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Production of Levan from Locally Leuconostoc *mesensteroides* isolates

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Abstract

Thirty isolates of *Leuconostoc mesenteroides* were isolated from different sources included fish intestine , raw milk , banana , carrots and sauerkraut . All isolates were tested for levan production using mucoidy and spectrophotometric method . Results showed that only 11 isolates had the ability to produce levan . Precipitation of levan was done by using seven non polar organic solvents included ethanol , acetone , methanol , diethyl ether , isopropanol , chloroform and toluene separately , the precipitation of levan by ethanol , acetone , methanol , diethyl ether , isopropanol , chloroform , toluene were recorded as levan dry weight 1.482 , 1.477 , 1.350 , 1.252 , 1.479 , 1.239 , 1.261 with levan production yield 7.4% ,7.3 % , 6.7 % , 6.2 % , 7.3 % , 6.1 % , 6.3% respectively .The optimum conditions for levan production were studied included temperature , incubation time , pH , inoculum size , sucrose concentration , culture medium . The optimum conditions for production were at 30 ºC for 24 h at pH 7 with 4 % inoculum size and 10 % sucrose concentration and the best culture medium for levan production was sucrose medium .

Key Words: Levan polymer , *Leuconostoc mesenteroides* , precipitation , optimum conditions

INTRODUCTION

Leuconostoc spp. is a genus of gram - positive bacteria , placed within the family of Leuconostocaceae and were first isolated in 1878 by Cienkowski [1 , 2] . Its traditionally found in association with plant matter , fermenting vegetables , milk , dairy p roducts , and wines and meats , usually nonpathogenic acid - tolerant organisms with optimal temperature 18 and 25°C [3 , 4] . *Leuconostoc* species along with the other lactic acid group can produce various compounds such as organic acids , hydrogen peroxide , diacetyl and bacteriocins during lactic fermentations , these components not only give desirable effects on food taste , smell , color and texture , but they also inhibit undesirable pathogenic microorganisms [5] , also *Leuconostoc* spp. have the ability to produce exopolysaccharides (EPS) , these biopolymers possess commercial potential , because of the wide variety of industrial applications of EPS [6] .

Levan is fructose polymer that synthesized from sucrose by a wide range of microorganisms [7] , its non - toxic , biologically active , extracellular polysaccharide [8] . Bacterial levan often have molecular weights over 500,000 Da , and commonly branched that result in offering a broad spectrum of applications [9] . Levans belong to a larger group of commercially important polymers referred to as fructans , which are used as a source of prebiotic [10] . Synthesis of levan is catalyzed by a group of enzymes referred to as levansucrases using sucrose as substrate [8 ,11] . Some microbial levan exhibit biological activities such as antitumor , antidiabetic and immunostimulating activities [12] . In medicine levan used as blood plasma volume expander , and drug delivery [13] , in blood plasma volume expander , levan can replace the normal blood protein in providing osmotic pressure which is useful for preventing shock from hemorrhagic , burns and surgery [14] , also animal studies have been shown that levan can lower blood cholesterol [9] , while in drug delivery , levan has been used as a coating material in drug delivery formulation [15] .

MATERIALS AND METHODS

Microorganisms

Leuconostoc mesenteroides isolates

Thirty isolates of *Leuconostoc mesenteroides* were isolated from different samples included fish intestine , raw milk , fresh fruits (Banana and carrot) and sauerkraut then identified throughout cultural , microscopical and biochemical test according to [16] and Vitek 2 system.

Screening of *Leuconostoc mesenteroides* for levan production

Mucoidy method

The levan screening medium (1 g trypton , 0.5 g yeast extract , 1.5 g agar , 0.25 g K2Hpo4 , 10 g NaCl , 20 g sucrose , pH adjust to 7.2) was inoculated after sterilization with 24 h old culture of *Leuconostoc mesenteroides* isolates and incubated at 30°C for 24 h , the slimy mucoid appearance of isolates was recorded as levan producer [17] .

Spectrophotometric method

The levan of selected *L. mesenteroides* isolates which recorded as producer isolates was estimated by the spectrophotometric method [18] . Levan screening medium without using agar - agar was inoculated by *L. mesenteroides* 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 30 °C for 24 h . After incubation the culture medium was centrifuged at 10000 rpm for 10 min , the biomass removed and the supernatant used to estimate levan concentration . The O.D was measured at 400 nm with the spectrophotometer , the equation was used to determine the levan concentration in the culture medium :

$$y = 0.1645x - 0.035$$

where y is the absorbance at 400 nm , and x is levan concentration expressed in mg / mL [18] .

Levan production

This process was done according to the procedure described by [19] , 250 ml Erlenmeyer flasks containing 100 ml of levan production medium (sucrose medium) (2.5 g Y east extract , 200 g Sucrose , 0.2 g MgSO4.7H2O , 5.5 g K2HPO4 to 1 liter of distilled water , the pH was adjusted to 7 then autoclaved) was inoculated with 2 % of *L. mesenteroides* ssp. *cremoris* (LF_5) suspension containing (9×108 cfu /ml) , incubated at 30ºC for 24 h .

Precipitation of levan

After 24 h of incubation , the culture were centrifuged at 10000 rpm for 10 min to obtain the pellets which is used as source of cell dry weight , the pellets washing twice with distilled water and drying at 80ºC . The supernatant was used for precipitation of levan by adding 1.5 volumes of seven non polar organic solvents separately to detected the best solvent for levan precipitation . These

solvents included ice - cold absolute ethanol , acetone , methanol , diethyl ether , isopropanol , chloroform , toluene separately . The precipitated pellets were washed twice by distilled water and collected by centrifugation at 10000 rpm for 10 min . The levan dry weight determined after oven - drying at 110ºC for 24 h and the levan production yield was calculated according to equation [20]

Levan production yield (%)

= levan concentration in dry weight (g/100ml) x 100
Initial sugar

Determination of optimum condition for levan production by *L .mesenteroides* ssp. *cremoris*

Effect of temperature

This process was done by incubation of *L. mesenteroides* ssp. *cremoris* (LF_5) at various temperatures in the range of (25 , 30, 35) ^oC for 24 h , after incubation the precipitation was done by using ice - cold ethanol . The levan dry weight , cell dry weight and levan production yield (%) were estimated for each temperature [20] .

Effect of incubation time

To determine the best incubation time for levan production , *L.mesenteroides* ssp. *cremoris* (LF_5) was incubated at the best temperature for (24 , 48 , 72) h separately . After incubation the precipitation was done by using ice - cold ethanol . The levan dry weight , cell dry weight and levan production yield (%) were estimated for each incubation time .

Effect of initial pH

For studying the effect of pH of the culture medium on levan production , the medium was adjusted at pH to (5.0 , 6.0 , 7.0 , 8.0 , 9.0) separately before sterilization . The culture medium incubated at the best temperature and incubation time , the precipitation was done by using ice - cold ethanol . The levan dry weight , cell dry weight and levan production yield (%) were estimated for each PH .

Effect of inoculum size

To determine the effect of inoculum size on levan production , the culture medium was inoculated with various inoculum size (2 , 4 , 6 , 8 ,10) % of inoculum (9×10^8 CFU/ml) , the medium was adjusted to the best pH and the cultivation culture media were incubated at the best temperature for the best incubation time . The precipitation was done by using ice - cold ethanol . The levan dry weight , cell dry weight and levan production yield (%) were estimated for each inoculum size .

Effect of sucrose concentration

To determine the best sucrose concentration for levan production the culture medium was inoculated at various sucrose concentration (10, 20 , 30, 40 , 50) % , the medium was adjusted to the best pH , the cultivation culture media were incubated at the best temperature for the best incubation time , inoculum size .The precipitation was done by using ice - cold ethanol , the levan dry weight , cell dry weight and levan production yield (%) were estimated for each sucrose concentration .

Effect of culture medium

For studying the effect of culture medium on levan production , two types of media were used , date extract medium and banana extract medium compared with levan production medium at 10% sucrose , each of the medium was adjusted to the best pH and the cultivation culture media were incubated at the best temperature for the best incubation time , inoculum size , aeration . The precipitation was done by using ice - cold ethanol . The levan dry weight , cell dry weight were estimated .

RESULTS AND DISCUSSION:

Screening of *Leuconostoc mesenteroides* for levan production

Mucoidy method

All 30 isolates of *L. mesenteroides* were tested for levan production . The detection and screening of levan production were recorded according to slimy mucoid colonies on the surface of levan screening medium . Results showed in the table (1) that only 11 isolates were slimy mucoid colonies , 9 isolates from 11 isolates were produce strong slimy mucoid colonies , and 2 isolates from 11 isolates were produce moderate slimy mucoid colonies figure (1) .

Table (1) : Screening for levan production by *Leuconostoc mesenteroides* isolates

Bacterial isolates	Mucoidy and ropiness
Leuconostoc mesenteroides ssp. *dextranicum* (LB 1)	–
Leuconostoc mesenteroides ssp. *cremoris* (LB 2)	–
Leuconostoc mesenteroides ssp. *cremoris* (LB 3)	–
Leuconostoc mesenteroides ssp. *cremoris* (LC 1)	–
Leuconostoc mesenteroides ssp. *cremoris* (LC 2)	–
Leuconostoc mesenteroides ssp. *cremoris* (LC 3)	–
Leuconostoc mesenteroides ssp. *dextranicum* (LF 1)	++++
Leuconostoc mesenteroides ssp. *mesenteroides* (LF 2)	+++
Leuconostoc mesenteroides ssp. *dextranicum* (LF 3)	–
Leuconostoc mesenteroides ssp. *mesenteroides* (LF 4)	++++
Leuconostoc mesenteroides ssp. *cremoris* (LF 5)	++++
Leuconostoc mesenteroides ssp. *cremoris* (LF 6)	–
Leuconostoc mesenteroides ssp. *cremoris* (LF 7)	–
Leuconostoc mesenteroides ssp. *cremoris* (LF 8)	–
Leuconostoc mesenteroides ssp. *cremoris* (LF 9)	+++
Leuconostoc mesenteroides ssp. *cremoris* (LF 10)	++++
Leuconostoc mesenteroides ssp. *cremoris* (LS1)	++++
Leuconostoc mesenteroides ssp. *cremoris* (LM 1)	–
Leuconostoc mesenteroides ssp. *cremoris* (LM 2)	–
Leuconostoc mesenteroides ssp. *cremoris* (LM 3)	++++
Leuconostoc mesenteroides ssp. *cremoris* (LM 4)	–
Leuconostoc mesenteroides ssp. *cremoris* (LM 5)	++++
Leuconostoc mesenteroides ssp. *cremoris* (LM 6)	–
Leuconostoc mesenteroides ssp. *cremoris* (LM 7)	–
Leuconostoc mesenteroides ssp. *cremoris* (LM 8)	++++
Leuconostoc mesenteroides ssp. *cremoris* (LM 9)	–
Leuconostoc mesenteroides ssp. *cremoris* (LM 10)	–
Leuconostoc mesenteroides ssp. *dextranicum* (LM 11)	–
Leuconostoc mesenteroides ssp. *dextranicum* (LM 12)	–
Leuconostoc mesenteroides ssp. *mesenteroides* (LM 13)	++++

++++ : high production of levan ; +++ : moderate production of levan ; - : no production of levan ; LB: isolated from banana ; LF: isolated from fish intestine; LM: isolated from raw milk ; LC: isolated from carrot; LS :isolated from sauerkraut

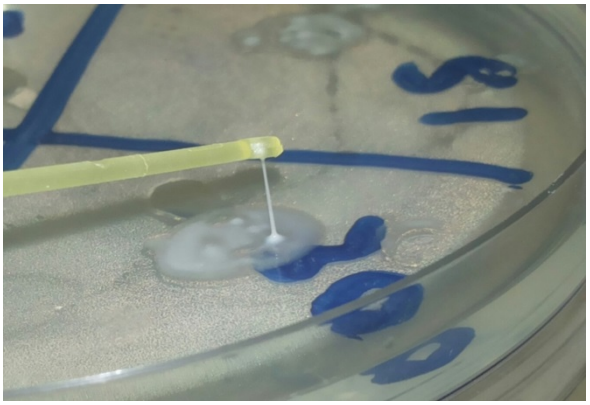

Figure (1): Slimy mucoid colonies of *Leuconostoc mesenteroides* isolates cultured on levan screening medium .

The presence of EPS associated with bacterial cells can be recognized by the formation of mucous colonies in solid medium [21] . Paulo *et al.* [22] reported that the presence of EPS produced by bacterial cells contributes to slime colonies formation in solid medium and increased viscosity in liquid medium . The production of exopolysaccharides in lactic acid bacteria recognized by detection the slime and mucoid of the colonies [23] . Han *et al*. [13] isolated levan producing strain a nd identified later as *Leuconostoc citreum* , and observed that there is a sticky string when the colony was picked up with a loop . Mamay *et al.* [24] reported that the existence of levan was detected by the slimy appearance on solid media .

Spectrophotometric method

After selecting the *L. mesenteroides* isolates that gave high productivity of levan , levan concentration was determined by using spectrophotometric method , results showed that the bacterial isolate that gave high concentration of levan was *L. mesenteroides* ssp. *cremoris* (LF_5) with concentration about 9.234 mg /ml , while the other isolates range from (1.683 - 8.869) mg / ml (Table 2) .

Table (2): Concentration of levan produced by *Leuconostoc mesenteroides*

Bacterial isolates	O.D at (400nm)	Levan concentration (mg /ml)
Leuconostoc mesenteroides ssp. *dextranicum* (LF_1)	0.500	2.826
Leuconostoc mesenteroides ssp. *mesenteroides* (LF_2)	0.312	1.683
Leuconostoc mesenteroides ssp. *mesenteroides* (LF_4)	1.406	8.352
Leuconostoc mesenteroides ssp. *cremoris* (LF_5)	1.554	9.234
Leuconostoc mesenteroides ssp. *cremoris* (LF_9)	0.784	4.553
Leuconostoc mesenteroides ssp. *cremoris* (LF_{10})	1.494	8.869
Leuconostoc mesenteroides ssp. *cremoris* (LFC_1)	0.564	3.215
Leuconostoc mesenteroides ssp. *cremoris* (LM_3)	1.454	8.626
Leuconostoc mesenteroides ssp. *cremoris* (LM_5)	1.484	8.808
Leuconostoc mesenteroides ssp. *cremoris* (LM_8)	0.476	2.680
Leuconostoc mesenteroides ssp. *mesenteroides* (LM_{13})	1.464	8.686

LF :isolated from fish intestine ; LC: isolated from carrot ; LM: isolated from raw milk

Precipitation of levan

Precipitation of levan from *L. mesenteroides* spp. *cremoris* (LF_5) was done by using different types of non polar organic solvents . Results showed that the precipitation of levan by ethanol , acetone , methanol , diethyl ether , isopropanol , chloroform , toluene were recorded as levan dry weight 1.482 , 1.477 , 1.350 1.252 ,1.479 , 1.239 , 1.261 with levan production yield 7.4% ,7.3 % , 6.7 % , 6.2 % ,7.3 % , 6.1 % , 6.3% respectively (Table 3) . The ethanol , acetone and isopropanol solvents gave close results . Choudhury *et al.* [25] studied several organic solvent to examined their efficacy on pullulan precipitation from fermentation broth , and found that ethanol gave the higher yield of polymer from fermentation broth compared to other organic solvent and also reported that diethyl ether and chloroform did not result in significant precipitation of the polymer . Yang *et al.* [26] and Bajpail *et al.*]27] used ethanol to precipitate EPS from various sources and showed better results than other organic solvents .

Table (3) : Precipitation of levan from *L. mesenteroides* ssp. *cremoris* (LF_5) using different types of solvents

Solvent	Levan dry weight (g/100ml)	Levan production Yield (%)	Cell dry weight (g/100 ml)
Ethanol	1.482	7.4 %	2.256
Acetone	1.477	7.3 %	2.249
Methanol	1.350	6.7 %	2.200
Diethyl ether	1.252	6.2 %	2.240
Isopropanol	1.479	7.3 %	2.247
Chloroform	1.239	6.1 %	2.169
Toluene	1.261	6.3 %	2.150

Determination of optimum condition for levan production by *L . mesenteroides* ssp. *cremoris*

Effect of temperature

The selected isolate *L. mesenteroides* ssp. *cremoris* (LF_5) was incubated at different temperatures (25 , 30 , 35) ^oC for determination the optimum temperature of levan production . After incubation , the dry weight (g/100 ml) of levan , levan production yield (%) and cell dry weight (g /100ml) were recorded for each temperature . Results showed that the optimum temperature for levan production was at 30 ^oC which in this temperature the levan dry weight was 1.482 g /100 ml with a yield 7.4 % and cell dry weight 1.781 g/100 ml , and at 25^oC the levan dry weight was 1.101 g/100 ml with a yield 5.5 % and cell dry weight 2.256 g /100 ml , while at 35 ^oC the levan dry weight was 1.223 g /100 ml with a yield 6.1 % and cell dry weight 1.901 g/100 ml (Figure 2) .

Kang *et al.* [28] studied expression and cloning of levansucrase from *L.mesenteroides* in *Escherichia coli* , and found that the optimum temperature and of this enzyme for levan formation were 30 °C . High fermentation temperatures (30°C to 42°C) result in inhibition the production of levansucrase which responsible for levan formation by *Zymomonas mobilis* [29] . Santos *et al.* [30] and Lorenzetti *et al.* [31] used 30 ^oC for production biopolymer levan from *Z. mobilis* . Benhadria *et al* . [32] reported that the optimal temperature for EPS synthesis was 30°C for the strains *Pediococus damnosus* and *Lactobacillus rhamnosus*.

Effect of incubation time

After optimum temperature for levan production . The effect of incubation time was studied by incubated the bacterial isolate *L. mesenteroides* ssp. *cremoris* (LF_5) in levan production medium with a different incubation times (24 ,48 ,72) h . Results showed that the optimum incubation time for levan production was 24 h , which at this time the levan dry weight was 1.482 g/100 ml with a yield 7.4 % and cell dry weight 2.256 g /100 ml , while at 48 h the levan dry weight was 1.320 g / 100 ml with a yield 6.6 % and cell dry weight 2.215 g /100 ml , at 72 h the levan dry weight was 1.101 g /100 ml with a yield 5.5 % and cell dry weight 2.201 g / 100 ml (Figure 3) . The maximum levan production was reported for 24 at 30°C [29] . Dos Santos *et al.* [33] studied the characterization and optimization of levan production by *Bacillus subtilis* , and found that the best incubation time started from 16 h . Mamay *et al.* [24] reported that the best production of levan from *B. licheniformis* when the culture medium containing sucrose was incubated for 24 h .

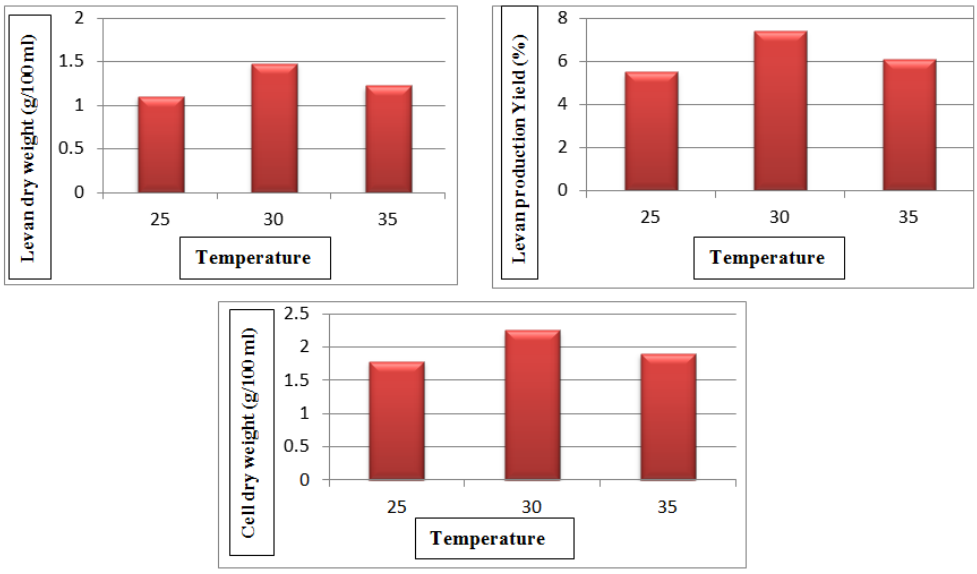

Figure (2):Effect of temperature on levan production by *Leuconostoc mesenteroides* ssp. *cremoris* (LF_5)

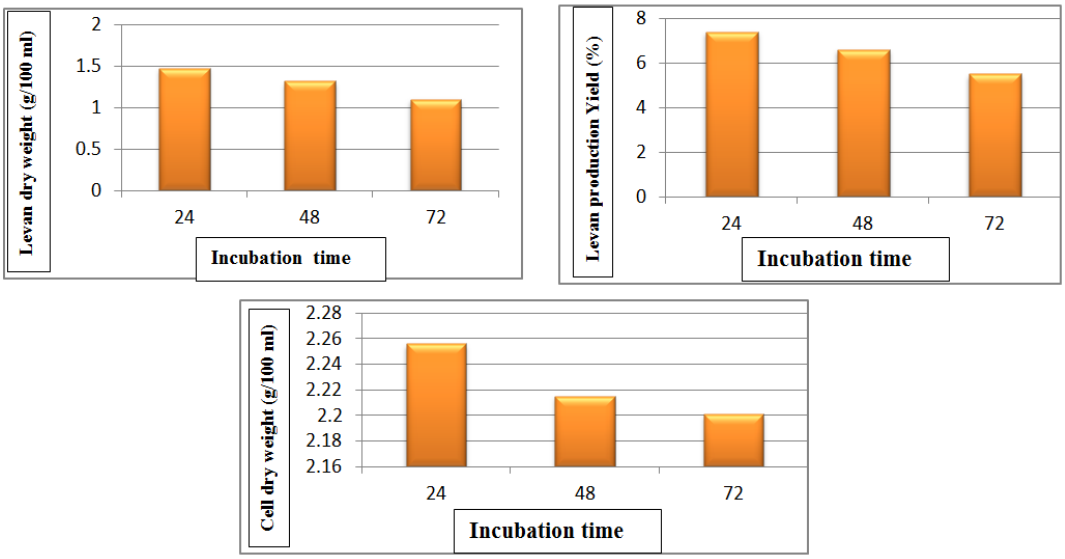

Figure (3) : Effect of incubation time on the levan production by *Leuconostoc mesenteroides* spp. *cremoris* (LF_5)

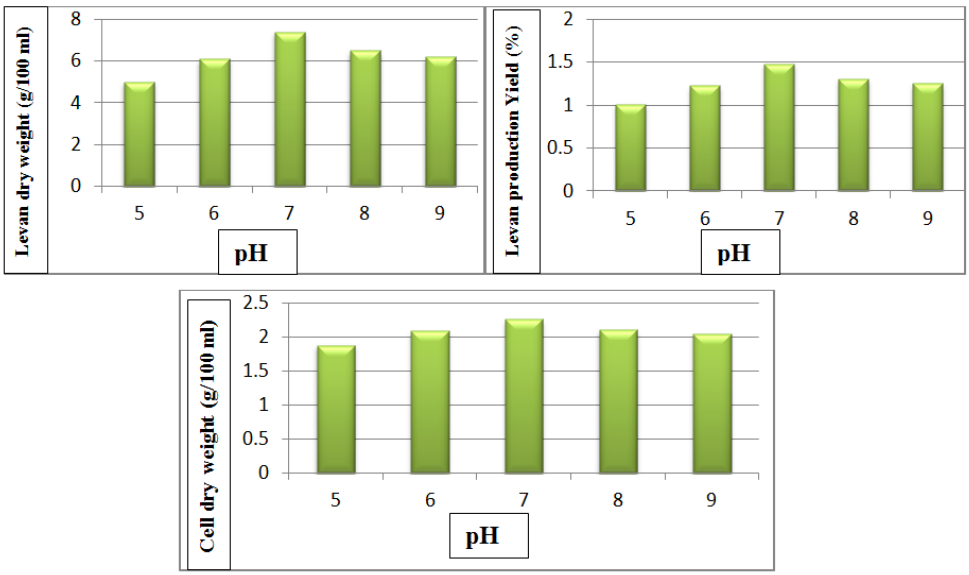

Figure (4) : Effect of pH on the levan production by *Leuconostoc mesenteroides* spp. *cremoris* (LF_5)

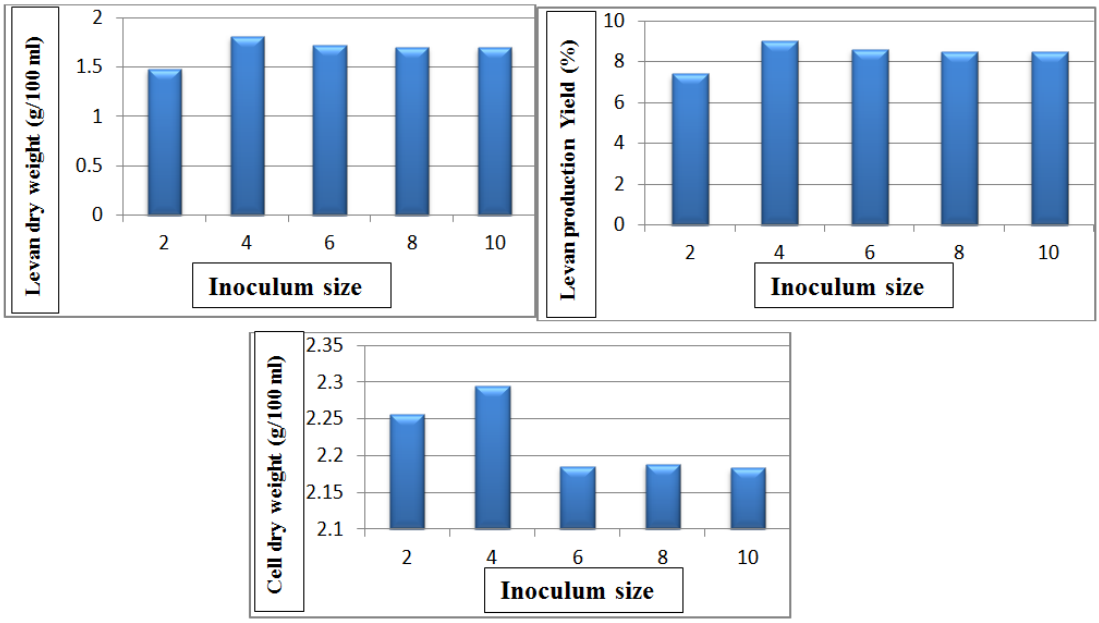

Figure (5) : Effect of inoculum size on the levan production by *Leuconostoc mesenteroides* spp. *cremoris* (LF_5)

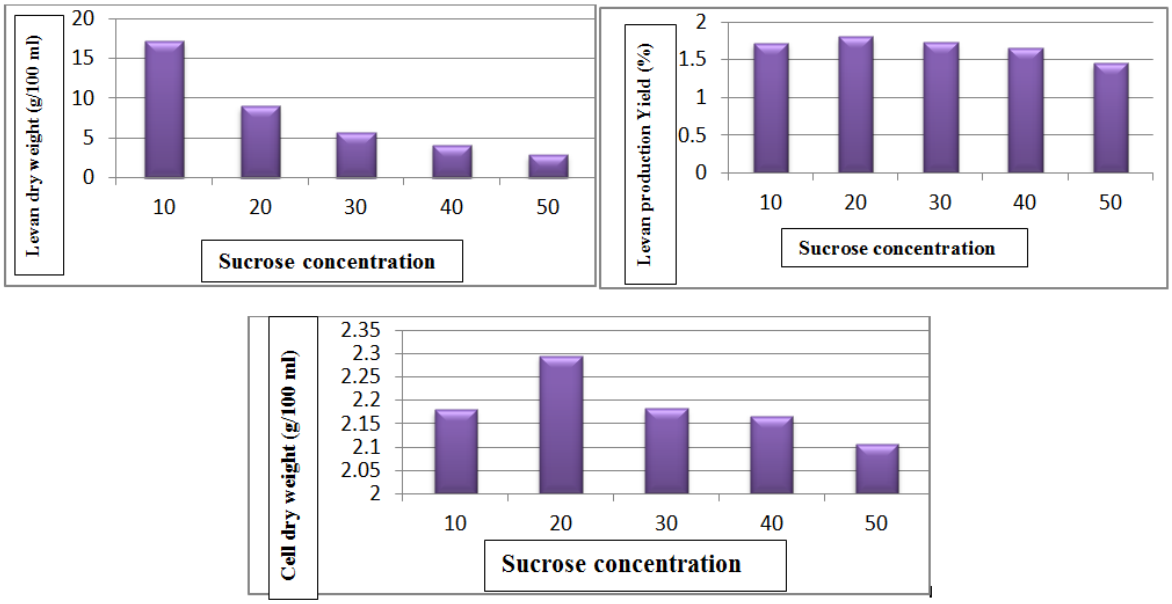

Figure (6) : Effect of sucrose concentration on the levan production by *Leuconostoc mesenteroides* spp. *cremoris* (LF_5)

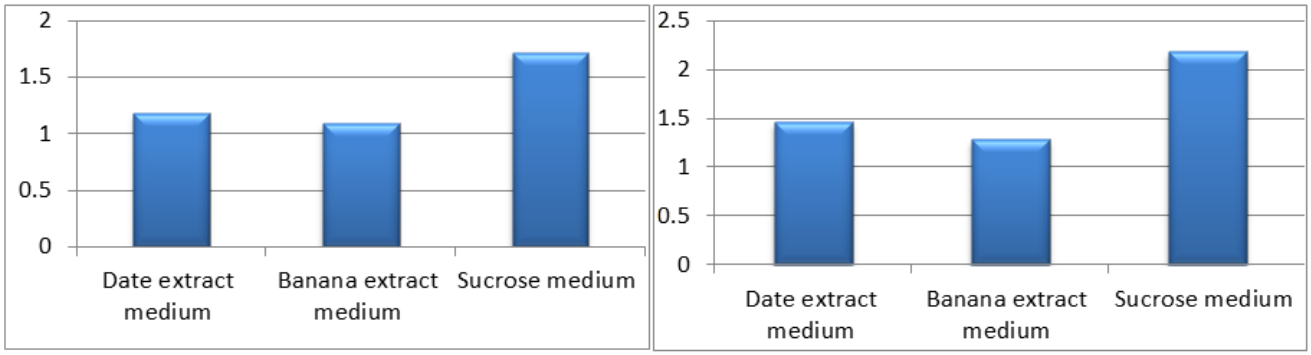

Figure (7) : Effect of culture medium on the levan production by*Leuconostoc mesenteroides* spp. *cremoris* (LF_5)

Effect of initial pH

After incubation time selection , bacterial isolate *L. mesenteroides* ssp. *cremoris* (LF_5) was incubated with varies pH numbers (5 ,6 ,7, 8 , 9) for optimum pH selection to levan production . Results showed that the optimum pH for levan production was at 7 which in this number the levan dry weight was 1.482 g /100 ml with a yield 7.4 % and cell dry weight 2.256 g /100 ml , while at pH (5) was 1.010 g / 100ml with a yield 5 % and cell dry weight 1.876 g /100 ml , at pH (6) was 1.228 g / 100ml with a yield 6.1 % and cell dry weight 2.090 g /100 ml , in pH (8) was 1.301 g /100 ml with a yield 6.5 % and cell dry weight 2.104 g/100 ml , and at pH (9) was 1.250 g /100 ml with a yield 6.2 % and cell dry weight 2.050 g /100 ml (Figure 4) . pH was considered as an important factor for the polysaccharide production , since high or very low values may repress levansucrase , the enzyme responsible for the biopolymer formation [29] . Senthil and Gunasekaran [34] reported that no levan production at pH 4 , Dahech *et al*. [35] mentioned that less levan production occurred at acid pH and also reported that the optimum pH for levan production lies within 7 and 8 . Abou- taleb *et al* . [20] found that cell dry weight and levan production gradually increased as the pH values increased from 5.5 to 6.5 and also mentioned that the levan production was gradually decreased as the pH values increased from 7.0 to 8.5 .

Effect of inoculum size

The effect of inoculum size on levan production was studied . *L. mesenteroides ssp. cremoris* (LF_5) was incubated with various inoculum size (2 , 4 , 6 , 8 , 10) % (9×10^8 cell\ ml) . Results showed that the best inoculum size for levan production was at 4% , which the dry weight of levan was 1.810 g / 100ml with yield 9 % and cell dry weight 2.295 g /100 ml , where in 2 % the dry weight was 1.482 g / 100ml with yield 7.4 % and cell dry weight 2.256 g /100 ml , while in 6 % it was 1.721 g /100 ml with yield 8.6 % and cell dry weight 2.185 g /100 ml , at 8% it was 1.700 g /100ml with yield 8.5 % and cell dry weight 2.188 g/100 ml , and in 10% the dry weight was 1.704 g /100 ml with yield 8.5 % and cell dry weight 2.183 g /100 ml figure (5) .

Hamedi *et al*. [36] found that the optimal inoculum size for exopolysaccharide production by *Agaricus blazei* was in inoculum size 3% . Onilude *et al*. [37] mentioned that there was a gradual increase in dextran production as the inoculum size increase from 2% to 6% , and then gradual decrease when reach inoculum size 8 % and 10 % . Yang *et al*. [26] successfully extracted polysaccharides from *Cordyceps militaris* at inoculum size 4 % . Salim , [38] reported that the best inoculum size for dextran production from *L. mesenteroides* spp. *mesenteroides* was4 % . Demirci *et al*. [39] showed that increase 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% .

Effect of sucrose concentration

After optimum inoculum size selection , bacterial isolate *L. mesenteroides* ssp. *cremoris* (LF_5) was inoculated at varies sucrose concentration (10 , 20 , 30 , 40 , 50) % . Results showed that the best sucrose concentration for levan production was at 10% , which the dry weight of levan was 1.720 g /100 ml with yield 17.2 % and cell dry weight 2.180 g/100ml , where in 20% the dry weight was 1.810 g /100 ml with yield 9 % and cell dry weight 2.295 g/100 ml , while in 30 % it was 1.733 g /100 ml with yield 5.7 % and cell dry weight 2.183 g /100 ml , at 40 % it was 1.661 g /100ml with yield 4.1 % and cell dry weight 2.166 g /100 ml and in 50% the dry weight was 1.451 g /100 ml with yield 2.9 % and cell dry weight 2.107 g /100 ml (Figure 6) .

Sucrose concentration has been identified as the most effective factor that controlling the molecular weight of the levan [40] . Santos *et al*. [30] mentioned that the levan yield was a highest in sucrose concentration 15% compared with the other sucrose concentration (20 , 25 , 30) % . The molecular weight of levan decreased with increased sucrose concentration up to 40 % [41] . González –Garcinuño *et al*. [18] reported that levan production decreases by increasing sucrose concentration , When the sucrose concentration was increased above 300 g /L , levan production decreased .

Effect of culture medium

The effect of culture medium on levan production was studied . *L. mesenteroides* ssp. *cremoris* (LF_5) was incubated at two different medium date extract medium and banana extract medium compared with levan production medium (sucrose medium) . Results showed that the best culture medium for levan production was in levan production medium , which the levan dry weight 1.720 g /100 ml and cell dry weight 2.180 g/100 ml , where in banana extract medium the levan dry weight 1.090 g/ 100 ml and cell dry weight 1.280 g/ 100 ml , while in date extract medium , the levan dry weight 1.180 g/100 ml and cell dry weight 1.460 g /100 ml (Figure 7) .

The low levan values obtained when date extract was used possibly due to the presence of combination of several sugars in date extract (sucrose , glucose and fructose) that inhibited the cell growth and metabolites production , dates containing different chemical components such as sugars , proteins and minerals , the excess of these minerals may affect levan production causing inhibition in levan formation and produce a poor quality product [42 , 43] . Ghnimi *et al*. [44] reported that the fructose content in Zahidi date was 35.9 g /100 ml followed by glucose 30.1 g /100 ml then sucrose 11.6 g/100 ml .

CONCLUSION:

In conclusion , the locally isolates of *Leuconostoc mesensteroides* had ability to produce levan polymer at different conditions and the best production was at 30 ºC for 24 h at pH 7 with 4 % inoculum size and 10 % sucrose concentration and the best culture medium for levan production was sucrose medium .

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