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Studies on structural and physical characteristics of a novel exopolysaccharide from *Pseudozyma* sp. NII 08165

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ABSTRACT

The aim of this work was to study the production of exopolysaccharide (EPS) from a novel ustilaginomycetes yeast strain *Pseudozyma* sp. NII 08165. The culture produced 3.5 g/l EPS on fourth day of fermentation in a glucose-based medium. The structural characterization revealed that the EPS was a polymer of glucose, galactose and mannose in the ratio of 2.4:5.0:2.6 with a molecular weight of 1.7 MDa. The pseudoplastic behaviour of aqueous EPS with a thermal stability up to 220 °C indicated its potential utility as a thickening or gelling agent in food industry. SEM studies of the EPS showed that it had compact film-like structure, which could make it a useful in preparing plasticized films. The AFM studies showed that EPS had spike-shaped microstructure. Physical properties of the exopolysaccharide determined further indicated its possible potential in different industrial applications.

1. Introduction

Exopolysaccharide (EPS) with interesting physiochemical properties are secreted by microorganisms of different taxonomic groups. The physiological roles of EPS are not yet unravelled but they are implicated in pathogenicity, biofilm formation, quorum sensing, etc. [1]. Microbial exopolysaccharides possess several industrial applications, some are being used as thickeners, emulsifiers and stabilizer in food industry [2] and certain others exhibit biological activity with potential to be used in therapeutic and pharmaceutical industries [3].

Many yeasts belonging to the genera *Cryptococcus* [4], *Rhodotorula* [5], *Sporobolomyces* [6] and *Candida utilis* [7] are reported as EPS producers. EPS production is considered as one of the adaptation strategies for Antarctic yeast [8]. Yeasts commonly give high yields of EPS which can be easily separated from the culture broth compared to those produced by bacteria, this makes them more suitable for their commercial production [9]. Pullalan, a water soluble glucan gum produced by black yeast *Aureobasidium pullulans*, has commercial applications in both food and oral care industries [10]. The exopolysaccharide produced by yeast like fungus *Tramella* consist of a mannan backbone and has immunoregulatory property [11]. *Sporobolomyces salmonicolor* produces glucomannan having pseuodplastic behaviour and has potential to be used as thickener in food industry [12]. The distinct emulsification property, high compatibility on macrophage cell line and protective effect against toxic activity of Avarol makes the *Sporobolomyces* glucomannan an attractive candidate for applications in cosmetic industry [12,13]. Many psychrophilic yeast strains like *Cryptococcus flavus*, *Cryptococcus laurenti* were found to be good producers of EPS and the EPS from *C. laurenti* showed pronounced emulsification activity along with other hydrocolloids [4,14].

Pseudozyma are ustilaginomycetous anamorphic yeasts and are usually associated with plants. *Pseudozyma* are one of the commercially important yeasts as they produce squalene [15] itaconic acid [16], erythritol [17] and mannosylerythritol lipids [18]. *Pseudozyma* sp. NII 08165 was found to be a good producer of glycolipid biosurfactants with the potential use as laundry additives [19]. In this paper, we investigated the production of exopolysaccharide by *Pseudozyma* sp. NII 08165. The rheological properties and physiochemical characteristics of EPS were studied to find out its potential application. To the best of our knowledge, this is the first report on structural characterization and physiochemical characterization of EPS by *Pseudozyma*.

2. Materials and methods

2.1. Microorganism

The strain *Pseudozyma* sp. NII 08165 was isolated from an aerial sampling on lipase screening plate at Biotechnology division of

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NIIST. Stock cultures were prepared in PDA slants and stored at 4 °C. The organism was maintained by sub-culturing at every four weeks. Phylogenetic analysis was performed for the strain identification.

2.2. Media preparation and culture conditions

Basal medium containing (%) 4 glucose, 0.3 $NaNO_3$, 0.03 $MgSO_4 \cdot 7H_2O$, 0.03 KH_2PO_4, 0.1% yeast extract (pH 6.0) was used for EPS production. This medium was established as growth medium for *Pseudozyma* sp. [20]. The culture was incubated for four to six days at 30 °C with 200 rpm agitation.

2.3. Production of exopolysaccharide by Pseudozyma sp.

Cell growth, EPS yield and viscosity of the culture broth were studied at regular time intervals. Cell growth was determined by taking an aliquot of the medium (10 ml), centrifuging it at 12,000 rpm and at 4 °C for 20 min and drying the cell pellet at 80 °C for 24 h. To determine the EPS yield, EPS from cell-free culture broth (obtained as above after centrifugation) was precipitated by mixing the supernatant with cold ethanol (4 °C, 1:2, v/v) for 4 h. The precipitate (EPS) was lyophilized (SCAN VAC, Cool Safe 110-4 PRO) and then dry weight was determined. The apparent viscosity of cell-free culture broth was measured by visco-rheometer (Rheolab MC1, Model-749558, Physica).

2.4. Isolation of EPS

The culture broth was centrifuged at 12,000 rpm at 4 °C for 20 min. Two volumes of cold ethanol (4 °C) was added to the supernatant and kept at 4 °C for 4 h. The precipitated EPS was resuspended in distilled water and dialyzed against two changes of deionised distilled water. The protein and nucleic acid contents of the EPS were analyzed by Bradford assay [21] and UV spectrophotometry (Nanodrop ND-1000), respectively.

2.5. FTIR analysis

The functional groups of *Pseudozyma* EPS were recorded by Fourier Transform Infrared spectroscopy using Bruker AlphaT IR spectrophotometer. Sample was prepared by grinding with KBr pellets and it was scanned from 500 to 4000 cm^{-1}.

2.6. Sugar analysis

To determine the monosaccharide composition of *Pseudozyma* EPS, 5 mg EPS was first hydrolyzed with 2 M H_2SO_4 at 100 °C for 3 h. The hydrolysate was then neutralized with calcium carbonate and filtered through 0.22 μm syringe filter. The monomers were determined by HPLC (Shimadzu) using Aminex HPX-87P carbohydrate analysis column (Biorad) with RI detector. The mobile phase used was deionised water at a flow rate of 0.6 ml/min.

2.7. Molecular weight estimation

Gel filtration chromatography was performed to determine the molecular weight of *Pseudozyma* EPS. EPS sample was loaded into gel filtration column packed with Sepharose 6B (Sigma–Aldrich, India) and elution of EPS was monitored by phenol-sulphuric acid assay [22]. Molecular weight was estimated from the standard graph which was plotted using standard dextrans (Sigma–Aldrich, India).

2.8. Rheological analysis of aqueous EPS solution

The dynamic viscosity of aqueous EPS was investigated by visco-rheometer (Rheolab MC1, Model-749558, Physica). The flow behaviour kinetics was established by Oswald-de-Waele model [23].

2.9. Thermogravimetric analysis

The thermal behaviour of *Pseudozyma* EPS was studied by thermo-gravimetric analysis (TGA) using TG-DTA 6200 (SII Nanotechnology Inc., Japan). The compound was subjected to a temperature range of 30–1000 °C under nitrogen atmosphere at a rate of 10 °C/min and the corresponding weight loss was determined.

2.10. Scanning electron microscopic analysis

Scanning electron microscopy (SEM) was done to study the surface morphology of *Pseudozyma* EPS. EPS solution (1 mg/ml) was added to aluminium stubs and air dried. The sample was gold sputtered using SC7620 Sputtercoater device and analyzed by scanning electron microscopy (Zeiss Evo-18 Special Edition).

2.11. Atomic force microscopy

Aqueous EPS solution (1 mg/ml) was added to freshly cleaved mica surface and kept for drying. Atomic force microscopic images were recorded under ambient conditions using a NTEGRA system (NT-MDT) operating with a tapping mode regime. Micro-fabricated TiN cantilever tips (NSG10) with a resonance frequency of 299 kHz and a spring constant of 20–80 N m^{-1} were used.

3. Results and discussion

3.1. Microorganism

The yeast isolate was identified by the phylogenetic analysis of the sequenced region of ITS1, 5.8S rRNA and ITS2 region [19]. The strain was deposited in NII culture collection and designated as *Pseudozyma* sp. NII 08165 and the sequence was submitted in NCBI Genbank with accession no JN969989.

3.2. Production of exopolysaccharide by Pseudozyma sp.

We compared EPS production in two media containing glucose and sucrose as reported by Poli et al. (2010) and Morita et al. (2007), respectively [13,20]. When *Pseuodzyma* sp. NII 08165 was grown in sucrose medium, EPS production was much lower than glucose containing medium (>1 g/l). Thus, glucose was used as the carbon source in further studies. Then EPS production was evaluated using different concentrations of glucose (1–5%). EPS yield increased with increase in concentration of glucose (data not shown) and the maximum production of EPS (3.5 g/l) was achieved with 4% glucose concentration (data not shown).

The cell growth, EPS yield and viscosity of the culture broth were monitored for a period of six days. EPS production reached the maximum on the 4th day of incubation (3.5 g/l); thereafter it decreased (Fig. 1). The viscosity data followed the similar pattern with maximum on 4th day (63 mPa s) and then declined, showing that the apparent viscosity of the culture broth was directly correlated with EPS production indicating that EPS was the single most important contributor to the viscosity of the culture medium. Results of cell growth, however, showed different pattern, with increase till 5th day and then decline (Fig. 1).

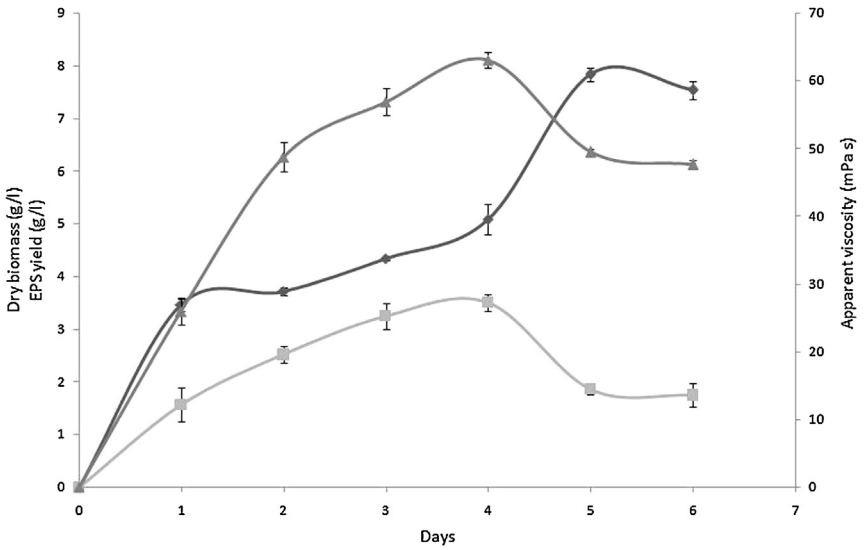

Fig. 1. Time course study of the growth and EPS production by *Pseudozyma* sp. NII08165. EPS synthesis (g/l; closed squares), biomass (dry weight, g/l; closed rhombus) and apparent viscosity (mPa s; closed triangles) of *Pseudozyma* sp. NII 08165 in batch culture.

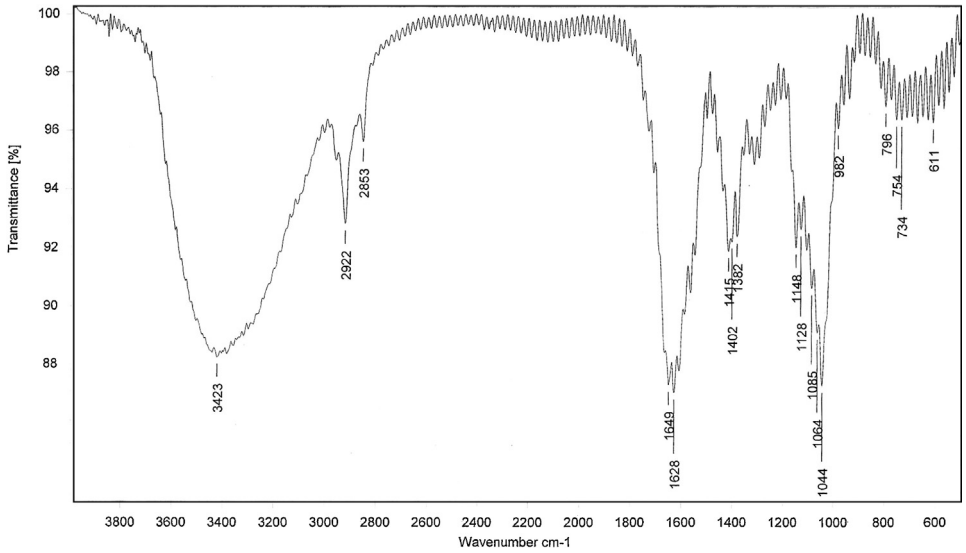

Fig. 2. FTIR spectrum of *Pseudozyma* EPS showing the typical polymer structure and functional groups of EPS.

3.3. Isolation of EPS and its characterization

The EPS recovered by ethanol precipitation was relatively pure and had only negligible amount of protein and nucleic acid (data not shown). The EPS was subjected to structural, physiochemical and morphological characterization to explore its potential applications.

3.4. FTIR analysis

The FTIR spectrum of *Pseudozyma* EPS was analyzed and absorption bands were assigned to reveal the typical polymeric structure of the carbohydrate (Fig. 2). A broad stretching in the region 3423 cm^{-1} was observed which represented the stretching vibration of the hydroxyl groups of carbohydrate. This is the characteristic absorption band of carbohydrate ring and is responsible for the water solubility of EPS [24,25]. The absorption bands at 2922 and 2853 cm^{-1} represented the C—H stretching of methyl and methylene groups [26]. The absorption band found in the region 1650–1540 cm^{-1} usually represents the stretching vibrations of enol and amide groups [27,28]. The stretching of C=O group was indicated by absorption band at 1628 cm^{-1}. Similarly, the peaks at 1415–1382 cm^{-1} could be assigned to >C=O stretch of the COO^- groups and C—O bond from COO^- groups [26,29]. A sharp absorption band at 1044 cm^{-1} represents the —C—O stretching vibration [29]. The wave number region from 1200 to 800 cm^{-1} is the fingerprint region and can be used to characterize different polysaccharides [30,31]. The monosaccharide constituents of pectic and hemicellulosic polysaccharides like galactose, mannose and glucose shows the strongest IR bands at 1078 cm^{-1}, 1070 cm^{-1} and 1035^{-1} respectively [32]. The absorption bands in the region 983–1200 cm^{-1} suggested the presence of sugar monomers such as glucose, galactose and mannose in the *Pseudozyma* EPS. In the anomeric region, absorption band at 796 cm^{-1} revealed the possible presence of alpha glycosidic linkages. On the contrary, absence of band at 870–890 cm^{-1} indicated that there could be no β-glycosidic linkage in the EPS [33]. The absorption bands observed at 734 cm^{-1} could be attributed to C—O—C bending vibration [34].

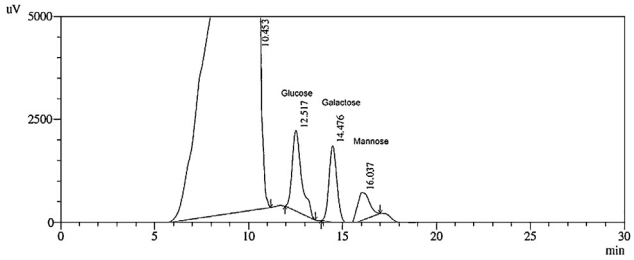

Fig. 3. HPLC analysis of acid hydrolyzed *Pseudozyma* EPS showing the sugar monomers-glucose, galactose and mannose.

3.5. Sugar analysis

Sugar analysis revealed that *Pseudozyma* EPS was a heteropolysaccharide of glucose, galactose and mannose (Fig. 3). The relative proportion of sugars determined in the EPS was 2.4:5.0:2.6 (glucose:galactose:mannose). The quantity and quality of the EPS produced by microbes are dependent on the carbon source and culture conditions [35]. A strain of *S. salmonicolor* produced glucogalacto mannan in the medium containing sucrose as carbon source [13]. The EPS of *C. flavus* contained mannose, glucose, xylose and galactose [4]. *Rhodotorula acheniorum* produced two fractions of EPS which were of mannan, while the EPS of *Rhodotorula glutinis* contained neutral sugars and uronic acids [5,36]. Difference in the sugars and their relative proportions contributes to the unique structures of EPS and their varying properties.

3.6. Molecular weight estimation

Pseudozyma EPS was found to have a molecular weight of 1.7 MDa. Generally, yeast EPS are high molecular weight polymers. Exopolysaccharide from a yeast, *S. salmonicolor* was reported to have a molecular weight of >1 MDa, while *C. flavus* produced EPS with molecular weight of 1.01 MDa [4,13].

High molecular weight of *Pseudozyma* EPS could be the reason for its high viscosity and ready-to-precipitate nature in ethanol. High viscosity of *Pseudozyma* EPS can be exploited in various industrial applications and its ready-to-precipitate nature in ethanol gives good product recovery thereby making downstream processing easy and efficient.

3.7. Rheological analysis of aqueous EPS solution

The rheological analysis of EPS solutions is important as it helps to determine the possible applications of EPS as thickeners, stabilizers, emulsifiers, gelling agents, etc. [36]. The dynamic viscosity of aqueous EPS was measured and the flow index value was determined by Oswald-de-Waele Model.

Oswald-de-Waele Model, $\tau = KD_r^n$

where τ = shear stress (Pa), D_r = shear rate (S^{-1}), K = consistency index (Pa s^n), n = flow index value.

The flow index value, 'n' for the *Pseudozyma* EPS solution was 0.422, which indicated the pseudoplastic nature of EPS. The apparent viscosity of the solution decreased with increasing shear stress revealing a non-Newtonian-shear thinning behaviour (Fig. 4). The pseudoplastic nature of yeast EPS had already been reported by Pavlova with *S. salmonicolor* [6,36]. Pseudoplasticity of biogums enhances sensory qualities such as flavour release and mouthfeel in food products and guarantees a high degree of mixability and pourability. Industrially important exopolysaccharides such as xanthan gum exhibit high viscosity with pseudoplastic behaviour which makes it an effective thickener and stabilizer in the food industry [2]. Rheological properties of scleroglucan made its

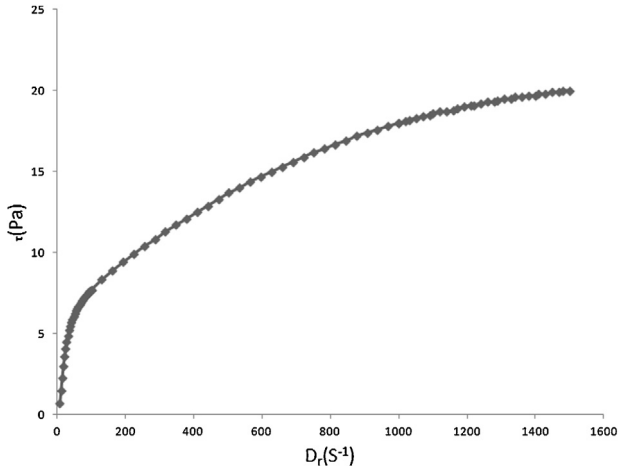

Fig. 4. Rheological behaviour of the aqueous solution of *Pseudozyma* EPS. The viscosity of the solution decreased with an increasing rate of shear stress revealing a non-Newtonian-shear thinning behaviour.

candidature for enhanced oil recovery applications [37]. Texture and gel quality of yoghourt is usually determined by the rheological properties of EPS produced by lactic acid bacteria during fermentation [38]. The rheological analysis of *Pseudozyma* EPS solution revealed its high viscous and pseudoplastic non-Newtonian fluid behaviour which implicate its potential as thickening and gelling agent in various industries.

3.8. Thermogravimetric analysis

It was clear from thermogram that *Pseudozyma* EPS underwent a weight loss of approximately 20% till the temperature of 120 °C, which corresponded to the elimination of surface bound water molecule (Fig. 5). This revealed the high water retention ability of *Pseudozyma* EPS. This property could be attributed to the presence of large number of carboxyl groups, as each carboxyl group is bound to a water molecule [39]. The exopolysaccharide was thermally stable up to 220 °C and the degradation temperature (T_d) was found to be 250 °C. The thermal degradation happened in two phases, the first being rapid till 300 °C with an observed weight loss of 45% and the second phase starting from 300 °C proceeded gradually at a slow pace. The slow decomposition might be due to the presence of some thermally stable saccharide moiety. The compound was fully decomposed at 895 °C. This result is in consensus with the EPS obtained from a basidiomycetes fungus, *Trameter*

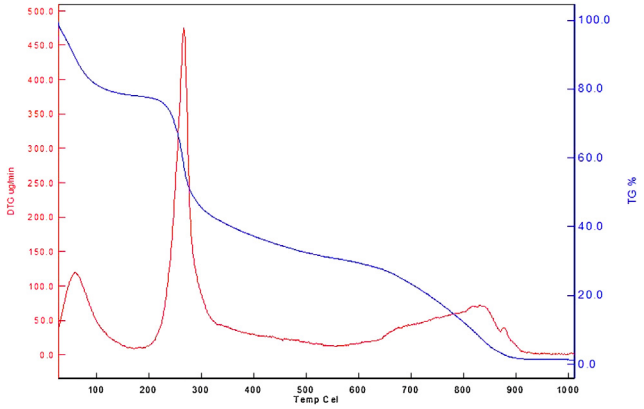

Fig. 5. Thermogravimetric analysis (TGA) of *Pseudozyma* EPS showing the thermal stability up to 220 °C and indicating a bistage thermal degradation.

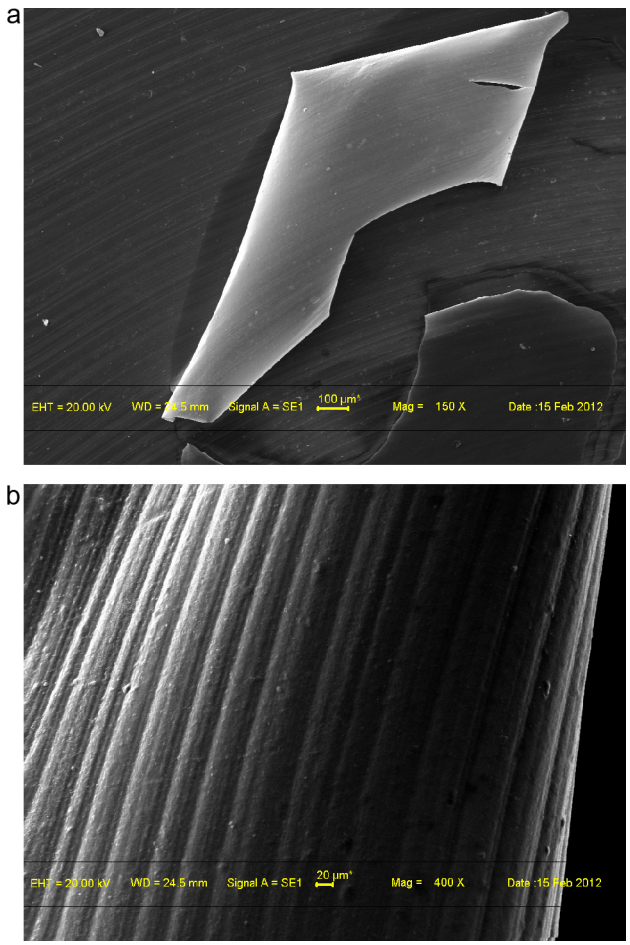

Fig. 6. Scanning electron micrograph (SEM) revealing the surface morphology of *Pseudozyma* EPS. Compact, non-porous film like structure at 150× magnification (a) and roof-tile like structure at higher magnification – 400× (b).

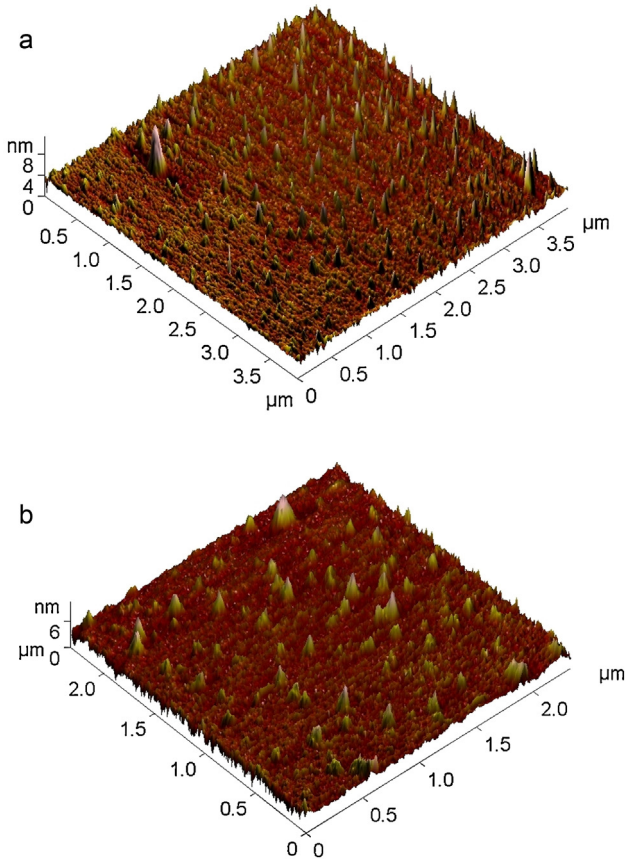

Fig. 7. Atomic force micrograph (AFM), 3D images of *Pseudozyma* EPS at concentration 10 μg/ml (a) and 100 μg/ml concentration (b). The microstructure of *Pseudozyma* EPS contained spike shaped lumps which became irregular at higher concentration.

vericolor which followed bistage thermal degradation with stability up to 280 °C [40]. The degradation temperature (T_d) of EPS from *S. salmonicolor* was 280 °C [13]. Thermostability of exopolysaccharide is an important characteristic considered for industrial applications, especially in food industry as the manufacturing and processing of several food preparations are usually carried out at high temperatures. *Pseudozyma* EPS, being thermostable along with its rheological properties is an ideal candidate for the food industry.

3.9. Scanning electron microscopic analysis

The scanning electron micrograph of the *Pseudozyma* EPS is given in Fig. 6. The *Pseudozyma* EPS formed a film like structure with smooth and rigid surface. Upon 400× magnification, the EPS appeared to have a compact roof tile like structure. The EPS did not possess any porous nature. All these properties project it as a good candidate for preparing plasticized films. The EPS from *Lactobacillus plantarum* was observed to have a compact structure and was proposed suitable for making plasticized films [26]. In a study by Ahmed et al., wherein SEM scan showed the integral surface structure of EPS from *Lactobacillus kefiranofaciens* which was an important feature required for plasticized film making [41]. Exopolysaccharides are one of the promising polymers for manufacturing bioplastics as it makes the process environmentally-friendly and safe.

3.10. Atomic force microscopy

Atomic force microscopy (AFM) was employed for the three dimensional analysis of surface structure and surface roughness of *Pseudozyma* EPS. AFM analysis revealed spike shaped lumps of varying size (Fig. 7). The lumps were proposed to be formed by inter- and intra-molecular aggregation of polysaccharide [33]. The surface structure seemed to be dependent on the EPS concentration. The smaller lumps of 3 nm height and comparatively larger ones with 5–6 nm height were observed at smaller concentration (10 μg/ml), where as irregular shaped spikes were observed at higher EPS concentration (100 μg/ml). The surface structure of EPS from *Lactobacillus* species contained round lumps and chains at low concentration and lumps and chains were irregular at higher concentrations [26]. Electron microscopy of EPS produced by *Bacillus pumilus* revealed the web like network structure which could be due to the intense affinity of EPS molecules for each other, thereby resulted in its excellent viscosifying and thickening properties [42]. The molecular structure of *Pseuodzyma* EPS revealed by AFM could suggest its strong affinity for water molecules and pseudoplastic nature.

4. Conclusions

Pseudozyma sp. NII 08165, a novel isolate secreted viscous compound, which was determined to be an interesting exopolysaccharide. The structural, physiochemical and morphological characterizations revealed the possible industrial applications of *Pseudozyma* EPS. *Pseudozyma* EPS was found to be a high

molecular weight polymer of 1.7 MDa with glucose, galactose and mannose as monomers. Its excellent rheological property along with thermal stability can be exploited in food industry. The compact microstructure suggested the potential application of *Pseudozyma* EPS in preparing plasticized films. Further work is needed to investigate the in vitro activity and ecological role of *Pseudozyma* EPS. These findings suggest the necessity of further exploration of ustilaginomycetes yeast for the production of commercially important EPS.

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