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RESEARCH ARTICLE

# Exopolysaccharides production and characterization of *Fusarium mangieferae* from mango (*Mangifera indica*) with some commercial potential applications

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ABSTRACT

Nine common fungi were isolated from different fruits plant such as amla (*Phyllanthus emblica*), banana (*Musa acuminate*) and mango (*Mangifera indica)*. The most promising fungus producing exopolysaccharide (EPS) was Fusarium mangieferae which was identified according to microscopic morphological features and confirmed by IARI, Delhi. Three main spectroscopic analyses (FTIR, TLC and GC-MS) were employed to characterize the EPS extracted from marine-derived *Fusarium mangieferae* . The monosaccharide composition of EPS determined by TLC indicates it is a homopolysaccharide composed of glucose. The FT-IR analysis proves the presence of biologically important functional groups and alpha glycosidic linkage between individual glycosyl residues. The GC-MS chromatography showed that the EPS consist of one peak of glucose hence *Fusarium mangieferae* EPS is homopolysaccahride. EPS of *Fusarium mangieferae* showed antibacterial activity against Klebsiella aerogenes is 29 mm and also have emulsification and bioflocculation activity.

**Keywords:** Exopolysaccharide, emulsification, bioflocculation, postharvest

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## INTRODUCTION

Microorganisms can produce exopolysaccharides (EPSs) with a wide range of properties and applications, including antitumor therapies, food packaging materials, and wound dressings. Fungi produce EPS to protect their cells against desiccation, phagocytosis, phage attack, toxic compounds, predation by protozoans, and osmotic stress, as well as to recognize cells (Ravella et al., 2010; Dudman, 1977). Terrestrial fungi have proved to be a rich source of new biological natural products. Because of their characteristic properties with reference to temperature, nutrients, competition and salinity, they have developed specific secondary metabolic pathways compared with other habitat fungi. Terrestrial filamentous fungi have proved to possess tremendous source of new secondary metabolites such as exopolysaccharide which have posses many industrial and medical application, as some recent studies prove too (Yang et al., 2008; Wang et al., 2008; Ismail and Nampoothiri,

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2010; Liu et al., 2011). Fungal EPSs have been recognized as high value bio-macromolecules for the last two decades. The purpose of the present research was to screening terrestrial EPS producing fungi and also characterization of EPS by using FTIR, TLC and GC-MS. Antibacterial, emulsification and bioflocculation activities were analysed.

## MATERIALS AND METHODS

### Isolation and Identification of fungi

Fungal samples from Capsicum annuum and Solanum tuberosum fruits plant source namely were collected. Cut up small pieces of the infected material, soak them in a 0.1% solution of mercuric chloride for 30 seconds, wash them three times with distilled water, and transfer them to solid PDA (potato dextrose agar) petri plates (Hemmi and Issigami, 1953; Khair et al., 2007; Akhtaret al., 2014). The inoculated plates were incubated at 25°C for 4-6 days in a BOD incubator (Phalisteen et al., 2008). Serial dilution and single spore inoculation method (Choi et al., 1999; Akhtar et al., 2014) were used to develop subculture of the isolates on potato dextrose agar medium. The culture was maintained on maintenance media i.e. PDA. Cultures of the test pathogen were stored at 4$^{o}$C on potato dextrose agar slants and subcultured regularly after 7 days. In order to characterize their growth properties and EPS production, the cultures were fermented in a rotary shaker for 14 days with enriched media.The selected exopolysaccharide producing isolate was identified on the basis of their morphology using standard keys (Alexopoulos and Mims, 1979). The cultures was sent to IARI (Indian Agricultural Research Institute), Delhi for species/strain level identification.

### Isolation of isolated cultures for the Extracellular polymer production

Pure cultures isolate were screened by Shake flask method for exopolysaccharides production. The cultures were inoculated in 150 ml of enriched media by Shamy and Nehad, (2010) and incubated on a rotary shaker at 29°C and 150 rpm for 2 weeks. The media used for exopolysaccharide production contained ( gm/l) Glucose 40 gm; Yeast extract 1.0 gm; peptone 0.5 gm; $K_2HPO_4$ 0.5 gm; $MgSO_4.7H_2O$ 0.5 gm. After 2 weeks of incubation, the culture was centrifuged at 4000 rpm for 15 min. (Shamy and Nehad, 2010) and the supernatant was collected for EPS isolation and settled fungal cells was used for biomass calculation. EPS was recovered by adding chilled 95% ethanol at a ratio of 1:3 to the clear supernatant and keeping it overnight at 4$^{0}$C. The precipitated EPS was weighed, dialyzed against deionized water for 2 days at 4 $^{0}$C (Rusa-Miadiedo et al., 2002) and dried at 40-60 $^{0}$ C to calculate the dry weight of EPS produced by the selected isolates. The dried EPS was used for further assays. The exopolysaccharide secreted by isolates was quantified by the phenol sulphuric acid method (Dubois et al., 1956) using glucose as the standard sugar and by the Folin Lowry method (Lowry et al., 1951) using BSA as the standard protein.

### Purification of EPS

The crude exopolysaccharides (100mg/5ml in deionized water) obtain from the isolates SM MRL 17 and 22 were subjected to anion exchange chromatography on DEAE Cellulose 52 column ( 25×1 cm) pre equilibrated using de ionized water and eluted with a volume of deionized water followed by a continuous gradient of NaCl from 0.0 to 0.5 M in deionized water with a flow rate of 45ml h$^{-1}$. Fractions (5ml) were collected and an aliquot (0.1ml) was tested for total carbohydrate by phenol sulhphuric acid method (Dubosis et al., 1956). The respective exopolysaccharide fractions were pooled and dialyzed overnight against deionized water and finally dried at 60$^{0}$ C.

**Analysis of Monosaccharide Composition by TLC**

1 mg EPS were hydrolysed with 1 ml of 4M TFA for 3hr at 100 $^{0}$ C. Sugar were identified by TLC using standard sugars for indentification. TLC was developed with n-propanol: water (85:15) as solvent system. The separated monosaccharides were visualized by derivatization of the chromatogram. Derivatization was done by spraying the chromatogram with freshly prepared aniline-diphenylamine-orthophosphoric acid reagent ( 4 ml of aniline, 4gm of diphenylamine, 200 ml of acetone and 20 ml of 85% phosphoric acid) followed by baking the plates for 15-20 minutes at 80-100$^{0}$C ( Dawson et al., 1986) for colour development.

**FTIR Analysis**

The structure of exopolysaccharide was studied by recording the FTIR spectra using a FTIR imaging system (Bruker 3000Hyperion Microscope with Vertex 80, Germany). The dried exopolysaccharides were grounded with KBr powder and pressed into pellets for FTIR spectra measurement in the frequency range of 400-4000$^{-1}$cm.

**GC-MS Analysis**

The structure of exopolysaccharide was studied by GC-MS was performed on Agilent 6890 plus Gas Chromatogram fitted with a flame ionising detector (FID).

**Commercial application of isolated exopolysaccharides**

**Bioflocculation Activity**

4.5 ml of Kaolin suspension (5 gm/litre) was added to each test tube and mixed with 0.25 ml of $CaCl_2$ solution (90 mM). 100 µl. of the test exopolysaccharide was added this mixture and vortexed for 30 sec and allowed to stand for 5 min at room temperature. The optical density of the upper phase was measured at 550 nm with spectrophotometer. Guar gum and Polyacrylamide will be used for comparison. A control experiment was carried out in the same manner with 100 µl. distilled water.

**Flocculating activity = (B-A) x 100 / B**

**A** = O.D at 550nm of flocculent sample

**B** = O.D at 550nm of distilled water

**Antibacterial Activity**

Antimicrobial activity of test exopolysaccharide was done by using Agar-well diffusion method (Attaie et al., 1987). Molten agar was poured in sterilized petriplate and allowed to solidify. It was subsequently seeded with the test organism (20µl) by spreading it on the surface with a sterile spreader then 6mm wide well was bored in this agar plate and filled with 60µl of the exopolysaccharide solution (0.1mg/ml).The plates were incubated at 25°C-30°C and zone of inhibition was measured after 24 hrs.

**Bioemulsicifying activity**

Emulsification activity of the isolated exopolysaccharide was tested using the method of Kurane and Nohata, 1991). Refined commercially available vegetable oils like olive oil ( Figaro brand), Soyabean oil (Fortun brand), Mustard oil ( Tanker brand), sunflower oil(Sweekar brand) and sesame oil (Tilam brand) were used as a source of lipid for assaying emulsification activity. One percent solution of exopolysaccharides isolated from the respective selected isolates was prepared in deionized water. Equal volume of respective vegetable oils and exolpoysaccharides (1:1 v/v) in graduated eppendrof tubes were shaken for 10min at 150 rpm on a rotary shaker to make a lipid emulsion. The emulsion was centrifuged at 2000g for 5 min and the thickness of the emulsified layer was measured after 24hours. The lipid emulsifying activity was expressed as percentage of the volume of emulsified layer per the volume of the whole layer. The emulsifying activity of standard polysaccharide like xanthan and guar gum against vegetable oils like olive, sunflower, soyabean, sesame and mustard oils were also tested for comparison of emulsification activity( Fig..)

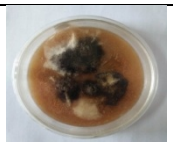
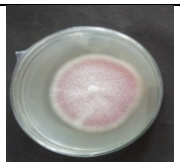
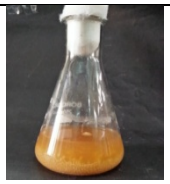
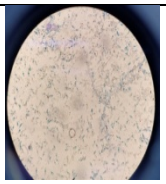
$$\text{Emulsification Index (\%)} = \frac{\text{Volume of emulsion layer}}{\text{total Volume}} / \text{X } 100$$

**RESULTS**

Among 9 strains isolated from fruits plant (Table 1) the strain MRL SM 8 produced significant amount of extracellular polysaccharide.According to Alexopoulos and Mims (1979), fungal isolates are tentatively identified up to generic levels based upon colony forms, hyphal morphology, spore characteristics, and reproduction structures.

Microscopically the isolates MRL SM 8 was Fusarium specie. The MRL SM 8 aerial mycelium is white and floccose. Microconidia are various in shapes, oval to allantoids with 0 to 1 septate. Marcoconidia are long and slender, usually 3-5 septate.The identification of fungi at the species level was confirmed by IARI, Delhi for strains identification done by Dr. Deeba Kamil. The MRL SM 8 was Fusarium Mangifera ( Table 1).

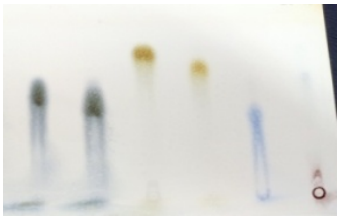
**Table 1: Isolation of *Fusarium mangifera***

| Sample | Isolation of fungi (6-7 days) | Pure Plate | Secondary observation | Microscopic | Identification |
|---|---|---|---|---|---|
|  **Mango** (Mangifera indica |  |  |  |  | Fusarium Mangifera:<br><br>Its aerial mycelium is white and floccose. Microconidia varial in shape, oval to allantoids with 0 to 1 septate. Marcoconidia are long and slender, usually 3-5 septate |

Shake flask method was used to grow the isolates at 300 C and 150 ml of enriched media was used for inoculation by Shamy and Nehad. The isolates were allowed to grow using Shake flask method at $30^0$C. The cultures were inoculated in 150 ml of enriched media by Shamy and Nehad, (2010). It was also observed that the enriched media became viscous due to formation

of exopolysaccharide in aerobic condition during fungal growth. The biomass and EPS yield (in gm) of isolates SM MRL 1 and 10 were collected after 2 weeks are 4.18 and 4.93 (Biomass), 0.117 and 0.113 ( EPS) respectively.

Crude exopolysaccharide was partially purified by repeated fractional precipitation with ethanol and dialysis. The dialyzed exopolysaccharide loaded on anion-exchange column Cellulose DEAE-5. The partially purified exopolysaccharides from the isolates SM- 15 showed one elution peak which eluted at 0.2 M NaCl indicating the homogeneity of exopolysaccharides i.e., it may contain only one type of polymer. TLC was used to analyze the monomeric composition of EPS produced by Fusarium mangieferae (SM MRL 8) after hydrolysis with acid. When sprayed with a detection reagent, the standard monosaccharide turned dark blue in the presence of glucose.The Rf value of the bands obtained by hydrolysate corresponded to glucose. This implied that the polysaccharide is a homopolysaccahride (Fig.1)



8 Glu Rha Arb Mal Gluc acid

Isolated exopolysaccharide

8- *Fusarium mangieferae*

Standard

Glu- Glucose
Rha- Rhamnose
Arb- Arbinose
Mal- Maltose
Glu acid- Glucoronic acid

**Fig. 1: Thin Layer Chromatography of Exopolysaccharide**

FTIR is a useful analytical technique to determine functional groups and covalent bonding information. It works on the fact that bonds and groups of bonds vibrate at certain frequencies. Using IR spectra for carbohydrate analysis, the α and β -conformers can be distinguished from each other in the continuum vibrational bands, since the α and β-configuration are well separated in the 950 to 750 cm region, where the α conformers lie between 870 and 840 cm, while the β -conformers are about 890 cm apart (Kacurakova et al., 2001; Yang et al., 2009).Polysaccharides have bonds around 3400cm-1, 2939 $cm^{-1}$, and 990-1200 $cm^{-1}$, which are O-H bonds and C-H bonds of $CH_2$ groups, respectively (Fig. 2). The spectrum peak at 3444cm indicates that the molecule has OH and NH2 groups (Desouky et al., 2008).

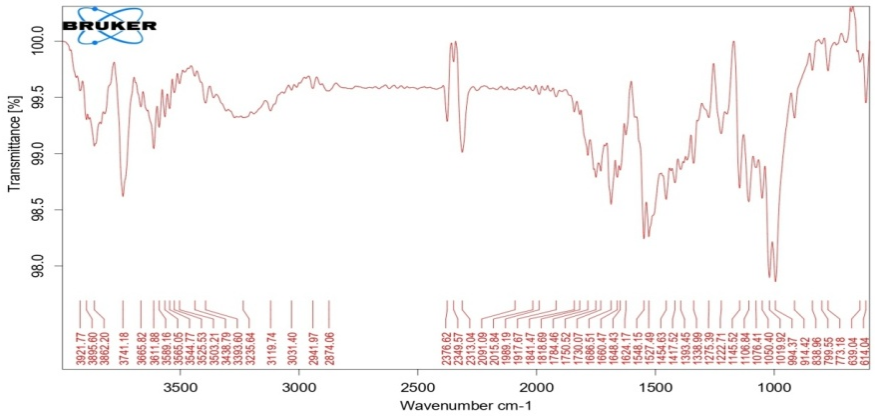



**Fig 2: FTIR of isolated Exopolysaccharide**.

Glycosyl composition analysis performed by GC-MS was showed homo exopolysaccharides in SM MRL 15. SM MRL 15 is homopolysaccharide observed only one peak of glucose (Fig. 3).

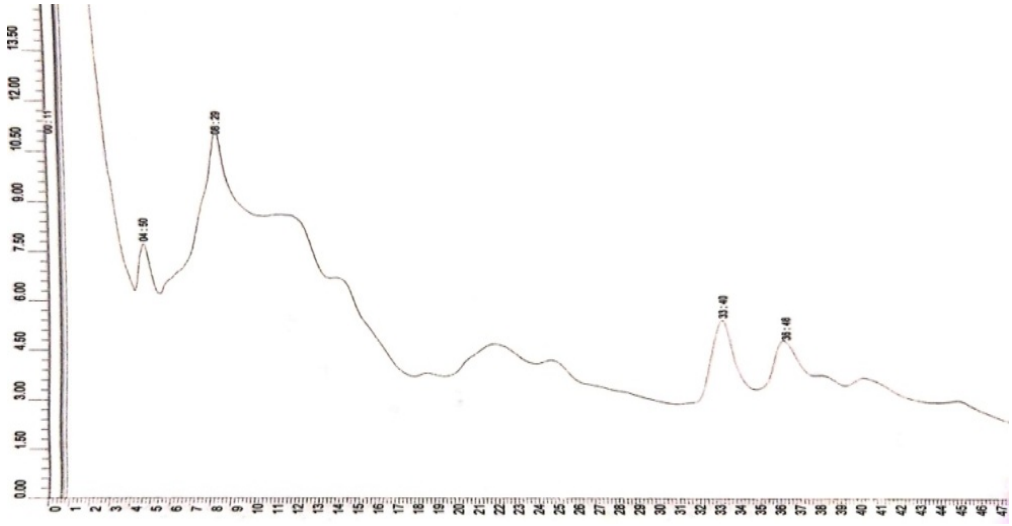


**Fig 3: GC-MS of isolated Exopolysaccharide**

Fungal exopolysaccharides are widely used in food, pharmaceutical, medical, cosmetic, and agricultural sectors. The application of exopolysaccharides in the flocculation process will be a significant to the health and eco-friendly usage in waste water treatment processes. It was observed that isolate exopolysaccharide have flocculation activity (Table 2). Maximum flocculation activity within 10min. was observed in case of exopolysaccharides from isolates MRL SM 8 (*Fusarium mangiferae*) (90.83 $\pm$ 0.63value). The flocculation activities of these exopolysaccharide were comparable with the standard polysaccharides such as xanthan gum, guar gum. The flocculation activity was seen to increase with increase in the concentration of exopolysaccharides. Maximum flocculation activity was observed in the concentration range of 10mg/ml. Thus, the result indicates that these exopolysaccharides can be used is a good novel substituent for existing bioflocculants for industrial applications.

**Table 2: Flocculation Activity of Exopolysaccharides/ Standard Polysaccharides**

| Exopolysaccharide of Fungi | Percent Flocculation activity at various concentration of EPS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2mg/ml | SD | 4mg/ml | SD | 6mg/ml | SD | 8mg/ml | SD | 10mg/ml | SD |
| *Fusarium mangiferae* | 50.48 | 0.59 | 60.27 | 1 | 72.67 | 0.53 | 85.66 | 0.63 | 90.83 | 0.63 |
| Xanthum gum | 69.48 | 0.38 | 73.85 | 0.31 | 78.81 | 0.38 | 85.16 | 0.17 | 93.01 | 0.31 |
| Guar gum | 59.03 | 0.56 | 69.18 | 0.38 | 74.81 | 0.76 | 83.94 | 0.21 | 89.65 | 0.22 |

Antibacterial activity of exopolysaccharides from selected isolates was assayed agar well diffusion method (Aitte et al., 1987). It was observed that all the selected test bacterial strains showed growth inhibition on nutrient agar plates with fungal exopolysaccharides. The maximum inhibition showed by the *Fusarium mangiferae* (SM MRL 8) against Klebsiella aerogenes is 29 mm (Table 3). Hence, exopolysaccharides produced from selected isolates also used in medical fields as an antibacterial activity.

**Table 3: Antibacterial activity of Exopolysaccharides/ Antibiotics**

| Exopolysaccharide of fungi | Inhibition zone | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Staphylococcus aureus*** | **SD** | ***Bacillus subtillis*** | **SD** | ***Pseudomonas putida*** | **SD** | ***Klebsiella aerogenes*** | **SD** | ***Proteus vulgaris*** | **SD** |
| *Fusarium mangiferae* | 18 | 0.5 | 15 | 0.7 | 19 | 1 | 29 | 1 | 12 | 1 |
| Ciproflaxin | 39 | 0.5 | 38 | 0.5 | 43 | 0.7 | 36 | 1 | 32 | 1 |
| Tetracycline | 36 | 1 | 45 | 0.5 | 34 | 1 | 30 | 1 | 40 | 1 |

**Table 4: Flocculation Activity of Exopolysaccharides/ Standard Polysaccharides**

| Exopolysaccharide of Fungi | Percent Emulsification activity at various concentration of EPS | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Olive oil** | **SD** | **soybean oil** | **SD** | **Mustard oil** | **SD** | **Sunflower oil** | **SD** | **Sesame oil** | **SD** |
| *Fusarium mangiferae* | 65.54 | 0.15 | 75.53 | 0.11 | 70.22 | 0.035 | 71.10 | 0.67 | 62.73 | 0.04 |
| Xanthum gum | 81.03 | 0.24 | 75.13 | 0.10 | 72.30 | 0.04 | 77.43 | 0.07 | 73.58 | 0.08 |
| Guar gum | 73.51 | 0.09 | 75.51 | 0.08 | 73.75 | 0.06 | 84.71 | 0.09 | 66.58 | 0.13 |

## DISCUSSION

Exopolysaccharides producing fungi have been isolated from plants, plants sources, soil, water, agriculture waste, hydrothermal vent, waste water, sewage sludge, and groundwater; they are also isolated from extreme environments, such as deep-sea, Antarctic ecosystems, saline lakes and geothermal springs. Fungal isolates from fruit plants sources Amla (*Phyllanthus emblica*), Banana (*Musa acuminate*) and Mango (*Mangifera indica*). This is the first study which reports the novelty of samples used for isolating significant exopolysaccharide producers. These isolates were Alternaria sp., from the infected sample. All fungal isolates showed diversity in colony form, colour, elevation or margin. During initial growth, some fungal colonies on PDA medium were cottony and became darker as the culture aged. The identification of fungi based on morphology character is often misleading. Therefore, confirmed identification isolates were sent to IARI, Delhi.

The exopolysaccharide produced by the fungi is water-soluble and viscous in the medium. *Fusarium mangiferae* produced significant amount of EPS have been reported in the literature (Nehad and Shamy, 2010; Mahapatra and Banerjee, 2013; Al-Manhel, 2017).

EPS of *Fusarium mangiferae* is homopolysaccharide present glucose. Further FTIR analysis of the isolated exopolysaccharides revealed the presence of major functional groups like carboxyl and hydroxyl groups. The presence of these functional groups in the exopolysaccharide may enhance their application in the various industrial areas.

Biosurfactants have several advantages over synthetic ones, such as biodegradability, low toxicity, biocompatibility and digestibility, as well as their use in food processing, cosmetics, and maximizing oil recovery. On a pH 3.5 growth medium, *Mucor rouxii* produces biopolymer flocculants using beet-molasses as a carbon source. For its crude EPS product, *Mucor rouxii* produced excellent flocculation properties which, EPS effectively aggregated and precipitated the soil or charcoal particles over a five-minute period (Abu-Elreesh et al., 2014; Abdel-Aziz et al., 2011). Exopolymers of curvilaria sp. DFH1 strains serve as effective flocculants for kaolin suspensions (Gadallah et al., 2014). *Myxobacteria Sorangium* cellulosum exopolysacachride exhibit flocculation activity as detected by modified Kurume et al. (1994a) (Zhang et al., 2002).

Antibacterial substances or compounds kill or slow the growth of bacteria (Kohanski et al,. 2010). In 1942, Selman coined the term "antibiotic" to describe any substance produced by a microbe to inhibit growth of other microbes (Waksman Sa, 1974; Hughes and Fenical, 2010). In a study, crude exopolysaccharides of *Ganoderma carnosum, Polyporus arcularius, Cerrena unicolor, Laetiporus sulphureus, Coprinus comatus, Lenzites betulina* and *Clavariadelphus truncatus* showed high antimicrobial activity against *Bacillus subtilis* (NRRL B-3711) and *Staphylococcus aureus* (ATCC 25923) (Demir and Yamac, 2008). Exopolysaccharides isolated from the white rot fungus *Ganoderma applanatum* have been shown to have antibacterial properties against *Vibrio fischeri* and *Staphylococcus aureus*.

Many microbial exopolysaccharides have the ability to be used as bio-emulsifier because they can stabilize emulsions between water and hydrocarbons. The use of bio-emulsifiers have been considered advantageous over to synthetic emulsifiers due to their physicochemical properties, biodegradable and higher foaming non-toxic nature(Freitas et al., 2009 and Lopes et al., 2014). Bio based emulsions could be utilized for targeted delivery of specialized bioactive agents and functional foods, to combat diseases and to promote and sustain good health. Hence, bio based emulsions could play a role in wide variety of natural and manufactured materials used in many fields, including the pharmaceutical, cosmetics and food industries (Lin and Mei, 2000; Mc Clements, 2005; Islam et al., 1997). The emulsification activity (% EA) of the indigenous Aureobasidium pullulans RYLF10 EPS was quite fair with vegetable and olive oils used in their study. The emulsion of the test EPS with 27 types of olive oil can potentially be used in various food applications where olive oil is used (Yadav et al. 2014). The potential usefulness of the biosurfactant produced by a flamentous fungus *Fusarium* sp., *Penicillium* sp. and *Trichoderma* sp. isolated from soil samples from the Amazon region and they can be used in high salt-concentration conditions, for example, in tertiary oil recovery activities (Sena et al., 2018).

## CONCLUSION

The greatest potential of fungal exopolysaccharides is related to their use in high value market niche, such as cosmetics, pharmaceuticals, food and biomedicine, in which traditional polymers fail to fulfill with the required degree of purity or lack some specific functional properties. Such markets will provide opportunities for the development of upcoming fungal

exopolysaccharides providing that they have unique desirable physicochemical may find application in various industrial sectors like cosmetics, oil recovery industries as emulsifiers, biomedicines, waste water treatment, food industries etc.

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