Physicochemical characterization of exopolysaccharides conjugated to phenolic compounds: a novel acidic exopolysaccharide containing tartaric acid derived from *Rhodotorula taiwanensis*

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Background
Polysaccharides that are derived from different sources, in particular those from microorganisms, constitute a hot topic in contemporary research thanks to their high-value applications in different biotechnological sectors.
Objective
Considering limited existing studies concerning yeasts, the current study was designed to search for promising exopolysaccharide (EPS)-producing yeasts from samples obtained from different biological sources, adopting the strategies of isolation and screening.
Materials and methods
The present study focused on isolation and screening of EPS-producing yeasts from samples obtained from different biological sources, namely, soil rhizosphere, rotten fruits, local beverages, dairy products, and mixture pickles; identification of the selected promising yeast isolates phenotypically and genetically; extraction and chemical composition of crude exopolysaccharides (C-EPSs) in terms of their contents of carbohydrate, protein, and phenolics; and physicochemical characterization of the partially purified exopolysaccharides (PP-EPSs) by high-performance liquid chromatography (HPLC), Fourier transformation infrared, proton nuclear magnetic resonance, thermogravimetric analysis, X-ray diffraction, scanning electron microscope, and energy-dispersive X-ray analysis.
Results and conclusion
The most potent isolates that provided the highest yields (2.5 and 2.25 g/l) were identified phenotypically and genetically as *Rhodotorula mucilaginosa* A1 and *Rhodotorula taiwanensis* G1. The chemical compositions of C-EPSs of both strains differed in terms of their contents of carbohydrate, protein, and phenolic components. HPLC analysis of the phenolic compounds of C-EPSA1 revealed the presence of eight different constituents, of which quercetin followed by kaempferol, hesperetin, and gallic acid represented 99.81%. However, C-EPSG1 contained only seven, in a much smaller quantity. HPLC analysis demonstrated that both PP-EPSs were acidic heteropolysaccharides; PP-EPSA1 consisted mainly of 69.52% fructose and 30.48% uronic acids. PP-EPSG1 is probably unique; it showed remarkable differences as it contained tartaric acid (1.22%) besides glucose (50.04%), fructose (39.65%), and uronic acid (9.09%). Spectral analyses of both PP-EPSs confirmed their polysaccharide nature through the presence of characteristic functional groups and glycosidic linkage regions. PP-EPSs were semicrystalline in nature, similar in porosity and surface smoothness, and showed resistance to high temperatures. Elemental analysis indicated the participation of both PP-EPSs in five elements (O, C, N, S, and P) in close proportions; PP-EPSA1 contained Ca as an additional element.

Keywords:
exopolysaccharides, Fourier transformation infrared, high-performance liquid chromatography, *Rhodotorula mucilaginosa*, *Rhodotorula taiwanensis*, thermogravimetric analysis, radiograph diffraction analysis physicochemical characterization

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Introduction

Plants and algae that are known as the major photosynthetic sources of biopolymers are now rendered inconsequential due to being negatively

affected by climatic and geological environmental conditions. Since the discovery of microbial bioactive exopolysaccharide (EPSs), both bacteria and fungi are considered valid alternative sources to sustain and prevent the depletion of photosynthetic producers. Besides, these microbes are characterized by rapid growth and high yield of biopolymers without complicated extraction technology. Moreover, EPSs produced by bacteria and fungi compete positively with plant and algae in purity, chemical quality, bioactivity, significance, and potency [1–4].

A vast array of microorganisms are able to synthesize and produce polymers outside the cell as secondary metabolites. These biopolymers are mainly composed of polysaccharides, proteins, phosphate, sulfate, N-acetyl-amino sugars, acetyl groups, and DNA. Microbial polysaccharides as a biopolymer signify a class of important products that are of increasing concern for several sectors of the industry. They are eco-friendly, sustainable, biodegradable, and biocompatible and have higher steadiness to extreme conditions [3,5–8].

Microbial EPSs offering a great assortment with regard to chemical structure and composition that specify their biological activities and biotechnological potentialities [9–11]. In medicine, microbial EPSs have the capacity to attain a distinct biological effect as an antioxidant, antiulcer, antitoxin, antitumor, immune stimulator, anticoagulant, antithrombotic, antiviral, antimutant, vaccines, and adjuvants [2,12–16].

In comparison with bacterial EPS, there are a limited number of studies related to yeasts, even though yeasts are known to produce larger amounts of EPSs that are easy to separate from the growth medium [14,17,18]. The productivity, structure and physical properties of EPSs are strain, medium composition, and culture condition dependent [12,17,19,20]. The current study was designed with the aim of isolating promising EPS-producing yeasts.

Materials and methods

Sample collection

A total of 17 samples from various biological sources were rounded up for yeast isolation; three rhizosphere samples from soils cultivated with banana, clover, and onion, located at Tanash, El Waraq, and Ossem, Giza, Egypt; seven samples of rotten apple, cherry, guava, mango, pomegranate, strawberry, and tangerine; four samples of dairy products, Kareesh cheese, yoghurt, raw milk, and whey; two local beverages, hibiscus and liquorice; and one sample of pickle mixture.

Isolation and pure culture maintenance

Each samples was first homogenized in sterile saline solution (0.9% NaCl) at ratio of 1 : 9 g/ml, and then 10-fold serial dilutions were performed. A portion of 1 ml from each dilution was plated on yeast extract peptone dextrose (YPD) agar medium [21] composed of 2% dextrose, 2% peptone, 1% yeast extract, and pH 5.5. Plates were incubated at 28–30°C for 2–4 days, and then the mucoid yeast colonies were preliminary recorded as EPS producers, picked up, and purified through repeated streaking plate method and subcultured on YPD agar following the same procedures. Pure yeast cultures were stored at 4°C till use and subcultured once every 2 months.

Screening of yeast isolates for exopolysaccharide production

The purified mucoid isolates were screened for EPS production. Flasks were loaded at 40% (v/v) of fermentation medium, which composed of g/l: 2.0 $(NH_4)_2SO_4$; 1.0, KH_2PO_4; 0.5, $MgSO_4.7H_2O$; 0.1, $CaCl_2$; 0.1, NaCl and 1.0, yeast extract; pH 5.5 and supplemented with 50 g glucose after sterilization as EPS production medium. Such medium was inoculated with standard inoculum of each isolate individually and allowed to grow at 28–30°C for 96 h on a rotary shaker at 200 rpm [22].

Preparation of standard inocula

Yeast isolates were subcultured on YPD slants at 28–30°C for 2 days. The standard inocula were prepared by transferring a loop from each slant culture to 250-ml conical flask containing 100 ml of YPD broth medium. Inoculated flasks were incubated at 28–30°C and 180 rpm for 48 h on a rotary shaker (Thermo Scientific Maxq481 RI HP Incubator Shaker, 401 Millcreek Road, Marietta, Ohio 45750, United States). An aliquot of 5 ml (2.5×10^7 CFU/ml) of each was centrifuged at 5000 rpm for 10 min under aseptic conditions, and then the sedimented cells were washed twice with sterile saline solution and used as standard inoculum to inoculate 100-ml volume of production medium.

Determination of pH and yeast biomass

At the end of the incubation period, the final pH of the fermentation cultures was measured using pH meter (Adwa pH meter). To determine yeast biomass, each culture was centrifuged at 6000 rpm for 20 min. Supernatant was poured out, and the precipitate cells were washed with sterile distilled water, and recentrifuged twice, followed by drying to constant weight at 105°C (SML 32/250 Zelmed Drier, Poland); the result of biomass yield was expressed as cell dry weight (CDW) g/l medium.

Extraction of crude exopolysaccharides

The crude exopolysaccharide (C-EPSs) were isolated from the cultivated medium though methodology of Cerning *et al*. [23], which was modified by Haroun *et al*. [24].

Phenotypic and molecular characterization of potential yeast strains

Based on the yield of EPSs, the most productive yeast isolates were characterized following the directions given conventionally by other studies [25–27]. The purified isolates were classified based on vegetative and sexual reproductive characteristics along with morphological and physiological features. The methodology adopted by Maldonade *et al*. [28] was followed for studying shape, cellular dimension, type of cell division, and formation of ring, film, sediment, pseudohyphae, mycelium, and ballistospores. Physiological and biochemical tests included carbon and nitrogen utilization; growth at different temperatures; amyloid composite synthesis; urea hydrolysis; and coloration of diazonium blue B. The obtained results were compared with the reference data of yeast species previously designated in the literature [25,27].

To obtain accurate and reliable identification, sequence analysis of the variable D1/D2 domain of the large subunit (26 S) ribosomal rDNA was performed. The divergent D1/D2 domain of 26 S rDNA was amplified with primers NL-1 (5'-GCA TAT CAA TAAGCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAGACG G-3') [29]. The PCR was done by agarose gel electrophoresis. The amplified product was then purified using Gene JET PCR Purification Kit (Thermo) and sequenced in GATC Biotech AG Company (Jakob-Stadler-Platz 7,D-78467 Konstanz, Germany). The 26S sequence of the isolated yeasts was used for a BLAST search in the EMBL/GenBank database. Based on Kurtzman and Robnett [29] statement, yeast strains showing nucleotide substitutions greater than 1% in the D1/D2 domain of the large subunit ribosomal ribonucleic acid gene are usually specified as different species. The BLAST database of National Center for Biotechnology Information (*http://www.ncbi.nlm.nih.gov/blasthttp://www.ncbi.nlm.nih.gov/blast*) was used to match the obtained resolved sequence with known 26S rDNA sequences [30]. The sequences were deposited in the GenBank database, and the accession numbers were obtained. A phylogenetic tree was constructed using the neighbor joining [31] and maximum composite oscillation methods using the MEGA 5.0 software (*http://megasoftware.net/http://megasoftware.net/*) as described by Tamura *et al*. [32].

Chemical composition of crude exopolysaccharides

Carbohydrate and protein

The colorimetric phenol-sulfuric-acid-method [33] was used to determine the carbohydrate content. Total carbohydrate content was calculated by reference to a calibration curve of a standard solution of glucose (Sigma). Total proteins were determined according to the method of Bradford [34], with bovine serum albumin (Sigma) as the standard.

Determination and characterization of phenolic content of crude exopolysaccharides

Total phenolic compounds (TPCs) were determined using the Folin-Ciocalteu (FC) method [35] with minor modification [36]. Total phenolic contents were estimated by reference to a calibration curve of a standard gallic acid and expressed as mg of gallic acid equivalents (GAE) per g dry substrate (mgGAE/g). Phenolic compounds were analyzed using high-performance liquid chromatography (HPLC; Agilent 1260 series), identified, and quantified by matching their spectra and retention times with those of standards as performed by Khalil *et al*. [37].

Partial purification of exopolysaccharides

C-EPSs were partially purified following the methodology of Cerning *et al*. [23]. The produced EPSs were dissolved in 10% trichloroacetic acid with stirring overnight to remove protein. The precipitated proteins were removed by centrifugation at 6000 rpm for 15 min at 4°C; the pH of the resulted supernatants was adjusted to 7.0 and EPSs were reprecipitated with 2× volumes of chilled ethanol and centrifuged as previously mentioned. Finally, the precipitated EPSs were resuspended in distilled water with gentle heating (at <50°C) and then dialyzed using Dialysis Tubing (12 000–14 000 Da 50 dialysis membrane, Medicell International, UK) for 96 h with two changes of distilled water per day at 4°C. Thereafter, the dialyzed contents were freeze dried (Benchtop Freezone Freeze Dryer Labconco, Model C50). The partially purified exopolysaccharides (PP-EPSs) were stored at 4°C for physicochemical and biological studies.

Physicochemical characterization of partially purified exopolysaccharides

Monosaccharide and uronic acid composition

Overall, 20 mg of PP-EPS samples was dissolved in water, heated to rehydrate, and then hydrolyzed with 3 ml of 2 N trifluoroacetic acid (TFA) at 120°C for 8 h in sealed small glass tubes. Afterward, the tubes were opened, evaporated to dryness with addition of methanol to remove TFA. EPS hydrolysates and standard sugars including fructose, glucose, sucrose,

and lactose were analyzed by HPLC (column Phenomenex Luna NH2 250×4.6 mm). Column temperature was kept constant at 30°C; the mobile phase consisted of acetonitrile: HPLC grade water (80 : 20, v/v), and the sample was eluted at a flow rate of 1.3 ml/min. Monosaccharide contents of the EPS samples were detected by refractive index detector; the data integration was done by Clarity Chromatography Software.

Uronic acid also was detected in PP-EPS hydrolysates by HPLC; the separation was performed on Rezex column for organic acids analysis (300×7.8 mm). Column oven temperature was kept constant at 65°C, the mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.6 ml/min at 24°C, and 20 µl of each sample was injected at a time. The UV detector was set at 214 nm, and the data integration was done by Clarity-Chrom software.

Fourier-transform infrared analysis

The Fourier transformation infrared (FT-IR) spectrum analysis was carried out using FT-IR, Bruker, Model Japan, and resolution: 4 cm^{-1}. PP-EPS sample was ground with spectroscopic grade potassium bromide powder and then pressed to create a disc of potassium bromide (1 mm pellet) for FT-IR analysis at the frequency range of 400–4000 cm^{-1}.

Proton nuclear magnetic resonance analysis

Overall, 5 mg of the PP-EPS samples was dissolved in 0.75 ml of heavy water (D_2O) and placed in a 5-mm proton nuclear magnetic resonance (NMR) tube [38]. The ^{1}H-NMR spectra were detected at 25°C using a BRUKER AV 500 MHz spectrometer (USA) in the ppm range of 0.5–6.0.

Thermogravimetric analysis

The thermal gravimetric analysis of the PP-EPS samples was performed using a thermal system (TG-DTA/DSC Model: Q600 SDT). The compound was subjected to a temperature range of 30–1000°C under nitrogen atmosphere at a heating rate of 10°C/min and the corresponding weight loss was determined. Based on the relationship between the weight loss and the heating rates against temperature, TG-DSC thermogram graphs were plotted.

X-ray diffraction

X-ray diffraction (XRD) patterns were performed to determine the crystalline natures of the EPSs on radiograph powder diffractometer using PANanalytical X'Pert PRO equipped with secondary monochromatometer and Cu-radiation (λ=1.54056 Å) at the Central Laboratories Sector, the Egyptian Mineral Resources Authority. The diffraction scan was carried out at diverse ranges of two-theta angles (10–90°C) under running conditions of 45 kV and 35 mA, and scanning speed of 0.04°/s [39]. Dried EPS sample was prepared on a quartz substrate, and intensity peaks of diffracted X-ray were continuously recorded with scan step time of 1 s at 25°C, and diffracted X-ray were calculated with Bragg's law: $d=\lambda/2 \text{ Sin } \theta$) and relative intensities (1/1°). The obtained charts and relative intensities were compared with the database files of the International Center for Diffraction Data using Score Plus Software.

Scanning electron microscope and energy dispersive X-ray

The microstructure and surface morphology of EPS was determined by a Jeol JEM-2100 electron microscope at an accelerating voltage of 20 kV. The EPS was settled in 2.5% of glutaraldehyde (Sigma) and then dehydrated by ethanol and then EPSs were wrapped with gold by a sputter coater (Scancoat six, Oxford) and scrutinized under the scanning electron microscope (SEM) operating at 20 keV. Micrographs were recorded at higher magnification to ensure clear images [40]. Energy dispersive X-ray (EDX) is an elemental spectroscopy method that works together with electron microscopy to assay the elemental composition of EPS samples. The EDX procedure is performed at an accelerating voltage of 20 kV. Such technique allows an effective, speedy, and accurate determination that can be imaged by the electron microscope in a nondamaging means.

Statistical analysis

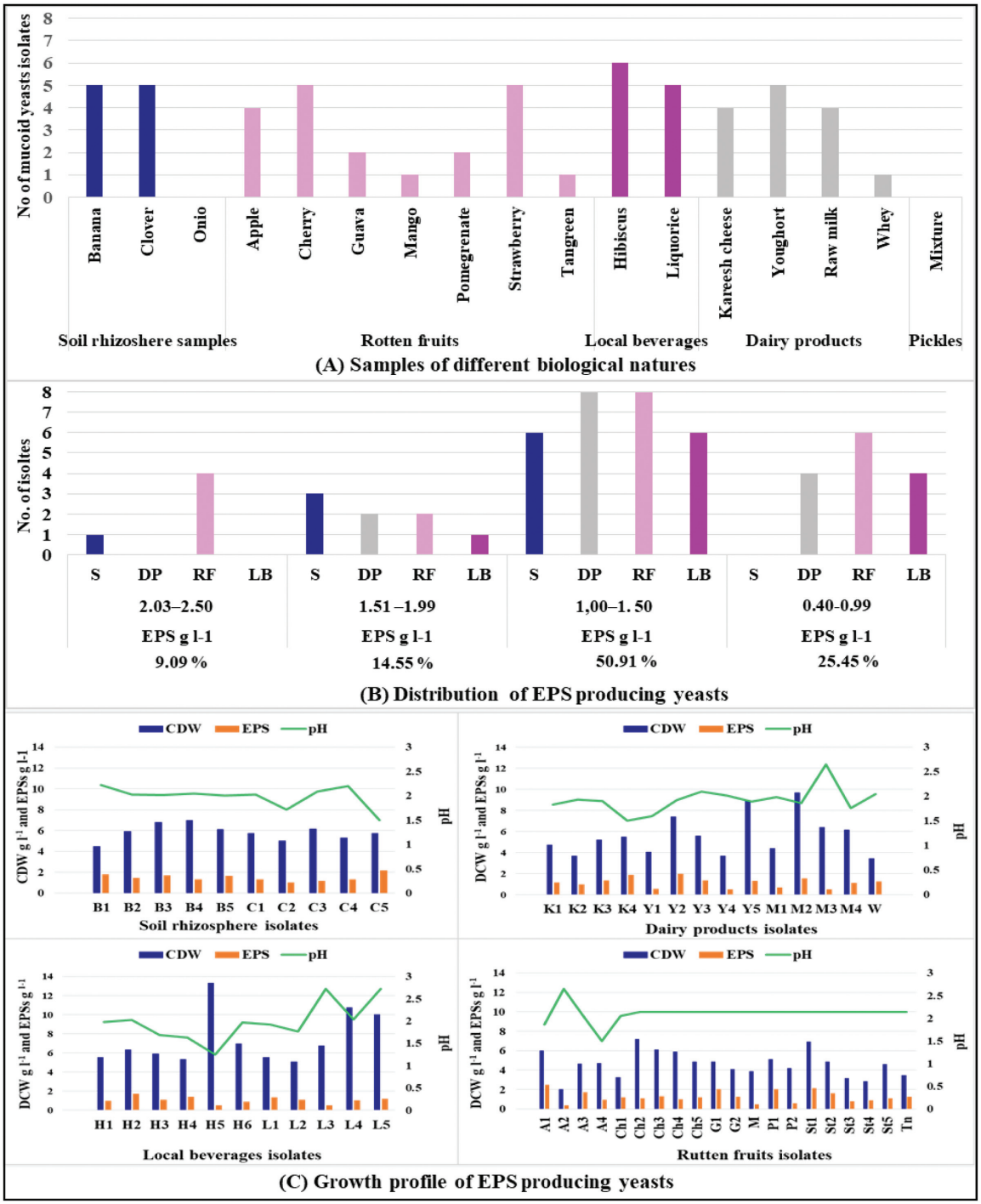
All of the assessments mentioned in this study were conducted in triplicate. Least significant difference test was applied at 0.05 confidence level to confront the significant difference between means of replicates using statistical analysis software Costat Program, Version 6.4.

Results and discussions

EPSs of microbial origin have potential bioapplications in the medical, pharmaceutical, and many industrial sectors including food. In recent times, yeast's EPSs have attracted specific interest thanks to their appreciable yield and effortless extraction from the growth medium compared with other microbial sources [14,41]. Through the present work, 17 samples from different biological sources were microbiologically analyzed for isolating promising

EPS-producing yeasts. All the samples generated putative EPS-producers except onion rhizosphere soil and mixture pickle samples (Fig. 1a). The 55 recovered yeast isolates were screened to check for their ability to produce EPSs. The results revealed that all of these isolates produced EPSs but with variable performance; their yield ranged from 0.4 to 2.5 g/l. Of those, five (9.09%) isolates attained the highest yield (2.0–2.5 g/l), and eight (14.55%) yielded EPSs within the range from 1.51 to 1.99 g/l. The majority of isolates (28, 50.91%) yielded EPSs over the range of 1.0 and 1.50 g/l; the lowest yield (0.40–0.99 g/l) was produced by 14 (25.45%) yeast isolates (Fig. 1b). Like other secondary metabolites,

Figure 1



Isolation and distribution (a, b) of preliminary EPS producing yeasts (c). Growth profile of yeast isolates: EPSs yield ($LSD_{0.05}$=0.11), biomass yield as cell dry weight (CDW, $LSD_{0.05}$=0.19) and pH ($LSD_{0.05}$=0.10) by recovered yeasts from different biological natures. S, soil; Dp, dairy products; RF, rotten fruits; LB, local beverages; B, banana; C, clover; K, kareesh cheese; Y, yoghurt, M, raw milk; W, whey; H, hibiscus; L, liquorice; A, apple, Ch, cherry; G, guava; M, mango; P, pomegranate; St, strawberry; Tn, tangerine. EPS, exopolysaccharide; LSD, least significant difference.

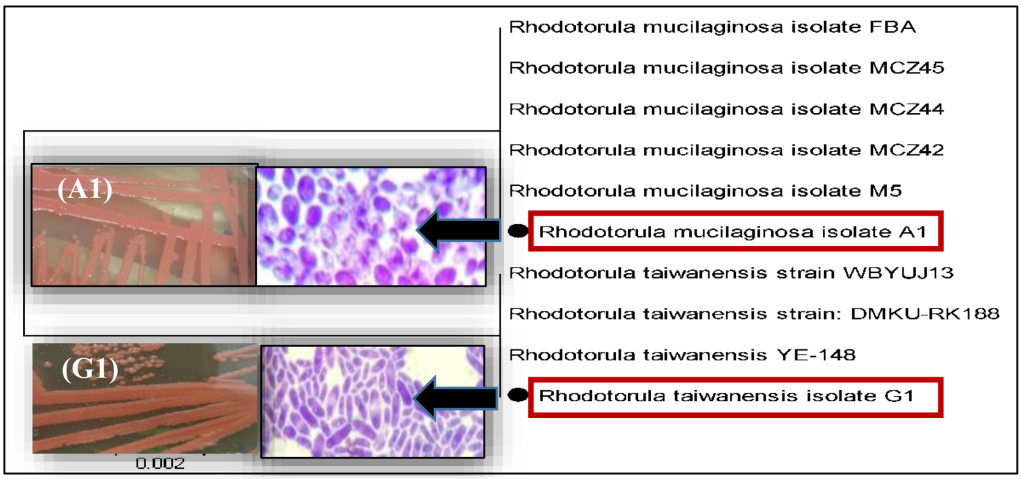
EPS productivity was strain, medium composition, and growth conditions dependent [42,43]. Biomass yields (CDW) ranged between 2.02 and 13.34 g/l and seemingly were not associated with the EPS yields; the greatest CDW production was obtained only from a single isolate of hibiscus (H5) despite its low EPS yield of 0.50 g/l. It is palpable that EPSs production by different yeast isolates is accompanied by a reduction of the pH values of production medium (Fig. 1c). The postfermentation pH values were dropped from the initial pH of 5.5 to below 3.0 by all tested isolates. Vigorous acidification of the postculture medium in the course biosynthesis of EPSs by yeasts has been observed in several studies. Such steep drop in pH is attributed to the fast depletion of ammonium ions and subsequently the discharge of H ions from yeast cells [22,42,44]. Based on highest EPS productivity, two promising isolates (one from rotten apple, A1, and the other from rotten guava, G1) were selected for further study.

Phenotypic and genotypic characterization of selected yeast strains

The macro-morphological and micro-morphological characteristics as well as carbon assimilation of the tested strains revealed an obvious disparity between them in terms of colony color, cell shape, and assimilation of lactose, rhamnose, and trehalose. On YPD agar plates, the isolate A1 developed mucoid, smooth surface, and pink-colored colonies with complete margin; under microscopic observation, cells were round or oval forming budding structures. However, the colonies of the isolate G1 were orange colored, smooth mucoid with complete margin; the cells appeared elongated under microscopic observation (Fig. 2). Both isolates had the ability to grow at temperatures over the range of 20–37°C, did not produce spores, and failed to ferment glucose and inositol but had the ability to assimilate D-glucose, D-xylose, sucrose, glycerol, and maltose. The strains were able, as well as, to assimilate L-lysine and nitrate but failed to assimilate nitrite. They reacted positively with the diazonium blue B reagent, were resistant to cycloheximide, and had the capacity to secrete urease enzyme. Following the traditional keys of identification, results of morphological, cultural, and physiological properties strongly suggested that the isolates A1 and G1 belonged to genus *Rhodotorula*.

However, adequate identification is difficult because of the great phenotypic intraspecific diversity. Therefore, molecular technique would provide more lucidity to differentiate neatly nigh species [45,46]. Therefore, the phenotypic classification of the two species was confirmed genetically with 100% similarity as *Rhodotorula mucilaginosa* and *Rhodotorula taiwanensis*, and the nucleotide sequences were banked in the GenBank database under the accession numbers of MZ496289 and MZ496291, respectively. According to the phylogenetic analysis (Fig. 2), one of the isolates was placed in the same clade of the strains *R. mucilaginosa* M5, MCZ42, MCZ44, MCZ45, and FBA. The other strain was placed in the same clade of the strains *R. taiwanensis* YE-148, DMKU-RK188, and WBYUJ13. In general, the collection of detailed macro-morphological and micro-morphological and physiological characterization and sequencing D1/D2 of 26 S rDNA yeast gene as a molecular marker are considered an integral approach for a reliable taxonomy [47].

Figure 2



The macro-morphological and micro-morphological characteristics and neighbor-joining phylogenetic trees of the isolates A1 and G1.

Table 1 Chemical composition (%) of exopolysaccharide A1 and exopolysaccharide G1 produced by *Rhodotorula mucilaginosa* and *Rhodotorula taiwanensis*, respectively

	Carbohydrate	Protein	TPCs	Fructose	Glucose	Gala. acid	Tart. acid	Gluc. acid
EPSA1	36.60	15.32	4.322	69.52	–	23.43	–	7.05
EPSG1	46.89	54.20	3.939	39.65	50.04	6.89	1.22	2.20

EPS, exopolysaccharide; Gala., galacturonic; Gluc., glucuronic; Tart., tartaric; TPC, total phenolic compounds (expressed as g_{GAE}/100 g dry substrate).

The basic components of crude exopolysaccharides

Carbohydrates and proteins

Preliminary chemical structures of EPSs are not homogenous and mainly contain carbohydrates, proteins, nucleic acids, lipids, and phenolic compounds [39,48]. Total carbohydrate contents derived from the microbial crude extracellular polymers ranged from 40% to more than 90% with proteins up to 17.19% [49,50]. The basic chemical composition of the produced C-EPSs exhibited significant differences. The main component of EPSA1 synthesized by *R. mucilaginosa* was carbohydrate (36.60%) followed by protein (15.32%), whereas the basic chemical analysis of that secreted by *R. taiwanensis* (EPSG1) was protein (54.20%) followed by carbohydrate (46.98%) (Table 1). The ratio of carbohydrate to protein was 2.39 compared with 0.87, in that order. Such ratio was found to vary greatly depending on the extraction method, the EPS-producing microorganism, or even those synthesized by the same strain under various culturing conditions [51,52]. High protein content up to 40–50% was detected in C-EPS synthesized by deep-sea marine bacterium [53]. *Fusarium coccophilum* BCC2415 exopolymer contained more than 69% protein and less than 20% sugars, whereas EPSs of the other studied fungal strains contained sugars over the range of 40 and 90%, and the protein content ranged from 1 to 10% [49]. High protein content might be attributed to the trapping of considerable amounts of exoenzymes in the EPS [54] or to an overvaluation of the protein content because humic substances and their phenolic compounds are not taken into account [55]. Oil emulsifying capacity of EPSs depends mainly on its constituents of carbohydrate and attached protein [56].

Phenolic components

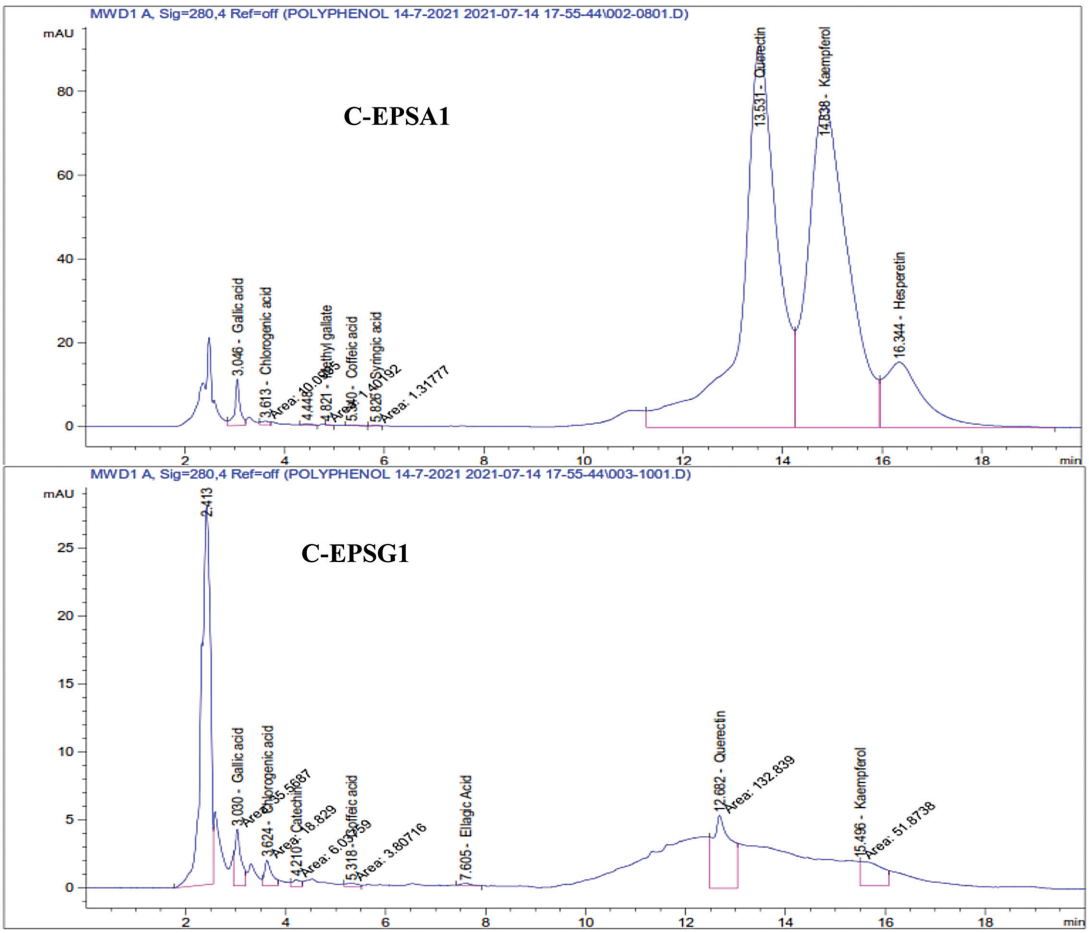
Phenolic compounds are a diverse group of bioactive compounds with various applications in nutraceutical, pharmaceutical, and cosmetic industries. Although plant is the main source of phenolic compounds, different microorganisms were also found to synthesize such compounds as secondary metabolites through two general routes: the shikimic acid and the acetic acid pathways [57–60]. Many phenolic compounds are readily combined with water-soluble polysaccharides through covalent or noncovalent bonding in the food system [61,62]. Limited studies revealed the conjugation of phenolic compounds with microbial EPS [48,63]. The results obtained from the current study highlighted the presence of certain phenolic compounds coupled with *Rhodotorula* C-EPSs. TPCs estimated spectrophotometrically by the FC method were 43.22 and 39.39 mgGAE/g for C-EPSA1 and C-EPSG1 derived from *R. mucilaginosa* and *R. taiwanensis*, respectively (Table 1). The free phenolic components of C-EPSA1 determined by HPLC were 33.22 mg/g, of which flavonoids accounted for more than 97% (quercetin, kaempferol, and hesperetin accounted for 17.05, 13.75, and 1.63 mg/g, respectively). Hydroxybenzoic as one of the main groups of phenolic acids accounted for more than 2%, where gallic acid was recorded in the highest concentration (0.724 mg/g) compared with syringic acid (0.004 mg/g) and methyl gallate (0.001 mg/ g). Chlorogenic and coffeic acids, as free hydroxycinnamic acids, were detected at 0.054 and 0.003 mg/g, respectively (Table 2 and Fig. 3). In contrast, the FC method showed low selectivity compared with HPLC owing to the overestimation of the TPCs conjugated with C-EPSG1 of *R.*

Table 2 High-performance liquid chromatography analysis of phenolic compounds conjugated with crude exopolysaccharide A1 and crude exopolysaccharide G1 (mg/g) derived from *Rhodotorula mucilaginosa* and *Rhodotorula taiwanensis*, respectively

Free phenolic contents	C-EPSA1	C-EPSG1
Gallic acid	0.724	0.195
Chlorogenic acid	0.054	0.060
Catechin	ND	0.026
Methyl gallate	0.001	ND
Coffeic acid	0.003	0.006
Syringic acid	0.004	ND
Ellagic acid	ND	0.008
Quercetin	17.053	0.302
Kaempferol	13.749	0.1000
Hesperetin	1.627	ND
Total HPLC	33.22	0.696
Total phenolic compounds (FC) (mg_{GAE}/g dry substrate)	43.22	39.39

C-EPS, crude exopolysaccharide; FC, Folin-Ciocalteu; HPLC, high-performance liquid chromatography.

Figure 3



HPLC analysis of free phenolic components of C-EPSA1 and EPSG1 derived from *Rhodotorula mucilaginosa* and *Rhodotorula. taiwanensis*, respectively. C-EPS, crude exopolysaccharide; HPLC, high-performance liquid chromatography.

taiwanensis. Only 1.671 mg/g of TPCs (39.388 mg/GAE g) was determined by HPLC, of which only 41.66% of peak areas were identified (0.696 mg/g) and 58.34% were not identified (0.975 mg/g). Such discrepancy may be, on the contrary, due to the high protein content (542 for C-EPSG1 versus 153.2 mg/g for C-EPSA1); together with other reducing substances, they are considered an important interference factor that leads to an overestimation of the spectroscopic phenolic compounds [64,65]. On the contrary, more complex phenolic compounds could not be identified [57]. Overall, C-EPSG1 contained only seven phenolic components, in much smaller amounts, namely, quercetin then gallic acid and kaempferol (0.302, 0.195, and 0.100 mg/g, respectively). Chlorogenic (0.06 mg/g) and coffee acids (0.006 mg/g) were detected in amounts, somewhat, like those of C-EPSA1. Ellagic acid was detected (0.008 mg/g) as a dark derivative of gallic acid (Table 1 and Fig. 3).

Physicochemical characterization of partially purified exopolysaccharides

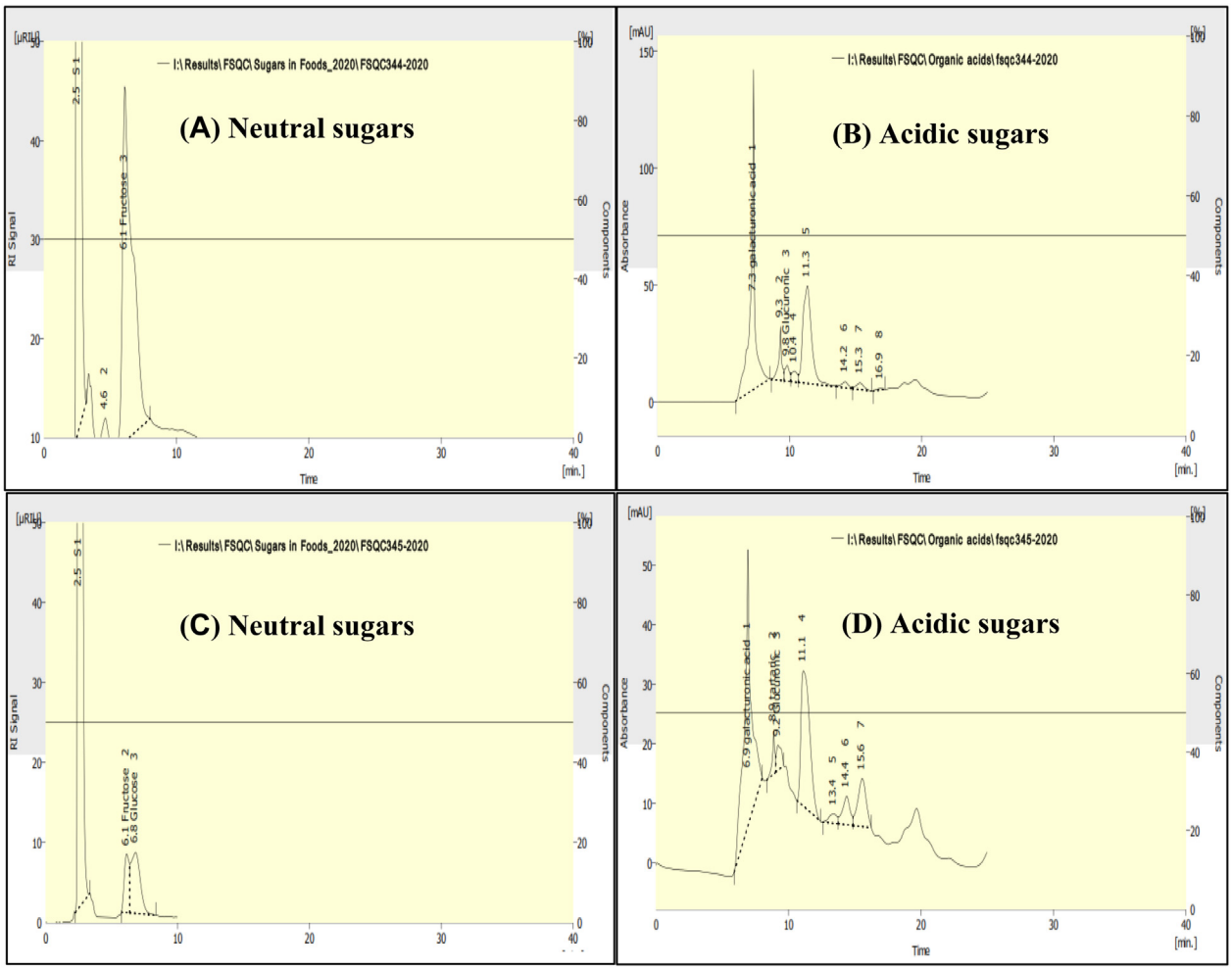
Monosaccharide composition

Hydrolysis time and temperature were the key factors affecting the liberation degree and stability of monosaccharides, whereas TFA concentration had a limited effect. Great loss ratios of peaks area of different released monosaccharides were observed at 110°C for 6 h; the acidic sugars had the highest loss (>50%), then natural sugars (~30–40%), and basic sugars (~20%) [66]. In the current study, the monosaccharide configurations of the EPSs derived from *R. mucilaginosa* and *R. taiwanensis* were hydrolyzed under condition of 2 N TFA at 121°C for 8 h and analyzed by HPLC. Matching the retention time with the reference standard sugars, the data obtained (Fig. 4 and Table 1) demonstrated that the PP-EPSs were acidic heteropolysaccharides and mainly composed of neutral sugars

(69.52–89.69%), whereas the acidic components accounted from 10.31 to 30.48%. Monosaccharide composition of EPSA1 differed, to a great extent, from that of EPSG1. HPLC chromatography (Fig. 4a) for EPSA1 showed two peaks confirming the presence of two neutral sugars, one (at 4.6 min) is unknown and the other was fructose (6.5 min), where it represented the most abundant component (69.52%). Among the eight peaks that appeared at different retention times (Fig. 4b), only two were identified as uronic acids; the galacturonic and glucuronic acids accounted for 23.43 and 7.05%, respectively, of PP-EPSA. Glucose (50.04%) and fructose (39.65%) constituted the predominant components of EPSG as neutral sugars (Fig. 4c). However, among the seven acidic components present (Fig. 4d), galacturonic acid (6.89%), tartaric acid (1.22%), and glucuronic acid (2.20%) were only identified. As previously observed, the monosaccharide compositions of EPSs are widely varied among the producing species and even strains. Such differences disclose the specificity of each microbial strain in producing its own EPS and thus defining its functionality [67,68]. To our knowledge, there are no data available regarding the production and/or monosaccharide composition of EPS synthesized by *R. taiwanensis*. Its polysaccharide seems to be unique in containing tartaric acid. Through a literature review, tartaric acid has not been identified as a component of EPSs produced either by yeasts or bacteria; thus, this is likely to be a novel EPS. Only one early study described an unusual structure of an acidic polysaccharide isolated from Pleurochrysis carterae, which contained glucuronic, galacturonic, tartaric, and glyoxylic acids in equal molar ratios [69].

The monosaccharide composition of *Rhodotorula* EPSs, in this study, was quite different from those produced by some other species belonging to this genus as reported previously. Except for the EPS derived from *Rhodotorula glutinis* KCTC 7989 which was identified as a uronic acid/mannose-rich exopolymer

Figure 4

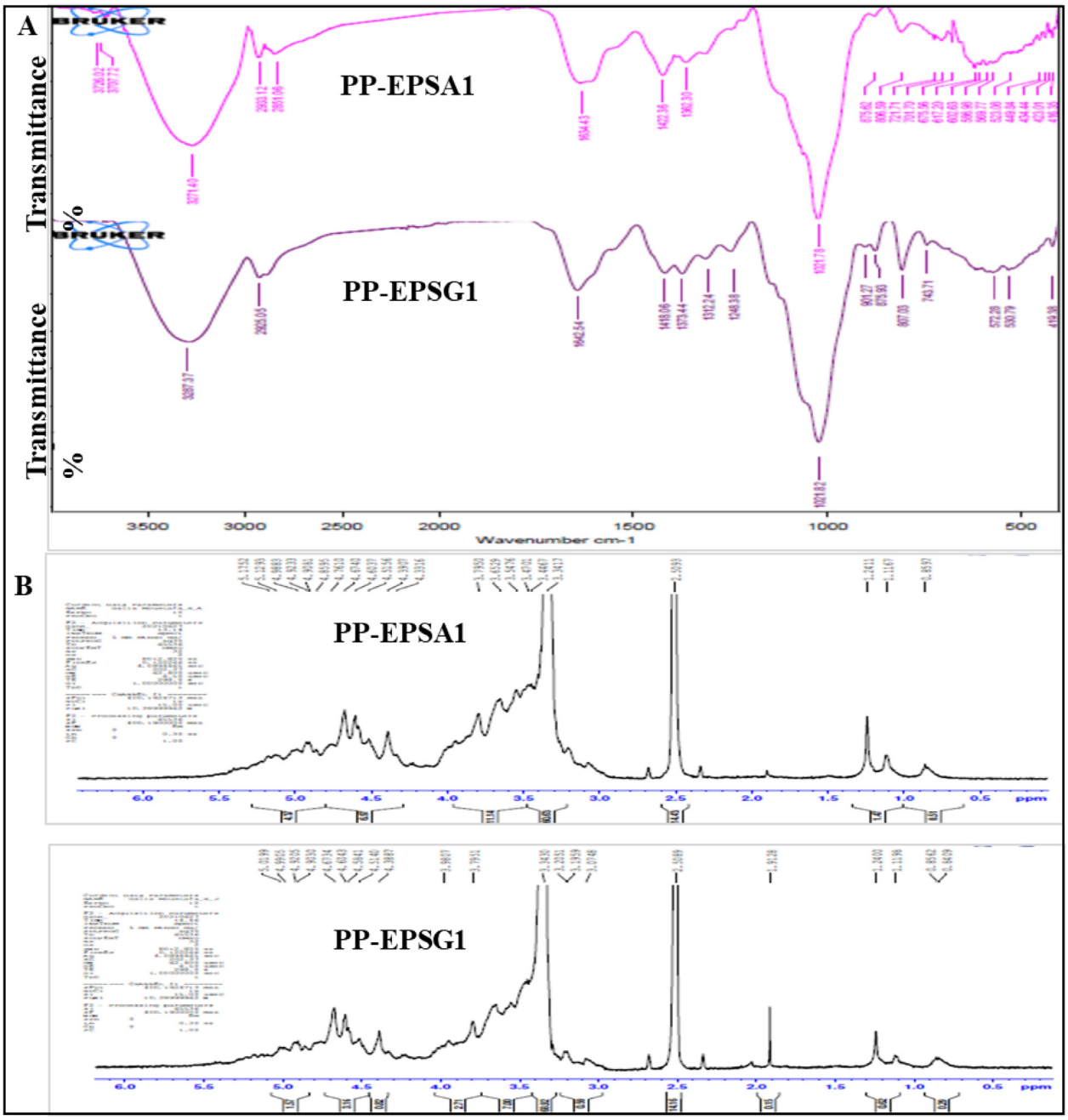


HPLC chromatograms of the monosaccharide of PP-EPSA1 produced by *Rhodotorula mucilaginosa* (a and b) and PP-EPSG1 produced by *Rhodotorula taiwanensis* (c and d). HPLC, high-performance liquid chromatography; PP-EPS, partially purified exopolysaccharide.

[44], the EPSs produced by other strains of *Rhodotorula* including *R. mucilaginosa* were heterogeneous and characterized by the presence of mannose mainly among the neutral sugars and the absence of acidic sugars [12,70–73]. The differences in EPS composition may be owing to the meager separation of monosaccharides by different chromatographic analyses besides analysis conditions [74]. In a prior study conducted by Dogan *et al.* [39] to characterize and quantify monosaccharides of EPS derived from *Bacillus licheniformis* B22 using LC-MS and HPLC techniques, differences in both amount and monosaccharide type were observed. Glucose was determined by both analyses, whereas mannose, rhamnose, galactose, arabinose, and xylose were only identified by LC-MS, and maltitol, fructose, sorbitol, and xylitol were only determined by HPLC.

In general, the monosaccharide composition determines the main structure that eventually affects the polysaccharide hydrodynamic configuration, and thus, its rheological properties. A strong association

Figure 5



FT-IR (a) and ^{1}H-NMR (b) spectra of PP-EPSA and PP-EPSG derived from *Rhodotorula mucilaginosa* and *Rhodotorula taiwanensis*, respectively. FT-IR, Fourier transformation infrared; ^{1}H-NMR, proton nuclear magnetic resonance; PP-EPS, partially purified exopolysaccharide.

was observed between EPS content of uronic acid and its flocculation and antioxidant activities as well as heavy metal binding capabilities [75,76]. Thus, our acidic polymers can be exploited in various fields of biotechnology.

Fourier transformation infrared analysis

The FT-IR spectra of the PP-EPSA1 and PP-EPSG1 derived from *R. mucilaginosa* or *R. taiwanensis,* respectively, are presented in Fig. 5a, which display a variety of typical absorption bands of polysaccharides as extracted from existing literatures. The characteristic absorption bands appearing at 3271.40 and 3287.37 cm^{-1} were attributed to -OH stretching group, which characterize the carbohydrate ring in EPSs. Incidence of a large number of -OH increases their affinity for binding water molecules, which is responsible for the solubility of the EPS [72,77]. The absorption bands at 2933.12–2851.06 cm^{-1} specified weak (–CH–) stretching, conforming to the methylene group [12,77–79]. The peaks appearing at 1634.43 and 1642.25 cm^{-1} were ascribed to the asymmetric extending vibration of the carbonyl group (C=O) and also amide I (C-N) [80,81]. The bands emerging around 1422.36 and 1248.38 cm^{-1} may be derived from the C–O stretching frequency and deformation vibrations of O–H (alcohols and phenols) [67,80]. Peaks at wavenumbers of 1362.30, 1373.44, 1312.24, and 1248.38 indicated the typical absorption of carboxyl or carboxylate groups [79,81,82]. Strong absorption peaks at 1021.78 and 1021.82 cm^{-1} pointed to the glycosidic bond C–O–C and pyranose ring, which affirmed the existence of polysaccharides [82–84]. The absorption in the area between 807 and 901 cm^{-1} pointed out the occurrence of sulfate groups (S=O and C-O-S) [80,85,86]. The negative charges of the EPSs that were conferred by sulfate groups and COO^- make such product of medical and biotechnological importance [76,87]. Occurrence of the α-configuration of pyranose glycosidic residue was specified at the bands appeared between 807.03 and 901.27 cm^{-1} [88]. Furthermore, the distinctive weak absorptions within the area 701.70 and 743.71 might point out toward the occurrence of β-glycosidic linkages [68,89]. Moreover, weak peaks emerging at low frequencies of wavenumbers below 700 cm^{-1} might specify the incidence of glycosidic bonds in the polysaccharide [68]. Altogether, these spectra corroborate the presence of carbohydrates in the EPSs.

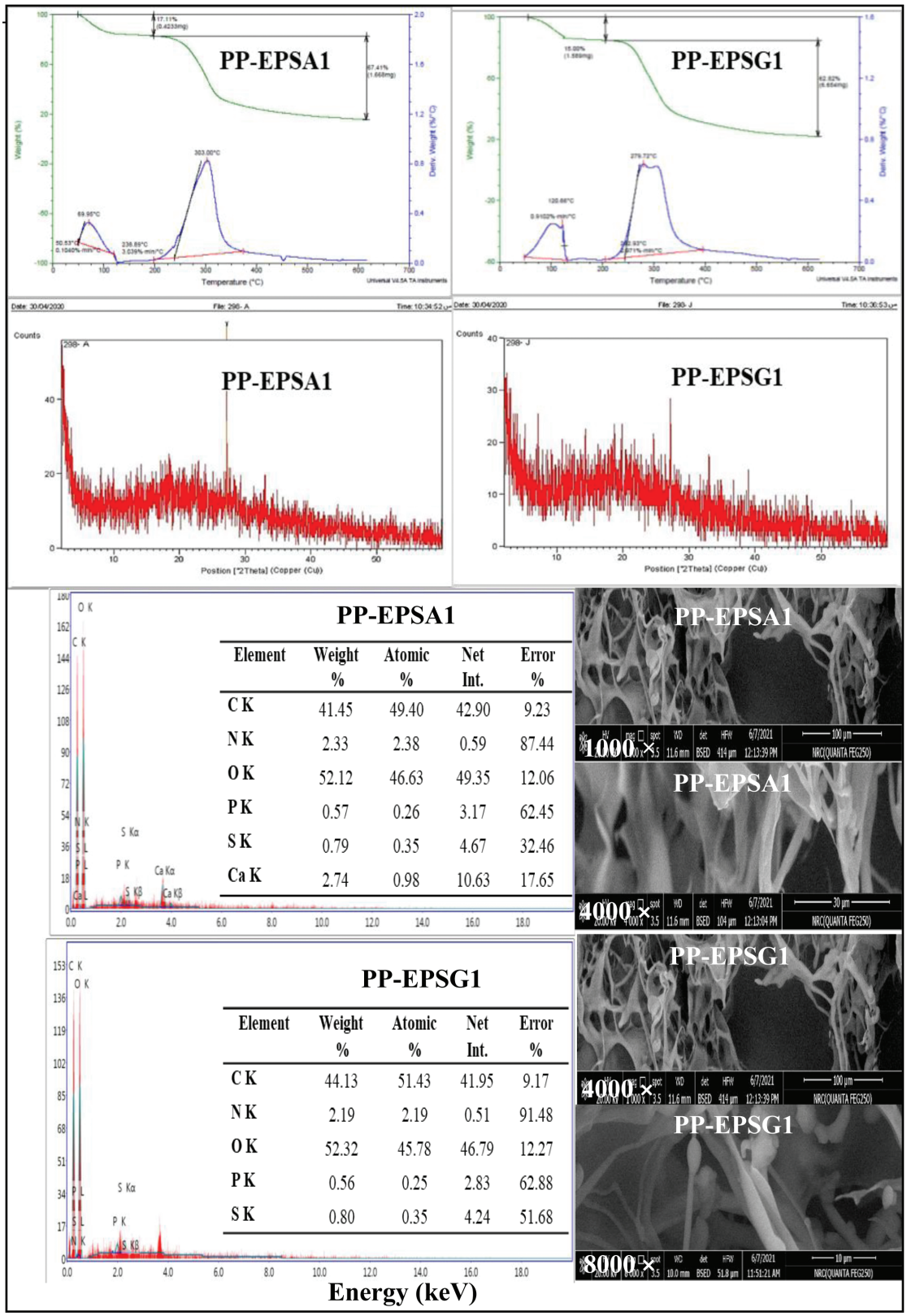
Proton nuclear magnetic resonance analysis

The ^{1}H-NMR spectra of the PP-EPSA1 and PP-EPSG1 produced by *R. mucilaginosa* and *R. taiwanensis*, respectively, are illustrated in Fig. 5b. The peaks were allocated comparing with the chemical action data described in the literature. In general, the ^{1}H-NMR spectra of polysaccharides predominantly comprised three major regions characterizing such compounds; the signals located within each region often serving as signatures to characterize polysaccharide structures [38,78,90]. In the present study, both PP-EPSs showed almost similar chemical shifts, indicating the presence of the common characteristic of polysaccharides. The anomeric region (δH 4.3–5.17) revealed the incidence of α-anomeric and β-anomeric protons; the types of bond are frequently 1→3, 1→4, or 1→6 attributable to the presence of chemical shifts between δH 4.3 and δH 4.8 ppm and δH 4.9 to δH 5.17 ppm, respectively [78,91]. Regarding glycosidic linkage type/position, such chemical shifts pointed toward the existence of diverse monosaccharides in repeating units [92]. The peak at 4.85 ppm in EPS produced by *R. mucilaginosa* A1 might be attributed to H-1 of β-D–mannan as described for *R. mucilaginosa* YR-2 by Takita *et al.* [93] and *R. mucilaginosa* sp. GUMS16 by Hamidi *et al.* [72]. Moreover, the major signals at δH 5.17, δH 4.98, δH 4.92, and δH 5.019, δH 4.99, δH 4.903 ppm, respectively (Fig. 5b), were assigned as α-(1→6) linked glucan as described by Maina *et al.* [94]. An *et al.* [95] attributed the signals at δH 4.993 and δH 5.047 ppm to the presence of H1 of glucose and mannose units, respectively, in the glucomannan derived from *Amorphophallus panomensis*; thus, it could be suggested that the unidentified sugar in PP-EPSA1 structure is a mannose. The ring proton region ranging between δH 3.3 and δH 4.5 showed signals corresponding to many sugar residues; such region is characteristic for polysaccharides. As reported by Poli *et al.* [96] and Alexandersson and Nestor [97], the signals at δH 3.2 and δH 4.4 ppm are thought to be protons of the pyranose or furanose rings. Finally, the alkyl region ranged between δH 1.1 and 1.9. Kavita *et al.* [90] attributed the signals at δH 1.1–1.5 ppm to the presence of a methylene group. Such remark was found to be compatible with the characteristic absorption bands of both EPSs at 2933.12–2851.06 cm^{-1} in the FT-IR spectra of the present study. Moreover, strong signals at δH 2.5 ppm in both EPSs confirmed the presence of acetyl group, as previously decided by Kavita *et al.* [90] and Biswas *et al.* [98].

Thermogravimetric analysis

The thermogravimetric profiles of the PP-EPSA1 and PP-EPSG1 produced by *R. mucilaginosa* and *R. taiwanensis*, respectively, are shown in Fig. 6a. It is

Figure 6



Physicochemical characters: thermogravimetric mass loss at heating rate of 10°C/min (a); (b) X-ray diffractograms; (c) elemental analysis by energy dispersive X-ray analysis and scanning electron micrograph of PP-EPSA1 and PP-EPSG1 produced by *Rhodotorula mucilaginosa* and *Rhodotorula taiwanensis*, respectively. CK, carbon; NK, nitrogen; OK, oxygen; PK, phosphorus; SK, sulfur; Ca, calcium. PP-EPS, partially purified exopolysaccharide.

clearly observed that the degradation of both PP-EPSs occurred at two stages. At first, 17.11 and 15.00% losses in weights of EPSs, respectively, were observed at around 200°C. Upon increasing the temperature, a great loss of EPSs was observed at more than 200–600°C to become 67.41 and 62.82%, respectively. The initial weight loss is due to the loss of the polymer's water absorbed that increases with increasing the number of carboxylic groups [99]. Besides, the great dropping in weights at temperatures higher than 200°C was attributed to the degradation of the polymer by breaking the bonds between C–O and C–C and subsequently the evolution of CO, CO_2, and H_2O [16,100].

For each EPS, two different peaks appeared at 69.95 and 303.00°C, with an average of 238.89°C, and 120.66 and 279.72°C, with an average of 282.93°C, for PP-EPSA1 and PP-EPSG1, respectively (Fig. 6a). Each range of the exothermic process showed a considerable thermal change of amorphous state of EPS into a crystalline state [101,102]. In general, the thermal decomposition process includes three stages termed dehydration, decomposition, and chemical bond reorganization [103]. The present study clearly showed the resistance of PP-EPSs produced by *R. mucilaginosa* A and *R. taiwanensis* G to high temperatures and thus the possibility of its commercial application, especially in the field of food industry.

X-ray diffraction analysis

XRD is an influential means to assess the crystallization/amorphous characteristics of the polysaccharides. The crystallinity of polysaccharide is recognized by the strong, narrow, and sharp peaks, whereas the small broad peaks indicate the amorphous component of the polymer [16,81,104]. As shown in Fig. 6b, the XRD patterns of PP-EPSA1 and PP-EPSG1 produced by *R. mucilaginosa* and *R. taiwanensis*, respectively, disclosed the presence of 'bulge-shaped diffraction peaks,' which mainly characterize the amorphous nature and low crystallinity of EPSs. The analysis of XRD charts exhibited the presence of a distinctive diffraction peaks at 27.1590 and 27.1942°C with d-spacing of 3.28073Çº and 3.28190 Çº, respectively (Table 3). Generally, the restriction of crystallinity indicates both PP-EPSA1 and PP-EPSG1 are semicrystalline or poorly crystalline polymer. These results match well with those previously obtained by many researchers [16,39]. Although the crystalline areas in a polymer act as a mainstay and upgrade its thermodynamic properties, the amorphous nature makes it able to dissolve rapidly providing great solubility and bioactivity. This superiority in the bioactivity is considered a positive advantage of the amorphous form of the polymer [81,105].

Surface morphology of exopolysaccharides and elemental analysis

The surface morphology and topography of the PP-EPSA1 and PP-EPSG1 derived from *R. mucilaginosa* and *R. taiwanensis* were scanned by SEM at ×1000–×8000. As seen from the Fig. 6c, at both magnifications ×1000 and ×4000, the microstructure of EPSA1 appears as smooth porous web-like structure with smooth surface. Interestingly, there is a clear difference between the EPSA1 and EPSG1 in microstructure, but they are similar in porosity and surface smoothness. SEM images of the EPSG1 at ×4000 and ×8000 (Fig. 6c) revealed rod-shaped with some spherical and irregular strip-like structures with smooth surface and porous nature. The dissimilarity in microstructure and topography of different EPSs have previously been reported [72,106]. It has already been concluded that the porous nature and surface smoothness of EPSs are promising advantages in industrial applications. The porosity of EPS increases the water-holding capacity; thus, it can be used as a thickener, texturizer, viscosifier, stabilizer, drug delivery, and excipient in the field of food and pharmaceutical industries [102,107,108]. Besides, the surface smoothness of EPSs is beneficial for making food packaging films and plasticized bio-films [109,110].

EDX is an elemental spectroscopy technique that permits for a quick, effectual, and accurate analysis

Table 3 X-ray diffraction patterns of partially purified exopolysaccharide A1 produced by *Rhodotorula mucilaginosa* and partially purified exopolysaccharide G1 produced by *Rhodotorula taiwanensis*

PP-EPSs	Peak's number	Position at 2θ	d-spacing Å	Height (intensity)	FWHM left at 2θ	Rel. Int. (%)
PP-ERSA1	1	27.1590	3.28073	24.12	0.1440	100.00
PP-EPSG1	1	27.1492	3.28190	18.63	0.1440	100.00

FWHM, full width at half maximum, it is measured at 50% of intensity height of peak on 2θ axis; PP-EPS, partially purified exopolysaccharide. The diffracted X-ray were calculated with Bragg's law using the International Center for Diffraction Data base file program.
d-spacing describes the distance between planes of atoms that give rise to diffraction peaks. Each peak in a diffractogram results from a corresponding d-spacing.

to determine the elemental composition of any material that can be imaged in an electron microscope. The quantitative elemental compositions of PP-EPSA1 and PP-EPSG1 produced by *R. mucilaginosa* and *R. taiwanensis*, respectively, are shown in Fig. 5c. The EDX microanalysis revealed the similarity between both PP-EPSs, where oxygen and carbon were the major elements with weight percentages of 52.12 and 52.32, and 41.45 and 44.13, respectively. The high bulk % of those two elements highlighted that both PP-EPSs are comprised mainly of carbohydrates. Such results were harmonious with those obtained from the elemental analyses of EPSs from *L*actobacillus *delbrueckii* subsp. *bulgaricus* NCFB 2483, *L. delbrueckii* ssp. *bulgaricus* B3, *Fomitopsis meliae* AGDP-2, and *Lactiplantibacillus plantarum* EI6 [74,79,111,112]. Nitrogen was also present in close proportions, scoring 2.33 and 2.15% in both PP-EPSs, respectively (Fig. 5c). Other elements including phosphorous (0.57, 56%) and sulfur (0.79, 80%) were also found in similar proportions in both EPSA and EPSG; the only variance between them was the presence of calcium in the former in a relatively high ratio (2.74%) and its absence from the latter. As specified by Synytsya *et al.* [113], polysaccharide-rich/purified samples should not contain nitrogen or may show only tiny proportion up to 1%. The presence of N in proportions greater than 1% may be derived from conjugated proteins with EPs that was not able to be separated during deproteinization and dialysis processes [74,112,114]. Furthermore, Zaghloul and Ibrahim [112] stated that the presence of nitrogen and phosphorus at levels of 2.15 and 0.99%, respectively, suggested the deficiency of deproteinized and dialyzed EPS produced by *L. plantarum* to linked protein and phospholipids. High Ca^{2+} concentration might be attributed to the higher content of acidic sugars in PP-EPSA1 and subsequently the anionic groups that bind with cations in the medium [115]. Generally, elements other than O, C, and H were always present in trace amounts and irregularly among the elemental compositions of the different polysaccharides as extracted from the literature [11,68,110].

Conclusion

The results obtained provide valuable information and support the production of biologically active compounds by yeasts. C-EPSs derived from *R. mucilaginosa* A1 and *R. taiwanensis* G1 were conjugated with protein and phenolic compound production. Phenolic components as natural antioxidants increase the chance to avoid using synthetic antioxidants that are added to foods and pose a health risk. The presence of the attached protein in high concentration increases its ability to emulsify oils. PP-EPSs were acidic heteropolysaccharides composed of neutral sugars (69.52–89.69%) and acidic ones (10.31–30.48%). PP-EPSG1 is likely to be a novel EPS as it contained tartaric acid (1.22%) besides uronic acid. The properties of PP-EPSs in terms of their resistance to high temperatures, porous nature, surface smoothness, and the net negative charge of EPSs conferred by anionic acids and sulfate groups give them promising advantages in the medical, pharmaceutical, food, and other industrial sectors.

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Authors contribution: F.M.R., H.K.E., M.A.M., F. A., and A.E.M. conceptualized and designed the research. D.M.M. conducted experiments. D.M.M., M.A.M., and F.M.R. analyzed the data. F.M.R. and M.A.M. wrote the original manuscript. All authors have read and approved the manuscript.

Conflicts of interest

There are no conflicts of interest.

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