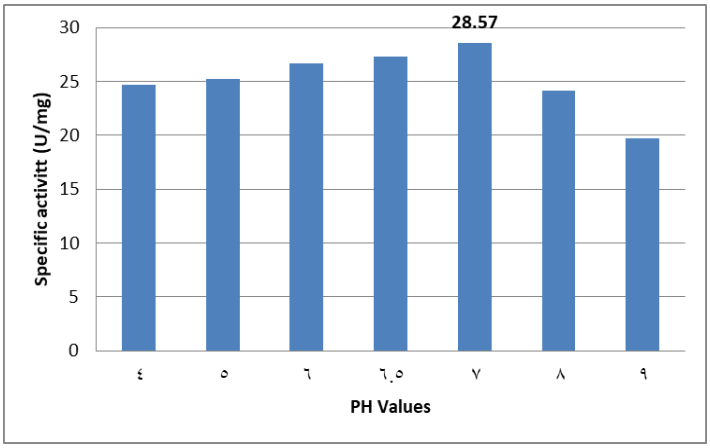


**Figure 4. Levansucrase production by *Lactobacillus plantarum V11* at different pHs**

Effect of different incubation periods

The results revealed that Lactobacillus plantarum V11, after 24 hrs. of incubation, gave specific activity of 30.18 U/mg, and the levansucrase production decreased with increasing the incubation time and reached (26.02) after 48hrs.of incubation. While after 72h, levansucrase activity started to decline Figure 5.

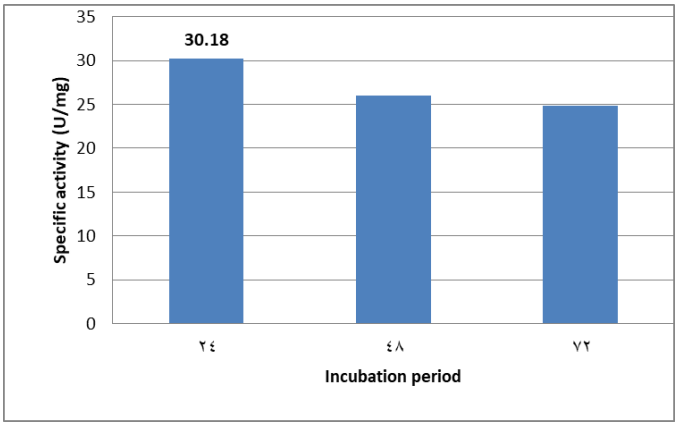


**Figure 5. Levansucrase production by *Lactobacillus plantarum V11* at different incubation periods.**

Effect of inoculum size

The effect of inoculum size on levansucrase activity was studied. Lactobacillus plantarum V11 was incubated with various inoculum sizes (1-10)%. Results showed that the best inoculum size for levansucrase activity was 4%, with specific activity at 33.52 U/mg Figure 6.

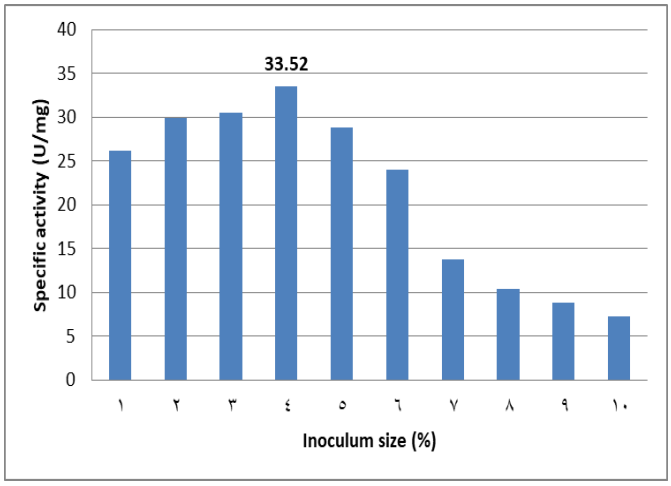


**Figure 6. Effect of inoculum size on the levansucrase production by *Lactobacillus plantrum*V11.**

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 levansucrase activity was at 40%, with the specific activity at 35.18 U/mg, as shown in Figure 7.

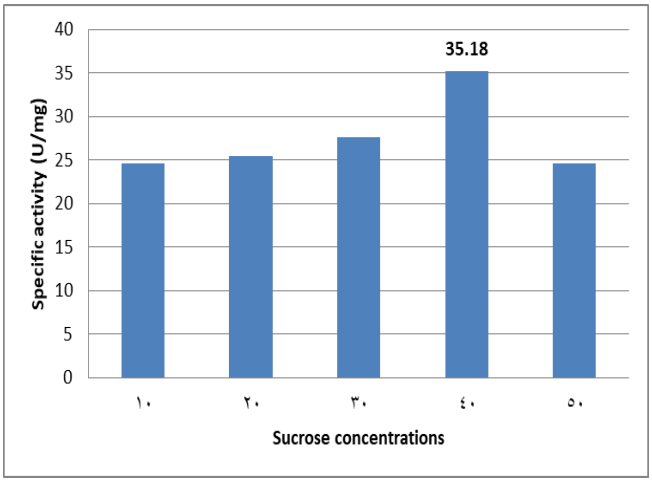


**Figure 7. Effect of sucrose concentration on the levansucrase production by *Lactobacillus plantarum*V11. Levan is a polymer of fructose synthesized by levansucrase using sucrose as the substrate (16).**

Effect of nitrogen sources

The effect of different nitrogen sources on levansucrase activity was studied. The results showed that peptone was the best for levansucrase activity from Lactobacillus plantarumV11 With 20.43 U/ml. Specific activity reached 36.48 U/mg, followed by potassium nitrate, tryptone and yeast extract, the second nitrogen sources suitable for the levansucrase production with specific activity 35.78, 34.41 and 34.30 U/mg, respectively. In contrast, the other nitrogen sources represented the poor nitrogen sources for levansucrase production, as shown in Figure 8. Nitrogen source is a major nutrient after carbon essential for the growth of microorganisms in more significant amounts.

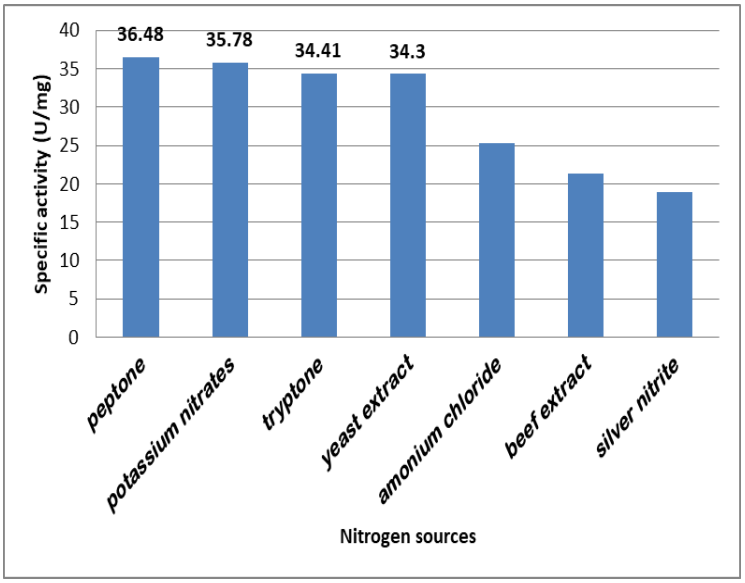


**Figure 8. Levansucrase production by *Lactobacillus plantarum*V11 at different nitrogen sources.**

Effect of carbon sources

Six different natural carbon sources were used for determining the optimal carbon source for levansucrase activity by Lactobacillus plantarumV11. The result showed that among six various plant extracts, the date extract was the best carbon source and gave higher specific activity, reaching 42.03 U/mg and beet extract with a specific activity of 40.21 U/mg, While the remaining media gave specific activity between (37.12-39.35 U/mg). as shown in figure 9.

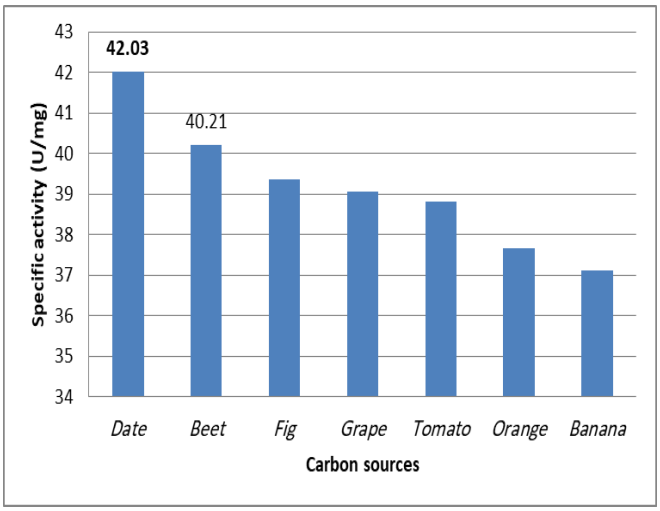


**Figure 9. Levansucrase production by *Lactobacillus plantarum* V11 at different carbon sources.**

## DISCUSSION

The easiest way to assess EPS production is to visually observe the phenotypic characteristics of the colonies: slimy or ropy phenotypes. The slimy phenotype is characterized by mucilaginous colonies. In contrast, the ropy phenotype is characterized by forming long filaments when an inoculation loop is lifted from the colony surface or cell pellet. The screening methods to evaluate the microbial ability to produce exopolysaccharides are based on the cultivation of the LAB in

a medium enriched with different sugars (glucose, fructose, sucrose, galactose, or lactose) [14].

Levansucrase-possessing bacteria have mucoid colonies when grown on a sucrose-containing agar plate due to levan synthesis. This feature can detect and identify bacteria that produce a levansucrase [15]. The enzyme exhibited its induction in the presence of sucrose used as a substrate. Colonies showed slimy mucoid appearance of levan polysaccharide production [16].

It is well-known that the pH and temperature significantly affect enzyme activity. Generally, the optimal reaction pH of levansucrase from microorganisms is between 4.0 and 7.0, while the optimal reaction temperature is very different [16].

The levansucrase performed best at pH 6.5 and retained >80% activity in the pH range of 6−7. The maximum level of levan production was found at pH 7 but decreased at pH 7.5 [16,17].

The results indicated that bacterial growth, as well as enzyme production, was greatly influenced by both physical and chemical conditions. It was revealed that high enzyme titers were achieved after 24 hours of incubation in a medium [18]

The optimal inoculum size for exopolysaccharide production by Agaricusblazei 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% [10].

Nitrogen is an essential part of protein, nucleotides, enzymes and a cofactor that plays a vital role in the metabolism [12].

The intra- and extracellular levansucrase (LS) activities produced by bacteria were promoted by supplementing the sucrose medium with yeast and peptone as nitrogen sources. Supplementation of the selected media with yeast or peptone resulted in a significant increase in LS production [12].

EPS production showed that the amount of EPS produced by plantarum in the dating medium was high. Microorganisms utilize carbon sources for their growth and in the production of levan and levansucrase. Carbon source is an important factor impacting the quality and quantity of levan and levansucrase production. Many carbon sources are used for levan and levansucrase production, such as sucrose, glucose, fructose, galactose, maltose, mannose and mannitol [19,20,21].

## CONCLUSIONS

*Lactobacillus plantarum* isolated from the vaginal of healthy women was a better producer of levansucrase activity. This research was to detect the levansucrase activity in *Lactobacillus* spp., besides screening and optimizing the production condition of levansucrase.

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