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Analysis and Characterization of Purified Levan from *Leuconostoc mesenteroides* ssp. *cremoris* and its Effects on *Candida albicans* Virulence Factors

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Abstract

The objective of the current study is to characterize levan purified from *Leuconostoc mesenteroides* ssp. *cremoris* and the detection of its effects against the growth and virulence factors of *C. albicans*. The purification and characterization of levan produced by local isolates was done using thin layer chromatography (TLC) and High performance liquid chromatography (HPLC) analysis which determined and proved that purified levan is a homopolysaccharide consisting only of fructose. The determination of functional group analysis of levan was studied using Fourier transform infrared spectroscopy (FTIR). The results showed that the bond region in 857.60 cm-1 is typical to carbohydrates (identification of polysaccharides). The melting point was also determined. Levan began to liquefy at 224 ºC, and was completely melting at 330 ºC. The antifungal activity of levan was determined against fourteen isolates of *Candida albicans* taken from oral and vaginal specimens. The MIC for all of the C. *albicans* isolates was between (50 –100) mg/mL. The effects of purified levan (at sub MIC) on *C. albicans* virulence factors which included phospholipase, haemolysin production, biofilm formation and hyphal transition were determined. The results showed that purified levan had the ability to inhibit the virulence factors of C. *albicans* with significant differences compared with the control. The change in the P_z value of most isolates was recorded and turned from strong isolates to moderate isolates. On the other hand, the inhibition of haemolysin production was recorded. The highest inhibition percentage was 58.53 %, while the lowest percentage was 53.84 %. The purified levan showed inhibitory effects on *C. albicans* biofilm formation in microtiter plates and acrylic denture resin specimens at different incubation times of 24, 48, and 72 hours. In the microtiter plates, the highest inhibition percentage of biofilm formation was 70.58 % at a seventy-two- hour incubation time, whereas for the acrylic denture resin specimens, the highest inhibition percentage of biofilm formation was 68 % at a seventy-two-hour incubation time. Also, the inhibition of hyphal transition was recorded, and the highest inhibition percentage of hyphal transition was 59.26 %. In conclusion, purified levan showed inhibitory effects on the growth and virulence factors of *C. albicans.*

Keywords: Levan, *Leuconostoc mesenteroides*, Inhibitory effect, *Candida albicans*, Virulence factors

1. Introduction

Levan is a fructose polymer synthesized from sucrose by a wide range of microorganisms, and it is a non-toxic, biologically-active, extracellular polysaccharide (Franken *et al*., 2013).Bacterial levans often have molecular weights over 500,000 Da, and are commonly branched which results in offering a broad spectrum of applications (Öner *et al*., 2016). Levan belongs to a larger group of commercially-important polymers referred to as fructans, which are used as a source of prebiotics. The synthesis of levan is catalyzed by a group of enzymes referred to as levansucrases using sucrose as substrate (Hill *et al*., 2017). Some microbial levans exhibit biological activities such as antitumor, antidiabetic, and immune-stimulating activities (Lu *et al*., 2014). In medicine, levan is used as a blood and plasma volume expander and drug delivery. In blood and plasma volume expansion, levan can replace the normal blood protein in providing osmotic pressure which is useful for preventing hemorrhagic shocks, as well as burn and surgery shocks (Rehm, 2009).

Candida albicans is a member of the normal human microbiota (Jabra -Rizk *et al*., 2016). The ability of *C. albicans* to infect such diverse host niches is supported by a wide range of virulence factors which include biofilm formation, phenotypic switching, and the secretion of hydrolytic enzymes (Brunke *et al*., 2016). The effect of levan on yeast- hyphal transition associated with tissue invasion and the escape from phagocyte cells after host internalization was estimated, and it is considered as an important virulence factor (Jacobsen *et al*., 2012).This study is conducted with the objective of characterizing levan purified from local *L. mesenteroides* ssp. *cremoris*, and for the detection of its activity against the growth and virulence factors of *C. albicans*.

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2. Materials and Methods

2.1. Microorganisms

2.1.1. Leuconostoc mesenteroides ssp. cremoris Isolates

Leuconostoc mesenteroides sp. *cremoris* was isolated from local fish intestines and was identified using cultural, microscopical, and biochemical test as well as the Vitek 2 system.

2.1.2. Candida albicans Isolates

Fourteen *C. albicans* isolates were taken from oral and vaginal specimens. All isolates were subjected to the cultural, microscopical, and biochemical tests as well as the Vitek 2 system.

2.2. Levan Production

This process was done according to the procedure described by (Abou-Taleb, *et al*., 2015). 250 mL Erlenmeyer flasks, containing 100 mL of a levan production medium (2.5g Yeast extract, 200g Sucrose, 0.2g $MgSO_4.7H_2O$, 5.5 g K_2HPO_4 added to one liter of distilled water, with the pH adjusted to seven then autoclaved), were inoculated with 2% of *L. mesenteroides* ssp *cremoris* suspension containing (9×10^8 CFU/mL), and was incubated at 30ºC for twenty-four hours.

2.3. Precipitation of Levan

After twenty-four hours of incubation, the culture was centrifuged at 10000 rpm for ten minutes to obtain the pellets which are used as source for cell dry weight. The pellets were washed twice with distilled water, and dried at 80ºC. The supernatant was used for the precipitation of levan by adding 1.5 volumes of ice - cold absolute ethanol for levan precipitation. The precipitated pellets were washed twice by distilled water, and were collected by centrifugation at 10000 rpm for ten minutes. The levan dry weight was determined after oven-drying at 110ºC for twenty-four hours (Abou-Taleb, *et al*., 2015).

2.4. Purification of Levan Produced by L .mesenteroides ssp. cremoris

The precipitated levan polymer at optimum conditions was re-suspended in demineralized water at 4ºC over the duration of sixteen hours, and was then dialyzed (MWCO 14,000 Da) overnight against demineralized water. The polymer was precipitated with two volumes of 96 % ice – cold ethanol, centrifuged for ten minutes at 10.000 rpm (Abou-Taleb, *et al*., 2015). The O.D was measured at 400 nm to estimate levan concentration at each step of the purification according to the equation described by González - Garcinuño *et al*. (2017):

$$y = 0.1645x - 0.035$$

where y is the absorbance at 400 nm, and x is the levan concentration expressed in mg / mL The purified levan was dried at (40-45) ºC., and was kept for further analysis .

2.5. Characterization of Levan Purified from L. mesenteroides ssp. Cremoris

2.5.1. Thin-Layer Chromatography (TLC)

Levan (0.01 gm,) purified from *L. mesenteroides* ssp. *cremoris*, was hydrolyzed in 5 % HCl (v/v) and was heated for an hour in a water bath at 100ºC. Equal weights (0.01gm) of glucose, sucrose, and fructose were dissolved in 1mL of 1% ethanol. Thin-Layer Chromatography (TLC) has been performed by using silica. The position and distance of the spots were determined, and the relative flow (R_f) had been calculated as described by (Radhi *et al*., 2013):

$$R_f = \frac{\text{Distance moved by substance}}{\text{Distance moved by the solvent front}}$$

2.5.2. High Performance Liquid Chromatography(HPLC)

The analysis of Levan by high performance liquid chromatography (HPLC) has been carried out at the Ministry of Science and Technology in Baghdad, Iraq. The instrument operates using mobile phase containing 15 mM NaOH spiked with 1 mM barium acetate; the flow rate was 1.5 mL/min, temperature was 40 ºC, and the injection volume was 20 µL .

2.5.3. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) has been carried out using (Bruker – Tensor 27 with ATR unit) in the Physics Department at the Collage of Sciences of Mustansiriyah University in Baghdad, Iraq. The instrument operates in the wavenumber range of (400 – 4000 cm^{-1}) that measures the amount of IR radiation reflected or transmitted through a sample. The results obtained are in the form of a graphical chart, in which the X – axis represents the wave number, while the Y-axis represents the transmittance % .

2.5.4. Melting Point

A melting-point test has been carried out using (Melting Point, Digital, Advanced, SMP30). The capillary tube that was sealed at one end was filled with levan up to 2-3 mm, and was then inserted into a melting point apparatus and heated. At this point, two temperatures were recorded: the temperature at which the substance began to liquefy, and the temperature at which it became completely liquefied.

2.6. Antifungal Activity of Levan Purified from L. mesenteroides sp. cremoris against Candida albicans Isolates

The antifungal activity of levan purified from *L. mesenteroides* ssp. *cremoris* against *C. albicans* isolates was determined by the microdilution method in 96 – well flat-bottom microplate titer on the basis of minimum inhibitory concentration (MIC) values. The experiment was done according to the procedures described by Salman *et al*. (2018) with slight modification.

2.7. Effect of Levan Purified from L. mesenteroides sp. Cremoris on Candida albicans Virulence Factors

2.7.1. Inhibition of Phospholipase Production

The inhibition of Phospholipase production by levan was detected. The *C. albicans* isolates were grown in the presence and absence of levan at the sub MIC concentration; 20 µL of C. *albicans* suspension compared to 0.5 McFarland was added to 80 µL of a Sabouraud dextrose broth (SDB) and mixed with 100 µL of levan; while the control contained only 180 µL of broth and 20 µL of *Candida* suspension. Then 10 µL from all of the isolates was placed on the surface of the egg yolk medium, and was left to dry at room temperature. The plates were

then incubated at 37◦C for twenty-four hours; after incubation, the P_z value was calculated as follows (Zarei *et al*., 2010):

$$\text{Phospholipase activity } Pz = \frac{\text{Diameter of colonies (mm)}}{\text{Diameter of the zone of opacity + colonies}}$$

2.7.2. Inhibition of Haemolysin Production

The inhibition of haemolysin production by levan was detected using the procedure described by (Lee *et al*., 2014), and the experiment was done in triplicate. *C. albicans* isolates were grown in the presence and absence of levan at the sub MIC concentration; 20 µL of *C. albicans* suspension compared to 0.5 McFarland was added to 80 µL of SDB and mixed with 100 µL of levan, while the control contained only 180 µL of SDB and 20 µL of the *C. albicans* suspension without levan, incubated at 37 ºC for twenty-four hours.

Candidal cultures (treatment and control) at 100 µL were added to 10 mL of red-blood cells that were previously prepared and were incubated at 37 ºC for one hour. Centrifugation was applied after incubation at 12,000 rpm for ten minutes to collect the supernatants. The optical densities of the supernatants were then measured at 543 nm. Inhibition of haemolysin production percentage was calculated according to the equation described by Chevalier *et al*. (2012) with slight modification.

$$\text{\% Inhibition of haemolysin production} = \frac{\text{O.D control} - \text{O.D treatment}}{\text{O.D control}} \times 100$$

2.7.3. Inhibition of Biofilm Formation in Microtiter Plates

The effect of levan on the biofilm formation of *C. albicans* was studied using 96 flat-bottom well microtiter plates. The *C. albicans* isolates were grown at 37 ºC for 24, 48, and 72 hours in the presence and absence of levan at the sub MIC concentration (the experiment was done in triplicate). Inhibition of the biofilm formation percentage was calculated according to the equation described by Chevalier *et al*. (2012).

$$\text{\% Inhibition of hyphal transition} = \frac{\text{No. of hyphae in control} - \text{No. of hyphae in treatment}}{\text{No. of hyphae in control}} \times 100$$

2.7.4. Inhibition in Acrylic Denture Resin Specimens

The effect of levan on the biofilm formation of *C. albicans* was studied using acrylic denture resin specimens **(**obtained from Dentistry College at Mustansiriyah University in Baghdad, Iraq); the experiment was done in triplicate. The acrylic denture resin specimens were kept for twenty-four hours in the levan solution at sub MIC concentration. The control acrylic specimens were kept in water according to the procedure described by Salman (2018) with modification. Inhibition of the biofilm formation percentage was calculated according to the abovementioned equation described by Chevalier *et al*. (2012).

2.8. Inhibition of Hyphal Transition

The effect of levan on yeast- hyphal transition was estimated by the cultivation of *C. albicans* in the hyphal transition medium (YEP broth).The experiment was done in triplicate. *C. albicans* isolates were grown at 37 ◦C for twenty-four hours in the presence and absence of levan at sub MIC concentration. 20 µL of *Candida* suspension compared to 0.5 McFarland was added to 80 µL of YEP broth, and mixed with100 µL of levan, while the control contained only 180µL of YEP broth and 20µL of the candidal suspension. Yeast and hyphae were counted using a hemocytometer slide by observation under an optic microscope. The numbers of hyphae were determined, and the inhibition of the hyphal transition was calculated by comparing it with the control. The inhibition of the hyphal transition was calculated according to the equation described by kumar, *et al*., (2011) with modification**.**

$$\text{\% Inhibition of hyphal transition} = \frac{\text{No. of hyphae in control} - \text{No. of hyphae in treatment}}{\text{No. of hyphae in control}} \times 100$$

2.9. Statistical Analysis

SPSS software version 21/ France was used for data analysis by one -way ANOVA- test to calculate the *P*- vale between the test groups' means in the study.

3. Results

3.1. Purification of Levan Produced by L .mesenteroides ssp. cremoris

The purification of levan produced by *L .mesenteroides ssp. cremoris* was done for the sake of this study. After production under optimum conditions, the concentration of levan was estimated at each of the purification steps. The results showed that levan concentration increased at each step of the purification process. The concentrations recorded were: (9.234, 13.7933, 15.6291, 17.9696) mg /mL before precipitation, after precipitation, after dialysis, and at the last precipitation respectively (Figure 1). The total dry weight of levan after purification was 7.621 g / L.

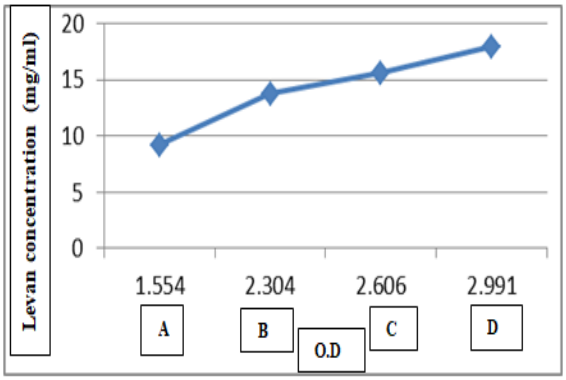

Figure 1. Concentration of levan produced by *L. mesenteroides spp. cremoris* at different steps of purification, A: before precipitation, B: after precipitation, C: after dialysis, D: after last precipitation.

3.2. Characterization of Levan Purified from L. mesenteroides ssp. cremoris

3.2.1. Thin-Layer Chromatography (TLC)

Levan contents produced by L. *mesenteroides* ssp. *cremoris* were analyzed by TLC to determine their components of monosaccharides. The R_f values of fructose were identical or so close to the acid hydrolyzed levan. The R_f value of levan was 0.42, while the R_f values of sucrose, fructose, and glucose were 0.32,0.42 and 0.37, respectively. This result indicates that the purified levan from *L. mesenteroides ssp. cremoris* consisted solely of fructose (Figure 2).

Figure 2. Thin - Layer Chromatography analysis of levan purified from *L. mesenteroides* ssp. *cremoris*

3.2.2. High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography) HPLC) was used to separate, identify, and quantify each component in the mixture. The results revealed that the levan consisted solely of fructose, which indicates that the purified levan was of the homopolysaccharide type (Figure 3).

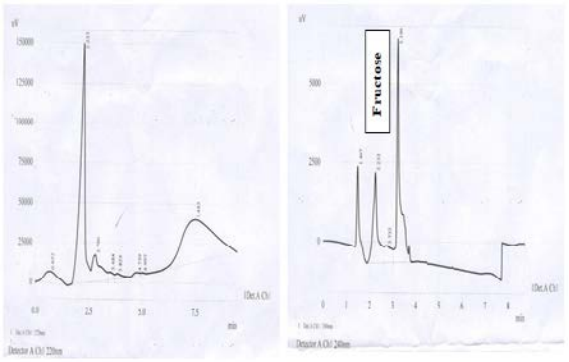

Figure 3. A: HPLC analysis of levan produced by *L. mesenteroides* ssp. *cremoris*; B: HPLC analysis of standard sugar

3.2.3. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) spectra analysis was used to detect the functional groups of purified levan from *L .mesenteroides ssp. cremoris*. The characteristic peaks of levan indicated that the purified levan was of the polysaccharide type. The band in the region of 3271.26^{cm-1} represents hydroxyl (O-H) stretching, while the band in region of 2932.40 $^{cm-1}$ is for (C – H) stretching vibration. The band region of 1634.83 $^{cm-1}$ is typical for C=O stretching, while the band region found in the 1417.22 $^{cm-1}$ and 1217.17^{cm1} are due to C-H plan deformation and aromatic skeletal stretching. The bond region in 857.60 $^{cm-1}$ is typical for carbohydrates (identification of polysaccharides) (Figure 4).

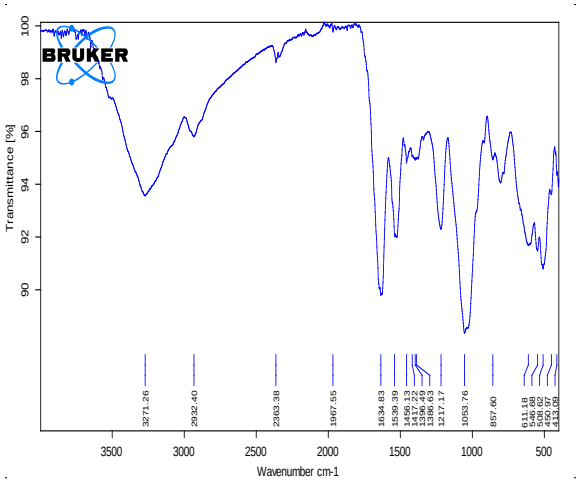

Figure 4. FTIR spectra of levan produced by *Leuconostoc mesenteroides* ssp. *cremoris*.

3.2.4. Melting Point

Melting point analysis was used to detect the temperature at which the compound began to liquefy. Also it can provide the information about the purity of the sample. The results showed that the purified levan from *L. mesenteroides* ssp. *cremoris* began to liquefy at 224 ºC, and at 330 ºC levan was completely melting. Purified levan was characterized as a white crystalline powder highly soluble in water.

3.3. Antifungal Activity of Levan Purified from L. mesenteroides ssp. cremoris against Candida albicans Isolates

The Antifungal activity of the biopolymer levan was determined on the basis of minimum inhibitory concentration (MIC) values. MIC at concentrations ranging between 200 and 0.09 mg /mL was determined for all of the *C. albicans* isolates. The results showed that the MIC for all C. *albicans* isolates was between (50 – 100) mg/mL.

3.4. Effect of Levan Purified from L. mesenteroides ssp. cremoris on Candida albicans Virulence Factors

3.4.1. Inhibition of Phospholipase Production

In the current study, levan had a high effect on the phospholipase production of all *C. albicans* isolated from both oral and vaginal candidiasis. Phospholipase activity decreased in all of the *C. albicans* isolates. The P_z value reached 0.81 compared to the control. The results of the statistical analysis showed significant differences (P = 0.001) between the treatment and control groups of all the isolates (Table 1).

Table 1. Inhibition of phospholipase production by levan purified from *L.mesenteroides ssp.cremoris.*

Candidal isolates	P_z value		
	Control Mean ± SD	Treatment Mean ± SD	*P* Value
C. albicans (CO_1)	0.68 ± 0.01	0.73 ± 0.02	0.001
C. albicans (CO_2)	0.68 ± 0.03	0.74 ± 0.02	0.001
C. albicans (CO_3)	0.65 ± 0.03	0.72 ± 0.03	0.001
C. albicans (CO_4)	0.70 ± 0.03	0.73 ± 0.02	0.001
C. albicans (CO_5)	0.69 ± 0.03	0.76 ± 0.05	0.001
C. albicans (CO_6)	0.69 ± 0.05	0.77 ± 0.05	0.001
C. albicans (CO_7)	0.71 ± 0.08	0.75 ± 0.03	0.001
C. albicans (CO_8)	0.79 ± 0.04	0.81 ± 0.02	0.001
C. albicans (CO_9)	0.70 ± 0.01	0.74 ± 0.06	0.001
C. albicans (CV_1)	0.69 ± 0.04	0.72 ± 0.04	0.001
C. albicans (CV_2)	0.70 ± 0.05	0.73 ± 0.02	0.001
C. albicans (CV_3)	0.70 ± 0.02	0.77 ± 0.04	0.001
C. albicans (CV_4)	0.70 ± 0.02	0.77 ± 0.03	0.001
C. albicans (CV_5)	0.69 ± 0.02	0.72 ± 0.03	0.001

CO: Oral isolates; CV: vaginal isolates; *P* = 0.001: significant differences

$P_z = 1$ → Negative for phospholipase

$P_z = 0.80\text{-}0.89$ → Weak phospholipase

$P_z = 0.70 - 0.79$ → Moderate phospholipase

$P_z = <0.70$ → Strong phospholipase

3.4.2. Inhibition of Haemolysin Production

The inhibitory effect of levan purified from *L. mesenteroides ssp. cremoris* on haemolysin production from *C. albicans* isolated from oral and vaginal candidiasis was observed in this study. The haemolysin activity decreased in all the *C. albicans* isolates, and the optical density of hemoglobin released from lysed erythrocytes ranged between (0.17 and 0.18) compared with the control (0.39 and 0.43). The results of statistical analysis showed significant differences ($P \leq 0.01$) between the treatment and control groups of all the isolates. The highest inhibition percentage for haemolysin production was 58.53 %, while the lowest inhibition percentage was 53.84 % (Figure 5).

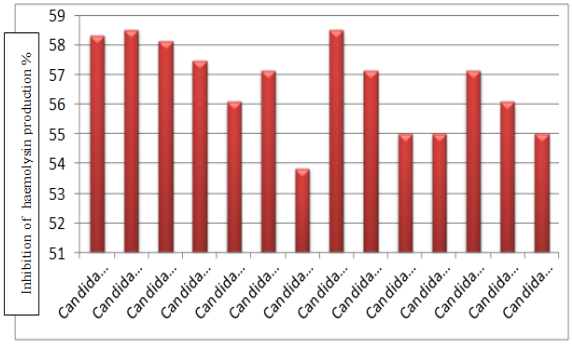

Figure 5. Inhibition percentage of haemolysin production of *C. albicans* by levan purified from *L. mesenteroides sp. cremoris*

3.4.3. Inhibition of Biofilm Formation in Microtiter Plates

In this study, levan is shown to have an inhibitory effect on the biofilm formation of all *C. albicans* isolated from both oral and vaginal candidiasis. The biofilm formation decreased in all of the *C. albicans* isolates at different incubation times of 24, 48, 72 hours compared to the control. The highest inhibition percentage for biofilm formation was 70.58%, while the lowest percentage was 50 % at seventy-two-hour incubation time for *C. albicans* isolated from oral cavities. As for *C. albicans* isolated from vaginal infections, the highest percentage was 59.37 %, while the lowest inhibition percentage was 51.85 % (Table 2).

Table 2. Inhibition percentage of biofilm formation by levan purified from *L. mesenteroides ssp. cremoris* in different incubation time **.**

Candidal isolates	Inhibition of biofilm formation %		
	24 h	48 h	72 h
C. albicans (CO_1)	43.75	50	60
C. albicans (CO_2)	69.09	69.35	70.58
C. albicans (CO_3)	35.71	42.10	56.66
C. albicans (CO_4)	57.14	57.57	63.04
C. albicans (CO_5)	33.33	44.44	55.17
C. albicans (CO_6)	33.33	47.61	50
C. albicans (CO_7)	62.79	63.44	66.05
C. albicans (CO_8)	30.76	35.29	55.17
C. albicans (CO_9)	36.84	46.42	51.28
C. albicans (CV_1)	35.71	42.10	55.17
C. albicans (CV_2)	52.94	54.54	59.37
C. albicans (CV_3)	25	31.25	51.85
C. albicans (CV_4)	33.33	45.83	55.55
C. albicans (CV_5)	40.62	46.15	52

CO: oral isolates; CV: vaginal isolates

3.4.4. Inhibition in Acrylic Denture Resin Specimens

In this study**,** the inhibition effect of levan purified from *L. mesenteroides* ssp. *cremoris* on the biofilm formation of *C. albicans* isolated from oral candidiasis in acrylic denture resin specimens was studied. Purified levan had a high effect on the biofilm formation of *C. albicans* (CO_2) in acrylic denture resin. Biofilm formation decreased in C. *albicans* (CO_2) at different incubation time (24, 48, and 72) hours compared to the control. The results of the statistical analysis showed significant differences (*P*= 0.001) between the levan treatment groups and the control (Table 3).

Table 3. Inhibition of candidal biofilm formation by levan purified from *L. mesenteroides ssp. cremoris* in acrylic denture resin specimens

Incubation time	O.D at (450 nm)		
	Control Mean ± SD	Treatment Mean ± SD	*P* value
24h	0.48 ± 0.01	0.18 ± 0.01	0.001
48h	0.59 ± 0.01	0.21 ± 0.01	0.001
72h	0.72 ± 0.01	0.23 ± 0.02	0.001

$P \leq 0.01$: significant differences

The inhibition percentage of biofilm formation was calculated. The results showed that the highest inhibition percentage of biofilm formation was 68% at a seventy-two- hour incubation time (Figure 6). On the other hand, at a forty-eight-hour incubation time, the highest inhibition percentage for biofilm formation was 64.4%, while the lowest inhibition percentage for biofilm formation was 62.5 % at a twenty-four- hour incubation time (Figure 7).

Figure 6. Inhibition of *C. albicans* biofilm formation in acrylic denture resin specimens by levan purified from *L. mesenteroides* ssp. *cremoris* at a seventy-two- hour incubation time.

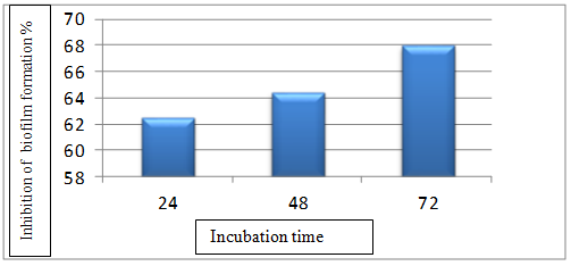

Figure 7. Inhibition percentage of *C. albicans* biofilm formation in acrylic denture resin specimens by levan purified from *L. mesenteroides* ssp. *cremoris*

3.5. Inhibition of Hyphal Transition

The inhibitory effect of levan purified from *L. mesenteroides ssp. cremoris* on the hyphal transition of *C. albicans* isolated from oral and vaginal candidiasis was recorded for this study. The hyphal transition decreased in all of the *C. albicans* isolates with significant differences ($P = 0.001$) between treatment and control of all the isolates (Table 4).

Table 4. Inhibition of hyphal transition by levan purified from *L. mesenteroides ssp. cremoris.*

Candidal isolates	No. of hypha		
	Treatment (Mean ± SD)	Control (Mean ± SD)	*P* Value
C. albicans (CO_1)	26.67 ± 7.64	48.33 ± 7.64	0.001
C. albicans (CO_2)	23.33 ± 2.89	40.00 ± 5.00	0.001
C. albicans (CO_3)	21.67 ± 7.64	48.33 ± 7.64	0.001
C. albicans (CO_4)	25.00 ± 5.00	43.33 ± 7.64	0.001
C. albicans (CO_5)	23.33 ± 2.89	43.33 ± 7.64	0.001
C. albicans (CO_6)	20.00 ± 0.00	46.67 ± 7.64	0.001
C. albicans (CO_7)	23.33 ± 5.77	43.33 ± 7.64	0.001
C. albicans (CO_8)	26.67 ± 5.77	43.33 ± 7.64	0.001
C. albicans (CO_9)	25.00 ± 5.00	41.67 ± 7.64	0.001
C. albicans (CV_1)	20.00 ± 5.00	43.33 ± 7.64	0.001
C .albicans (CV_2)	21.67 ± 2.89	45.00 ± 5.00	0.001
C. albicans (CV_3)	23.33 ± 2.89	45.00 ± 5.00	0.001
C. albicans (CV_4)	20.00 ± 5.00	40.00 ± 5.00	0.001
C. albicans (CV_5)	18.33 ± 2.89	45.00 ± 5.00	0.001

CO: oral isolates; CV: vaginal isolates; $P \leq 0.01$: significant differences

The inhibition percentage of hyphal transition was calculated for this study, and the highest recorded inhibition percentage for hyphal transition was 59.26 %, while the lowest inhibition percentage was 38.44 % (Figure 8).

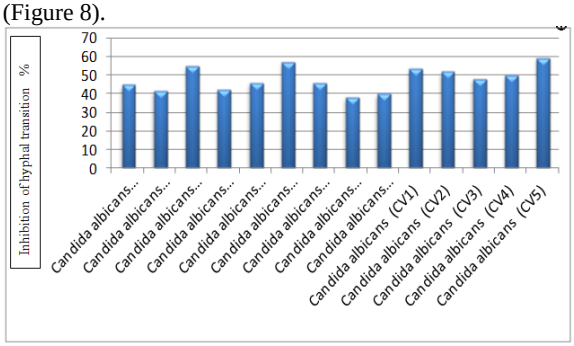

Figure 8. Inhibition percentage of hyphal transition of *C. albicans* by levan purified from *L. mesenteroides ssp. cremoris.*

4. Discussion

Levan is a homopolysaccharide composed of fructose units. It is a type of fructan that belongs to a group of fructose- based oligo- and polysaccharide (Fedewa and Rao, 2014). Moosavi-Nasab *et al.* (2010) reported that the R_f value of acid-hydrolyzed levan from *Micro-bacterium laevaniformans* was identical to that of fructose, and also found that levan was composed solely of fructose. Radhi *et al.* (2013) mentioned that levan was composed mainly of fructose residues when analyzed by thin-layer chromatography (TLC).

Mamay *et al.* (2015) mentioned that the FTIR spectra analysis of levan produced from *B. licheniformis* showed that the stretching of O- H vibrations appeared at the wavelength around 3300 $^{cm-1}$, while the band at around 2900 represents C-H stretching, and that the peak at the wavenumber of 1660 $^{cm-1}$ is typical for C=O stretching. Also Nasir *et al.* (2015) analyzed levan secreted from *Halomonas* and *Chromobacter* by FTIR and found out that the region of typical carbohydrates at the finger prints was at the wave number range of 1000-800^{cm-1}. Levan has quite

interesting biochemical properties, and good thermos-stability with a melting point of 225°C (Divya and Sugumaran, 2015).

Polysaccharides exhibit activities against *C. albicans*. Tøndervik *et al*. (2014) showed that oligo-saccharide. Oligo G is an alginate having the ability to reduce fungal cell growth. Martins *et al*. (2017) mentioned that polysaccharides from *Agaricus brasiliensis* can stimulate human monocytes to capture *C. albicans.*

Pritchard *et al*. (2017) found out that alginate oligosaccharides have the ability to inhibit phospholipase in *C. albicans.* Martinez *et al*. (2010) reported that chitosan, which is a polymer isolated from crustacean exoskeletons, can inhibit candidal biofilm formation *in vivo*. Salman (2018) observed the anti-biofilm effect of biosurfactant from *L. mesenteroides* against *C. albicans* in acrylic disc.

Polysaccharides have been shown to possess a broad spectrum of antimicrobial and antibiofilm activities against fungi and bacteria. Chevalier *et al*. (2012) successfully developed a day-care mouthwash specifically for the dry mouth which consisted of a healthy biofilm, including endogenous bacteria and salivary mucins with an external polysaccharide coating to protect the oral mucosa, and to create an oral environment unfavorable to *Candida* proliferation, causing inhibition of the yeast- hyphal transition and iron chelation. Pritchard *et al*. (2017) have successfully used alginate oligosaccharides to induce marked alterations in hyphal formation that result in reducing the invasion of *C. albicans* in the epithelial cell animal experiment model.

5. Conclusion

In conclusion, the purified levan produced from the *Leuconostoc mesenteroides* ssp. *cremoris* isolates had an inhibitory effect on the growth and virulence factors of *Candida albicans.*

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