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Functional evaluation of exopolysaccharide from *Pseudozyma* sp. NII 08165 revealed the potential thickening and emulsifying applicability

Kuttuvan Valappil Sajna[1#]*, Rajeev Kumar Sukumaran[1], Lalitha Devi Gottumukkala[1], Sankar Sasidaran[2]& Ashok Pandey[1^]

[1]Microbial Processes and Technology Division; [2]Functional Materials Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram-695 019, Kerala, India

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Exopolysaccharides (EPSs) have huge commercial potential as additives in food, personal care and pharmaceutical formulations. The physicochemical properties, in particularly rheological behaviour of an EPS, determine its potential application. In the present study, we investigated the structural, rheological, emulsifying, flocculating and water holding properties of *Pseudozyma* EPS in relation to its potential for industrial applications. *Pseudozyma* sp. NII 08165 was previously reported to produce an EPS of 1.7 MDa with high viscosity and pseudoplastic behaviour. Congo red assay indicated an ordered helical conformation of theEPS that could impart many desirable attributes. Comparative analysis of the EPS with respect to xanthan gum revealed that it had significant thickening efficiency and moderate suspending ability. Rheology of *Pseudozyma* EPS was stable up to 60°C and over the pH range 4.0-9.0. EPS exhibited superior flocculating activity and its emulsifying activity was as good as that of xanthan gum. The *Pseudozyma* EPS also demonstrated adequate water holding capacity. These results revealed that the *Pseudozyma* EPS could be used as a potential thickener, emulsifying agent or flocculating agent for diverse applications.

Keywords: Flocculating activity, Rheology, Water holding capacity

Microbial exopolysaccharides have immense biotechnological potential and are capable of replacing the traditionally used plant and algal-based hydrocolloids, owing to their ease of production, consistent quality, and superior performance[1]. EPS are widely used as a thickening or gelling agent in industries owing to the rheology modifying properties. EPS, rich in -OH functional groups can impart good solvation in aqueous solution. Due to their intra- and inter-molecular interaction, the addition of EPS leads to the stabilization of emulsions and help in film formation. It can also prevent the agglomeration of ice particles and improve the organoleptic properties of food formulations[1,2]. Xanthan gum, an EPS produced by *Xanthomonas campestris*, has been widely used in many industries as a thickener and emulsion stabilizer. Thermal stability and hydrophobic nature of some of the EPS make them potential biolubricants, which can be used in oil drilling and recovery process[3]. An edible coating made of EPS (eg. gellan) has been developed for food packaging applications[4]. Value-added products can be obtained from EPS with unconventional monosaccharide composition. A good example is fucose, a rare sugar with anti-inflammatory and anticarcinogenic properties can be obtained from FucoPol[5]. Owing to the safety and biocompatibility, many EPS are used in the biomedical field for the purpose of drug delivery, tissue engineering and regenerative medicine. Biocellulose and Kefiran exhibit good scaffolding properties with porous structure and can be used for wound dressing[6]. Some EPS with biological properties such as antioxidant activity, hypoglycemic or hypocholesterolemic activity can be used as nutraceuticals[7]. An exopolysaccharide, DeinoPol produced by *Deinococcus radiodurans* is a promising high-value medical polymer due to its protective abilities against reactive oxygen species (ROS) induced cellular damage[8]. Some probiotic EPS such as *Bifidobcterium* EPS exhibited anticancer activity in cancer cell lines by inducing cell cycle arrest and cell apoptosis[9].

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*Correspondence:
Phone: +91 9847336102 (Mob.)
E-mail: Sajnak@iisc.ac.in; Sajna.scie@gmail.com
#Present add.: [#]Department of Biochemistry, IISc, Bengaluru, Karnataka, India
[^]Centre for Innovation and Translational Research, CSIR-IITR, Lucknow, UP, India

Yeasts are the major industrially relevant microbes used for producing many biotechnological products such as ethanol, SCP, and heterologous proteins. Their commercial utility surpasses other groups of microbes used for industrial applications[10]. Though many yeasts belonging to the species, such as *Candida*, *Rhodotorula*, *Cryptococcus*, *Sporobolomyces* have been reported to produce EPS, utilization of yeast for commercial production of EPS is still limited as there is a clear knowledge gap in the characterization and applicability of yeast EPS[11].

Pseudozyma species are commercially used for production of lipase and biosurfactants. Various other metabolites produced by *Pseudozyma* species are itaconic acid, squalene, and enzymes responsible for bioplastic degradation[12-14]. *Pseudozyma* sp. NII 08165 is a versatile yeast, producing metabolites depending on the carbon source in the culture medium. The organism produced mannosylerythritol lipids — a glycolipid class of biosurfactants from culture medium containing vegetable oil; and EPS from the glucose-containing medium. Structural studies revealed that *Pseudozyma* EPS was a high molecular weight polymer of glucose, galactose, and mannose with a compact film-like structure[15,16]. In the present study, we investigated the structural, rheological, emulsifying, flocculating and water holding properties of *Pseudozyma* EPS for its suitability in diverse applications.

Materials and Methods

Production of EPS by *Pseudozyma* sp. NII 08165

Culture medium containing (g/L) 40 (w/w) glucose, 3.0 $NaNO_3$, 0.3 g/L $MgSO_4$. $7H_2O$, 0.3 KH_2PO_4, 1.0 yeast extract (pH 6.0) was used for the production of EPS[17]. The inoculum was prepared by inoculating *Pseudozyma* sp. NII 08165 into 50 mL potato dextrose broth in 100 mL conical flask and incubated at 30°C and 200 rpm agitation for two days. The production medium was inoculated with 10% w/v inoculum and incubated at 30°C and 200 rpm agitation for four days. EPS was extracted as reported previously[16].

Conformation of *Pseudozyma* EPS by Congo red assay

The conformational structure of *Pseudozyma* EPS solution was determined by measuring the change in the absorption maximum (λ_{max}) of the dye Congo red in the presence and absence of polysaccharide preparations. *Pseudozyma* EPS solutions were prepared at 0.1 % with different NaOH concentrations from 0 to 0.4 M. 91 μM Congo red (99%, Sigma Aldrich, USA)was added to these solutions. The absorption spectra were recorded from 400 to 600 nm at 25°C with a UV-160A (Shimadzu) spectrophotometer. 0.1% xanthan gum (Sigma Aldrich, USA) was taken as a positive control. As a negative control, solutions of pure dye were used at the same NaOH concentrations[18].

X-Ray diffraction (XRD) analysis

The crystallinity index of EPS was determined by X-ray diffraction method as described previously[19] using an X- pert pro X- Ray diffractometer (PANalytical, Netherlands). The X-ray diffractograms were recorded from 0 to 80° with a step size of 0.03° using a Cu - Kα radiation X-ray (λ= 1.54 Å) generated at a voltage of 40 kV and a current 30 mA.

Thickening efficiency

Thickening efficiency was measured by determining the viscosity at various concentrations of EPS[2]. EPS solution was prepared from 0 to 2% concentration and the viscosity was measured by visco-rheometer (Rheolab MC1, Model- 749558, Physica). Xanthan gum was used a control.

Suspending ability

Suspending ability was determined by measuring the yield value. EPS solution (2%) was prepared and the viscosity was measured by visco-rheometer (Rheolab MC1, Model- 749558, Physica). Yield value is calculated by using the following equation[2].

$$\text{Yield Value (mPa)} = 2{*}r_1\,(\eta_1 - \eta_2) \qquad \text{Eq. (1)}$$

η_1, η_2 are apparent viscosities obtained at two different spindle speed, r_1 and r_2 only when r_2/r_1= 2. Two percent xanthan gum was used as a control.

Effect of temperature and pH on rheology of EPS

The stability of EPS over a wide range of temperature and pH was studied using viscometric analysis[20]. An aqueous solution of EPS was prepared at a concentration of 1%. The effect of temperature was studied by incubating EPS solution at 20, 40, 60, 80and 100°C for 3 h, followed by measuring the viscosity of solutions. The pH stability was studied over a pH range of 2.0-12.0 by dissolving EPS in the appropriate buffers. The stability was investigated by measuring the viscosity by visco-rheometer (Rheolab MC1, Model 749558, Physica).

Flocculating activity

The flocculating activity was measured by using the method as described by Lim *et al*.[21]. Activated carbon was used as a testing material, which was suspended in distilled water at a concentration of 5 g/L. In a test tube, 10 mL of activated carbon suspension was added and mixed with 0.1 mL of $CaCl_2$ solution (6.8 mM). To this mixture, various amounts of EPS were added and vortexed for 30 s and allowed to stand at room temperature (28.8°C) for 10 min. The turbidity of the upper phase was measured at 550 nm. A control experiment without the EPS was also pursued in the same manner. The flocculating activity (%) was calculated according to the following equation:

$$\text{Flocculating activity} = (B-A)/B \times 100 \qquad \text{Eq. (2)}$$

whereas B-absorbance of control, A- absorbance of the sample.

Emulsifying activity

The emulsifying activity of EPS was determined according to Bramachari *et al.*[22]. Briefly, lyophilized EPS (0.5 mg) was dissolved in 0.5 mL deionized water and volume was made up to 2.0 mL using Phosphate buffered saline (PBS). The sample mixtures were vigorously vortexed for 1.0 min after the addition of hexadecane. The absorbance was read immediately before and after vortexing (A_0) at 540 nm. The decrease in absorbance was recorded after incubation at room temperature for 30 and 60 min (At). A control was run simultaneously with 2.0 mL of PBS and 0.5 mL of hexadecane without EPS. The emulsification activity was expressed as the percentage retention of the emulsion during incubation for the time, t.

$$\text{Emulsifying activity} = At/A0 \times 100 \qquad \text{Eq. (3)}$$

The emulsifying activity of xanthan gum was also determined and compared with that of *Pseudozyma* EPS.

Water holding capacity

Water holding capacity (WHC) of *Pseudozyma* EPS was determined according to Ahmed *et al.*[23]. For this, 0.2 g EPS was suspended in 10 mL of deionized water on a vortex mixer. The dispersed material was centrifuged at 12000 rpm for 25 min. Unbound water that was not held by EPS material was discarded. All EPS material was dropped on pre-weighed filter paper for complete drainage of water. The weight of precipitated EPS was recorded. The percentage of WHC was calculated by the following equation:

$$\text{WHC (\%)} = (\text{[total sample weight after water absorption]/total dry sample weight}) \times 100 \qquad \text{Eq. (4)}$$

Statistical analysis

All experiments were carried out at least in triplicates and data obtained presented as means ± SD. All the data were subjected to one-way ANOVA. Results were considered significant at $P<0.05$ throughout the present study.

Results and Discussion

Conformation studies of *Pseudozyma* EPS by Congo red assay

Congo red assay is a rapid method for detecting the helical conformation in the polysaccharides. It has been reported that polysaccharides with a helical conformation form a complex with Congo red in dilute alkaline solution while polysaccharides with random coil conformation do not form complexes with Congo red. The principle of this assay is the formation of polysaccharide-Congo red complex, which results in the shift of maximum absorption wavelength (λ_{max}) of Congo red[18]. Changes in λ_{max} of the dye Congo red in the combination of *Pseudozyma* EPS at different concentrations of NaOH were measured (Fig. 1). Xanthan gum was taken as a positive control as it was reported to have a rigid helical structure[1]. At low concentration of NaOH, λ_{max} of Congo red-EPS solution shifted to longer wavelength (522 nm). Thereafter, λ_{max} dropped gradually reaching the same value as of the control. A similar observation was noted in the case of xanthan gum also. Hydrogen bonds are relevant for the dye-fibre interaction, and λ_{max} displacement is caused by the order-disorder transition that might be due to the breakage of hydrogen bonds.

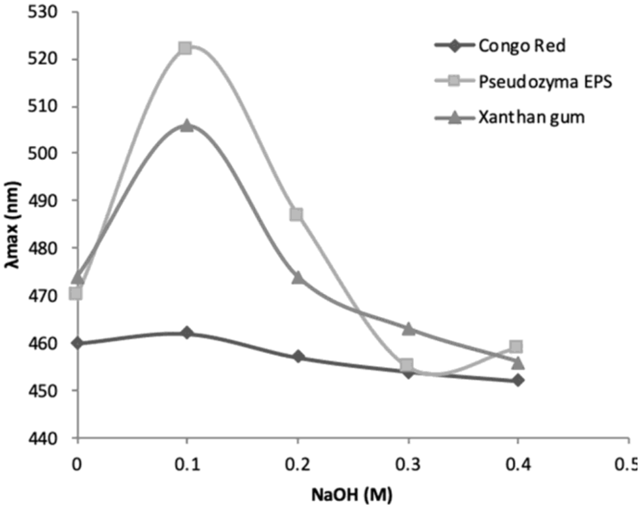

Fig. 1 —Shift in λmax of Congo red-*Pseudozyma* EPS complex and Congo red-xanthan gum complex at different NaOH concentrations

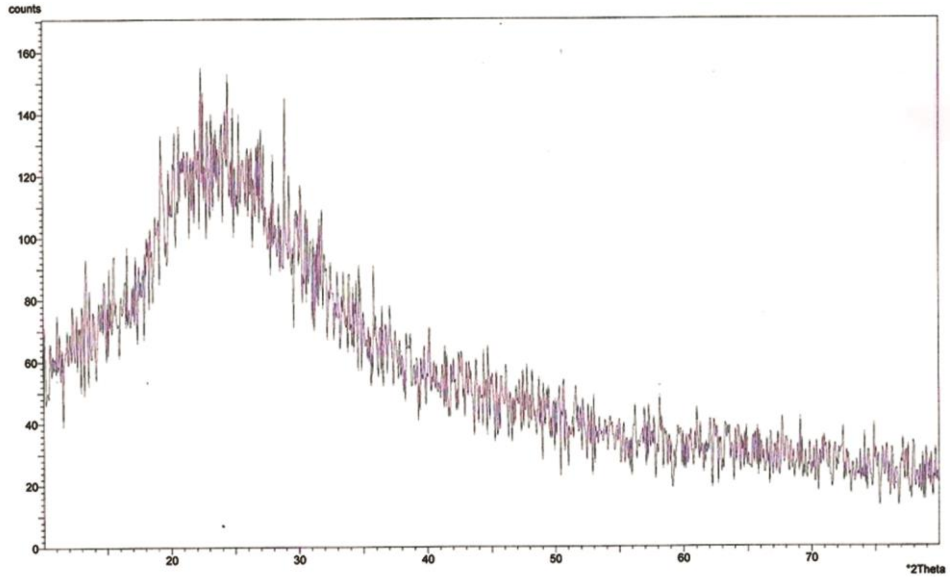

Fig.2 — XRD profile of *Pseudozyma* EPS

Hence, it can be concluded that *Pseudozyma* EPS adopted an ordered hydrogen bond dependent helical conformation in neutral and slightly alkaline aqueous solutions. Under strong alkaline condition (>0.2 M NaOH), the tertiary structure was denatured into random coil conformation.

Molecular conformation determines the viscoelastic behaviour of the polysaccharides in solution. Helical conformation of polysaccharide contributes to its rigidity and molecular stiffness. The helical structure of xanthan gum is responsible for its ability to bind with other molecules used in food and drug formulations[1]. Velasco *et al*.[18] reported that the tertiary structure of β-glucan from *Pediococcus parvulus* was an ordered hydrogen bond dependent helical conformation in neutral and slightly alkaline solution, which denatured at high concentration of NaOH. Ordered polysaccharide improved organoleptic properties of food formulations like thickness and mouthfeel and resulted in perfect flavour release[24].

X-Ray diffraction (XRD) analysis

X-ray diffraction is a powerful tool used for phase identification of the materials. No characteristic diffraction peaks were detected in the XRD pattern of *Pseudozyma* EPS. Hence, the compound was fully amorphous in nature (Fig. 2). Singh *et al*.[25] suggested that it was difficult to interpret the broad amorphous peaks of amorphous EPS while it is easy to interpret narrow crystalline peaks and calculate the crystallinity index.

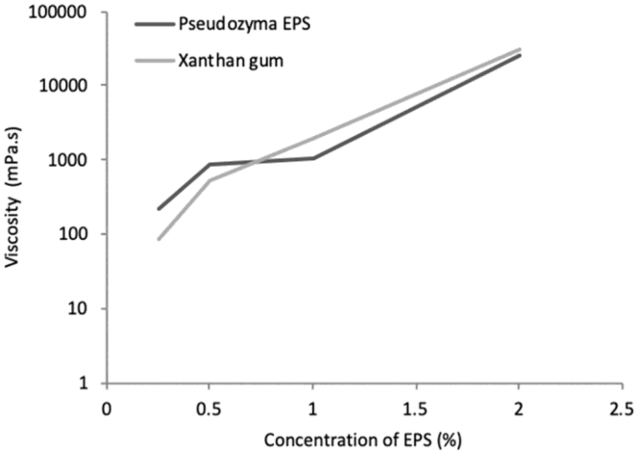

Fig. 3 — Viscosity *vs*. concentration of EPS plot to determine the thickening efficiency

Thickening efficiency

Fig. 3 illustrates the effect of concentration of EPS and xanthan gum on their viscosity. One of the major applications of EPS is the thickening of an aqueous system, and thickening efficiency determines the concentration ideal for thickening. At a concentration of 0.25% (w/v), *Pseudozyma* EPS resulted in a moderate viscosity of the solution. An addition of *Pseudozyma* EPS at a concentration of 0.5% contributed to a significant increase in viscosity, which showed its potential as a thickening agent for the water-based system. *Pseudozyma* EPS at a concentration of 2% caused a high viscosity with a gel-like consistency. Beyond 2%, EPS was not fully soluble in the aqueous system. Hence, the concentration ideal for EPS to be used as a thickener could be from 0.5-2%. These findings were similar to that of xanthan gum, an efficient thickening agent used for industrial purposes, and thickening efficiency of *Pseudozyma* EPS was comparable to that of xanthan gum.

A pronounced increase in viscosity at a range of concentrations of 0.2-1% has been reported in the case of Vanzan, a commercial brand of xanthan gum[26]. There is a positive correlation between the thickening efficiency of the EPS produced by *Lactococcus lactis* and the viscosity of the fermented milk[27]. Primary structure, size, and composition of EPS play a significant role in thickening properties. Hence, the structural modification of EPS changes the thickening efficiency as side chains increase the stiffness of polymer chains[28]. Thickening efficiency reveals the performance properties of a polymer in an aqueous system. It can also be used to determine the degree of thickening to bring out the ideal viscosity of the final product and find out which hydrocolloids are ideal for thin formulation with maximal stability and minimal viscosity.

Suspending ability

Suspending ability is an important parameter to be considered from the application point of view of a polymer and is expressed in terms of yield value. Yield value is the minimum force that must be applied to a fluid to start disrupting the cohesive polymer network imparted by rheology modifier so that flow can occur. Simply, it is the initial force to initiate the flow. Greater the yield value, more stable the suspension would be. Yield value of *Pseudozyma* EPS was 4173 mPa, while xanthan gum showed a value of 9246 mPa at 2% concentration. Xanthan gum has already been reported to have excellent yield value compared to other commercial hydrocolloids. It has been reported that at a 1% concentration, yield values of xanthan gum, guar gum, hydroxymethyl cellulose, locust bean gum, carboxymethyl cellulose and sodium alginate are 11300, 4000, 830, 360, 410, 210 mPa, respectively. Xanthan gum also exhibited a notable yield value of 500 mPa even at low concentration of 0.3%, while all the above hydrocolloids do not exhibit significant values at this concentration[2]. Even though the yield value of *Pseudozyma* EPS was less than that of xanthan gum, it was still significant which could make it a possible stabilizing agent in the case of suspension formulation.

EPS from *Sphingomonas paucimobilis* GS-1 on cross-linking with chrome alum exhibited a strong suspending ability and other rheological properties and had the potential to be used in oil drilling applications[29]. Many EPS lack data on suspending ability. Structural and rheological properties of EPS such as rigid conformation and formation of the weak network in the solution account for the high yield value, which contributes to its remarkable ability to stabilize dispersion, such as suspensions and emulsions[30].

Effect of temperature and pH on rheology of EPS

Effects of temperature and pH on the rheology of EPS were studied. *Pseudozyma* EPS was having the viscosity of 756, 742, 586, 245 and 30 mPa.s at the temperature 20, 40, 60, 80 and 100°C. Rheology of *Pseudozyma* EPS was stable up to 60°C. Compared to ambient temperature, EPS retained 78% of its viscosity at 60°C, 32% activity at 80°C and 4% activity at 100°C. When the viscosity was monitored online at respective temperature, the viscosity was much higher at the lower temperature and there was a significant drop in viscosity at the higher temperature, which could be explained by the temperature dependence of liquid viscosity (data not shown). Viscosity remained more or less constant between pH 4.0-9.0. The viscosity values at the pH 2, 4, 7, 9 and 12 were 332, 729, 738, 742 and 623 mPa.s. When compared to the viscosity at neutral pH, EPS retained around 84% viscosity at pH 12.0, while viscosity was 45% at pH 2.0. Since it was stable over the pH 4.0-9.0, *Pseudozyma* EPS could be used in both acid and alkaline systems.

Temperature and pH play a huge role in the flow behaviour of EPS. Viscosity decrease with increase in temperature due to the altered intramolecular arrangements or modified 3D structure, with loose polymer structure. Hydrolysis of glycosidic linkages at high acidic pH could result in lower viscosity. At higher temperature and pH, the activation energy of the colloids will be decreased, which is related to the cohesion and stickiness of the sugar dispersions. Apart from molecular conformation, the secondary structure of EPS, for example, the side chains, contribute to the stability of EPS at high temperature and a wide range of pH. Though viscosity decreases with increasing temperature, viscosity values of many EPS can be restored after the cooling, as the conformational transition of EPS from a rigid ordered state to flexible disordered state that happens under the influence of temperature is mostly reversible. Some EPS formed a gel-like solution of high viscosity only at acidic pH, while other EPS are highly viscous over a wide pH range of 3.0-11.0. Hence, temperature and pH stability of EPS is dependent on molecular structure[31-33].

Flocculating activity of EPS

Flocculating activity assay was performed for two different sets of *Pseudozyma* EPS concentration (0.001-0.01, and 0.01-0.1 mg/L) to determine the concentration of EPS at which high flocculating activity was obtained. The high flocculating activity was in the range of 0.01-0.1 mg/L of EPS. The flocculating activity was compared with that of xanthan gum (Fig. 4A). Optimal EPS concentration for the maximal flocculating activity was 0.01 mg/L and flocculating activity of xanthan gum was maximum in the range of 0.06 to 0.1 mg/L. Hence, *Pseudozyma* EPS exhibited higher flocculating activity than that of xanthan gum and was promising as a potential flocculating agent.

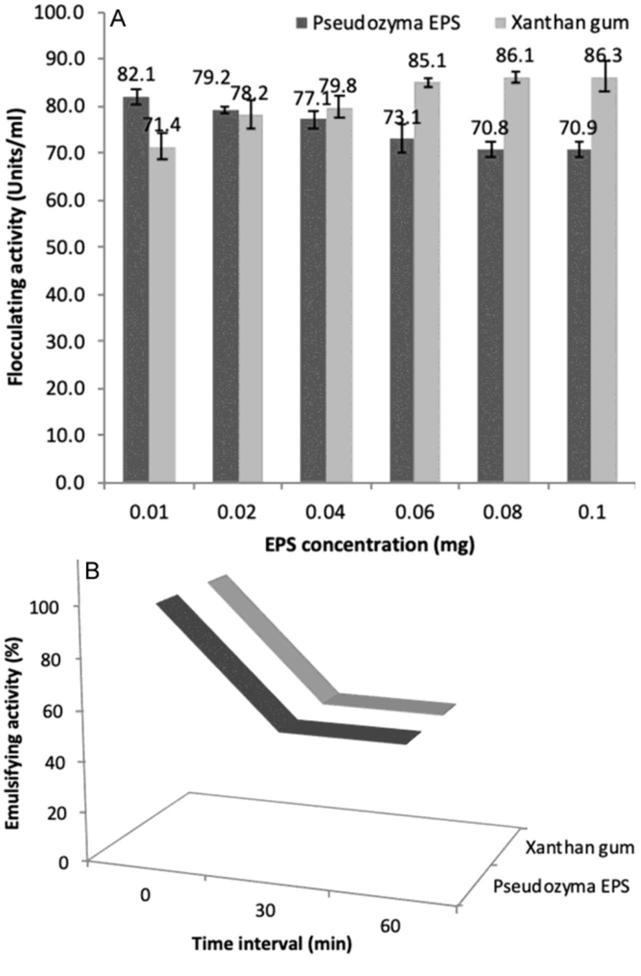

Fig. 4 — (A) Flocculating activity; and (B) Emulsifying activity of *Pseudozyma* EPS and xanthan gum

In flocculation, free functional groups present in the EPS act as bridges, which aggregate the suspended particles forming a floc. The concentration of flocculent should be optimized in flocculation as excess flocculants adsorb and destabilize the particle, resulting in poor flocculation. Flocculating agents are widely used for wastewater treatment, drinking water purification, and various downstream processes. Microbial flocculants are preferred over the chemical flocculants due to their non-toxicity and biodegradability. EPS produced by a *Zoogloea* strain had remarkable flocculating activity, which was better than that of xanthan gum[19]. Glucan-DM5 exhibited higher flocculating activity than the commercial hydrocolloid guar gum and possessed the potential to be used as bio-flocculent in the dairy industry for making cheese from curd[34]. Bioflocculants producing microbes could be effective heavy metals biosorption agents[35].

Emulsifying activity

This assay measures the ability of EPS in retaining the emulsion of hydrocarbons in water. The emulsifying activity of *Pseudozyma* EPS against n-hexadecane was compared with that of xanthan gum (Fig. 4B). *Pseudozyma* EPS retained54.46±3.6% and 54.23±2.7% activity after 30 and 60 min, respectively, whereas xanthan gum retained 53.81±4.0% and 53.45±3.4% after 30 and 60 min, respectively. Emulsions produced by *Pseudozyma* EPS were as stable as those of xanthan gum. The emulsification activity of *Pseudozyma* EPS was comparable to that of xanthan gum and could be considered as a potential emulsifier for food and cosmetic applications. Polymeric stabilizers such as xanthan gum are necessary for formulating oil-in-water emulsions, in skincare products.

Lower toxicity, higher biodegradability and improved performance at high temperature, pH and salinity are some of the advantages of using microbial emulsifiers over the synthetic emulsifiers. The emulsifying activity of EPS is imparted by the functional groups present in the structure[25]. Glucan-DM5, an EPS synthesized by *Lactobacillus plantarum* DM5 showed emulsifying activity superior to commercial hydrocolloids such as guar gum and sodium alginate[35]. Yeast EPS such as mannan and glucomannan exhibit strong emulsifying activity.

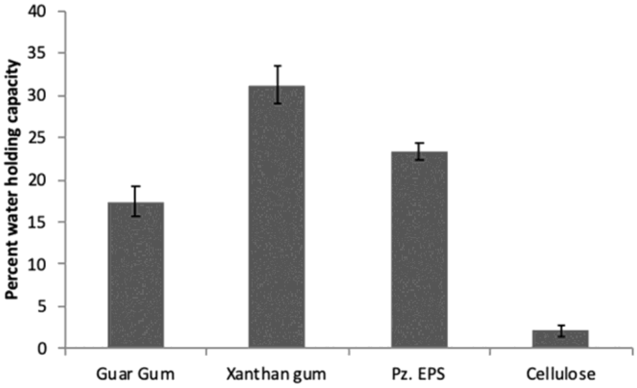

Fig. 5 — Water holding capacity of *Pseudozyma* EPS and different hydrocolloids

Because of its distinct emulsion stability when compared to the commercial emulsion stabilizers such as Rofetan and Arlacel, glucomannan was proposed for the preparation of emulsion creams[36]. Bhatnagar *et al*.[37] reported a sugar-rich exopolymer with significant emulsifying activity from cyanobacteria, *Nostoc calcicola*.

Water holding capacity of *Pseudozyma* EPS

Water holding capacity (WHC) is one of the hydration properties of hydrocolloids that allow the gum to hold the water. Fig. 5 illustrates the WHCs of different polymers. WHC of *Pseudozyma* EPS was 23%, whereas guar gum, xanthan gum, and cellulose exhibited 17, 31 and 2%, respectively. Though EPS exhibited lower WHC than xanthan gum, its WHC was better than that of guar gum and cellulose. Since the particle size and moisture content of the polymer significantly influences the hydration properties of hydrocolloids, crude lyophilized EPS from *Pseudozyma* which has good emulsifying activity compared to the commercial-grade xanthan gum may hold good as a potent product for applications that require significant water holding capacity.

Water holding capacity of EPS is attributed to the permeable structure of EPS, where it can bind to a large amount of water through hydrogen bonding[23]. Nehal *et al*.[38] suggested that water retaining ability of EPS is related to the distinct chemical composition and high pore size of EPS. Water holding capacity is one of the important properties of the hydrocolloids used in the food and cosmetic industry. Xanthan gum, guar gum, and modified cellulose products are commercially used hydrocolloids that enhance the water holding capacity of food, cosmetic and pharmaceutical preparations. High water holding capacity of hydrocolloids causes the reduction in evaporation rate, alteration in freezing rate and modification in ice crystal formation of food. Hydrocolloids are essential ingredients in the baking industry, which confers various desirable functions. One of the primary functions is to serve as an antistaling agent by improving the water holding capacity of the product[39]. Hydrocolloids with high water holding capacity are usually added to cosmetics such as moisturizing formulations and sunscreens. One of the prerequisites for a cosmetic ingredient for the facial mask products is its high water holding capacity, where it enhances the moisture uptake by facial skin[40]. High water holding capacity of bacterial cellulose produced by *Acetobacter xylinum* facilitated its potential application as absorbent pads[41].

Conclusion

An ordered helical conformation was found to be the structural feature of *Pseudozyma* EPS. Compared to xanthan gum, *Pseudozyma* EPS possessed significant thickening efficiency and moderate suspending ability. *Pseudozyma* EPS was stable over a range of temperature (20-60°C) and pH (4.0-9.0). The potential emulsifying, flocculating ability and water holding capacity of *Pseudozyma* EPS were evaluated. The pseudoplastic behaviour of EPS, along with significant thickening efficiency and suspending ability offered potential for its applications as a thickening agent or gelling agent in the food industry. Because of its excellent flocculating activity, *Pseudozyma* EPS could also be used as a flocculent in the management of wastewater and industrial effluents. *Pseudozyma* EPS possessed good emulsifying activity with good water holding capacity, which further emphasized on its potential applications in food, cosmetic and pharmaceutical industries. Thus, this study demonstrated that *Pseudozyma* EPS could be an attractive candidate for industrial applications.

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Conflict of Interest

Authors declare no conflict of interests.

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