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Broad-spectrum inhibition for biofilm producers in dental decay by dextrnase purified from Lactobacillus fermentum

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Abstract

The irreversible solubilization of tooth mineral by acid produced by particular bacteria adhering to the tooth surface in bacterial populations known as dental plaque causes dental decay. When cultivated on dextran as a substrate, a Lactobacillus fermentum bacterial strain isolated from dental decay can create extracellular dextranase. Using ammonium sulfate at 65 percent saturation,followed by ion exchange chromatography on DEAE-Cellulose column and gel filtration chromatography on sephadex G-100 column, dextranase was purified to homogeneity with a recovery yield of 39.40 percent and 3.43 fold purification. By swabbing over the gingival and subgingival region of dental decay, Gram positive bacteria such as Staphylococcus aureus, Streptococcus mitis, Streptococcus mutans, and Streptococcus oralis were isolated, and all of these isolates had varying amounts of biofilm development. All Gram-positive biofilm-forming bacteria (biofilm producers) were inhibited by purified dextrnase, with greater inhibition percentages of 69.09 percent and 65.5 percent against Streptococcus mutans and Streptococcus oralis, respectively. While the dextranase has a lesser inhibitory percentage of 30.7 percent and 33.2 percent against Streptococcus mitis and Staphylococcus aureus, respectively, it has a promising application for eliminating and preventing biofilm development, suggesting its prospective use in the dental industry.

Key words: Lactobacillus fermentum, dextranase, dental decay

Introduction

The breakdown of tooth mineral by acids produced from bacterial fermentation of sucrose and other dietary carbohydrates causes dental decay (1). These bacteria exist in dental plaque, which is a bacterial community that forms on the tooth surface. A cavitation on the tooth surface is a clinical indicator of dental deterioration (2). Cavitation, on the other hand, is a late event in the pathophysiology of decay, following a clinically apparent subsurface lesion known as a white spot and subsurface demineralization that can only be seen microscopically (3). Many factors contribute to dental caries, including host factors (teeth and saliva), dietary items, microbes, and time. Streptococcus and Lactobacillus are two well-known dental caries germs that are the major pathogens that cause tooth caries and deterioration. The presence of sucrose causes them to create insoluble glucans, which form an oral biofilm, often known as dental plaque, which starts the caries process (2).

Endohydrolysis of dextrans-1,6-linkages to oligosaccharides is catalyzed by dextranases (-1,6-D-glucan-6-glucanohydrolase; EC3.2.1.11) (4,5), so that it produces low-molecular-weight polysaccharides that have many applications in different feilds.

Dextranase is a protein that is widely employed in the in medicine, as well as sugar and food industries (1). In dentistry, the prospect of dextranases destroying the oral biofilm (commonly known as dental plaque) has drawn researchers' attention to the use of these enzymes to cure dental plaques (2,6).

Therefore thismain aims for this research were to isolationLactobacillus sp. producing dextranase, purification of dextranase and investigation of the its action as antibiofilm agent against biofilm producers in dental decay.

Materials and Methods

Samples collection

Forty five bacterial samples were collected fromoral cavity by swabbing cross the gingival and subgingival region of dental decay. These samples were suspended in Lactobacillus MRS broth and incubated ,anaerobically at 37^{o}C for 6h. Suspensions were made from each tube in1 ml of saline solution and were diluted 10-fold in the same medium. 100 µlof each dilution were spread on surface of plates containing solidified LactobacillusMRS agar, then plates were incubated,anaerobically ,for 48 h at 37^{o}C(7). These isolates were diagnostic by growing on Lactobacillus MRS agar, microscopic examination and biochemical tests. The phenotypic identification was boostedwithVitek-2 system (Bio-Merieux, France).

Screening of Lactobacillus spp. for dextranase production

All the bacterial isolates were inoculated in to the dextranase agar plates(pH5.5) containing 1% dextran T2000, 0.3% NaNo3, 0.05% KCl, 0.1% K2HPO4.3H2O, 0.05% MgSO4.7H2O, 0.001% FeSO4.7H2O, and 1.5% agar powder. The positive dextranase producing isolates after 48 hrs of incubation at 37°C anaerobically produced clear zone against the turbid surface(4), after that thediameter of the transparent area around the bacterial colonies was determined.

Extraction and secondary screening

The selected bacteria isolates were transferred into 10 ml of a medium contained the same dextranase agar contents except the agar and pH was adjusted to 5.5. The bacteria isolates were incubated anaerobically for 48 h at 37^{o}C. After centrifugation at 10000xg for 20 min the pellet of cells was removed and dextranase activity, protein content and specific activity were assayed for resulted supernatant.

Protein concentration and dextranase assays

Proteins concentration was measured by Bradford(8).dextranase activity was tests withmixing25µl enzyme solution with 25µl of 1%(w/v) dexan in 0.1M citratebuffer pH 5.0 and the incubation at 37ºC for 30 min. After that 50µl of Somogyi copper reagent was added then incubated for 5 min in water bath with80^{o}C. then cand 50 µl of arsenomolybdate reagent was added. Colorimetric measurements were performed at 500 nm(5). The gradient concentrations of maltosewere prepared by doing the standard curve. One unit of dextranase activity was defined as the amount of enzyme, which generated 1µ mole of maltose under the above conditions.

Purification of dextranase

Culture supernatant that was obtained by centrifugation of selected isolate subjected to fractionation by ammonium sulphate precipitation at 20-80% saturations. The precipitate was collected by centrifugation and suspended with 10 mM potassium phosphate buffer, pH 7.0) then the dialysis with potassium phosphate buffer for overnight. The dialyzed protein was loaded on a DEAE-Cellulosecolumn(2cm diameterx25cm long) and eluted with gradient of 0.2-0.6 M NaCl prepared in potassium phosphate buffer with flow rate of 0.6ml/min. The pooled and concentrated fractions that revealed the highest level of activity were loaded onsephadex G-100 column (2x80 cm). The column was eluted with same buffer with0.5 ml/min as flow rate. The concentration of protein at 280 nm and dextranase were determinedafter that the fractions with maximium activity were stored for further uses..

Isolation and quantitative screening of biofilm activities

The same bacterial samples were directly streaked on nutrient agar and incubated for 18-24h at 37C.The grown isolates identified by morphological and biochemical tests besides Vitek-2 system. These bacterial isolates were checked for formation of biofilm by microtiter biofilm assay as follow:

The isolates with Overnight culture in nutrient agar plate was transfered to Trypticase soy broth (TSB) and compared with (0.5) MacFarland standards. The primary inoculums were then inoculated in TSB with (1%) glucose into (96) wells flat bottom microtiter polystyrene plate (triplicate for each isolate). Plates were covered and incubated at (37ºC) for (24) hr. in aerobic conditions and anaerobically, the well is then decanted and washed three times with normal saline. After washing, fixed with methanol for (15)min. Subsequently, the wells were decanted and crystal violet stain(0.5%) was added for (20) min. The wells were again decanted and washed with D.W. Finally addition of absolute ethanol to the wells for extracting the stain. The absorbency of each well was readwith(450) nm in ELISA reader. Classification based on OD values obtained for the tested isolateswas used for the purpose of data simplification and calculation. The isolates which developed strong biofilm were chosen for further experiments (9).

Weak biofilm =<0.120

Moderate biofilm = 0.120-0.320

Strong biofilm = >0.320

Evaluation the effect of dextranase on biofilm formation

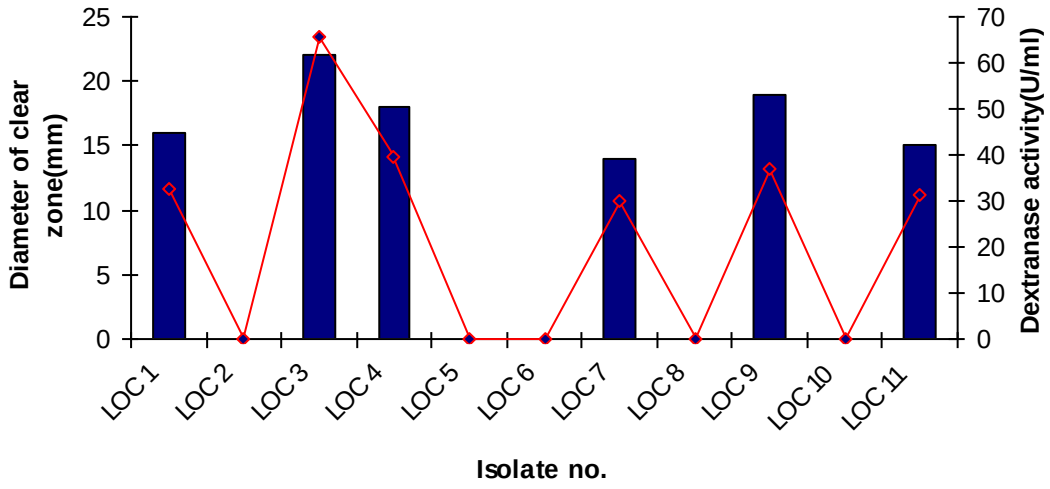
In order to evaluation of the dextranase activityagainst biofilm formation in microtiter plate method, 100µl of the purified dextranase in10 mM potassium phosphate buffer was mixed with 100 µl of chosen bacterial suspension, followed by incubation at 37C for 24h. The negative control included 100 µl of 10 mM potassium phosphate buffer instead of purified enzyme. After incubation period, the biofilm activity was repeated as mentioned previously.The percentage of biofilm formation for each pathogenic bacteria was measuredaccording to the(10):

Percentage of biofilm inhibition(%)=[O.D control-O.D treatment]/O.D control x100

Results and discussion

Detection dextranase production

ThirteenLactobacillus fermentum were collected out of 45 samples of oral cavity and these thirteenLactobacillus fermentumisolates initially subjected to rapid screening for extracellular dextranase production using dextran agar plates by formation a transparent area around the bacterial colonies.The results revealed that six isolates were found to be positive for dextranase activity by their growth on this medium and Lactobacillus fermentumOC_3revealed a high scale of dextrnase productivity and the level of activity reached to 22 mm(figure-1). In a research reported by (6) found that Aggrgatbacteractinomycetemcomitans, Porphyromonas gingivalis and several other periodontopathogens isolated from subgingival plaque sample by DNA microbial probe testing method. In contrast, several bacterial isolates belonged to the genus of Actinomyces. and Bacteroides produced extracellulardextran-degrading enzymes(11).



Figure(1): Primary and secondary levels of dexranase productivity for Lactobacillus fermentumisolates

Extraction and secondary screening

For selection the best dextranase producer, all six of Lactobacillus fermentumpositive isolates were subjected to secondary screening in liquid culture and it was found that Lactobacillus fermentumOC_3 produced the highest level of dextranase with activity (65.8)U/ml so that this isolate was selected for further studies as reported in figure(1).

A wild-type lactobacillus spp. strain were tested for productivity of anti-bacterial secondary biometabolites such as dextranase in submerged fermentation(12).In 2018(13)evaluated that the marine bacterium Catenovulum sp. showed an ability of produce of dextranase enzyme, also demonstrated(14) that the gene responsible on dextranase production was detected in Streptococcus sobrinus.

Purification of levanase

The extracted dextranase in the form of crude enzyme solution fromLactobacillus fermentumOC_3subjected to precipitation with using 20-80% saturation of ammonium sulfate.The results reported that 65% saturation was the best in dextranase precipitation with increasing the specific activity to 48.26U/mg. The dialysis step was aimed to throw the remaining impurities. The produced solution was loaded to a DEAE-Cellulose column. The elution with 0.2 to 0.6 MNaCl gradient led to observe three protein peaks of proteins and the dextraase activity was located in the second protein peak with specific activity to 71.72 U/mg(figure-2). The active fractions provided to sephadex G-100 columnand after the elution one protein peak appeared with dextranase specific activity 137.5 U/mg protein, 39.40% a yield and 3.34 fold of purification (figure-3) and as shown in table(1).

Column chromatography on DEAE-cellulose and Sephadex G-100 were used after the fractionation with ammonium sulfate for purification of dextranase from Catenovulum sp. with 29.6-fold and a specific activity of 23.09 U/mg protein(12). In contrast, using of ammonium sulphate precipitation, ion exchange with DEAE column and a Bio-Gel P-200 column in gel filtration used for purification of dextrnase produced by Streptococcus sobrinus(13).Another research reported by(14) found that the thermostable dextranase was purified from Talaromyces pinophilus by fractionation with ammonium sulfatefollowed by Sepharose 6B chromatography with 11.27% recovery.

Table-1: Serial steps of dextranase Purification from Lactobacillus fermentumOC_3

Purification step	Size(ml)	Dextranase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/ mg)	Total activity	Purification fold	Yield (%)
Crude extract	35	65.8	1.6	41.12	2303	1	100
$(NH_4)_2SO_4$ precipitation	16	72.4	1.5	48.26	1158.5	1.17	50.30
DEAE-Cellulose	12	78.9	1.1	71.72	946.8	1.74	41.11

Sephadex G-100	11	82.5	0.6	137.5	907.5	3.34	39.40

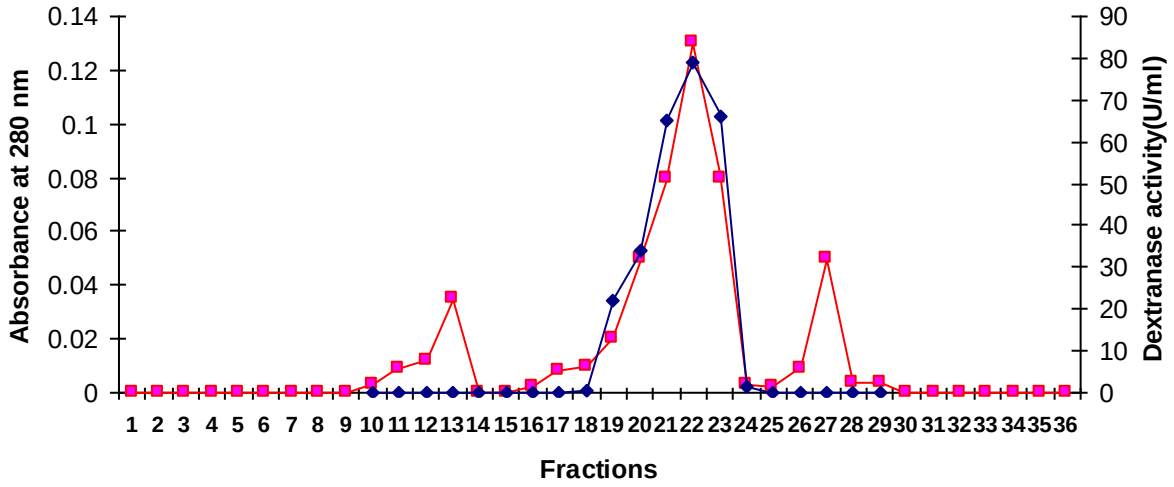


Figure (2): Dextranase Purification from Lactobacillus fermentumOC$_3$ by using ion exchange chromatography on DEAE-Cellulosecolumn

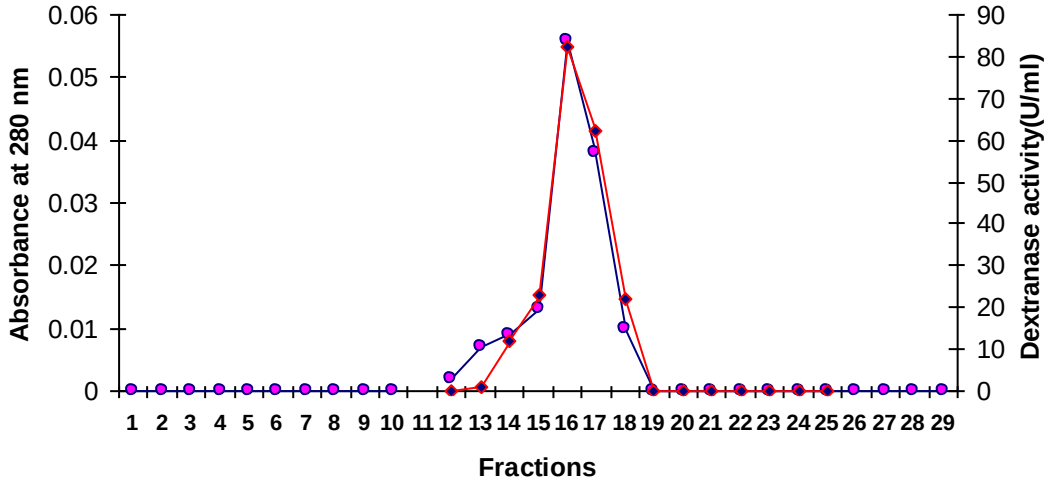


Figure (3): Dextrnase Purification from Lactobacillus fermentumOC$_3$ by using gel filtration chromatography on Sephadex G-100column

Isolation and quantitative screening of biofilm activities

The results showed ,after culturing of bacterial samples on nutrient agar , biochemical tests and the diagnostic by using Vitek-2 system, that Gram positive bacteria included 5 isolates ofStaphylococcus aureus, 4 isolates of Streptococcus

mitis, 2 isolates of Streptococcus mutans and 3 isolates of Streptococcus oralis were appeared by swabbing cross the gingival and subgingival region of dental decay..

The formation of biofilm by these pathogenic isolates were tested using microtiter plate method. Biofilm formation was found in all tested bacteria, only 3 isolates of Streptococcus mitis , 2 isolates of Streptococcus mutans and 2isolate of Staphylococcus aureus were strong biofilm formation. While 3 isolates of Streptococcus oralis, 1 isolates of Streptococcus mitis , 1isolate of Staphylococcus aureus were moderate biofilm formation and2isolate of Staphylococcus aureus produced it weakly as revealed in table(2).

Table(2): Biofilm formation by Gram positive bacteria isolated from dental decay

Bacterial isolate	Absorbency at 450nm
Streptococcus mutansOC_1	0.75
Streptococcus mutansOC_2	0.84
Staphylococcus aureus OC_1	0.38
Staphylococcus aureus OC_2	0.46
Staphylococcus aureus OC_3	0.28
Staphylococcus aureus OC_4	0.10
Staphylococcus aureus OC_5	0.68
Streptococcus mitisOC_1	0.62
Streptococcus mitisOC_2	0.71
Streptococcus mitisOC_3	0.78
Streptococcus mitisOC_4	0.22
Streptococcus oralis OC_1	0.21
Streptococcus oralis OC_2	0.23
Streptococcus oralis OC_3	0.17

According to the results there were 7(50%) isolates had strong biofilm, 5 isolates(35.7%)with moderate biofilm formation and only 2(15.8%) isolates were weak biofilm producers. Biofilm formation is a common bacterial survival way in hostile environments. Bacteria can build biofilms in a range of abiotic surfaces often seen in water systems, as well as in natural aquatic ecosystems (15,16). Differences in biofilm thickness could be attributed to a range of factors, including isolates' capacity to form a biofilm, primary number of adherent cells, and quality and quantity of quorum sensing signaling molecules (autoinducer) produced by each isolate (17- 25).

The effect of dextranase on biofilm production

The inhibitory effect of purified dextranase on biofilm formation by different genera from oral cavity was studied. the results revealed that higher inhibition percentage were against Streptococcus mutans and Streptococcus oralis with 69.09% and 65.5%,respectively, While lower inhibition percentage were against Streptococcus mitis and Staphylococcus aureus with30.7% and 33.2%, respectively as shown in table(3).

Table(2): Inhibition percentage of biofilm formation by dextranase purified from dental decay

Bacterial isolate	Inhibition of biofilm formation(%)
Streptococcus mutansOC_1	64.33
Streptococcus mutansOC_2	69.09
Staphylococcus aureus OC_1	52.7
Staphylococcus aureus OC_2	33.2
Staphylococcus aureus OC_3	53
Staphylococcus aureus OC_4	36.2
Staphylococcus aureus OC_5	45.8
Streptococcus mitisOC_1	41.1
Streptococcus mitisOC_2	36.3
Streptococcus mitisOC_3	30.7
Streptococcus mitisOC_4	35
Streptococcus oralis OC_1	62.8
Streptococcus oralis OC_2	65.5
Streptococcus oralis OC_3	60.1

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