Characterization and Biological Activities of an Alkali Soluble Exopolysaccharide from *Acetobacter*

Hema Chandran[1*] and Kanika Sharma[2]

[1]*Department of Botany, Microbial Research Laboratory, Mohanlal Sukhadia University, Udaipur-313001, Rajasthan, India.*
[2]*Department of Botany, Mohanlal Sukhadia University, Udaipur-313001, Rajasthan, India.*

Authors' contributions

This work was carried out in collaboration between both authors. Author HC Planned and performed the experiments, wrote the manuscript and prepared the final version of the manuscript. Author KS supervised the whole work. Both authors have read and approved the final version of the manuscript.

Original Research Article

ABSTRACT

Natural polysaccharides have received much attention due to the plethora of their applications. Acetobacter sp. MRL 5A (KX082688) produced an alkali soluble exopolysaccharide (20.39 g/L) on sucrose based media during the late log and early stationary phases. The exopolysaccharide was insoluble in water with high moisture-retention ability and produced viscous solutions and gels. The exopolysaccharide contained 40.3% glucose, 34.4% galactose, 22.2% mannose and 3.1% of arabinose monomers revealed by GC. The exopolysaccharide showed emulsifying, flocculating and in-vitro antioxidant activity similar to standard exopolysaccharides like dextran and xanthan. This EPS can be of potential use in food, cosmetic, pharmaceutical and biomedical fields.

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*Corresponding author: E-mail: pillaihema02@gmail.com;

1. INTRODUCTION

Bioprospecting is the most frequently used term for screening biological resources for the extraction of commercially important compounds. Among the commercially important microbial metabolites the demand for exopolysaccharides (EPSs) produced by microorganisms has significantly increased due to their various industrial applications in food, medicine and pharmacy [1,2]. Microbial polysaccharides are more productive, less resource intensive and rely on controlled fermentation processes, with more degrees of freedom to control product composition, yield and productivity [3].

Bacteria produce a wide range of exopolysaccharides with varied physicochemical properties and serve distinct biological functions. The usefulness of bacterial exopolysaccharides was recognized with the discovery of dextran and xanthan [4]. Bacterial exopolysaccharides are non toxic, possess high biodegradability, better compatibility with the environment, etc. They are widely used in the food industry as viscofying, stabilizing and emulsifying agents. They are also used as bioflocculants in many industrial sectors and in waste water treatment [5] and also reported to function as potent anti-oxidants by preventing oxidative damage [6]. The anionic nature of these polysaccharides makes them interesting candidates for various biomedical applications [7].

Considering applicability of exopolysaccharides in diverse fields, exploration of newer habitats and new exopolysaccharide producers are demanding. Since, bacterial diversity in the environment remains untapped; exploration of newer habitats may render organisms with novel metabolites of commercial value. During the past few decades, exploration of natural resources like sugarcane juice, breads, fermented vegetables, fermented beverage, dairy products etc. [8] resulted in identification of several new exopolysaccharide producers.

Screening of less tapped environmental samples may render a hope for isolating new exopolysaccharide producing bacteria. The solid/liquid wastes generated from juice processing units is extremely diverse because of the presence of sugars, minerals, organic acid, dietary fibre and phenolics etc. These compounds may favor the growth of bacteria making it one of the potential substrate for isolating exopolysaccharide bacteria of industrial importance.

The purpose of this research was to isolate and identify the exopolysaccharide producing bacterial isolates from juice processing shops waste water. The exopolysaccharide secreted by the isolate/isolates were recovered, purified and characterized to identify its monosaccharide composition. Apart from this few biological activities like emulsifying, flocculation and antioxidant activity were evaluated to illustrate potential industrial application of the EPS.

2. MATERIALS AND METHODS

2.1 Screening Exopolysaccharide Producing Isolates

Waste water from juice processing shops was collected in sterile vials, serially diluted, plated on nutrient agar plates and incubated at 30°C for 24 hrs. After incubation, the isolates with mucoid, ropy and glistening phenotypic colony morphology was restreaked on media suggested by Tallgren et al [9] with simple modifications and incubated at 30°C aerobically for 5 days for exopolysaccharide production. The media used for exopolysaccharide production contained (g/L) 40 g sucrose; 0.2 g $MgSO_4 .7H_2 O$; 9 g $K_2H PO_4$; 3 g KH_2PO_4; 2 g yeast extract; 2 g peptone; 2 g NaCl; 15 g agar.

2.2 Phylogenetic Identification of Isolate

MRL 5A was selected for further study as it produced significant quantity of exopolysaccharides compared to other isolates. Total genomic DNA was extracted from the isolate MRL 5A using a standard protocol as per the Micro seq. Kit (USA). The 16S r RNA gene was amplified from the genomic DNA by PCR using the universal bacterial primers. The obtained 16S r RNA gene sequence was compared with those of other bacteria in GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the phylogenetic affiliation of isolate MRL 5A.

2.3 Exopolysaccharide Isolation

The isolate MRL 5A was cultured in liquid media containing (g/L) 40 g sucrose; 0.2 g $MgSO_4 .7H_2 O$; 9 g $K_2H PO_4$; 3 g KH_2PO_4 ; 2 g yeast extract, 2.0 g peptone and 2.0 g NaCl for 5 days at 28°C on rotary shaker at 150 rpm. Cell free supernatant was obtained by centrifugation of culture broth at15,000 rpm for 20 min at 4°C, deproteinized using 5% trichloroacetic acid at

4°C under static conditions. The solution was re-centrifuged at 15000 rpm to remove the precipitated proteins and the supernatant was collected. Exopolysaccharide was precipitated from the deproteinized supernatant by addition of three volumes of chilled ethanol and left overnight at 4°C [10]. Precipitated EPS was dissolved in minimal amount of distilled water and then stored in a dialysis bag at 4°C, with four exchanges of distilled water every 3 h, to remove the small neutral sugars [11]. The dialysed suspension was further lyophilized to obtain the partially purified exopolysaccharide. Total neutral sugar content in the lyophilized EPS was estimated by phenol-sulfuric acid method using D-glucose as the standard [12]. Total soluble protein was measured according to Herbert et al. [13]. Uronic acid content from the hydrolysed EPS was determined spectrophotometrically using the Carbazole method [14].

2.4 Purification of Exopolysaccharide

The partially purified exopolysaccharides was insoluble in water even after boiling and sonication. Thus the crude exopolysaccharides (100 mg) was boiled in 1 M NaOH to solubilize exopolysaccharides [15]. Alkali soluble exopolysaccharides was then dialyzed and lyophilized. The lyophilized exopolysaccharides were subjected to anion exchange chromatography on a column of DEAE–Sephadex A-25 (25 ×1cm), pre-equilibrated using 0.01 M Tris–HCl (pH 8.0). The column was washed with equilibration buffer followed by successive elution with sodium chloride solution of increasing molarities (0.0 to 0.5 M) in a stepwise manner. Fractions (5 mL) were collected and monitored spectrophotometrically by phenol–sulfuric acid assay [12].

2.5 Characterization of Exopolysaccharide

2.5.1 FTIR analysis

FT-IR spectra of purified exopolysaccharide were recorded using KBr pellets on a Bruker 3000 Hyperion Microscope with Vertex 80, Germany. The dried exopolysaccharides were grounded with KBr powder and pressed into pellets for FT-IR spectra measurement in the frequency range of 400-4000 cm-1. The analysis was performed at Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology, Bombay.

2.5.2 Thin Layer Chromatography (TLC)

Two mg of purified exopolysaccharide was hydrolyzed with one ml of 4 M trifloruroacetic acid at 100^0C for 3 hrs. The hydrolyzate was cooled and evaporated to dryness under reduced pressure at 40°C and re dissolved in distilled water. 2 μL of the acid hydrolyzed exopolysaccharide and standard monosaccharides were spotted on TLC plates and allowed to dry completely. The plates were developed using freshly prepared propanol: water solvent system in the ratio 85:15. The chromatograms were further derivatized by spraying freshly prepared aniline-diphenylamine-orthophosphoric acid reagent followed by baking the plates for 15-20 min at 80-100°C for color development [16].

2.5.3 Gas Chromatography Mass Spectroscopy (GC MS)

Monosaccharide composition analysis of the exopolysaccharide was carried out as described by Hung et al. [17]. GC was performed on Agilent 6890 plus Gas chromatogram fitted with a flame ionisation detector (FID). For resolution HP - 5(0.32 mm x0.25 μm x 30 m) fused silica columns and temperature programme 150°C for 5 min, 2^0C/min- 200°C was used. Hewlett Packard 3398A Chemstation was used for quantitation of peaks. For GLC-MS analysis, Hewlett Packard 5890 series 2 gas chromatograph tandemly linked to JEOL-AX500mass spectrometer was used with electron impact ionization (EI) at 70eV and ion source temperature at 200°C. Separation was performed on HP-1(0.25 mm x0.25 μm x 25m) and HP-5 (0.25 mm x0.32 μm x 30 m) capillary columns using temperature programs of 150°C for 5 min, 2°C/min- 200°C. The volume of sample injection was 1 μL.

2.6 Biological Activities of Exopolysaccharide

2.6.1 Flocculating assay

A suspension of kaolin clay was used to study the flocculating activity of the partially purified exopolysaccharide according to the method of Kurane et al. [18]. Standard polysaccharides like xanthan, dextran and guar gum were also tested for flocculation activity.

Flocculating activity (%) = (B – A)/B X 100

A= Absorbance of exopolysaccharide
B= Absorbance of control

2.6.2 Lipid emulsifying assay

Emulsification activity of the partially purified exopolysaccharide was tested using the method of Kurane and Nohata [19]. The lipid emulsifying activity was expressed as percentage of the height of emulsified layer per height of whole layer. The emulsifying activity of standard polysaccharides like xanthan, dextran and guar gum against vegetable oils like olive, sunflower, soyabean, sesame and mustard oils were also tested for comparison of emulsification activity.

Emulsification Index (%) = (Height of emulsion layer/ Total Height) × 100

2.6.3 Antioxidant assay

Free radical scavenging activity for 1,1-diphenyl-2-picrylhydrazyl (DPPH) was assessed as an indicator of the antioxidant activity of the partially purified exopolysaccharide using the method of Shimada et al. [20]. The DPPH free radical scavenging activity of standard polysaccharides like xanthan and dextran were also tested.

Scavenging effect (%) = (Ac –As)/Ac× 100

Ac is the absorbance of the control, As is the absorbance of the sample

3. RESULTS AND DISCUSSION

3.1 Isolation of EPS-Producing Isolates

EPS-producing isolates were selected on the basis of phenotypic colony morphology: Ropy or slimy. Seven exopolysaccharide producing bacteria were obtained from various fruit juice processing waste water on the basis of phenotypic morphology. The isolate MRL 5A recorded highest exopolysaccharide production (20.39 g/l) among the obtained isolates and was thus selected for further studies. It was observed that the culture broth became viscous after 24 hrs of incubation period. The increase in viscosity of culture media was observed day by day which can be attributed to exopolysaccharide production by the isolate MRL 5A.

Review of literature reports EPS producing bacteria from dairy and non- dairy based fermented products, food samples like sugarcane juice, breads, fermented vegetables, fermented beverage called marcha and sourdough etc [21].

Juice shop waste water samples are reported to be rich in sugars, minerals, organic acid, dietary fibre, phenolics and nutrients in the form of nitrogen, phosphorus and potassium [22]. Exopolysaccharide formation is reported to be favored in the presence of excess carbohydrates at low temperatures [3]. The presence of the organic and inorganic compounds including trace elements and organic growth factors in the waste samples might have triggered EPS production in the bacteria making it one of the potential substrate for isolating EPS producers.

3.2 Phylogenetic Identification of Selected Isolate

The isolate MRL 5A shared 99% 16S rRNA gene sequence similarity with *Acetobacter cerevisiae* strain SCMA40 (KJ469783) and *Acetobacter* species AaM47a (JQ314092). In the neighbor joining phylogenetic tree, isolate MRL 5A was grouped within the genus *Acetobacter*. Thus, the isolate MRL 5A was phylogenetically affiliated with the genus *Acetobacter* and was named *Acetobacter* sp. MRL5A with the Genbank Accession number KX082688.

3.3 Exopolysaccharide Isolation

Maximum exopolysaccharide production (20.39 g/l) was observed within 120 hours, after which it started declining. The exopolysaccharide yield was observed to increase during the exponential growth phase and the yield declined when the culture reached its stationary growth phase (data not shown). The regulation of the EPS biosynthetic pathway is dependent on the carbon sources added to the growth medium [23]. The decline in EPS production may be due to the lack of available carbon source and it might have induced the bacteria to utilize EPS produced as a carbon source [24] for its further metabolism. Pham et al. [25] reported the decrease in exopolysaccharide production during late stationary phase due to the production of glycohydrolases that catalyzed the degradation of polysaccharide.

The partially purified EPS was off white in color and used for subsequent analysis. Chemical analysis of the partially purified EPS demonstrated the presence of neutral sugars and uronic acid. A diverse range of polysaccharides, with various sugars, uronic acids have been reported previously in exopolysaccharides [26].

3.4 Purification of Exopolysaccharide

Partially purified EPS upon anion-exchange chromatography on DEAE Sephadex A- 25 column yielded two fractions APS I and APS II at 0.20 M NaCl. Majority of exopolysaccharides synthesized by bacteria are polyanionic due to the presence of uronic acids, ketal-linked pyruvate or because of negatively charged functional groups like carboxyl, phosphoric, sulphate and hydroxyl groups [26]. The polyanionic character and the differences in charge density allow exopolysaccharides to be fractionated by gradient elution using anionic resins. The exopolysaccharide fractions eluted between the ranges of 0.2 M NaCl gradient. The carbohydrate content in APS I fraction was more compared to APS II and thus it was used for structure and characterization analysis. UV-Vis absorption spectrum at 260 nm or 280 nm showed no obvious absorption peaks indicating absence of nucleic acid or protein in the purified exopolysaccharides. Both fractions gave negative response to ninhydrin test indicting the absence of contaminating protein in the purified sample.

The results obtained in the present study are in accordance with the available reports. Sun et al. [27] purified exopolysaccharides from the Arctic Marine Bacterium *Polaribacter* sp. SM1127 with DEAE-Sepharose fast flow anion-exchange chromatographic column with 0–0.7 M NaCl gradient. The crude exopolysaccharides from *Bacillus megaterium* RB-05 was purified through an anion exchange chromatography of Cellulose DEAE-52 with 0.2 M NaCl [28].

3.5 Characterization of EPS

3.5.1 FT IR analysis

The FTIR spectrum of purified exopolysaccharide revealed strong and broad absorption peaks in between 3429- 3446 cm^{-1} indicating the presence of OH groups. The absorption peaks at 2924 - 2926 cm^{-1} is assigned to C-H stretching vibration. A strong peak at 1635-1660 cm^{-1} is also a C=O stretching vibration of a -CHO group, which is a characteristic absorption peak of uronic acid structures. A symmetric stretching band near 1460 - 1463 cm^{-1} indicates carbonyl groups (C=O). The absorptions at about 1056- 1059 cm^{-1} indicated the bending vibration of hydroxyl groups. The bands at 861 cm^{-1} confirmed α- glycosidic bonds in the polysaccharide (Fig. 1).
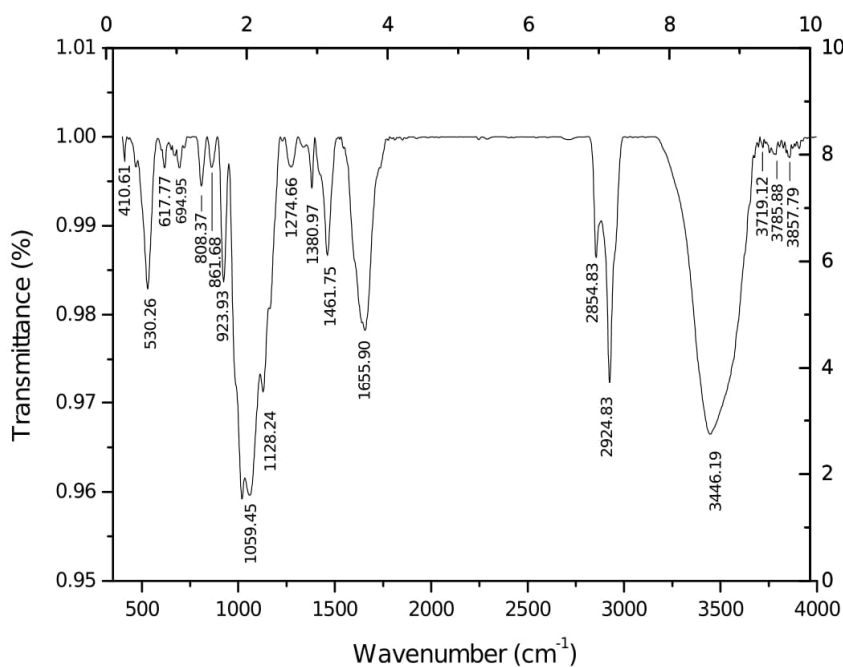

Fig 1 FTIR analysis of the exopolysaccharide from *Acetobacter* sp. MRL 5A

3.5.2 TLC and gas chromatography

TLC analysis of EPS revealed that it as a heteropolysaccharide composed of monosaccharides like glucose, galactose, mannose and traces of rhamnose and galacturonic acid. The derivatization reagent gave dark blue colored spots for glucose and galactose, yellow-green colour for rhamnose and pale violet colour for galacturonic acid. In TLC analysis glucose, galactose and mannose resolved poorly thus were not clearly distinguishable as single spots.

Glycosyl composition analysis performed by GC-MS showed that glucose was the dominating sugar followed by galactose and mannose and traces of arabinose (Fig. 2). Analysis of monosaccharide composition of EPS by GC revealed glucose, galactose, mannose and arabinose in relative percent of 40.3, 34.4, 22.2 and 3.1.

TLC and GC profile of EPS fractions confirmed the presence of multiple sugar monomers reflecting its heterogeneous nature. Imran et al. [29] reported that the monomer composition in EPS can vary among different strains of the same species. Glycosyl composition of exopolysaccharides from *Polaribacter* sp. showed the presence of *N*-acetyl glucosamine, mannose, glucuronic acid, with moderate amounts of galactose and fucose and minor amounts of glucose and rhamnose [27]. GC-MS

analysis of alditol acetates of exopolysaccharides from *Bifidobacterium animalis* reported the presence of fructose, mannose and glucose as the monosaccharide [30].

3.6 Biological Activities of Exopolysaccharide

3.6.1 Flocculation assay

Flocculating activity of exopolysaccharide increased with increasing concentration of exopolysaccharide. Maximum flocculation activity (95.12± 0.11%) was observed in the concentration range of 10 mg/ml within 10 minutes. The flocculation activities were comparable with that of the standard exopolysaccharides like xanthan and dextran at the same concentration (Fig. 3) which suggests that they can be used as a bioflocculants. The most important factors affecting flocculating activity are molecular weight and functional groups present in the flocculant. High molecular weight organics play a major role in bioflocculation when compared with low molecular weight organics [31]. Large molecular weight bioflocculant has sufficient number of free functional groups by which strong and large flocs can be formed. The presence of the carboxyl groups on the molecular chain of the biopolymer allows the chain to spread out as a result of electrostatic repulsion and the stretched molecular chains provide more effectual sites for particle attachment.
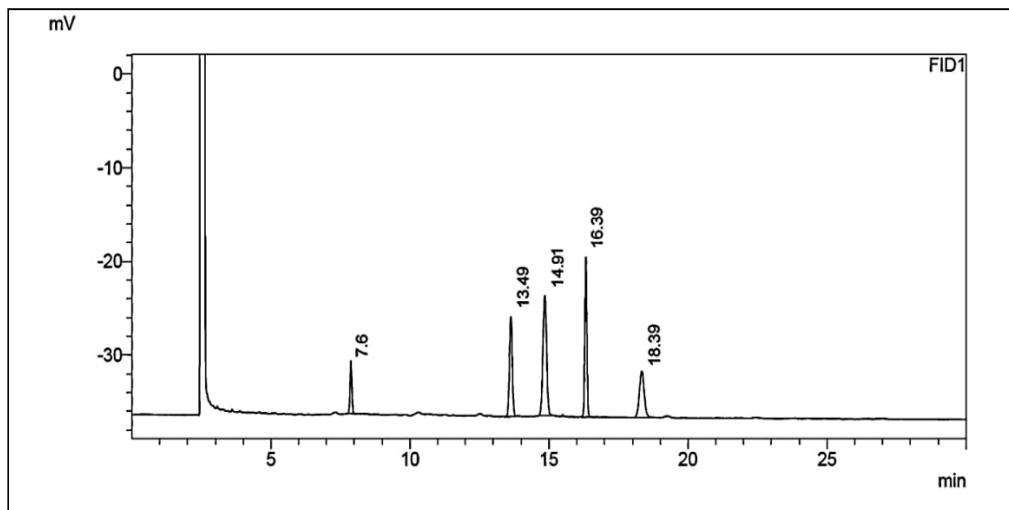

Fig. 2. GC MS chromatogram of exopolysaccharide hydrolysate from *Acetobacter* sp. MLR 5A
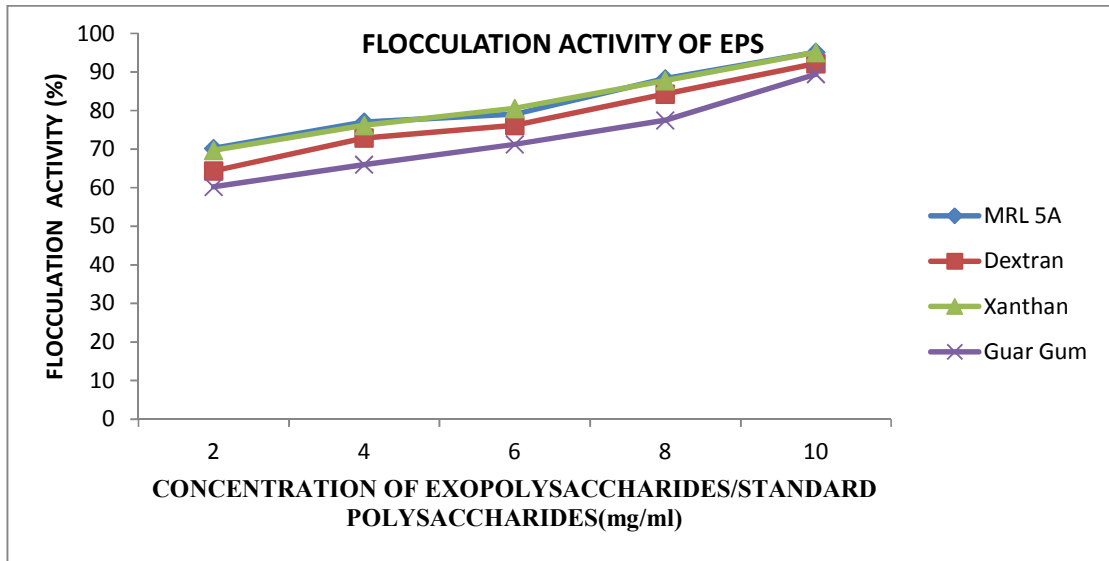*The results are represented as mean ± SD of the three independent data*
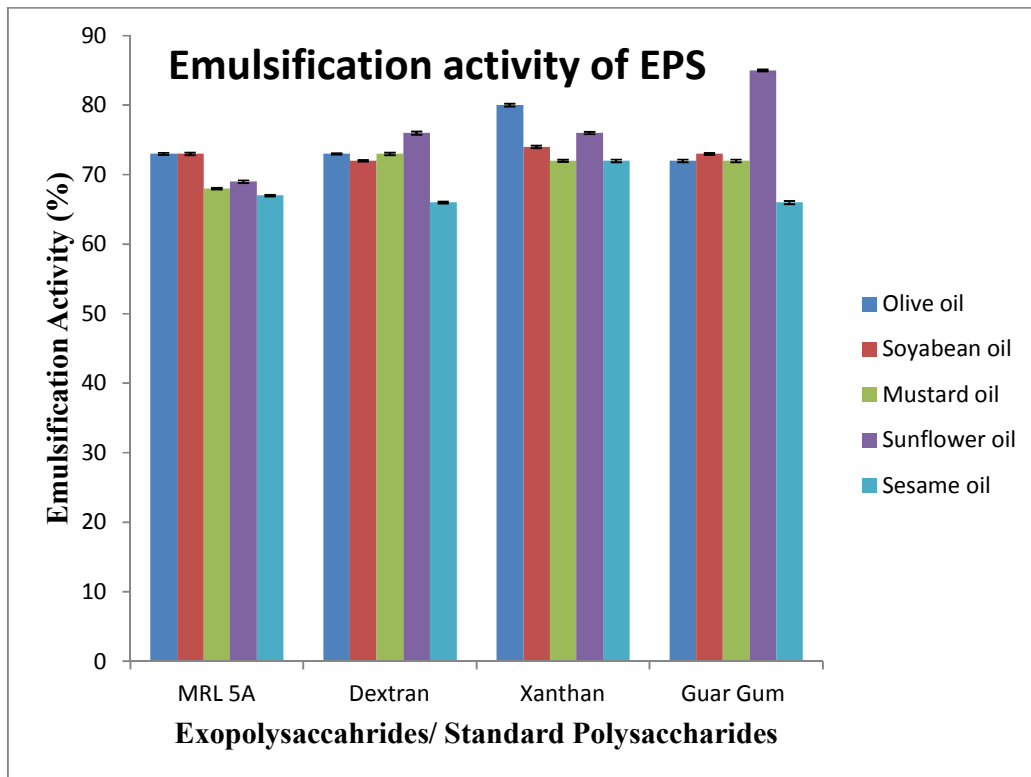
Fig. 3. Flocculation activity of exopolysaccharides

Fig. 4. Emulsification activity of exopolysaccharides
The results are represented as mean ± SD of the three independent data

3.6.2 Emulsification assay

The exopolysaccharides exhibited stable emulsions with emulsification indexes higher than 60% with all the vegetable oils tested (Fig. 4). The emulsions were observed to be stable till one month which suggests the usefulness of these EPS for making oil/water emulsions for
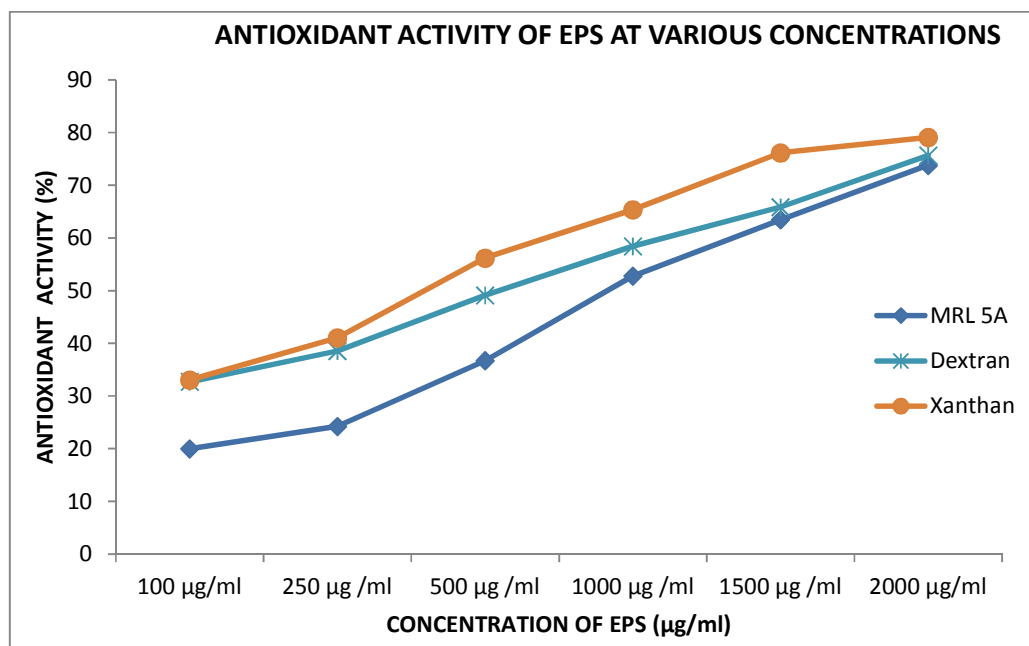
ANTIOXIDANT ACTIVITY OF EPS AT VARIOUS CONCENTRATIONS

Fig. 5. Antioxidant activity of exopolysaccharides
The results are represented as mean ± SD of the three independent data

cosmetics and food industries. The efficient emulsifying activity of exopolysaccharides is a function of their chemical composition [32]. For any compound to be a stable emulsifier, it should retain at least 50% of the emulsion after its formation [33]. Sahana et al. [34] reported high emulsifying activity of EPS from *Cronobacter* sp against hydrocarbons such as petrol, kerosene, xylene, palm oil, coconut oil, and olive oil with emulsification index greater than 60%.

3.6.3 Antioxidant assay

The obtained data indicate that EPS from MRL 5A has good antioxidant activity and is dose-dependent. The antioxidant activity was seen to increase with an increase in the concentration of exopolysaccharide (Fig 5). The exopolysaccharides showed 73.79 ± 0.31 % antioxidant activity at a concentration of 2 mg/ml while xanthan and dextran possessed 79.09 ± 0.40% and 75.66 ± 0.38 % antioxidant activity at concentration of 2 mg/ml respectively. The obtained results suggest that EPS can be used as a natural antioxidant, an alternative to synthetic antioxidants.

Polak-Berecka *et al* [23] reported that the structure and composition of EPS play a crucial role for their specific biological actions. The antioxidant activity of polysaccharides might be attributed to their hydroxyl groups and other functional groups, such as C=O, –COOH and –O–. These groups donate electrons to reduce the radicals to a more stable form or react with the free radicals to terminate the radical chain reaction [35]. Li and Shah [36] also reported dose-dependent DPPH free radical scavenging activity of crude exopolysaccharides from *L. helveticus* MB2-1.

4. CONCLUSION

In this study, *Acetobacter* sp. MRL 5A was isolated from a fruit juice processing effluent which produced good quantity of EPS. The EPS produced was similar to xanthan in its composition but insoluble in water and soluble in alkali. The EPS was characterized by FTIR, TLC and GC-MS, and its biological activities were subsequently investigated. Exopolysaccharide exhibited better emulsifying, *in vitro* antioxidant and flocculating activities which are relatively similar to that of commercial polysaccharides. Aforementioned results suggest that exopolysaccharide from *Acetobacter* sp. MRL 5A may find its application in various industrial sectors like cosmetics, oil industries, biomedicine, waste water treatment, etc. and in addition, further studies on process conditions are needed for the prospect of large-scale production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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