Exopolysaccharide production from agro-industrial wastes by lactic acid bacteria isolated from silage

Ahmed R. Henawy[a], Abdelhadi A. Abdelhadi[b], Asmaa A. Halema[b], Refae I. Refae[a], Olfat S. Barakat[a]

[a]Department of Microbiology, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt, [b]Department of Genetics, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt

Correspondence to Ahmed R. Henawy (MSc), Department of Agriculture Microbiology, Faculty of Agriculture, Cairo University, El-Gamaa St., P.O. Box 12613, Giza, Cairo, Egypt. Tel: +01011578381; fax: +(202)35717355; e-mail: ahmed.ragab1@agr.cu.edu.eg

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Background and Objectives
Lactic acid bacteria (L.A.B.) can produce exopolysaccharides (EPSs) using agricultural and industrial waste materials. This approach can prevent the harmful disposal and buildup of these wastes in the environment in addition to producing valuable products. Thirteen LAB-producing EPS isolates were selected, and the similarity and distance indices were determined between them through Rep-PCR DNA fingerprinting, and molecularly identified LAB from silage samples. Evaluation of the ability of the isolated strains to produce exopolysaccharides was carried out, in addition to the optimization of the polysaccharides from renewable resources.

Materials and methods
LAB-producing EPS isolates were molecularly identified by the 16S rRNA gene sequencing and deposited their DNA sequences to NCBI. EPS production using the examined 13 strains was carried out on MRS as a standard production medium and ranged between 1.53 and 7.53 g/l. Then, the highest significant EPS-producing strains i.e., *Lacticaseibacillus paracasei* strain LAB 64, *Lacticaseibacillus rhamnosus* strain LAB 160, and *Lacticaseibacillus rhamnosus* strain LAB 192 were further examined for EPS production from the agro-industrial wastes sugarcane molasses, salted cheese whey, and their mixture.

Results and conclusion
The maximum EPS production by the three strains was obtained in a mixture of molasses: whey (1/1 v/v). Calcium carbonate addition to the production mixture significantly improved EPS production in almost all cases and it is important to neutralize the media. Moreover, increasing the mixture sugar concentration of the fermentation mixture from 2% to 5% enhanced EPS production by all strains. In this regard, a 2-fold increment in EPS production was achieved by the *Lactic. rhamnosus* strain LAB 160 22.39 g/l. The extraction and analysis of the EPS product were carried out using both FT-IR and HPLC compared to an EPS standard. FTIR and HPLC analysis confirmed the polymer as an α-glucan, which was identified as dextran through a comparison between its retention time and the retention time of the dextran standard.

Keywords:
agro-industrial wastes, dextran, exopolysaccharides, FT-IR, HPLC, lactic acid bacteria

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Letter to the Editor of Egyptian Pharmaceutical Journal

Introduction

Exopolysaccharides (EPSs) from bacteria have been the focus of increasing research in recent years due to the significant use of natural polymers in a variety of industries. Chemistry, medicine, and the food business have all taken a special interest in bacterial EPSs because of their distinctive structural characteristics [1]. EPSs are frequently utilized as viscous, stabilizing, and emulsifying agents in the food sector due to their capacity to increase water holding capacity [2] to enhance the rheological properties, texture, and sensitivity of bread and dairy products i.e. cheese and yogurt [3]. EPSs have potential health benefits in addition to their technological attributes, such as anti-inflammatory, anticancer, antiviral actions antioxidant, and cholesterol-lowering effects [4–6].

Researchers' interest has been drawn to lactic acid bacteria (LAB) among EPS-producing bacteria because of their potent capacity to create EPSs. According to reports, the LAB strains from the genera *Streptococcus*, *Pediococcus*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Weissellale* can produce EPSs [7]. LAB are capable of producing EPSs with a

variety of various structures without posing any health risks and are considered safe microorganisms (GRAS-Generally Recognized As Safe) [8]. In LAB, EPSs are crucial in regulating the physicochemical properties of the cell surface [9], guarding against harmful environmental effects on bacteria, dehydration, phagocytosis, antibiotics, and phage attacks [10–12]. EPSs participate in the extracellular matrix's structural components, serving as a container for cells during cell membrane development [13].

Based on the EPS's constituent units, it can be split into two categories. Different monosaccharide units, such as xanthan and gellan, make up heteropolysaccharides. Homopolysaccharides, such as glucans and fructans, are made up of repeated units of a single type of monosaccharide (i.e., glucose or fructose). The fructans produced by LAB are levan and inulin, whereas the glucans produced most frequently are dextran, alternan, curdlan, pullulan, mutan, and cellulose [14]. In the food and pharmaceutical industries, these natural polysaccharides have been utilized as transporters, thickeners, encapsulants, binders, additives, and lubricants [15].

Dextran is a bacterial homopolysaccharide mainly composed of consecutive α-(1→6) linked glucose units [16–18]. The dextransucrase enzyme is necessary for dextran production to take place outside the bacterial cell [19]. The most frequently employed enzyme for dextran biosynthesis at the industrial level is dextransucrase [20,21]. In the pharmaceutical and food industries, dextran is a commonly used and readily available EPS with several applications [22]. Previous applications of dextran as the delivery system of a nano-based drug include the delivery of anticancer agents [23] and the targeted doxorubicin delivery to cancer cells [24]. Dextran is yet another intriguing possibility for oral insulin delivery [25]. Dextran has a high-water solubility and exhibits no cellular toxicity after drug delivery. Dextran is also completely digested by the body, which lowers the risk of renal failure and makes it a highly desirable nanomedicine, cell imaging system, nano-drug carrier, or nanobiosensor [22,26,27].

Agro-industrial wastes are produced in large quantities and at high rates, and they have been thoroughly researched to yield metabolites of industrial importance like alcohols, organic acids, microbial biomass, proteins, biohydrogen, methane, biobutanol, and EPS [28–30]. The use of a biobased economy has grown significantly in recent years, and it is critical to identify the most promising pathways to support scientific and technological advancements for waste valorization [31]. The aim of this work was directed to the isolation and screening of EPS-producing LAB, identification and characterization of the most promising EPS producer isolates using molecular techniques, studying some factors affecting their production, studying the effect of sugar and $CaCO_3$ concentrations on EPS production, and characterizations of EPS structure and polymer type using FTIR and HPLC analysis.

Material and methods

Silage and agro-industrial wastes

Conventional corn silage samples were collected from the deep inner layer of a small-scale farmer batch in Om-dinar farmer Embaba, Giza, Egypt in December 2019 in sterilized clean bottles. Egyptian sugarcane molasses was obtained in June 2021 from the Integrated Sugar Industries Company, El Hawamdia, Giza, Egypt. and clarified according to Dumbrepatil *et al*., [32]. The salted cheese whey was kindly provided by the Dairy Science Department, Faculty of Agriculture, Cairo University.

Isolation of lactic acid bacteria

Serial dilutions from each silage sample were prepared to get dilutions up to 10^{-7}. For the isolation of organisms, 1 ml of each dilution was plated onto De Man, Rogosa, and Sharpe (MRS) [33]. The plates were incubated for 48 h at 42°C. Single colonies were purified twice and the pure isolates were grown on MRS broth at 42°C for 24 h and stored at −20°C with 20% glycerol [34] or stored at −80°C until usage.

Screening for EPS-production by LAB isolates

To identify the mucoid visual appearance, individual colonies were transferred to modified MRS-agar plates containing 2% sucrose and cultured at 42°C for 48 h. The isolates were divided into three categories: strong, normal, and light based on how they appeared. By growing on a semi-defined medium (SDM-agar), the visually promising LAB isolates were further validated for ESP synthesis [35]. The capacity of chosen isolates to create EPS in various liquid media, including freshly activated 5% (v/v) inoculum in 250 ml screwcap bottles containing 100 ml of MRS broth at 42°C for 48 h. Using HCl 1M, all media were brought to a pH of 6.5 ±0.2 before being autoclaved for 15 min at 121°C.

Production of EPS by LAB isolates from agro-industrial wastes

MRS media was used in EPS production as a standard media. As well as agro-industrial wastes like sugarcane molasses and salted cheese whey were used in the

optimization and production of EPS using the highest EPS-producing isolates. Egyptian sugarcane molasses obtained in June 2021 from the El Hawamdia factory for the Integrated Sugar Industries Company, El Hawamdia, Giza, Egypt was clarified according to the method Dumbrepatil *et al.*, [32]. The Dairy Science Department at Cairo University's Faculty of Agriculture provided salted cheese whey. Sugarcane molasses was clarified according to the method Dumbrepatil *et al.*, [32] before use. The salted cheese whey was boiled for 20 min and chilled. After passing through muslin cloth and Whatman filter paper No. 1 [36], the sterilization of clear filtrate was done for 15 min at 121°C and stored at 4°C until use. Optimization of process parameters for the EPS production by the examined LAB isolates was carried out in batches in 250 ml screw-capped bottles containing ml working volume of either molasses, salted cheese whey, or molasses: whey mixture (1: 1 v/v) containing either 2% or 5% sugar concentration). The fermentation brew was supplemented with 0.5% yeast extract and inoculated with 5% (v/v) of the 48-h-old culture of the examined bacterial candidate then incubated at 42°C on a rotary shaker (150 rpm) for 48 h. To study the effect of sugar concentration on EPS-production by the examined bacterial isolates, another set of experiments were carried out as mentioned before with a 5% sugar concentration. Additionally, the effect of applying different calcium carbonate ($CaCO_{3)}$ concentrations i.e., 0, 0.5, 1, 2, and 5%(w/v) to the fermentation brew on EPS production by the different LAB isolates were examined using either MRS media as a control., molasses, whey, or their mixture.

Rep-PCR DNA fingerprinting

The genetic diversity analyses were carried out by enterobacterial repetitive intergenic consensus (ERIC), repetitive extragenic palindromic (Rep), and BOX sequence-specific primers [37] of thirteen LAB isolates and the sequences of the primers were mentioned in Table 1. Rep-PCR DNA fingerprinting was performed with the primers BOXA1R, ERIC2R, and $(GTG)^5$. DNA patterns were analyzed using PyElph 1.4 program. Dendrogram, similarity and distance index were carried out by the Past program.

Bacterial identification of LAB isolates by *16S rRNA* gene sequencing

Genomic DNA was obtained for molecular identification using the Simply^{TM} Genomic DNA Isolation Kit (Gene Direx, Inc. cat. no. SN023-0100, Taiwan) in accordance with the manufacturer's instructions. For the *16S rRNA* gene, two universal primers (27F and 1492R) were employed, and their sequences are reported in Table 1 [38]. PCR reaction proceeded in accordance with the manufacturer's instructions of the OnePCR^{TM} master mix (Gene Direx, cat. no. MB203-0100, Taiwan) and the annealing temperature of each primer was listed in Table 1. PCR product purification was performed utilizing ExoSAP-IT^{TM} PCR Product Cleanup Reagent (Applied Biosystems, USA, cat. no 78201) and sequencing the purified DNA at Sangon Biotech Co., Ltd, Macrogen, Korea. The alignment of *16S rRNA* gene sequences of the isolates and their closely related strains was done by Clustal Omega version 1.2.4 [42]. The submission of the bacterial isolates' sequences was already done to the GenBank database and the comparison with published sequences in the same database was utilized using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Trimming the ends was carried out by trimAl version 1.4.rev22 [43]. Highly homologous sequences were selected and aligned utilizing CLUSTAL OMEGA. A phylogenetic tree was constructed by MEGA 11 utilizing the Maximum Likelihood method under the Kimura 2-parameter model, bootstrapping was performed on 1000 bootstrap replications to assess the data.

EPS extraction and determination

At the end of the fermentation period with all batches as mentioned above, 100 ml of the tested bacterial cultures were pipetted out and centrifuged at 10,000 for 20 min. The filtrate was used to estimate the

Table 1 Primers used in this study

Genes	Primer sequence	Annealing temperature	Reference
16S rRNA			
27F	5′-AGA GTT TGATCMTGG CTC AG-3′	58°C	[38]
1492 R	5′-TAC GGY TAC CTT GTT ACG ACTT-3′		
Rep-PCR DNA fingerprinting			
BOXA1R	5'CTACGGCAAGGCGACGCTGACG3'	52°C	[39]
$(GTG)^5$	5'-GTGGTGGTGGTGGTG-3'		[40]
ERIC2R	5′-ATG TAA GCTCCT GG GGA TTC AC-3′		[41]

extracellular polysaccharide using the ethanol extraction method as described by Shi [44]. The filtrate was homogenized and heated at 95°C for 6 h. The extracts were filtrated through Whatman No.2 filter paper or centrifuge at 10,000 rpm for 5 min, then precipitated with 4 times 95% ethanol, stirred vigorously, and left overnight at 4°C. The precipitate of exopolysaccharide was recovered by centrifugation at 10,000 rpm for 15 min and discard the supernatant followed by drying the pellets at 60°C for 72 h.

Analysis of polymer

The obtained EPS polymer was characterized using both HPLC and FT-IR analysis. FT-IR analysis of the polymer sample was studied using NICOLET 380 FT-IR, Thermo Scientific (China) [45]. Quantitative and qualitative HPLC analysis were performed on partially purified EPS produced by the examined LAB strains to determine α-glucans (adopting the Waters Alliance 2695 Separations Module) using column Benson polymeric Bp100 Ca, Waters 2410 HPLC Refractive Index Detector with a mobile phase of DI H_2O, a flow rate of 0.4 ml/min, column temperature 85°C, sample size 20 µI (30 mg/ml), and column size 100×7.8 mm [46].

Statistical analysis

R programming language used for statistical analysis; the main effects' significance was determined by Analysis of variance (ANOVA) which was measured by LSD and was performed at ($P < 0.05$). All analysis was made by R using an Agricolae package for statistical analysis [47], and ggplot2 for data visualization [48]. All analyses were performed in triplicate, and the mean values were reported.

Results and discussion

Isolation and screening of EPS-producing bacteria for EPS production

One hundred bacterial isolates obtained from the conventional silage were isolated on MRS media. These isolates were screened for EPS production by cultivation on a modified MRS-agar, and SDM media to detect and evaluate their potential mucoid visual appearance. Among them only thirteen isolates showed different degrees of "ropiness" as shown in Table 2. The isolate with the mucoid and slimy colony was used as the source of tentatively considered an EPS-producer [49,50]. All thirteen isolates were Gram-positive nonspore-forming, bacilli, and catalase negative. Among these isolates, three grew *i.e.*, LAB 64, LAB160, and LAB 192 were the superior EPS producing up to 5.33, 7.53, and 5.40 g/l EPS, respectively, and could successively grow on MRS agar containing 6.5% NaCl as presented in Table 2. Therefore, these three isolates were selected for further investigation.

EPS production was examined on MRS liquid media using these thirteen isolates and was found that the isolates coded as (LAB 64,160,192) were the highest significant EPS production as shown in Table 2.

Genetic diversity analysis using Rep-PCR DNA fingerprinting

Rep-PCR DNA fingerprinting analysis was done with three primers of 13 LAB isolates. The total number of bands, polymorphic bands, unique positive bands, % of polymorphism, Polymorphic Information Content (PIC), discriminating power (DP), marker index, and resolving power (RP) were presented in Table 3.

Table 2 Some morphological and biochemical characteristics of the isolated bacteria and their EPS production in MRS media

Isolate code	Gram reaction and Cell shape	Colony mucoid appearance	Catalase test	Growth in NaCl 6.5% MRS	EPS production g/l
LAB 54	+ bacilli	+	–	–	2.43±0.30
LAB 64	+ bacilli	++	–	+	5.33±0.25
LAB 72	+ bacilli	+	–	–	3.33±0.25
LAB 76	+ bacilli	+/–	–	+	1.73±0.15
LAB 84	+ bacilli	+/–	–	+	1.53±0.25
LAB 153	+ bacilli	+	–	–	3.36±0.37
LAB 160	+ bacilli	++	–	+	7.53±0.30
LAB 192	+ bacilli	++	–	+	5.40±0.26
LAB 202	+ bacilli	+	–	–	2.63±0.15
LAB 209	+ bacilli	+	–	+	2.30±0.20
LAB 214	+ bacilli	+/–	–	–	1.70±0.26
LAB 222	+ bacilli	+	–	+	3.33±0.20
LAB 223	+ bacilli	+/–	–	–	1.53±0.30
L.S.D. $P_{>5\%}$					0.41

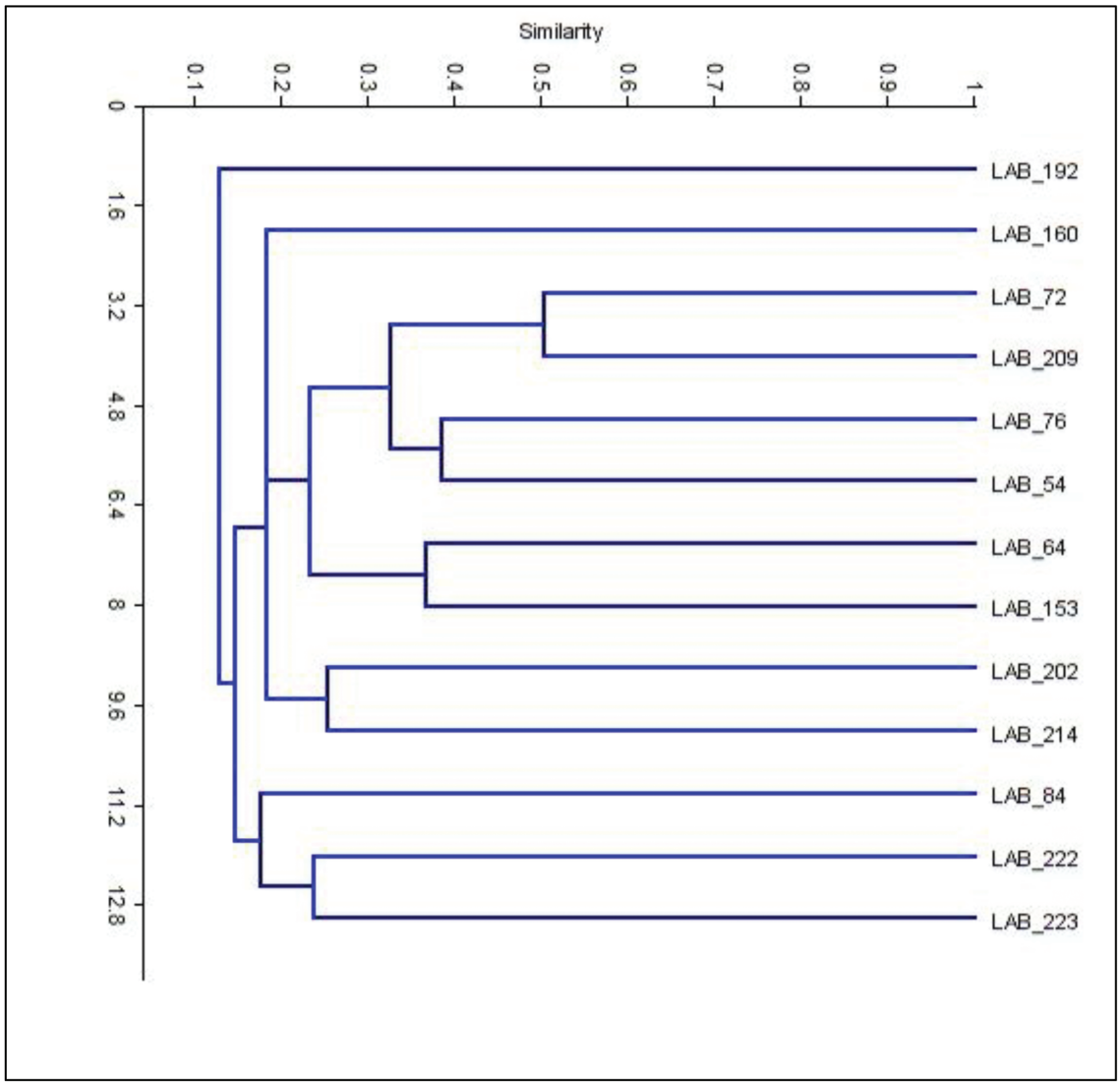
(Gram stain, Catalase test, and Growth in NaCl 6.5%) + = positive, –=negative, **(Mucoid visual appearance)** ++, strong colony mucoid visual appearance; +, normal mucoid visual appearance; +/– light mucoid visual appearance.

Also, the dendrogram based on UPGMA cluster analysis of Jaccard similarity values of combined Rep-PCR fingerprint patterns of the 13 LAB isolates was demonstrated in Fig. 1. Similarity and distance indices were calculated according to Jaccard similarity values and presented in Fig. 2. The total number of bands was 70 fragments and the number of bands by independent primer BoxA1R, ERIC2R, and $(GTG)^5$ was 23, 22, and 25, respectively. All the primers produced 100% polymorphic bands. No unique bands were identified. $(GTG)^5$ amplified the highest number of bands (25 bands). PIC values ranged from approximately 0.21 to 0.31. The marker index ranged from approximately 0.0017–0.0044 as it depends on the percentage of polymorphism and PIC [51]. Resolving power and discriminating power ranged from 7.3 to 10.5 and 0.92 to 0.97 respectively and this indicated that there is a genetic

Table 3 Data of rep-PCR DNA fingerprinting primers and the extent of polymorphism

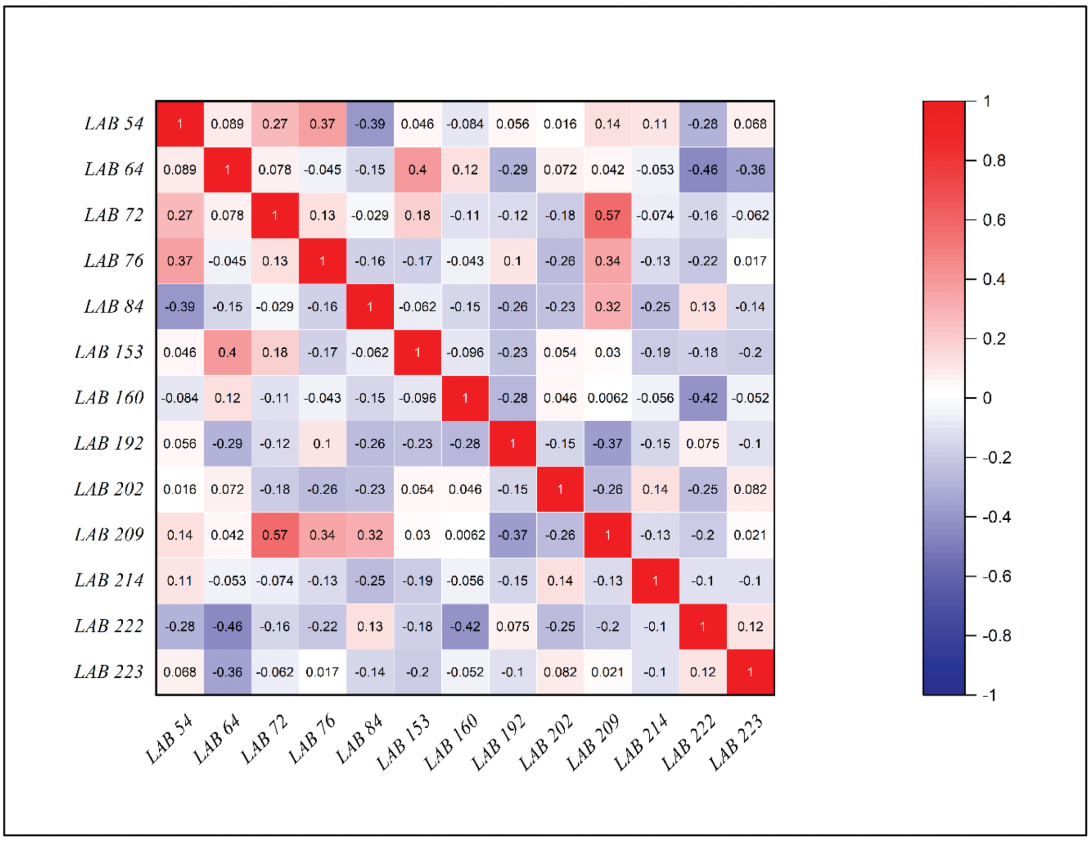
Primer name	Total bands	Polymorphic bands	Polymorphism (%)	Polymorphic Information Content **(PIC)**	Marker Index (MI)	Discriminating power (DP)	Resolving power (RP)
BOX A1R	23	23	100	0.276356	0.003154	0.956443	9.090909
ERIC 2 R	22	22	100	0.316966	0.004457	0.927024	10.58333
$(GTG)^5$	25	25	100	0.218983	0.001748	0.978728	7.333333
Combined	70	70	100	0.267091	0.005236	0.961105	17.33333

Figure 1



Dendrogram based on UPGMA cluster analysis of Jaccard similarity values of combined Rep-PCR fingerprint patterns of the 13 LAB isolates.

Figure 2



Similarity and distance indices according to Jaccard similarity values between the 13 LAB isolates.

diversity between the strains due to the high resolving power and discriminating power of these primers [52,53]. Hence, we can conclude that the 13 isolates may be different.

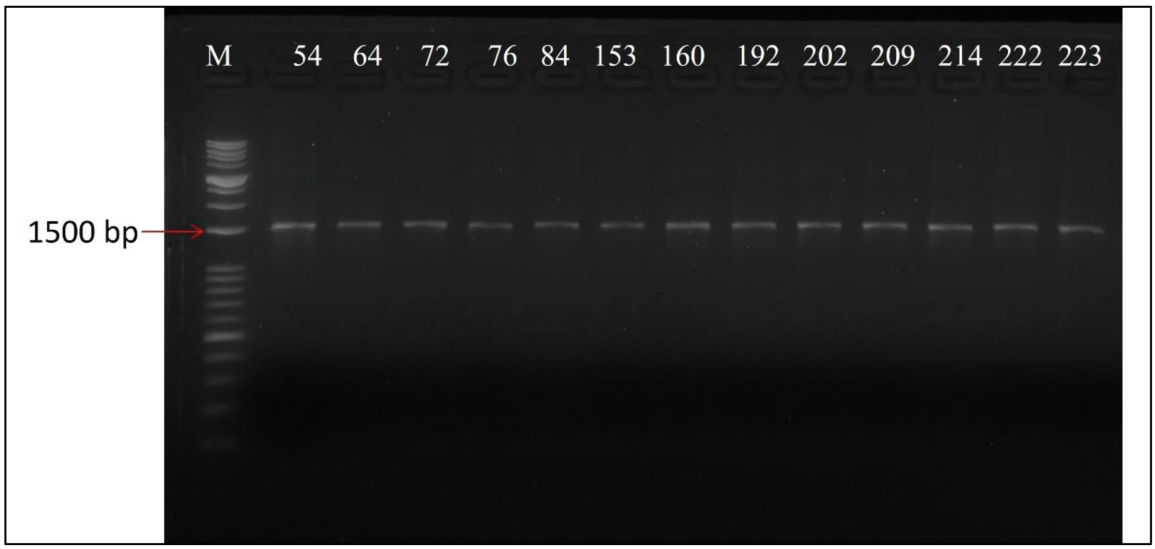
Molecular identification of efficient isolates in EPS production

The *16S Rrna* gene's universal primers were used to molecularly identify the thirteen LAB isolates. The *16S Rrna* gene's approximate 1500 bp was amplified as depicted in Fig. 3 and sequenced. Using BLAST tools, the nucleotide sequence obtained from the *16S Rrna* gene sequencing of the thirteen bacterial isolates was compared with the GenBank databases. Thirteen isolates' partial *16S Rrna* gene sequences were uploaded to the GenBank database. With the strain code LAB, all strains were submitted to NCBI, such as *Lacticaseibacillus paracasei* strain LAB 54, *Lacticaseibacillus paracasei* strain LAB 64, *Lacticaseibacillus rhamnosus* strain LAB 72, *Lacticaseibacillus paracasei* strain LAB 76, *Lacticaseibacillus rhamnosus* strain LAB 84, *Lacticaseibacillus rhamnosus* strain LAB 153, *Lacticaseibacillus rhamnosus* strain LAB 160, *Lacticaseibacillus rhamnosus* strain LAB 192, *Lacticaseibacillus paracasei* strain LAB 202, *Lacticaseibacillus casei* strain LAB 209, *Lacticaseibacillus paracasei* strain LAB 214, *Lacticaseibacillus rhamnosus* strain LAB 222 and *Lacticaseibacillus paracasei* strain LAB 223 under different accession numbers with the percentage of similarity as presented in Table 4. The thirteen strains' similarities varied from 94.35 to 100%. The phylogenetic tree was constructed using the *16S rRNA* gene sequences of the strains and strains that were closely related to them Fig. 4.

Production of EPS from agro-industrial wastes

Once the EPS production on MRS media was assessed, it was discovered that *Lacticaseibacillus paracasei* strain LAB 64, *Lacticaseibacillus rhamnosus* strain LAB 160, and *Lacticaseibacillus rhamnosus* strain LAB 192 exhibited the most substantial production of EPS. EPS production from two agro-industrial wastes (molasses and salted cheese whey) in addition to the mixture between the two agro-industrial wastes with a ratio (1:1) and the final concentration of sugars 2%. was used. These wastes were used as carbon source in the fermentation process due to their low cost and reduction of the environmental pollution resulting

Figure 3



Amplification of 16*S rRNA* gene of 13 LAB strains .1st lane corresponding to 1Kb DNA molecular marker (M).

from their accumulation [54]. Not all the strains have the same performance with these different wastes and the combination between them is presented in Table 5. LAB 64 produced the highest significant EPS concentration by using molasses (9.26 g/l) while, LAB 160 produced the highest significant EPS concentration by using the mixture between salted cheese whey: molasses (1:1) (9.66 g/l). Similarly, when using the mixture of salted cheese whey: molasses (1:1), LAB 192 gave the highest significant EPS concentration (7.33 g/l). Moreover it was observed that using the wastes mixture of salted cheese whey: molasses (1:1) resulted in the highest significant EPS production by the three strains compared to the individual wastes. The higher EPS production by all strains in the fermentation brew contained the mixture of the waste than in the molasses or whey fermentation batches might be ascribed to the fact that the mixture contains more mineral nutrients in addition to the high salt content in the whey [55,56]. Hence, all further investigations were run in batches containing a mixture of the two wastes.

Effect of $CaCO_3$ in the fermentation brew on EPS production by LAB

The effect of $CaCO_3$ addition on EPS production by the three LAB strains was studied using the mixed wastes as motioned before with different concentrations of $CaCO_3$ (0.5, 1, 2, and 5%), and MRS media as a control. The EPS production by *Lacticaseibacillus rhamnosus* strain LAB 160 was maximized by the application of $CaCO_3$ to the fermentation medium reaching up to 10.46 g/l in the MRS containing 0.5% $CaCO_3$ Table 6. However, there wereno significant differences influence of $CaCO_3$ application on the amount of EPS produced by all strains in the fermentation batches using MRS. In comparison with the fermentation batches using MRS, the application of $CaCO_3$ to the waste mixture fermentation batches significantly improved ESP production by LAB strains 64 and LAB192. However, the increases in the amounts of EPS produced by the LAB 160 due to $CaCO_3$ application were not statistically significant Table 7, 0.5% $CaCO_3$ was applied in all further investigations to the fermentation batches by all the examined strains.

In a previous report (57), $CaCO_3$ can be utilized as a neutralizing agent for minimizing substrate inhibition lactic acid fermentation processes in addition it was considered as a neutralizing agent [57]. In contrast, the addition of CaCO3 did not promote EPS production by LAB all the time nevertheless, the amount of EPS product was lowered in some cases causing a lowering in EPS production [58].

With increasing the sugar concentration in the form of either molasses, salted cheese whey, or their mixture compared to MRS to reach 5% with 0.5% $CaCO_3$, EPS concentration was approximately doubled as shown in Table 7. In a previous investigation [59], the increases in EPSs production by LAB were ascribed to the increases in the sugar concentration in the growth culture medium.

Analysis of EPS polymer produced by the promising LAB strains

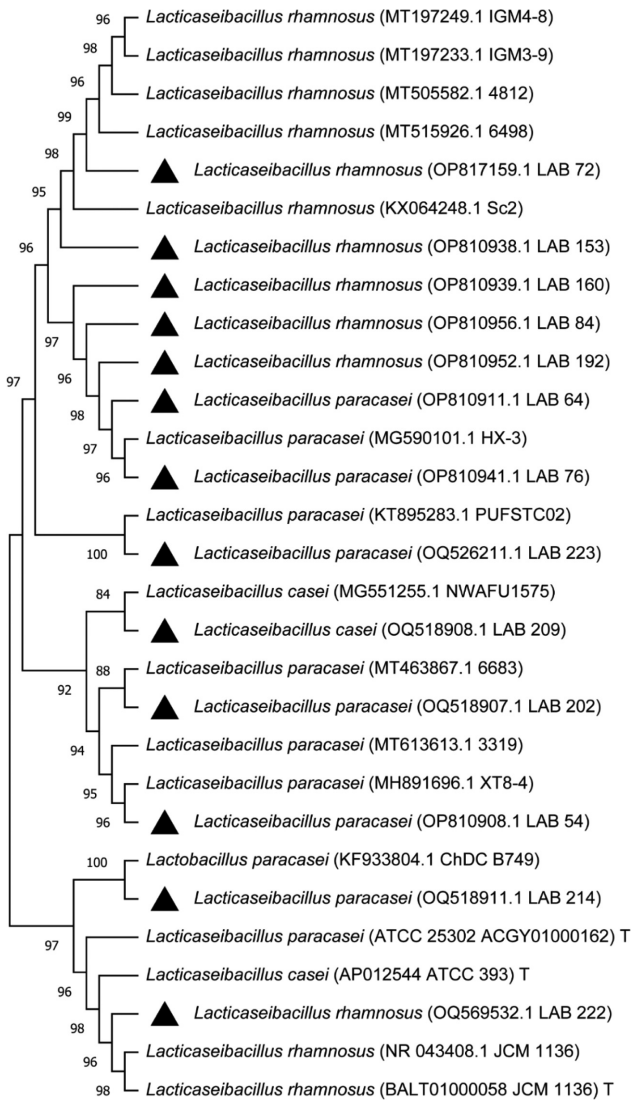
UV, and FTIR analysis for EPS

The pure EPS from strains LAB 64, 160, and 192 showed no absorption at 260 nm or 280 nm in

Table 4 Top-hit taxon, similarity percentage accession numbers and identification of the selected bacterial isolates

Isolate code	Top hit taxon	Similarity	Accession number	Identification
LAB 54	*Lactobacillus paracasei* strain XT8-4	99.64%	OP810908	*Lacticaseibacillus paracasei* strain LAB 54
LAB 64	*Lactobacillus paracasei strain* HX-3	94.35%	OP810911	*Lacticaseibacillus paracasei* strain LAB 64
LAB 72	*Lacticaseibacillus rhamnosus* strain IGM4-8	99.90%	OP817159	*Lacticaseibacillus rhamnosus* strain LAB 72
LAB 76	*Lactobacillus paracasei* strain 3319	99.16%	OP810941	*Lacticaseibacillus paracasei* strain LAB 76
LAB 84	*Lacticaseibacillus rhamnosus* strain Sc2	99.14%	OP810956	*Lacticaseibacillus rhamnosus* strain LAB 84
LAB 153	*Lacticaseibacillus rhamnosus* strain IGM3-9	99.91%	OP810938	*Lacticaseibacillus rhamnosus* strain LAB 153
LAB 160	*Lacticaseibacillus rhamnosus* strain 4812	97.22%	OP810939	*Lacticaseibacillus rhamnosus* strain LAB 160
LAB 192	*Lacticaseibacillus rhamnosus* strain 6498	97.65%	OP810952	*Lacticaseibacillus rhamnosus* strain LAB 192
LAB 202	*Lactobacillus paracasei* strain 6683	100.00%	OQ518907	*Lacticaseibacillus paracasei* strain LAB 202
LAB 209	*Lactobacillus casei* strain NWAFU1575	100.00%	OQ518908	*Lacticaseibacillus casei* strain LAB 209
LAB 214	*Lactobacillus paracasei* strain ChDC B749	100.00%	OQ518911	*Lacticaseibacillus paracasei* strain LAB 214
LAB 222	*Lacticaseibacillus rhamnosus* strain JCM 1136	100.00%	OQ569532	Lacticaseibacillus rhamnosus strain LAB 222
LAB 223	*Lactobacillus paracasei* strain PUFSTC02	100.00%	OQ526211	*Lacticaseibacillus paracasei* strain LAB 223

Figure 4



The phylogenetic tree of 13 LAB strains utilizing *16S rRNA* gene sequence. Bootstrapping was performed for a tree with 1000 replicates. Phylogenetic analyses were conducted in MEGA 11.

the UV-vis spectrum, demonstrating the absence of nucleic acid in the EPS sample. The FTIR spectrum of the EPS made from LAB160 Fig. 5 showed a broad and powerful stretching peak about 3417 cm^{-1} [60], which suggested that the polysaccharide included a significant amount of hydroxyl groups. C−H stretching vibration was the cause of the stretching band around 2931 cm^{-1} [61]. The stretch vibration of the C=O bond was the cause of the absorptions at 1632 cm^{-1} [62]. To characterizing polysaccharide structures, two key sections can be identified: the 950-750 cm^{-1} anomeric region and the sugar region, also known as the fingerprint area, measuring 1200-950 cm-1 [63]. Typical saccharide peaks were seen in the 1000–1200 cm^{-1} range. The vibration of the C−O−C and C−O bonds was said to be the cause of the bands in the 900–1150 cm^{-1} area [62]. A distinctive peak for the C−H arrangement in dextran was observed at 619 cm-1 [64].

HPLC analysis

Quantitative and qualitative dextran in EPS for all studied LAB strains (64,160, and 192), which were (14.50±0.45, 17.58±0.31, and 10.51±0.38) g/l, respectively on salted cheese whey, (22.39±0.26, 13.59±0.29, 13.48±0.43) g/l on molasses, and (19.51 ±0.31, 20.46±0.25, 15.56±0.38) on mixed wastes from (molasses: salted cheese whey 1:1), compared to control media MRS, which was (11.29±0.25, 9.34±0.20, 18.49 ±0.94) g/l Table 7 and Fig. 6 show as a total EPS extracted from fermented media. The R^2 value for the α-glucan standard curve was 0.998. Additionally, the results revealed that EPS from all examined LAB strains (64,160, and 192) showed independent peaks at the retention time (min), which were (12.44, 11.85, 12.27) min, respectively, compared to the retention

Table 5 Production of EPS from agro-industrial wastes using the three promising strains

	Polysaccharide concentration(g/l)		
Treatment	Salted cheese whey	Molasses	Salted cheese whey: molasses (1:1)
LAB 64	6.56±0.35 d	9.26±0.20 a	8.30±0.31 b
LAB 160	8.53±0.20 b	6.40±0.26 d	9.66±0.15 a
LAB 192	4.30±0.20 e	6.20±0.22 d	7.33±0.15 c

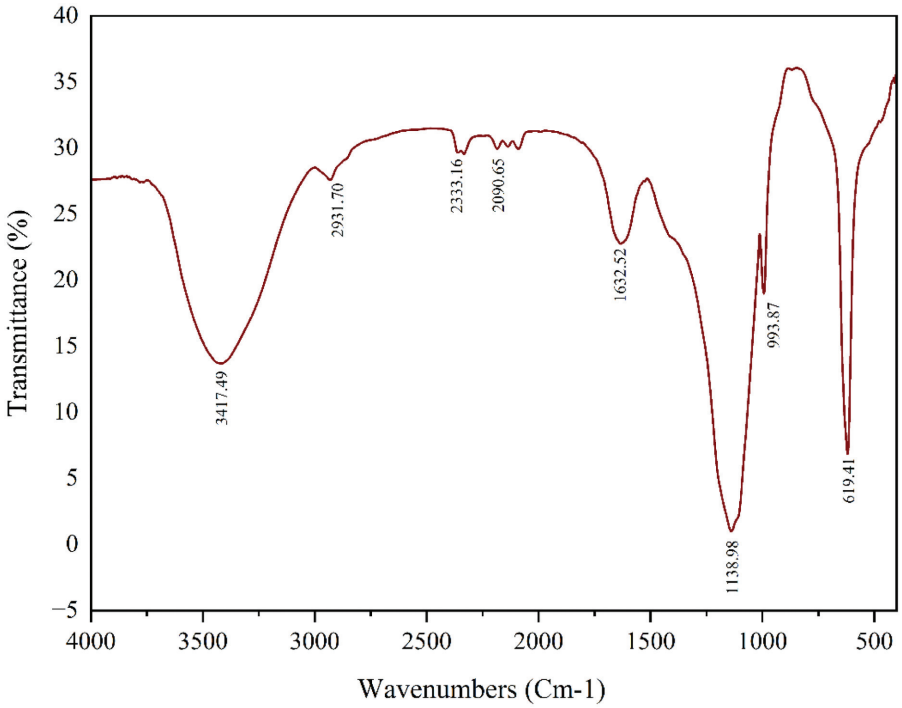
$LSD_{0.05}$ for wastes media = 0.40, Polysaccharide concentration means (g/l±SD)

Table 6 Polysaccharide production from (Waste mixture of 2% sugar with different concentrations of $CaCO_3$)

	Polysaccharide production g/l		
Treatment	LAB 64	LAB 160	LAB 192
MRS	5.97±0.31 c	10.09±0.33 a	7.95±0.44 b
MRS + 0.5% $CaCO_3$	6.16±0.21 c	10.46±0.33 a	7.76±0.03 b
Waste mixture+ 0.5% $CaCO_3$	7.34±0.46 ab	10.49±0.37 a	9.44±0.36 a
Waste mixture + 1% $CaCO_3$	7.35±0.21 ab	10.20±0.27 a	9.57±0.25 a
Waste mixture + 2% $CaCO_3$	7.82±0.18 a	10.40±0.23 a	9.68±0.17 a
Waste mixture + 5% $CaCO_3$	7.04±0.03 b	10.56±0.28 a	9.34±0.41 a

$LSD_{0.05}$ for $CaCO_3$ concentrations = 0.45, Polysaccharide concentration means (g/l±SD).

Figure 5



FTIR spectrum of exopolysaccharide produced by LAB160 strain as the highest producer of exopolysaccharide.

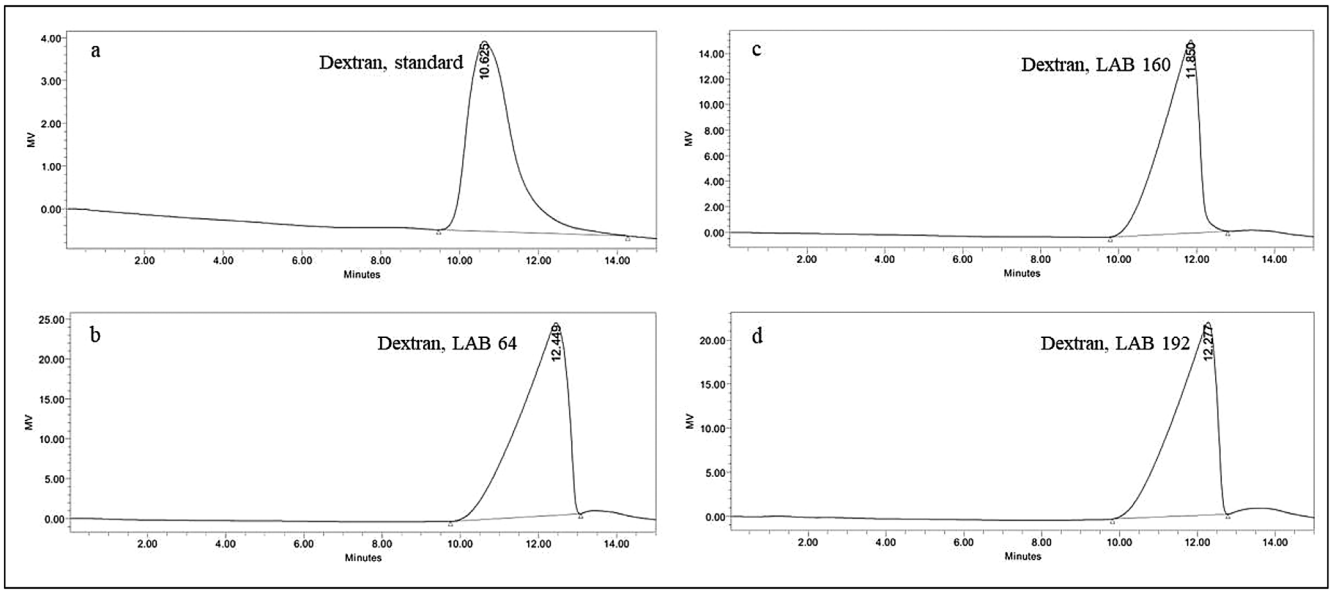
time for α-glucan (standard), which was 10 to 13 min as in Fig. 6 with dextran concentrations of 5.89, 12.37, 9.92 mg/ 100 mg EPS powder. In this regard, Li et al. [65]. Devised and validated a method for identifying and quantifying dextran 20 and sucrose with optical activity in the lyophilized thrombin powder. With the aid of HPLC, the monosaccharide makeup of EPS can be identified. To identify the monosaccharides, the EPS must first be hydrolyzed. At temperatures between (100–121°C), acids such trifluoroacetic acid (TFA), HCl, or H2SO4 can be employed to hydrolyze EPS [66]. Depending on preliminary screening for a selection of one isolate among three LAB isolates the bacterial strain, *Lacticaseibacillus rhamnosus* strain LAB 160 was selected for its superior production of EPS and α-glucan (dextran) was 22.46±0.25 g/l and 12.37 mg/ 100 mg of EPS powder, respectively as shown in Table 7, Fig. 6.

Table 7 Polysaccharide production from (wastes media 5%sugar, 0.5%$CaCO_3$)

	Polysaccharide production g/l		
Treatment	LAB 64	LAB 160	LAB 192
MRS*	6.16±0.21 f	10.46±0.33 d	7.76±0.03 f
Waste mixture*	7.34±0.46 e	10.49±0.37 d	9.44±0.36 e
MRS	11.29±0.25 d	9.34±0.20 e	18.49±0.94 a
Salt whey	14.50±0.45 c	17.58±0.31 b	10.51±0.38 d
Molasses: salt	19.51±0.31 b	22.39±0.26 a	15.56±0.38 b
Molasses	20.46±0.25 a	13.59±0.29 c	13.48±0.43 c

$LSD_{0.05}$ for bacterial strains on different types of wastes = 0.63, Polysaccharide concentration means (g/l±SD). *Sugar concentration is 2%.

Figure 6



HPLC spectrum of exopolysaccharide. a) standard dextran. b) exopolysaccharide produced by LAB 64 strain (dextran) with concentration 12.37 mg/100 mg. c) exopolysaccharide produced by LAB160 dextran with concentration 5.89 mg/100 mg. d) exopolysaccharide produced by LAB192 dextran with concentration 9.92 mg/100 mg.

Conclusion

Agro-industrial wastes such as sugarcane molasses and salted cheese whey can be utilized as a viable carbon source in the culture medium to produce EPS by the examined LAB strains. It is essential to note that employing agro-industrial wastes instead of manufactured sources in a culture medium reduces production costs. The biological production of EPS (dextran) is important in many food and pharmaceutical industries as well as using agro-industrial wastes in the production to protect the environment from their accumulation and invest their nutritional value in the production of valuable products. This work provides direction for expanding the hunt for low-cost materials to produce medium cultures.

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Conflicts of interest

There is no conflict of interest.

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