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ORIGINAL ARTICLE

The inhibitory effect of dextranases from *Bacillus velezensis* and *Pseudomonas stutzeri* on *Streptococcus mutans* biofilm

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ABSTRACT

Background and Objectives: Dental caries is a breakdown of the teeth enamel due to harmful bacteria, lack of oral hygiene, and sugar consumption. The acid-producing bacterium *Streptococcus mutans* is the leading cause of dental caries. Dextranase is an enzyme that can degrade dextran to low molecular weight fractions, which have many therapeutic and industrial applications. The purpose of the present study was to isolate a novel dextranase-producing bacteria from a source (molasses). The cell-free extracts containing dextranases were tested as antibiofilm agents.

Materials and Methods: Dextranase-producing bacteria were identified using phenotypic and genotypic methods such as 16S rRNA gene sequencing and enzymatic characterization.

Results: The highest six dextranase-producing bacterial isolates were *Bacillus* species. The best conditions for dextranase productivity were obtained after 72 hours of culture time at pH 7. The addition of glucose to the medium enhanced the production of the enzymes. The cell-free extract of the six most active isolates showed remarkable activity against biofilm formation by *Streptococcus mutans* ATCC 25175. The highest inhibition activities reached 60% and 80% for *Bacillus velezensis* and *Pseudomonas stutzeri*, respectively.

Conclusion: Therefore, our study added to the current dextranase-producing bacteria with potential as a source of dextranases.

Keywords: *Bacillus velezensis*; Biofilm; Dextranase; Molasses; *Streptococcus mutans*

INTRODUCTION

Dental caries or tooth decay is the breakdown of the teeth's enamel. The harmful bacteria found in the oral cavity play an essential role in the development of this decay. Several factors contribute to the increase in the rate of tooth decay, including lack of oral hygiene, sugar consumption, and smoking (1). Children are more exposed to tooth decay than adults, and previous studies have referred to an increase of its effect

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on people in the future due to the increased ingestion of sweeteners and insufficient contact with fluorides in some communities (2, 3). Sugars are the preferred substrate for the cariogenic microorganism that exist in dental plaque. *Streptococcus mutans* is among the leading players in dental caries, which is considered an acid producing bacterium; it participates through acid production in demineralizing the surface of enamel teeth and can progress in its crowns and roots (4, 5). Additionally, it produces polysaccharides that contribute to forming the oral biofilm matrix (5).

Several bacterial species contribute to the decay process, such as *Lactobacillus acidophilus, Lactobacillus fermentum*, and *Actinomyces viscosus* (6). Dextranases degrade dextran into simple sugars and can reduce the impact of dental caries and inhibit the formation of oral biofilms. Dextranases are glycosyl-hydrolases classified in the enzyme classification system as EC 3.2.1.11. The activity of dextranases is verified in microbial molasses and soil samples with high specificity to its substrate dextran (7). Dextranase is α-1,6 D-glucan-6-glucano hydrolases, also called glucanases, where they hydrolyze the α-1,6-linkages in dextran, beside the (α-1,2), (α-1,3) and (α-1,4) linkages that found in several dextrans, producing glucose units, isomaltose and isomalto-oligosaccharides (8).

The existence of a biofilm matrix decreases the permeation of anti-microbial agents and forms a barrier against antibiotics. Chemicals like fluoride products are active in removing dental plaque but are cytotoxic at relatively high concentrations (9). Therefore, finding new agents that can break down the biofilm is vital in treating dental caries, especially if it is a biologically-derived safe agent.

In this study, we isolated dextranase producing bacteria from sugar cane material; we tested their dextranolytic activity and evaluated the ability of their cell-free extract to inhibit the biofilm matrix produced by *S. mutans.* The current work characterized *Pseudomonas stutzeri* as a dextranolytic bacterium and provided characterization of biofilm inhibition generated *S. mutans.*

MATERIALS AND METHODS

Materials and culture media. Dextran was purchased from ACROS ORGANICS, New Jersey, USA. Sodium chloride, calcium chloride, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, phosphoric acid (85% w/v) and sodium hydroxide were purchased from El Nasr Pharmaceutical Chemical Co, Egypt. Glucose, iodine, crystal violet, 95% ethanol, sodium metabisulfite, phenol, sodium potassium tartrate, magnesium sulfate, acetic acid, and glycerol were obtained from Piochem for Laboratory Chemicals, Egypt. Yeast extract was obtained from LAB M, the UK; peptone was purchased from Condalab, Madrid, Spain; agar-agar was obtained from TM Media, India. Brain heart infusions were purchased from Himedia, India. The Coomassie brilliant blue G-250 and bovine serum albumin were from MP Biomedicals, LLC. DNS was obtained from Sigma Aldrich, US. Trypticase soy broth (TSB) was purchased from Himedia, India, and the nutrient broth was purchased from Oxoid Limited, Ireland.

Samples. Sixty-seven samples from sugar cane fields, sugar cane press facility as molasses, soil, juice, and traces were collected from Naga-Hammadi city, Qena governorate in Egypt, for use in the present study. The collected samples were stored at 4°C for further use in the isolation experiments (10). The sixty-seven samples were collected in sterilized 100 ml containers opened at the top for bacterial isolation. The containers were packed with 5 g of the collected samples, tightly closed, and covered.

Bacterial isolation, maintenance, and storage. For the selective isolation of bacteria producing dextranase, dextran was used as a substrate. Two media were used subsequently for isolation: The synthetic medium (Enrichment solution) as (w/v): 0.5% dextran, 0.1% K_2HPO_4 and 0.1% KH_2PO_4, pH 7.4. All components were sterilized by autoclaving at 121°C for 20 min. A 0.5 g was added aseptically added to 5 ml of the enrichment solution to isolate dextranolytic bacteria (11). After seven days of static incubation in ES at 37°C under aerobic conditions, the mixture was vortexed, and a sample was aseptically streaked on dextran agar plates. The plates contained the following as (w/v): 0.2% dextran, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.3% peptone and 0.3% yeast extract, pH 7.4 - 7.6. The inoculated plates were incubated at 37°C overnight; the growth and the dextranolytic activity were then observed, using Lugol's solution (Iodine reagent) to visualize the dextranolytic activity as a clear zone around the visible colonies on a dark brown background on the dextran agar plates. The detected

colonies were purified by several streaking until single pure colonies were obtained (11).

Gram staining test was done for each pure colony; the 67 isolates collected were filtered to the 6 highest active dextranolytic bacterial strains. The selected isolates were identified using a standard 16S rRNA analysis. For long-standing storage, all bacterial isolates were transferred to a 10 ml nutrient broth medium for overnight incubation; finally, aliquots were transferred to a sterile 1.5 ml Eppendorf tube. Glycerol was added to the stored bacterial samples in a final ratio of 20% v/v to be stored at -20°C for reuse (12).

S. mutans (ATCC 25175) was obtained from ATCC (American Type Culture Collection, USA) and used as a positive control in the biofilm inhibition experiment. *Lactobacillus reuteri* (DSM 20016) was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) to be used as a reference strain in the detection of enzyme activity experiment and enzymatic biofilm inhibition.

Identification of the dextranolytic bacterial isolates using 16S rRNA gene sequencing. DNA extraction was done according to the protocol provided by the Gene Jet genomic DNA purification kit (Thermo K0721, USA). The obtained DNA was subjected to PCR to amplify the 16S rRNA gene using Maxima Hot Start PCR Master Mix (Thermo K1051) as follows: gently vortex and briefly centrifuge Maxima® Hot Start PCR Master Mix (2X) after thawing, then add the following components in for each 50µl reaction at room temperature (Table 1).

PCR was done using Maxima Hot Start PCR Master Mix (Thermo K1051), Performing PCR using the recommended thermal cycling conditions outlined (Table 2).

The final PCR reaction tubes were cleaned using Gene JET™ PCR Purification Kit (Thermo K0701). Finally, the sequencing was performed on the PCR product on GATC Company using ABI 3730xl DNA sequencer using forward and reverse primers. The 16S rRNA gene was amplified using the following universal primers: forward primer,5'AGAGTTTGATCCTGGCTCAG3', and reverse primer 3'ACGGCTACCTTGTTACGACTT5'.

Dextranase activity assay. Of the selected isolates, the two bacterial isolates A and B were cultured separately in the production medium, containing glucose or dextran as the sole sources of carbon, according to the following composition (g/l): glucose or dextran 3, yeast extract 2.5, $MgSO_4$, 0.02, K_2HPO_4, 5.5, the medium was adjusted to pH 7 (10). The bacterial cultures were sampled at two-time intervals; 48 h and 72 h. The samples were centrifuged at 4°C, 14,000 ×g for 20 min using a cooling centrifuge (2-16k Sigma, Germany). The supernatants were then concentrated at 50°C under vacuum at 47 mbar and 100 rpm using a rotary evaporator (Rota vapor R-300 Buchi, G. Switzerland). The concentrated cell-free extracts were filter-sterilized using 0.22µm sterile filter (MS sterile syringe filter, Biomed Scientific, Sri Lanka) and stored at 4°C for further detection.

The measurement of dextranolytic activity was prepared as follows: 250 µl CFE was mixed with 250 µl of dextran solution (1gm of dextran dissolved in 0.1M potassium phosphate buffer with pH 7.2), and the reaction volume was completed to 1 ml using potassium phosphate buffer (13). The reaction was incubated at 37°C for 48 h and by the liberated end of the reaction, the reducing sugars were detected using the DNS reagent. 3,5-dinitrosalicylic acid (DNS) was prepared as follows: DNS 0.71 g and NaOH (1.32 g) were dissolved in 100 ml of distilled water with stirring, then K-Na tartrate (20.4 g), Na-meta bi-sulphite (0.55 g) and phenol (0.5ml) were added. One milliliter of the sample was mixed with a 3 ml DNS reagent. The mixture was boiled for 15 min at 100°C and then

Table 1. The components for PCR reaction

Maxima® Hot Start PCR Master Mix (2X)	25 µl
16SrRNA Forward primer	1 ul (20uM)
16S rRNA Reverse primer(of each 8 primer)	1 ul (20uM)
Template DNA	5 ul
Water, nuclease-free	18 µl
Total volume	50 µl

Table 2. PCR thermal cycles with time and number of cycles

Step	Temperature, C	Time	Number of cycles
Initial denaturation / enzyme activation	95	10 min	1
Denaturation	95	30s	35
Annealing	65	1 min	
Extension	72	1 min 30s	
Final Extension	72	10 min	1

left to cool. The developed color was measured using a spectrophotometer at 540 nm (13). One unit (1U) of dextranase activity was defined as the amount of enzyme that degrades dextran to produce 1 µmol of glucose equivalent per min at 37°C with pH 7 (14, 15). A calibration curve was constructed using standard glucose solutions and a series of dilutions from 0.05 to 1mg/ml (14).

The total protein concentration was determined as stated elsewhere (16), and crystalline bovine serum albumin was used as the protein standard.

Factors affecting dextranase productivity: The pH of the production medium. Three different pH values for production medium, 5, 7, and 9, were applied to the culture of the two highest activity isolates (Isolate A and Isolate B) after 3 days of incubation in the production medium (10, 17-19).

The inoculum density. For each specific volume of the production medium, the same refreshed isolates with OD_{600} = 0.31, were added separately as 5% and 10% v/v to each medium, and then all were collected to be assayed (20).

Carbon source in the production medium. Dextran or glucose was used as the sole carbon source in the production medium at a concentration of 0.5% or 0.3%, respectively. The dextranase activity was determined as mentioned above (10).

Fermentation time. The test was done by withdrawing samples from the production medium at different times ranging from one day to 3 days from incubation; each was centrifuged and filtrated to test dextranase activity against dextran (10, 17). The dextranase activity in each case was determined using (DNS) method and compared.

Factors affecting dextranase activity: Concentration of the cell-free extract. A volume of 50 ml of CFE was added to a 250 ml round bottom flask and connected to a rotary evaporator (Rotavapor R-300 Buchi, G. Switzerland). The CFE was evaporated at 50°C under vacuum at 47 mbar, until the solution reached 5 ml in volume. The concentrated residue was transferred to a 15 ml falcon tube and filter-sterilized through a 0.22µm sterile filter and then stored aseptically at 4°C. Aliquots were used to determine the dextranase activity using the DNS method.

The reaction time. The bacterial CFE was mixed with a 1% dextran solution as a reaction mixture and incubated at 37°C, for three different times: 1 h, 24 h, and 48 h, separately, then the three were tested with the DNS assay separately (21).

The exposure to high temperature. The different isolates were streaked on dextran plates and were incubated at 50°C for 24 h. And using the agar diffusion method, the wells on the surface of the dextran agar were inoculated with 50 µl of sterile concentrated cell- free extracts that evaporated at 50°C using the rotary evaporator, dextran plates were incubated at 37°C for 24 h. Enzymes activity on mediums were detected using Iodine solution and clear zones that formed were measured in mm (17-19).

Biofilm inhibition assay. The biofilm inhibition assay was performed according to Peterson et al. with some modifications (22). An overnight culture of *S. mutans* ATCC 25175 was incubated at 37°C under an aerobic condition in a 5 ml Tryptone soy broth (TSB) medium until it reached an OD600 value of 0.6. A volume of 500 µl from refreshed *S. mutans* was diluted with 5 ml of new sterile TSB medium to add a volume of 100 µl from the previous to the wells of the 96-well microtiter plate as biofilm formation control. A volume of 100 µl of CFE from the four isolates (test) was mixed with 100 µl biofilm control sample separately in each well, and the mixture of CFE and biofilm control sample was incubated for 24 h at 30°C. The plate was emptied and washed twice with 200 µl potassium phosphate buffer pH 7.2. The plate was dried in the oven at 60°C for 20 min, 200 µl of 0.1% crystal violet was added to each well for 15 min, then carefully washed using distilled water and left to dry in the air overnight. To resolubilize the dyed biofilms, a volume of 200 µl of 30% acetic acid was added to each well and then the whole plate was transferred to a new plate (23). Finally, the absorbance was measured using a spectrophotometer (Thermo Scientific Multiskan FC Microplate Photometer, UK) at 620 nm. The percentage of inhibition of the biofilm formation was calculated as follows: Inhibition percentage = (untreated sample OD_{595} – test sample OD_{595})/ untreated sample OD_{595} × 100 (24).

Scanning electron microscopy of the biofilm inhibition. The biofilms formed on the glass coverslips, either untreated or treated with the different

CFEs, were photographed using the SEM technique. Control was carried out as follows: A sample of *S. mutans* was pre-inoculated in TSB medium at 37°C overnight, then 0.5 ml of this culture was re-inoculated into a fresh sterile 15 ml TSB medium in 50 ml falcon tube, with adding sterile glass coverslips for incubation at 37°C for 24 h. Another two 15 ml falcon tubes were filled like control but added 0.5ml of CFE of Isolate A & Isolate B, and incubation was done as above. All test coverslips were collected for fixation, dehydration, mounting, and coating (25). The coverslips were examined by scanning electron microscope (JSM 6510 LA, JEOL - JAPAN).

Statistical analysis. The existing data of the biofilm inhibition assay and dextranase activity of the most promising isolates were the average of three independent replicates ± standard deviation. Significant differences among control and treatment means were tested using one-way ANOVA and two-way ANOVA using (Graph Pad Prism 6.01), with a significance level of 0.05.

RESULTS

Bacterial isolation and identification. We screened molasses and soil samples from different locations in the city of Naga-Hammadi for the presence of dextranolytic bacteria. We recognized 49 isolates out of all examined isolates on the basis of their ability to give a clear zone around the growth upon adding iodine solution as an indicator on agar-dextran plates. The colonies of the isolates were described morphologically and the gram staining was performed on the spore-forming bacteria that constituted the majority of the isolates (Table 3).

Phylogenetic analysis of the identified isolates was performed using MEGAX32 10.0.5 using the maximum likelihood method and the Kimura 2-parameter model (26). Isolate A showed 96.67% identity to the 16S rRNA gene from the *Bacillus velezensis* FZB42, and Isolate B showed 97.93% identity to the 16S rRNA gene from the *Pseudomonas stutzeri* CCUG 11256. Isolate C showed 97.61% identity to the 16S rRNA gene from the *Bacillus tequilensis* 10b, Isolate D showed 96.31% identity to the 16S rRNA gene from the *Bacillus thuringiensis* IAM 12077, isolate E showed 94.54% identity to the 16S rRNA gene from the *Bacillus subtilis* DSM 10, and isolate F showed 95.45% identity to the 16S rRNA gene from the *Bacillus Pacificus* MCCC1A06182 (Fig. 1).

Extracellular dextranase activity, initial screening. Using dextran agar plates, sixteen isolates were tested to detect their dextranolytic activities. The diameters of the most promising six isolates were recorded (Fig. 2A and B), and isolates A and B showed the best dextranolytic activity with high and good stability rather than other isolates. Additionally, dextranolytic activity was detected after concentration of CFE in dextran plates for isolate A, Isolate B, S. mutans and *L. reuteri*, as shown in (Fig. 2D and E), where Isolate A and B were the best over *L. reuteri* with remarkable activity of *S. mutans* activity. Total Protein concentration values are shown in (Fig. 3) of CFE of the most promising six isolates.

The effect of some factors on dextranase productivity: The pH of the production medium. Isolate A (*B. velezensis*) and isolate B (*Ps. Stutzeri*), which showed the highest dextranase activities, were grown at different pH values: 5, 7 and 9. For (Isolate A), the highest dextranase activity recorded was 11.80 mU/ml at pH 5, while that recorded for (Isolate B) was 11.19 mU/ml at pH 7 (Fig. 4).

The inoculum density. We tested the effect of two inoculum densities, 5% v/v and 10% v/v, added to the production medium. The results showed an approximately 3-fold increase in dextanolytic activities for all isolates tested, A, B, C, D, E, F, upon using the higher inoculum density (Fig. 2C).

The Type of carbon source. We tested the effect of two different carbon sources, 0.3% glucose or 0.5% dextran, on the dextranolytic activity (Fig. 2F). The enzymatic activities detected upon using glucose were higher than the dextran (Fig. 2 F).

Fermentation time. We tested CFE activity after 45 h and 69 h of fermentation for the six isolates. The activity increased with time; isolate F (*Bacillus pacificus*) exhibited 7.47mU/ml after 45 h of incubation and increased to 31.62mU/ml after 69 h; also, the rest of the isolates showed similar results (Fig. 5).

Factors affecting the dextranase activity: using concentrated CFEs. We compared CFE before and after concentration using the rotary evaporator, which

Table 3. Identification of the most promising six isolates using 16S rRNA gene sequencing, with more details related to the isolation location,16S rRNA % identity and isolate accession numbers.

Code	Isolate	Location of isolation Molasses	16S rRNA % identity*	Isolate accession**
Isolate A	*Bacillus velezensis* FZB42	Clay soil from sugar cane	96.67%	NR_075005
Isolate B	*Pseudomonas stutzeri* CCUG 11256	agricultural land	97.73%	NR_118798
Isolate C	*Bacillus tequilensis*10b	Molasses	97.61%	NR_104919
Isolate D	*Bacillus thuringiensis* IAM 12077	Molasses	96.31%	NR_043403
Isolate E	*Bacillus subtilis* DSM 10	Molasses	94.54%	NR_027552
Isolate F	*Bacillus Pacificus* MCCC1A06182	Molasses	95.45%	NR_157733

*16S rRNA % identity was obtained from GenBank for the strains with the highest percentage identity to the 16S rRNA gene sequences of the isolates.

**The accession numbers of the 16S rRNA sequences were obtained from GenBank for the different isolates.

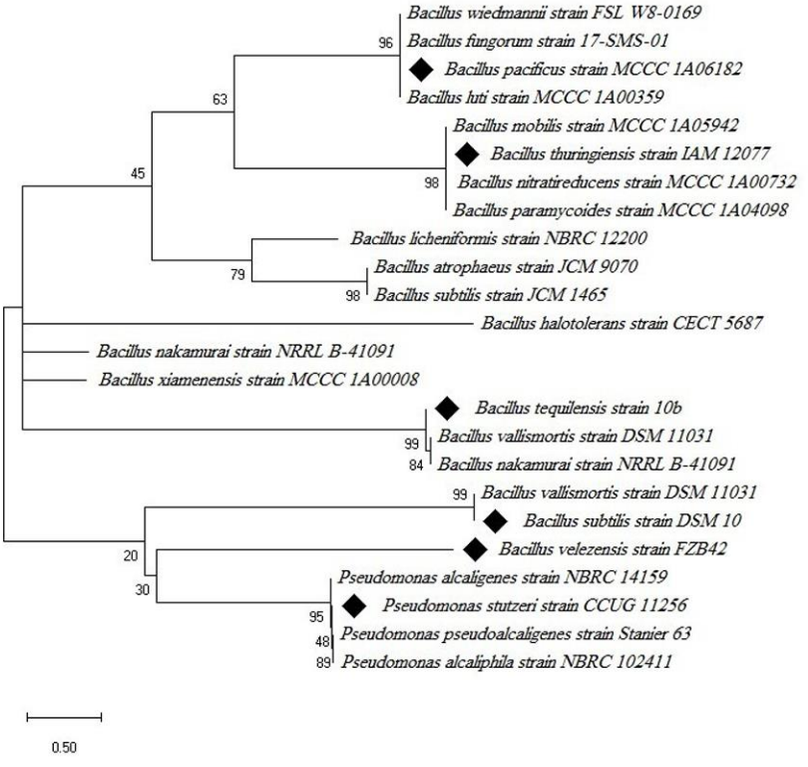

Fig. 1. The phylogenetic tree based on the 16S rRNA gene sequences showing the position and relationship between the most six active dextranolytic bacterial isolates reported in the current study marked with bold points and some identified related identified entries from the NCBI database.

showed a significant increase in the dextranolytic activities, approximately double (Fig. 3D and 6). For isolate A, activity after CFE increased by 2-folds, Isolate B and *L. reuteri* almost six fold (Table 4).

The effect of reaction time. For different isolates, by increasing the reaction time between dextran as substrate and CFEs, the resulting activity increases from 1 h to 24 h. Further experiments with slight modifications were conducted on concentrated CFE of Isolate A and Isolate B, and time was ranging from 1 h to 24 h till reached to 48 h, to show a gradual increase in the enzymatic activity (Fig. 7 and 8).

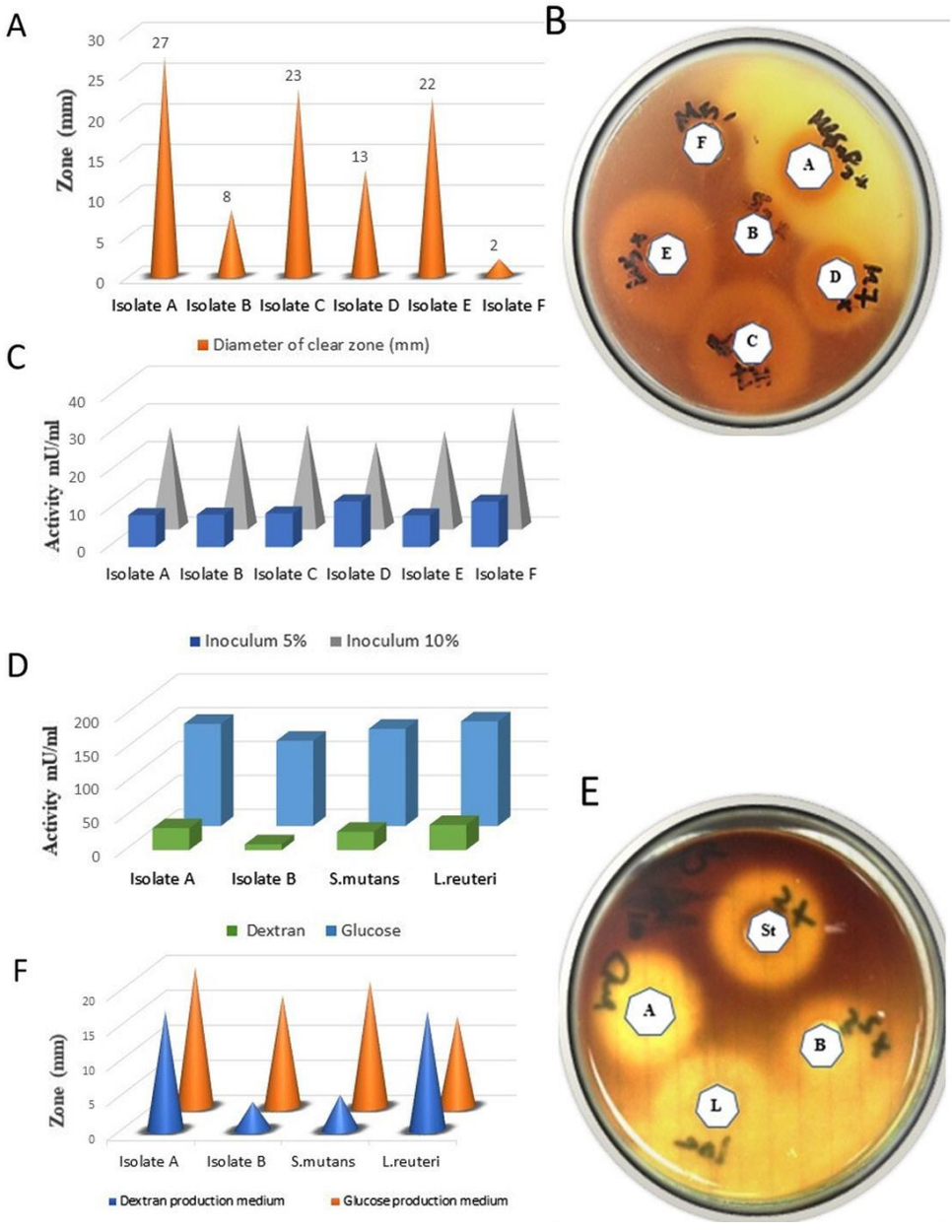

Fig. 2. The dextranolytic activities measured for CFEs from six different bacterial isolates and the diameters detected on the dextran agar plates (A, B). The effect of change of the inoculum volume on dextranolytic activity of six different isolates C. The dextranolytic activities of the concentrated CFEs of isolates A and B against *S. mutans* and *L. reuteri* CFE showing clear zones on the dextran plates were measured by millimeter (F). The effect of using two carbon sources in the production medium either dextran or glucose on the dextranolytic activities (F).

Effect of exposure to high temperature on vitality and enzyme activity. Testing of the ability of 16 different isolates to live and release their enzymes was carried out, as (Isolate A) *B. velezensis* showed positive enzymatic activity, the exposure to 50°C varies on its effect on different isolates, some of them survive and still enzymatically active, others can survive only with loss of the enzymatic activity, and the rest cannot survive at all, this was the first part of the high temperature effect.

The second part was as follows: the CFE of isolating A, B, *S. mutans* and *L. reuteri* that were extracted from glucose production medium that was better than dextran production medium after 48 h and 72 h from fer-

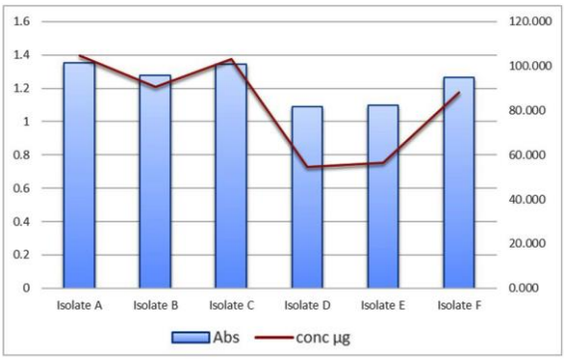

Fig. 3. Total Protein concentration values of CFE of the most promising six isolates.

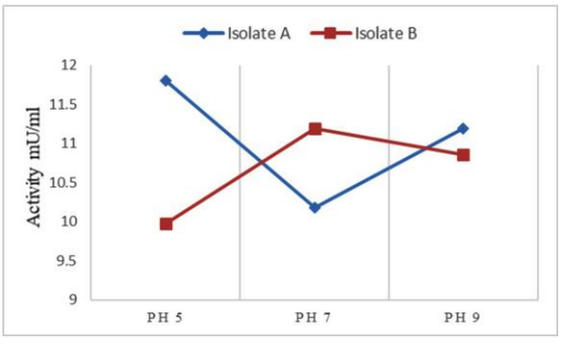

Fig. 4. Effect of different pH values of the production medium on the dextranolytic activities of isolates A and B.

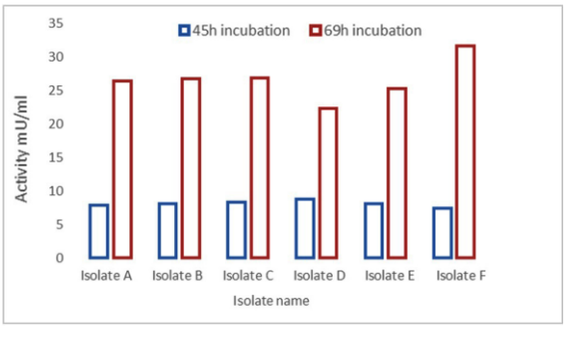

Fig. 5. The effect of fermentation time on dextranase activity of the selected isolates.

mentation were tested to show their ability on dextran degradation after exposure to 50°C in concentration, to show a positive activity with large remarkable clear zones on dextran plates as (Figs. 9 and 2 E).

Extracellular dextranase activity at optimized conditions. The dextranolytic activities of 16 isolates were assayed using the DNS method, which detects

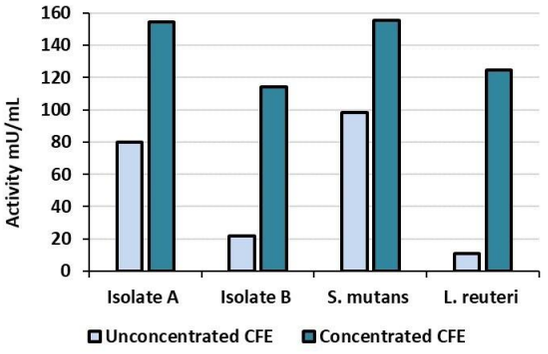

Fig. 6. Comparison between CFEs before and after concentration using the rotary evaporator for the CFEs of Isolate A, Isolate B, *S. mutans* and *L. reuteri.*

Fig. 7. Difference in CFE activity after 1h and 24 h from incubation with dextran substrate.

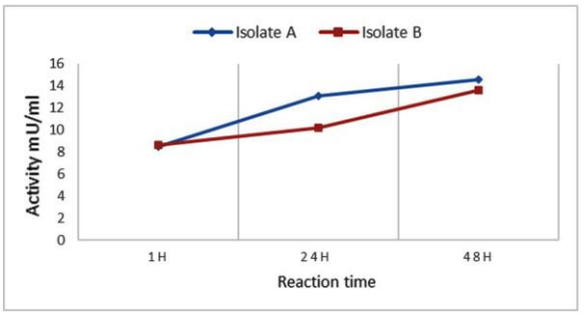

Fig. 8. The effect of the incubation time of CFE with dextran as a substrate tested for CFEs from the Isolates A and B.

the reduced sugars resulting from the dextranolytic activity. A calibration curve was constructed using a standard glucose solution. The activity recorded for isolate A was 8.22mU/ml. Optimized conditions for the four isolates resulted in the following Tables of dextranolytic activities: isolate A 28.5mU/ml; isolate B 30.19 mU/ml; isolate C 23.77mU/ml; isolate F 24.18mU/ml (Table 5). Clear zones of dextranolytic activities for isolates A, B, C, D, E and F with reor-

Table 4. Data are expressed as the mean ± S. D where: **Significant difference from the reference *L. reuteri* (P<0.01), *** Significant difference from the reference *L. reuteri* (P<0.001), **** Significant difference from the reference *L. reuteri* (P<0.0001).

Isolate code	Dextranase activity (mU/ml) before concentration		Dextranase activity (mU/ml) after concentration	
	Carbon source in fermentation medium			
Isolate A	**Dextran**	**Glucose**	**Dextran**	**Glucose**
	7.961 ± 0.61****	44.64 ± 1.30****	10.9 ± 0.63****	150.8 ± 0.49
Isolate B	3.758 ± 1.92	28.55 ± 1.07****	1.462 ± 0.14****	105.5 ± 1.95****
S. mutans	1.409 ± 0.21**	26.47 ± 0.44****	20.79 ± 0.57***	122.2 ± 1.89****
L. reuteri (control)	3.349 ± 1.93	69.89 ± 0.56	17.80 ± 0.59	151.9 ± 0.89

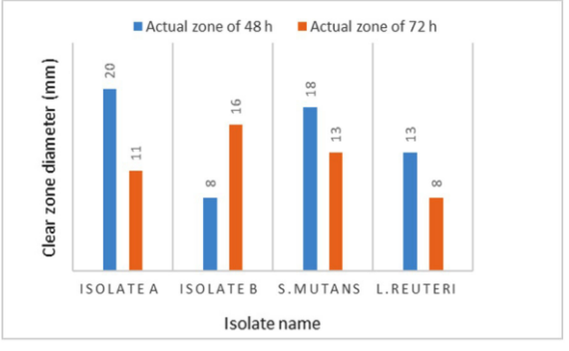

Fig. 9. Diameter of the clear zone in (mm) after CFE concentration extracted from the glucose production medium after 48 and 72 h from fermentation.

Table 5. Most promising isolates with dextranolytic activity without CFE concentration, preliminary evaluation of biofilm inhibition and optimum pH for activity compared to (*L. reuteri*) as a reference strain.

Isolate code	Activity mU/ml	% of biofilm inhibition	Optimum pH
Isolate A	28.50	43.46%	5&7
Isolate B	30.19	55.98%	5&7
Isolate C	23.77	51.23%	7
Isolate F	24.18	61.38%	7
L. reuteri (control)	26.61	35.81%	7

dered diameters are shown in (Figs. 2A and B).

Isolate A and isolate B showed higher dextranolytic activity compared to reference strain *Lactobacillus reuteri* (Fig. 2D).

Biofilm inhibition assay. The CFEs obtained from isolates A and B were tested for biofilm inhibition using *S. mutant* as a biofilm generating bacteria. Isolate A (*B. velezensis*) showed a 82.55% inhibition of the biofilm, while isolate B (*Ps. Stutzeri*) showed a 84.47% inhibition of the *S. mutant* biofilm (Figs. 10A, B, Table 5). Based on the biofilm assay and compared to the positive control, the effects of CFE on biofilm inhibition were remarkable, such as 82.55% and 84.47% inhibition when exposed to CFE of *B. velezensis* CFE. (Isolate A), the biofilm was treated with CFEs also before being concentrated by evaporation. All that was according to the intensity of crystal violet color shown in (Fig. 10), the absorbance readings, and the statistical analysis was done as demonstrated in Table 2 and Figs. 10A and B.

Scanning electron microscopy for *S. mutans* biofilm. The SEM photographed biofilms are shown in (Fig. 11), the control showed no visible structural deformities as nearly uniform unit with a highly condensed thick layer of cells and extracellular polysaccharide matrix. Although the biofilm treated with CFE showed less dense, and semi separated formed cells were seen (Fig. 11). The CFE inhibited biofilm formation and reduced the number of *S. mutans*. cells that adhered on the glass coverslips, where the formation of the biofilm on both surfaces was significantly reduced containing only a few spreading cells.

DISCUSSION

Many studies have reported that microbial dextranases effectively inhibit biofilm, especially in dental caries (10, 27). We here screened different types of sugar cane molasses, juice and soil for dextran lytic bacteria. Most of the samples were collected from places where dextran and complex sugars are expected to be at high levels.

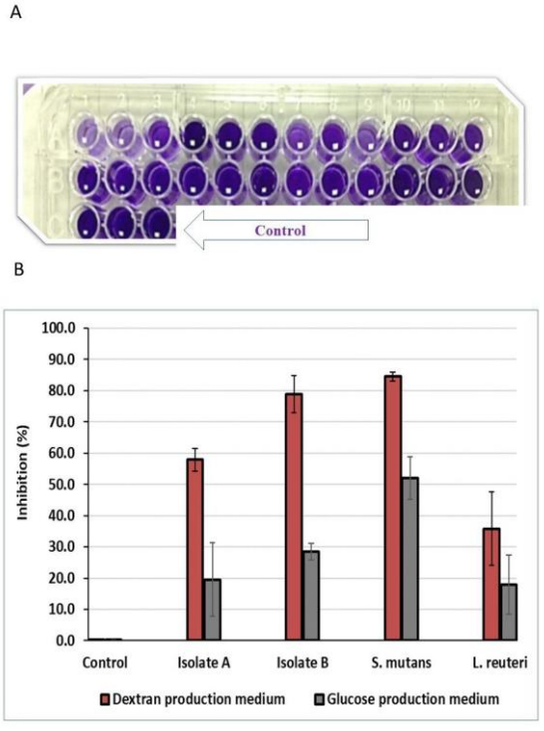

Fig. 10. Biofilm inhibition assay using 96-well micro-titer plate, the different CFEs were applied on a culture from *S. mutans*, the staining of the residual biofilm was done as described in the material and methods section, the brighter the final crystal violet color the more inhibitory effect produced (A). Percentages of biofilm inhibition using concentrated CFE from the Isolates A and B and from the *S. mutants* and *L. reuteri* (B).

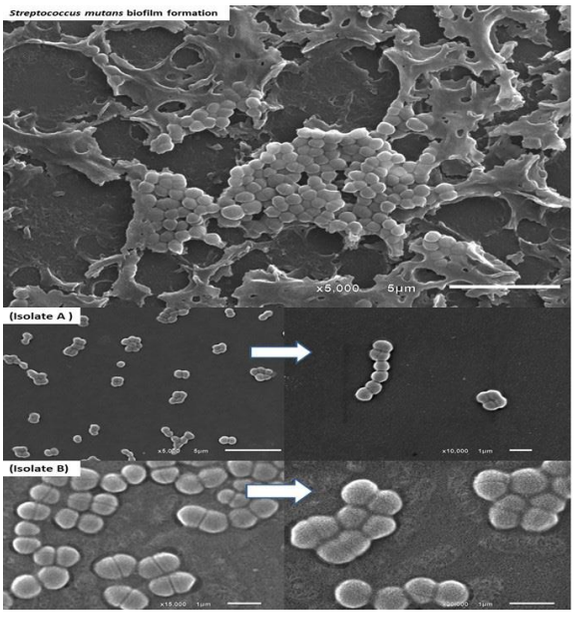

Fig. 11. The upper part: SEM micrographs of *S. mutans* biofilm formation on glass coverslip surfaces, Biofilms were grown in TSB in the presence of 0.5 ml of refreshed *S. mutans* at 37°C for 24 h. The image displayed was taken at magnifications (×5000). The selected image was chosen as the best representative of the amount of biofilm on the coverslips' surface. The lower part: SEM of biofilms that were grown in TSB in the presence of 0.5 ml of refreshed *S. mutans.* All images displayed were taken at magnifications (×5000 to×20 000). Electron microscopy image of *S. mutans* biofilm formed on glass coverslips treated with CFE at 37°C for 24 h, the cell-free extract was added to the biofilm as 0.5 ml of both: Isolate A and Isolate B, which showed less dense and semi separated formed cells with less biofilm formation on both sides, where it contained only a few spreading cells, without biofilm matrix formation around cells.

These sources included sugar cane seeding and cultivation soils, chunks of wastes of sugar cane, its juice and molasses, where molasses is considered the richest source. Molasses is produced as raw material after the sugar cane harvest at high temperature (28). Sugar cane agriculture is concentrated in Upper Egypt, especially the Qena governorate, which represents 48% of the total cultured sugar cane area in Egypt, where the Naga-Hammadi region has the highest production capacity (29). The successive enrichment steps using dextran-rich media supported the growth of dextranase-producing isolates. From sixty-seven samples, forty-nine isolates had a positive dextranase activity. Phenotypic and genotypic identification indicated that most isolates were Gram-positive bacilli, which marked the production of dextranase enzymes (11), and the 16S rRNA gene sequencing confirmed these results.

Isolates, *Ps. stutzeri* and *Bacillus* spp., have been reported earlier as potential microorganisms for dextranase production, in addition to *S. mutans* and *Lactobacillus reuteri,* which were standard strains that were used as a positive control in the current study, as suggested earlier (30). *S. mutans* was reported to produce dextranase enzyme, which was stable at 37°C and pH 7 (28, 31).

In addition, in our study, the tested *S. mutans* CFE against dental biofilm gave a remarkable inhibition effect. *Lactobacillus* sp. or probiotics are supposed to compete with pathogenic bacteria for nutrients with unknown mechanisms, hence the influence on the production of acid, peroxide and possible immune modification activities (1).

For dental caries inhibition applications, the stabil-

ity of enzyme activity and productivity is essential and can be affected by many factors, as studied and discussed in the methods section.

Because sugar cane molasses is considered a significant secondary product, most bacterial isolates could grow on molasses, showing that it can be a recommended carbon source. The use of enzymes has been considered an alternative strategy to fight various kinds of biofilm, mainly microbial ones. These enzymes are added to oral hygiene products such as mouthwashes, toothpaste, and chewing gums that can clean teeth with different mechanisms, such as removal of plaque by degradation of biofilm-attached extracellular matrix components or interference with biofilm formation. Enzymes can act gently and precisely on insoluble glucan hydrolases with dextranase and mutanase. The advantage of this strategy is that bacterial resistance towards the enzymes could rarely happen, in contrast to traditional chemical agents (3). Hence, our study focused on testing this strategy. According to Elvira et al., *Ps. stutzeri* produced intracellular dextranase with little excreted in the fermentation medium compared to *B. velezensis* and *L. reuteri*, indicating that moderate activity of *Ps. stutzeri* might be detected in cell-free extract (7).

The results in our study of the different factors on dextranase activity can help us to understand how the activity is positively or negatively affected, where the highest activity was verified after 69 h that reached 31.62mU/ml in the case of Isolate F and 26.81mU/ml for Isolate A and B, this is due to the consumption of all nutrients in the medium and subsequent degradation of dextran to oligosaccharides and then to disaccharides reached to small glucose units.

In addition, the bacterial cell multiplication and investments of the nutritional factors found in the fermentation medium.

The enzymatic activity is stable at a temperature as high as 50°C. This stability might be due to the induction by the thermophilic spore-forming bacteria *Bacillus.* Sp. (32). Dextranases produced from fungi have higher activities in temperature ranges from 50 to 60°C compared with dextranases made from bacteria that are around 40°C (33).

However, in our study, the bacterial dextranase activity was shown to be stable at a higher temperature of 50°C.

The maximum activity of *Ps. stutzeri* was detected at pH 7 but at pH 5 in the case of *B. velezensi*; this may be due to the difference in the stability of each enzyme versus the pH values. It was reported that dextranases with high pH values might be suitable for dental caries treatment because the enamel tolerates it more than other acidic products (19).

The enzyme activity was observed with an increase in inoculum volume from 5 to 10% (v/v), which led to an elevation in CFU and multiplication, increasing the release of enzymes into the production medium. Using glucose as a carbon source was more effective than dextran, as glucose is a simple and available carbon source, which does not need more effort exerted by microorganisms to be degraded or lysed. On the contrary, the complex polysaccharide dextran leads to an increase in the specificity of microbial substrates to glucose, besides the excess amount of glucose that cannot be utilized by microorganisms and accumulated in the fermentation medium. The residual glucose can then be reacted with DNS reagent and increased absorbance readings. Therefore, dextran was used as the primary substrate in this study. The gradual increase in the reaction time between CFE and the substrate had a good result in enzymatic activity, which may be due to the long exposure of a substrate to the enzymatic binding site, with gradual activity on substrate utilization and degradation.

The weak dextranase activity in some isolates might be due to the release of oligosaccharides or non-reducing sugars after dextran degradation, which cannot be detected by the DNS method that is less sensitive to oligo and di-saccharides than mono-saccharides (34).

The concentration of the cell-free extract was determined to reach the maximum activity of the crude enzymes and was achieved under the enzyme stability. To accurately measure dextranase activity, the assessment indicated that the prolonged time of enzyme exposure to the substrate at a constant temperature of 37°C led to increased enzymatic activity and substrate degradation.

However, in our study, the time of incubation, if exceeded after 48 h, may lead to degradation of the enzyme leading to loss of activity.

According to our study, *B. velezensis* and *Ps. stutzeri* were the highest enzyme producers with good stability, even significantly higher than the reference strain *Lactobacillus reuteri* (Fig. 6 and Table 5).

Indeed, the inhibition percentages reached 43% and 55% for the CFEs of isolates A and B, respectively and reached 60% and 80% after concentrating on the CFEs of the same isolates.

This study proposes that the CFE of isolates A and B contains dextranase and mutanase enzymes that can alter biofilm formation and polysaccharide accumulation, perhaps due to α-1,6 and α-1,3 glycosidic linkages -1,6 and -1,3 included in water-soluble glucan (WSG) and water insoluble glucan (WIG), since the biofilm of oral *Streptococcus* biofilm is made of α-(1,3)-glucan and α-(1,6)-glucan (Mutan and Dextran) that can be cleaved by mutanase and dextranase (19, 35). Mutanase enzyme was reported for biofilm inhibition produced from *Pseudomonas* and *Bacillus* spp. (36).

The CFE of *S. mutans* showed good activity reaching 85.69%, probably producing both glucosyltransferases, dextranase, and mutanase enzymes, which play at least two essential roles in the dental plaque ecology. They provide the plaque with nutrients by hydrolyzing glucans as latent storage of polysaccharides and modifying the molecular structure of glucans to be stronger, more fixed and adhesive on teeth surfaces. Therefore, dextranase, in this case, is considered an important virulence factor for *S. mutans* (31).

Besides, using dextranase cell-free supernatants rather than the whole microorganism or chemicals in the biofilm inhibition lowers the risks of spreading pathogens in the oral cavity and alters the microbial diversity in the application area without side effects.

CONCLUSION

This study determines that microbial sugar cane and related products had implications on the inhibition that plays a role in the formation of dental caries. CFE of isolate A (*B. velezensis*) and isolate B (*Ps. stutzeri*) are potential microorganisms for applications in dental plaque removal. This investigation aimed to assess the efficacy of crude enzymes on *S. mutans* biofilm. Consequently, oral hygiene products can involve dextranase enzymes produced from such species. Further experiments can be done to the current study and other possible effects on human health to develop a simple alternative therapy for caries.

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REFERENCES

1. Wasfi R, Abd El-Rahman OA, Zafer MM, Ashour HM. Probiotic *Lactobacillus* sp. inhibit growth, biofilm formation and gene expression of caries-inducing *Streptococcus mutans. J Cell Mol Med* 2018; 22: 1972-1983.
2. Vieira AR, Modesto A, Marazita ML. Caries: review of human genetics research. *Caries Res* 2014; 48: 491-506.
3. Pleszczyńska M, Wiater A, Bachanek T, Szczodrak J. Enzymes in therapy of biofilm-related oral diseases. *Biotechnol Appl Biochem* 2017; 64: 337-346.
4. Burt BA, Pai S. Sugar consumption and caries risk: a systematic review. *J Dent Educ* 2001; 65: 1017-1023.
5. Koo H, Schobel B, Scott-Anne K, Watson G, Bowen WH, Cury JA, et al. Apigenin and tt-farnesol with fluoride effects on *Streptococcus mutans* biofilms and dental caries. *J Dent Res* 2005; 84: 1016-1020.
6. Gambhir RS, Singh S, Singh G, Singh R, Nanda T, Kakar H. Vaccine against dental caries-an urgent need. *J Vaccines Vaccin* 2012; 3: 136.
7. Khalikova E, Susi P, Korpela T. Microbial dextran-hydrolyzing enzymes: fundamentals and applications. *Microbiol Mol Biol Rev* 2005; 69: 306-325.
8. Jaiswal P, Kumar S. Impact of media on isolation of dextranase producing fungal strains. *J Sci Res* 2011; 55: 71-76.
9. Lee K-H, Kim B-S, Keum K-S, Yu H-H, Kim Y-H, Chang B-S, et al. Essential oil of Curcuma longa inhibits *Streptococcus mutans* biofilm formation. *J Food Sci* 2011; 76: H226-30.
10. Zohra RR, Aman A, Zohra RR, Ansari A, Ghani M, Qader SAU. Dextranase: Hyper production of dextran degrading enzyme from newly isolated strain of *Bacillus licheniformis. Carbohydr Polym* 2013; 92: 2149-2153.
11. Khalikova E, Susi P, Usanov N, Korpela T. Purification and properties of extracellular dextranase from a *Bacillus* sp. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003; 796: 315-326.
12. Xavier JR, Madhan Kumarr MM, Natarajan G, Ramana KV, Semwal AD. Optimized production of poly (γ-glutamic acid)(γ-PGA) using *Bacillus licheniformis* and its application as cryoprotectant for probiotics. *Biotechnol Appl Biochem* 2020; 67: 892-902.
13. Teixeira RSS, Da Silva AS, Ferreira-Leitão VS, Da Silva Bon EP. Amino acids interference on the quantification of reducing sugars by the 3, 5-dinitrosalicylic acid assay mislead carbohydrase activity measurements. *Carbohydr Res* 2012; 363: 33-37.

14. Netsopa S, Niamsanit S, Araki T, Kongkeitkajorn MB, Milintawisamai N. Purification and characterization including dextran hydrolysis of dextranase from *Aspergillus allahabadii* X26. *Sugar Tech* 2019; 21: 329-340.
15. Huang R, Zhong L, Xie F, Wei L, Gan L, Wang X, et al. Purification, characterization and degradation performance of a novel dextranase from *Penicillium cyclopium* CICC-4022. *Int J Mol Sci* 2019; 20: 1360.
16. Kruger NJ. (2009). The Bradford Method For Protein Quantitation. In: Walker, J.M. (eds) The Protein Protocols Handbook. Springer Protocols Handbooks. Humana Press, Totowa, NJ. pp. 17-24.
17. El Banna HA, Gibriel AY, Mahmoud KF, Amin AA, Nessrien MN, Yassin N. Microbial production and characterization of dextranase. *Int J Curr Microbiol App Sci* 2014; 3: 1095-1113.
18. Esawy MA, Mansour SH, Ahmed EF, Hassanein NM, El Enshasy HA. Characterization of extracellular dextranase from a novel halophilic *Bacillus subtilis* NRC-B233b a mutagenic honey isolate under solid state fermentation. *E-J Chem* 2012; 9: 1494-1510.
19. Ren W, Cai R, Yan W, Lyu M, Fang Y, Wang S. Purification and characterization of a biofilm-degradable dextranase from a marine bacterium. *Mar Drugs* 2018; 16: 51.
20. Wardani SK, Cahyanto M, Rahayu E, Utami T. The effect of inoculum size and incubation temperature on cell growth, acid production and curd formation during milk fermentation by *Lactobacillus plantarum* Dad 13. *Int Food Res J* 2017; 24: 921-926.
21. Zohra RR, Aman A, Ansari A, Haider MS, Qader SAU. Purification, characterization and end product analysis of dextran degrading endodextranase from *Bacillus licheniformis* KIBGE-IB25. *Int J Biol Macromol* 2015; 78: 243-248.
22. Peterson SB, Irie Y, Borlee BR, Murakami K, Harrison JJ, Colvin KM, et al. (2010). Different Methods for Culturing Biofilms In Vitro. In: Bjarnsholt T, Jensen P, Moser C, Hiby N. (eds) Biofilm Infections. Springer, New York, NY. pp. 251-266.
23. O'Toole GA. Microtiter dish biofilm formation assay. *J Vis Exp* 2011; (47): 2437. 10.3791/2437.
24. Yoo Y, Seo D-H, Lee H, Cho E-S, Song N-E, Nam TG, et al. Inhibitory effect of *Bacillus velezensis* on biofilm formation by *Streptococcus mutans*. *J Biotechnol* 2019; 298: 57-63.
25. Tizro P, Choi C, Khanlou N. Sample preparation for transmission electron microscopy. *Methods Mol Biol* 2019; 1897: 417-424.
26. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018; 35: 1547-1549.
27. Finnegan PM, Brumbley SM, O'Shea MG, Nevalainen KMH, Bergquist PL. Paenibacillus isolates possess diverse dextran-degrading enzymes. *J Appl Microbiol* 2004; 97: 477-485.
28. Jiménez ER. Dextranase in sugar industry: A review. *Sugar Tech* 2009; 11: 124-134.
29. Hassan SF, Nasr MI. Sugar industry in Egypt. *Sugar Tech* 2008; 10: 204-209.
30. Allaker RP, Douglas CWI. Novel anti-microbial therapies for dental plaque-related diseases. *Int J Antimicrobl Agents* 2009; 33: 8-13.
31. Igarashi T, Morisaki H, Yamamoto A, Goto N. An essential amino acid residue for catalytic activity of the dextranase of *Streptococcus mutans*. *Oral Microbiol Immunol* 2002; 17: 193-196.
32. Driks A. Overview: Development in bacteria: spore formation in *Bacillus subtilis*. *Cell Mol Life Sci* 2002; 59: 389-391.
33. Jiao Y-L, Wang S-J, Lv M-S, Jiao B-H, Li W-J, Fang Y-W, et al. Characterization of a marine-derived dextranase and its application to the prevention of dental caries. *J Ind Microbiol Biotechnol* 2014; 41: 17-26.
34. Saqib AAN, Whitney PJ. Differential behaviour of the dinitrosalicylic acid (DNS) reagent towards mono-and di-saccharide sugars. *Biomass Bioenergy* 2011; 35: 4748-4750.
35. Otsuka R, Imai S, Murata T, Nomura Y, Okamoto M, Tsumori H, et al. Application of chimeric glucanase comprising mutanase and dextranase for prevention of dental biofilm formation. *Microbiol Immunol* 2015; 59: 28-36.
36. Sumitomo N, Saeki K, Ozaki K, Ito S, Kobayashi T. Mutanase from a *Paenibacillus* isolate: nucleotide sequence of the gene and properties of recombinant enzymes. *Biochim Biophys Acta* 2007; 1770: 716-724.