Elusive function of dental plaque polysaccharide produced from *Kocuria rosae* and it's molecular signature

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Background
Exopolysaccharides have been generally recognized as safe compounds, meaning that they do not represent a health risk when used. Owing to these properties, they have many applications in industrial areas and in healthcare. Our aim is to identify an extracellular polysaccharide (EPS)-producing strain from dental plaque bacteria and the verification of its polysaccharide's antitumor effect.

Materials and methods
Isolation of 22 dental plaque bacterial isolates from plaque samples of nine patients was carried out using pour plate method. The selection of the strain for molecular identification was done according to EPS production, whereas isolate no. 4 was identified by 16S rRNA sequencing analysis. Structure characterization of the EPS was described using UV and SEM images. The cytotoxic experiment was performed to investigate the inhibitory effect of different concentrations of EPS on the growth of cell line MCF7 human White breast adenocarcinoma.

Results and conclusion
Oral plaque bacteria vary greatly in their occurrence, depending on age, presence of systemic diseases, and personal oral hygiene. The amount of EPS produced from oral plaque bacteria also varies, though in general Gram-negative bacteria yielded larger amounts of EPS. Results revealed that isolate no. 4 is the most producer of EPS, identified as *Kocuria rosea* strain Y57, having 96% similarity with *Kocuria* spp. The biosynthesis of EPS from *K. rosea* using Luria–Bertani broth medium at 37°C for 24 h gave an EPS yield of 213 μg/ml. EPS from *K. rosea* is a powder with white color and is water soluble. Our results of in-vitro EPS assay against MCF7 human White breast adenocarcinoma released activity with LC_{50} 213 μg/ml.

Keywords:
anticancer, dental plaque, extracellular polysaccharides, SEM, 16S rRNA, UV

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Introduction

The human oral cavity is colonized by numerous and diverse microorganisms that are found to be commensally living but may cause dental caries and periodontitis, which are the two major oral diseases. Opportunistic microflora are increasingly known to be involved in the development of pathological processes in various systems and organs [1]. A complex community of microbes constitutes intraoral surfaces, which later form dental plaque [2]. Microbial extracellular polysaccharides (EPS) are soluble or insoluble biopolymers secreted by microbial cells forming a layer of protection for the cell surface and can be found in the fermentation medium [3,4]. Microbial EPS have numerous applications [5] including the applications of polysaccharides in field of the drug delivery systems [3,6] for important reasons: (a) the characterization and reproducibility can be obtained easily from natural sources [7]; (b) they can give sources to other materials by undergoing a wide range of enzymatic and chemical reactions; (c) EPS are suitable for biodegradation, biocompatibility, and have low immunogenic properties [8]; (d) the design of drug delivery systems can depend on substitute synthetic polysaccharides in ionic form partially, or totally [9]; (e) EPS have mucoadhesive properties [10]; (f) proteins, peptides, and other biomacromolecules can be conjugated or complexed with EPS [11]; and (g) EPS gels are easily formed. Polysaccharides are excellent materials for all aforementioned characteristics for the release of the 'smart' delivery systems, at the site of action, appropriate time, and in response to specific physiological stimuli [12,13].

The improvements of drug pharmaceutical properties, such as the distribution, solubility, and stability are a critical need for the decrease of adverse effects of both anticancer drugs and their co-solutes. The drug delivery systems include lipid base and solid lipid

particles, nanotechnology-based, carbon nanotubes and metal-based nanoparticles, and polymeric delivery systems or polymer small drug conjugates as in the case of EPS [14].

Conventional cancer therapies like surgery, radiotherapy, or chemotherapy remain the backbone of cancer therapy to date. However, not every cancerous tissue can be targeted by physical or chemical method. In this context, novel treatment options can be used in cancer therapy. Naturally derived drug-delivery systems like EPS can shuttle therapeutic compounds into the tumor.

Materials and methods

Written consent was obtained from each patient from the dental clinic (Dokki, Giza), and the study was carried out according to the regulations approved by the 'Ethical Committee of the College of Dentistry Research Center' [2]. The dental health status of each patient was assessed by a professional dentist.

Sample collection

Sampling was performed 2 h after eating in the morning. The sampling site was dried with a gentle air stream to avoid saliva contamination. Then, a sterile Gracey curette was used to collect the pooled supragingival plaque. Plaque was carefully removed with sterile curettes and placed into sterile Eppendorf tubes containing 10 mmol/l PBS (pH 7). Collected samples were snap frozen and stored at −80°C until analyzed.

Isolation and preliminary identification of plaque bacteria

Serial dilution was done for all plaque samples using the same buffer. A volume of 1.0 ml of each plaque sample in PBS (dilution 10^{-4}) was inoculated into Luria–Bertani (LB) agar medium by pour plate method in sterile 9.0 cm Petri dishes and incubated for 24–48 h at 37°C. The growing colonies were further transferred separately to LB medium plates, incubated for another 48 h at the same temperature, and then, each bacterial sample was morphologically examined using Gram stain.

Isolation and primary screening for polysaccharide-producing plaque bacteria

The following method was used for screening and isolating polysaccharide-producing bacteria according to Albalasmeh *et al.* [15].

Medium composition

Agrobacterium minimal medium containing [15 mmol/l $(NH_4)_2SO_4$, 40 mmol/l Na_2HPO_4, 20 mmol/l KH_2PO_4, 50 mmol/l NaCl, 1 mmol/l $MgCl_2$, 0.1 mmol/l $CaCl_2$ and 0.01 mmol/l $FeCl_3$]/1 l distilled H_2O, supplemented with 0.05% glucose, was used to measure the amount of polysaccharide in the supernatant of strains.

Methods

A loopful of each bacterial isolate was inoculated separately into 100 ml broth minimal medium, and cultures were grown at 37°C for 24 h in a shaker incubator adjusted to 120 rpm.

The supernatant of each bacterial broth culture was centrifuged at 5000 rpm for 5 min. The centrifuged supernatant was subjected to polysaccharide amount measurement:

A 2 ml aliquot was mixed with 1 ml of 5% aqueous solution of phenol in a test tube. Subsequently, 5 ml of concentrated sulfuric acid was added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they were vortexed for 30 s and placed for 20 min in a water bath at room temperature until color development. Then, light absorption at 490 nm is recorded on a spectrophotometer. Reference solutions are prepared in identical manner as above, except that the 2 ml aliquot of carbohydrate is replaced by DDI water. The water used in this procedure was redistilled, and 5% phenol in water (w/w) was prepared immediately before the measurements. Results of polysaccharide concentration presented as glucose concentration were calculated against glucose standard curve as shown in Fig. 1.

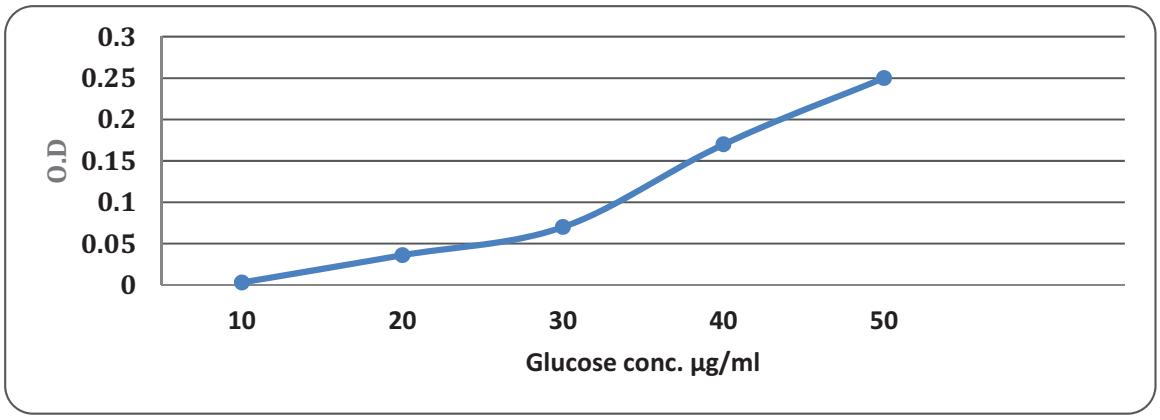
Collection of crude polysaccharide

Four liters of LB broth media was inoculated with each isolate and was incubated for 48 h at 37°C with shaking at 120 rpm. The bacterial growth was heated for 30 min at 80°C to kill the viable bacteria and then centrifuged for 30 min at 5000 rpm at 4°C. The supernatant that contains polysaccharides was precipitated by adding cold absolute ethanol to a final concentration of 80% (v/v) for 1–2 h at −20°C. The precipitate was collected by centrifugation for 30 min at 5000 rpm at 4°C, washed once in 80% ethanol for 30 min, and again centrifuged for 30 min. This washing step was repeated once with 96% ethanol. After centrifugation, the precipitate was dissolved in 2 ml of deionized water. The concentration of the exopolysaccharide was measured by phenol methods [16].

Molecular identification

The best EPS-producing bacterial strain was characterized by 16S rRNA sequencing analysis. Chromosomal DNA was extracted with Qiagen kit

Figure 1



Glucose standard curve.

according to the manufacture instruction. The PCR reaction of 16S rRNA was in a volume of 50 µl containing 1x green Taq PCR Buffer, 200 mmol/l of each dNTPs, 100 mg BSA, 10 pmole of each oligonucleotide primer, 2.5 U of green Taq DNA polymerase (Sigma), and 10 ng of DNA extract. PCR was performed by the following conditions: 1 min at 98°C followed by 35 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C. The 16S rRNA product was eluted, purified by Qiagen elution kit, and sequenced in Promega Company Laboratory (Cairo, Egypt).

The sequence was matched with previously published bacterial 16S rRNA sequences in the NCBI databases using BLAST. Selected sequences of other microorganisms with greatest similarity to the 16S rRNA sequences of bacterial isolate were extracted from the nucleotide sequence databases and aligned using MEGA6. Multiple Sequence Alignment was used to generate the phylogenetic tree. The 16S rRNA gene sequences of the bacterial isolates which reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers.

Isolation and purification of extracellular polysaccharides

A loopful of each bacterial strain was inoculated separately into 100-ml LB broth medium, and cultures were grown at 37°C for 24 h in a shaker incubator adjusted to 120 rpm. The EPS was isolated and purified according to Cerning *et al.* [17], with some modification. The growth cultures were heated at 100°C for 5 min to inactivate enzymes potentially capable of polymer degradation, and the cells were removed by centrifugation at 8000 rpm for 5 min at 4°C. The EPS was precipitated using two volumes of absolute ethanol. After standing overnight at 4°C, the resultant precipitate was collected by centrifugation at 8000 rpm for 20 min. The EPS was dissolved in deionized water, dialyzed against deionized water at 4°C for 24 h, and freeze-dried. The freeze-dried powder was dissolved in 10% (w/v) trichloroacetic acid to remove proteins. The supernatant was dialyzed at 4°C against deionized water for 5 days and freeze-dried. These preparations were referred to as purified EPS and were stored at 4°C.

Morphological characterization and structure analysis of the extracellular polysaccharides

Morphology of the pure freeze-dried EPS was examined using scanning electron microscope (Electron probe microanalyzer JEOL – JXA 840 A, model; Japan) analysis. Lyophilized powder of EPS was coated on gold particles, and microstructure was visualized under scanning electron micrograph at different magnification (×300, ×50, and ×900). Whole and surface view of EPS was taken [18].

The UV spectrum analysis of the purified EPS was recorded using T80+UV/VIS Spectrometer, PG Instrument Ltd (range: 190–1000 nm).

Cytotoxic effect on human cell lines

Cell viability was assessed by the mitochondrial-dependent reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to purple formazan [19].

All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (SG403INT; Baker, Sanford, Maine, USA). Cells were suspended in RPMI 1640 medium (for HePG2–MCF7 and HCT116–DMEM for A549 and

PC3), 1% antibiotic–antimycotic mixture (10 000 µ/ml potassium penicillin, 10 000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B), and 1% l-glutamine at 37°C under 5% CO_2. Cells were batch cultured for 10 days, and then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37°C for 24 h under 5% CO_2 using a water-jacketed carbon dioxide incubator (TC2323; Sheldon, Cornelius, Oregon, USA). Media was aspirated, fresh medium (without serum) was added, and cells were incubated either alone (negative control) or with different concentrations of sample. After 48 h of incubation, medium was aspirated, 40 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (2.5 µg/ml) was added to each well, and incubated for further four hours at 37°C under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200 µl of 10% SDS in deionized water was added to each well and incubated overnight at 37°C. A positive control which composed of 100 µg/ml was used as a known cytotoxic natural agent which gives 100% lethality under the same conditions [20].

The absorbance was then measured using a microplate multiwell reader (model 3350; BioRad Laboratories Inc., Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent *t*-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts, and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the following formula:

$$\left(\frac{\text{Reading of extract}}{\text{Reading of negative control}} - 1\right) \times 100.$$

A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program.

Results and discussion

Isolation, Gram staining, and primary screening for polysaccharide producing-bacteria

Plaque samples collected from nine patients were distributed in relation to the patient sex, age, and diagnosis, as recorded in Table 1. Isolates were coded as M for male and F for female. The patients' ages ranged between 28 and 68 years old.

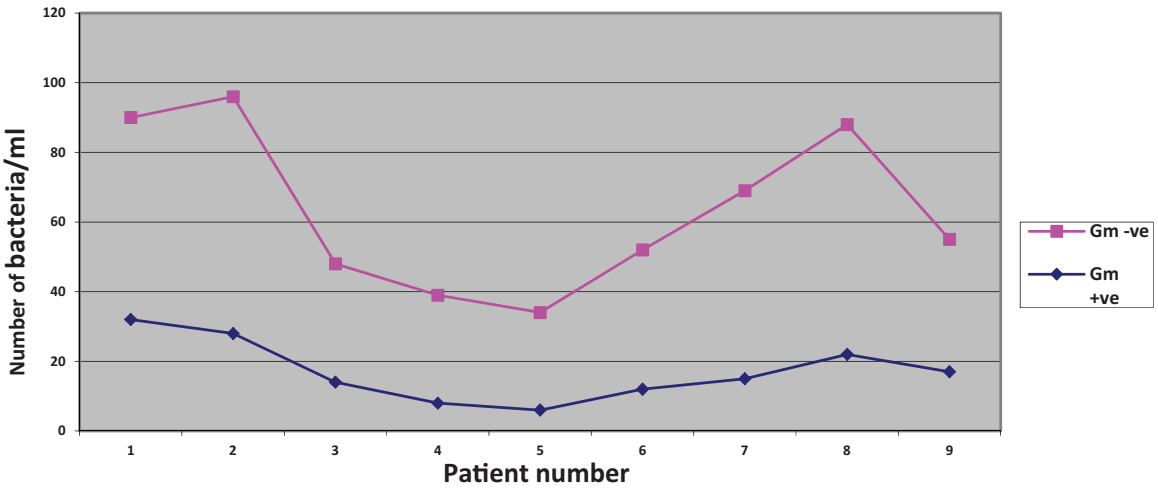
Each plaque sample was collected by a sterile curette and put in sealed Eppendorf tubes (2 ml) containing PBS at pH 7. Serial dilutions were made using the same buffer for each sample. Dilution of 10^{-4} was used to inoculate on LB agar medium in sterile Petri dishes by pour plate method, and then plates were incubated at 30–32°C for 24 h. Colonies of the produced bacterial isolates were differentiated on a morphological basis (mainly color, colony elevation, roughness, edges, and mucoid or not) and given consecutive numbers (1–22). The Gram staining of bacterial isolates from each patient indicated that age and systemic diseases affect the number of bacteria present and weather positive or negative, for patient numbers 1, 2 and 8 yielded the most bacterial number/ml because of the high age and moderate or poor oral hygiene. Moreover, the absence of a large number of teeth resulted in a less count of bacterial isolates, as in patient no. 7 (Fig. 2).

Table 1 Description of plaque samples collected from nine different patients

Number	Date	Sex	Age	Hygiene	Systemic diseases
1	18 April 2017	Female	64	Poor	Diabetes
2	18 April 2017	Female	64	Moderate gingivitis	High blood pressure
3	20 April 2017	Male	46	Poor	None
4	20 April 2017	Female	62	Moderate	None
5	21 April 2017	Male	33	Moderate	None
6	21 April 2017	Female	28	Moderate	None
7	21 April 2017	Male	68	Poor (many teeth lost)	High blood pressure-diabetes
8	24 April 2017	Male	65	Poor (gingivitis)	Parkinson's
9	24 April 2017	Male	49	Moderate	None

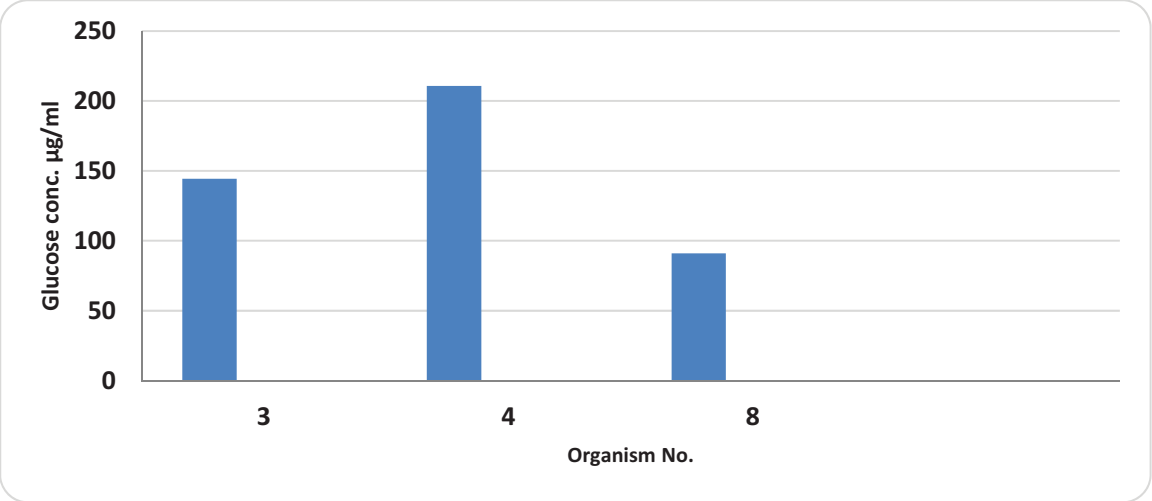
Three bacterial isolates nos 3, 4, and 8 were selected for further testing based on the measurement of the total sugar content (µg/ml glucose) of the LB-broth medium filtrate of each isolate (Fig. 3). The selection of the best strain to molecular identification was carried out according to EPS biosynthesis. The most active bacterial isolates strains (3, 4 and 8) were subjected to molecular identification.

Figure 2



Gram staining of the bacterial isolates.

Figure 3



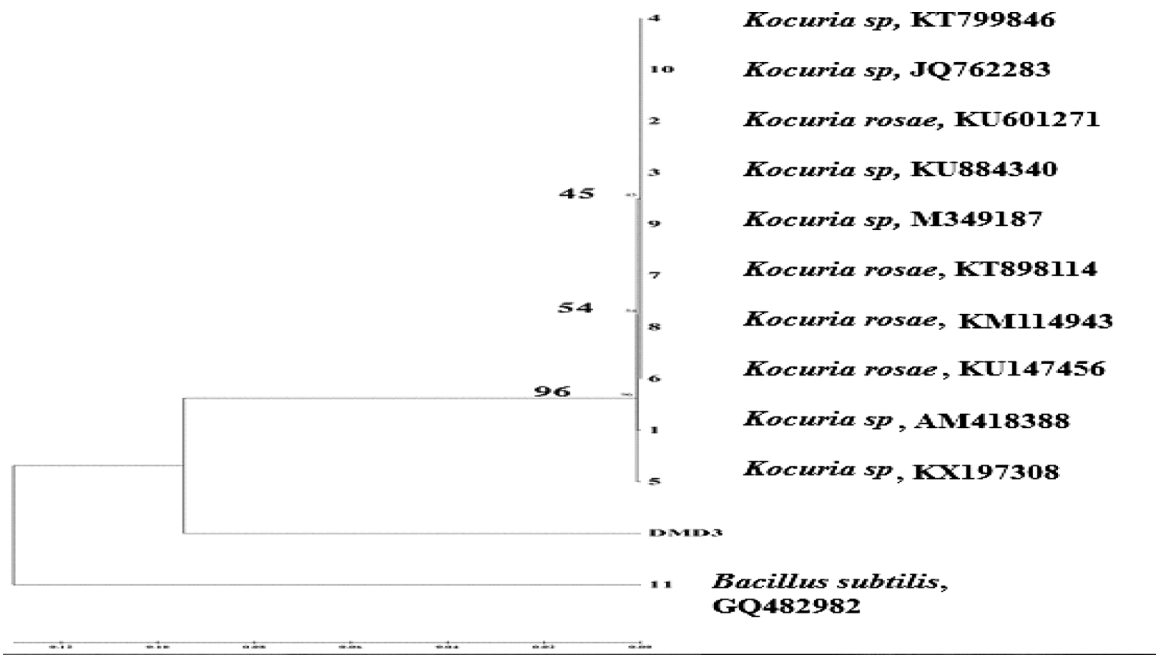
Isolation and primary screening for polysaccharide-producing bacteria.

Molecular identification of bacterial isolate no. 4

The chromosomal DNAs for bacterial isolate no. 4 were isolated by a versatile quic-prep. method for genomic DNA from Gram positive bacteria according to Pospiech and colleagues [21,22] with some modification. The 16S rRNA gene (~1500 bp) was amplified using universal primers: forward: AGA GTT TGA TCC TGG CTC AG and reverse: GGT TAC CTT GTT ACG ACT T. Then, the PCR was performed using primers designed to amplify about 1500 bp fragment of the DNA region of bacteria. Moreover, the Blast program (http://www.ncbi.nlm.gov/blast) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software [23]. The phylogenetic tree showed in Fig. 4 was displayed using the TREEVIEW program. The bacterial fragment sequence was as follows:

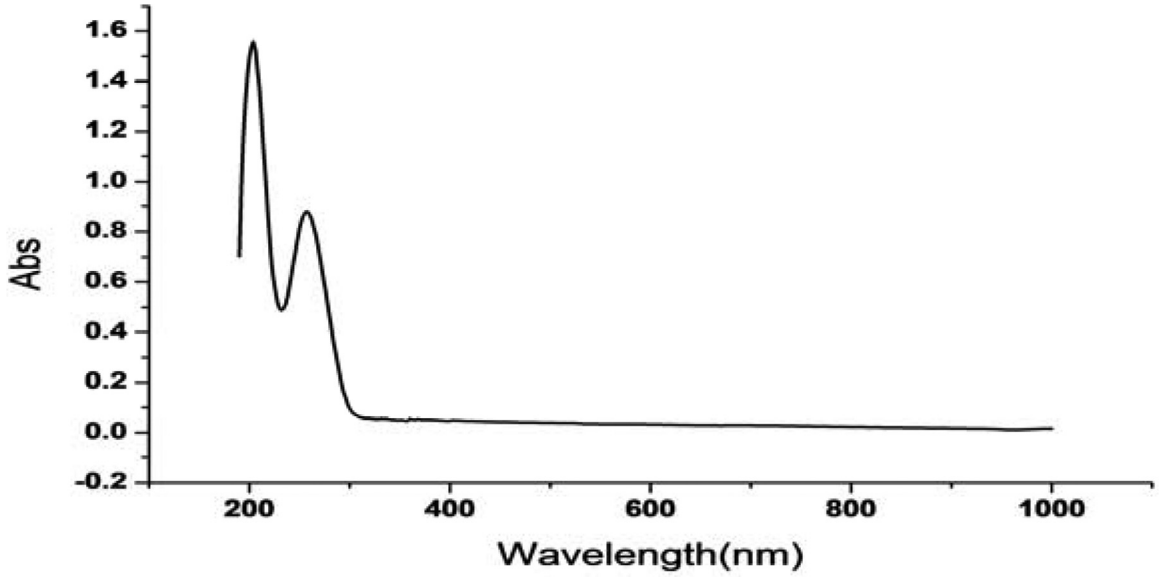
CCCGTCCCAGGGCTAACACGTGCTACAG
GGCTACACACGTGCTACAAGGGCTACAC
ACGTGCTAC AGGGCTACACACGTGCTAC
AAAGGCTACACACGTGCTACAAAGGCTA
CACACGTGCTACAAGGG TTCCCGCGGGC
TACAAGAAGTGCACAGATGGTACGAGGA
ACACCCATGGGCAAGGAAGGCCTCT GGC
TGTTACTGACGCTGAGGAGCGAAAGCAT
GGGGAGCGAACAGGATTAGATACCCTGG
TAGTCCATGCCGTAAACGTTGGGCACTA
GGTGTGGGGGACATTCCACGTTTTCCGC
GCCGTAGCTAACGCATTAAGTGCCCCGC
CTGGGGAGTACGGCCGCAAGGCTAAAAC
TCAAAGGAATTGACGGGGGCCCGCACAA
GCGGCGGAGCATGCGGATTAATTCGATG
CAACGCGAAGAACCTTACCAAGGCTTGA
CATTCACCGGACCGCCCCAGAGATGGGG
TTTCCCTTCGGTGTTGGTGGACAGGTGG

Figure 4



Phylogenetic tree: bootstrap test of phylogeny, UPGMA, sample 3. There is 96% similarity with *Kocuria* spp.

Figure 5



Ultraviolet spectra of the *Kocuria rosae* external polysaccharide (EPS).

TGCATGGTTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTCGTTCTATGTTGCCAGCACGT
GTATGGTGGGGACTCATAGGAGACTGCC
GGGGTCAACTCGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGTCTT
GGGCTACACACGTGCTACAA.

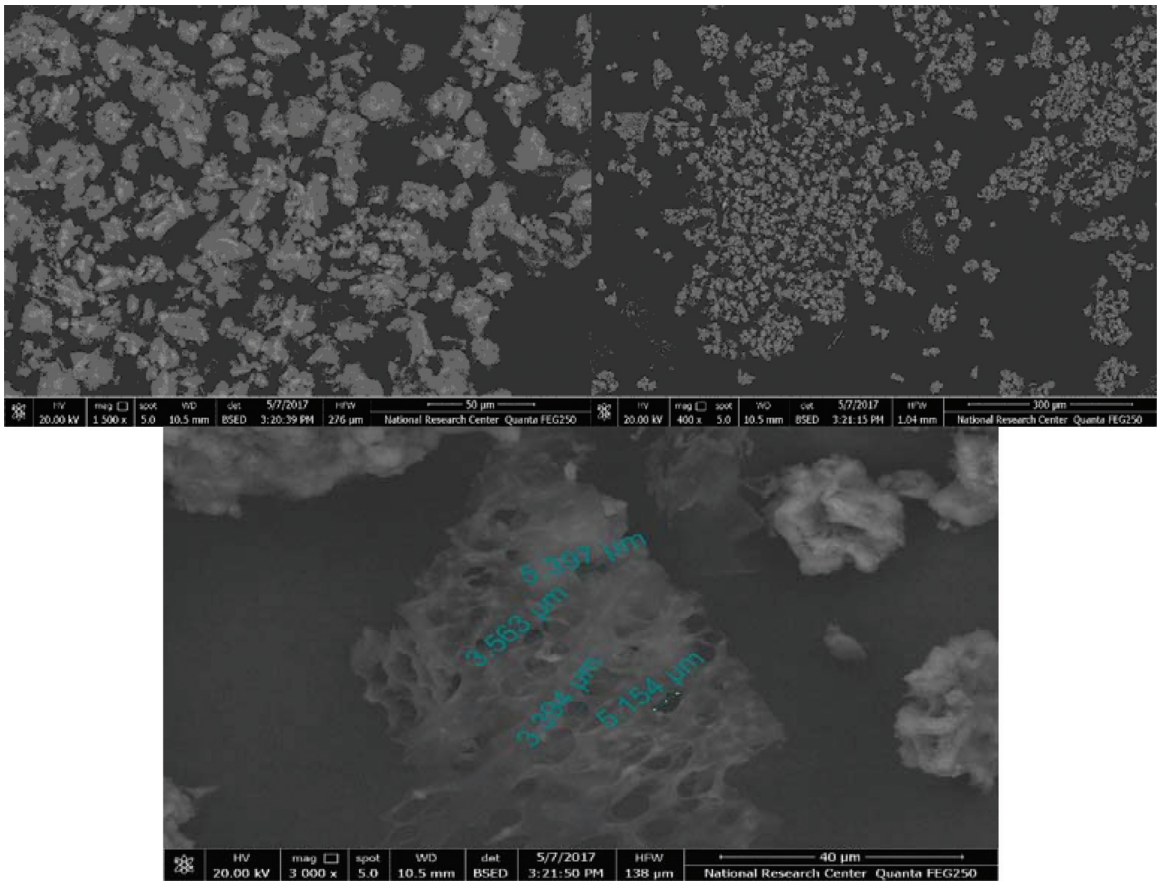
Moreover, the molecular identification detected that isolate no. 4 is *Kocuria rosea* strain Y57, with a similarity of 96% to *Kocurea* spp.

K. rosea is an opportunistic type of bacteria which can be identified in many systemic diseases but there is a small number of studies involving it because of the lack of data on their cariogenic associations [1].

Morphological characterization and structure characterization of the extracellular polysaccharides

K. rosea EPS UV spectrum revealed that two peaks were detected. Absorbance at 212 nm is characteristic for carbohydrates (Fig. 5), and 212 and 228 nm peaks

Figure 6



(a–c) SEM of *Kucoria rosae* external polysaccharide (EPS) powder surface at magnification power ×300, ×50, and ×40. (a) Crysallization of *K. rosae* EPS magnification power 300. (b) Flake shapes and gum mass EPS of *K. rosae*, magnification power ×50. (c) Biofilm membrane of *K. rosae* EPS magnification power ×40.

were reported for other polysaccharides, similar to the present results by Yun and Park [24].

K. rosea EPS structure characterization was described using SEM images, which show mainly three shapes like flakes, gum mass crystallize, and biofilm membranes with pores size between 3.3 and 5.3 µm (Fig. 5). *K. rosea* EPS is a whitish water-soluble powder. As it can be seen from the microstructure of EPS surface view that the produced EPS are compact in structure, first particles exhibit flakes-like structural unit and are highly compact (Fig. 6a–c). Therefore EPS have a potential as thickeners or as stabilizing agent for novel food products. Other particles are mostly seen in aggregates of irregular shapes and dimensions which are fibrous, gum-like, devoid of crystalline structure. The shape and structure or surface topology of the polysaccharide may be affected by the method of extraction and purification or preparation of the product.

Cytotoxic activity of the extracellular polysaccharides

K. rosea EPS exhibited antitumor activity against human White breast adenocarcinoma *in vitro*. EPS different concentrations (50.0, 25.0, 12.5, 5.0, and 0.0 µg/ml) have an inhibitory effect on growth of MCF7 (human White breast adenocarcinoma) and RPE1 (normal retina cell line).

Table 2 Antitumor activity of the external polysaccharide *in vitro*

Sample code	LC_{50} (µg/ml)	LC_{90} (µg/ml)	Remarks
MCF7 (human White breast adenocarcinoma)			
EPS	213	604.3	67.2% at 500 ppm
RPE1 (normal retina cell line)			
EPS	145.2	401.7	90.5% at 100 ppm

EPS, external polysaccharide; LC_{50}, lethal concentration of the sample which causes the death of 50% of cells in 48 h; LC_{90}, lethal concentration of the sample which causes the death of 90% of cells in 48 h.

Results presented in Table 2 revealed that, EPS inhibited the proliferation of the human White breast adenocarcinoma and normal retina cell line with LC_{50} of 213 and 145.2, respectively. These results indicated that EPS has potential antitumor activity against White breast adenocarcinoma but, if compared with its effect on RPE1 (normal retina cell

line) showed cytotoxic effect. The resistance mechanism was not clear up to date [25].

Conclusion

The natural products such as polysaccharides can be used as antitumor agent or carriers of other anticancer drugs. The polysaccharides produced by bacteria in the oral cavity can be used for anticancer therapeutic purposes. Regarding the different morphological structures of bacterial polysaccharides under SEM which are; flakes, gum mass, crystalline and biofilm membrane like structures with pores size between 3.3 and 5.3 micrometer, their effect on biological activity, and applications of biopolymers. These results also indicated that EPS has the most sensitive and a potential antitumor activity against White breast adenocarcinoma.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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