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Characterization and Antioxidant Activity of Exopolysaccharide Secreted by *Nostoc carneum*

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ABSTRACT
The physico-chemical characterization of the cyanobacterium *Nostoc carneum* extracellular polysaccharide (EPS) was studied. Two sugars moieties glucose (105.5 mg g^{-1} EPS) and xylose (215.2 mg g^{-1} EPS) were involved in the polysaccharide composition as well as the presence of sulfate, uronic acids and protein. The *in vitro* antioxidant assays (reducing power and DPPH) showed that *N. carneum* EPS possess antioxidant activity. Fourier Transformed Infrared (FT-IR) spectra of EPS showed a specific absorbance of O-H and –NH stretching, asymmetrical-symmetrical C-H stretching, Presence of sulfur containing functional groups and carboxylic acids. The thermal gravimetric and differential scanning calorimetric analyses confirmed that polysaccharide thermal stability was around 237°C. *Nostoc carneum* exopolymer showed pseudoplastic non-Newtonian fluid behavior in the aqueous solutions as well as increasing viscosity with increasing concentration.

Key words: Exopolysaccharides, *Nostoc carneum*, antioxidant activity, rheology, fourier transformed infrared thermal properties (TGA and DSC)

INTRODUCTION

Microbial Exopolysaccharides (EPSs) are biosynthetic polymers mainly consisting of carbohydrates secreted by bacteria (Freitas *et al*., 2009) and cyanobacteria (Parikh and Madamwar, 2006). According to Sutherland (2001) these exopolymers comprise 2 categories: Homopolysaccharides and heteropolysaccharides. Arskold *et al*. (2007) found that the heteropolysaccharides involve high molecular mass hydrated molecules consisting of numerous sugar residues and their biosynthesis is due to the coordinated action of various glycosyl transferases. These microbial Exopolysaccharides have features appropriate for economic purposes due to the presence of excessive number of different monomers, strong anionic nature as well as high hydrophobicity (Mota *et al*., 2013). During cellular metabolism many Reactive Oxygen Species (ROS) are produced. Presence of these ROS led to apoptosis, gene expression, cell signaling and ion transportation (Afonso *et al*., 2007). Therefore, antioxidants may have an important function in human protection from different oxidative damages associated to cancer, diabetes, cardiovascular disease and neurodegenerative diseases (Lin and Beal, 2003). The significance of these biopolymers may be as a result of their high application potential in food, cosmetic, pharmaceutical and oil industries, as well as their usage as thickening, stabilizing and emulsifying agents. In pharmaceutical industry they can be used as, as antiviral (Hayashi *et al*., 1996a, b; Singh and Das, 2011), anti-inflammatory agents and testing cytotoxicity of leukemia cells through apoptosis and ion absorption applications (Moreno *et al*., 2000; Shah *et al*., 2000; Singh and Das, 2011). This study is aimed at characterizing the physical and chemical properties as well as antioxidant activity of the EPS produced by *N. carneum* to ascertain their economic potentiality.

MATERIALS AND METHODS

Cyanobacterial strain isolation and identification: Cyanobacterial strain was isolated from cultivated soil in Mansoura District. Culture Purification was according to Andersen (2005), Desikachary (1959) and Van Landingham and Collins (1982).

Culture conditions: *Nostoc carneum* was grown in axenic cultures at 28±2°C. under continuous illumination (3000 Lux) in 500 mL conical flasks, containing 200 mL BG11media (Rippka *et al*., 1979) for 49 days incubation period.

Exopolysaccharide extraction: After culture centrifugation (4,500 g, 10 min) the EPS was precipitated by an equal volume of isopropanol and dried at 37°C (Reddy *et al*., 1996; Pawar *et al*., 2013).

Chemical characterization of EPS: Protein content was estimated according to Lowry *et al*. (1951). Carbohydrate content was estimated by phenol sulfuric acid method (Dubois *et al*., 1956). Meta-hydroxydiphenyl method (Blumenkratz and Asboe-Hansen, 1973; Filisetti-Cozzi and Carpita, 1991) was used to estimate the uronic acid content of exopolysaccharide. Sulfate content was estimated according to APHA. (1998). The EPS hydrolysis and composition analysis was achieved according to Chen *et al*. (1997) using a High Performance Liquid Chromatography (HPLC) system. Antioxidant activity assays: Ferric Reducing antioxidant power was measured according to method described by Qiao *et al*. (2009). The free radical scavenging activity of EPS was measured against 2 mM 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH)radicals using method of Hsu (2006).

Physical characterization of EPS: The UV-visible spectrum of EPS was recorded between 200 and 800 nm on a Lambda 35 spectrophotometer.

Fourier transform infrared spectrometry: FT-IR spectrum was recorded on The Mattson 5000FT-IR spectrometer in the frequency range of 400-4000 cm^{-1} (Wang *et al.*, 2004).

Thermal gravimetric analysis: The TGA was carried on a thermo analyzer of the type 50-H. The TGA was obtained in the range of 25-800°C under nitrogen atmosphere.

Differential scanning calorimetry: The pyrolysis pattern of the EPS was investigated using a differential scanning calorimeter (60-A). The thermo gram was obtained in the range of 25-250°C.

Rheological property analysis of EPS: The dynamic rheological measurement of extracted EPS solutions (5, 10 and 15 mg mL^{-1} EPS) was carried out on BROOKFIELDDV-3 Ultra Programmable Rheometer (Fernandes *et al.*, 1991).

RESULTS

Chemical composition of EPS: Under previously mentioned growth conditions, the EPS yield reached 1.121 g L^{-1} within 49 days. This EPS contained 54% carbohydrate. The monosaccharide portions composed of xylose and glucose in molar ratio 4.3:2.1. Protein, sulfate and uronic acids contents are shown in Table 1.

Antioxidant activity assays: The reducing capacity of *N. carneum* EPS using $k_3Fe(CN)_6$ reduction method (Fig. 1) increased with increasing EPS concentration. At the concentration of 10 mg mL^{-1}, the reducing capacity (absorbance at 700 nm) of EPS was 0.450 nm. The free radical scavenging activity of EPS (DPPH assay) demonstrated that EPS exhibits a similar curve of antioxidant activity compared to ascorbic acid. The IC_{50} value (32 µg mL^{-1}) of EPS is higher than that of ascorbic acid (24 µg mL^{-1}) (Fig. 2). The IC_{50} was low and results in high level of ascorbic acid equivalent antioxidant capacity AEAC (75 mg AA/100 g). This result indicated that *N. carneum* EPS has an inhibitory effect on the DPPH radical.

Physical properties of EPS: The extracted EPS was light yellow odorless powder, water-soluble giving clear homogeneous liquid. The ultraviolet scan spectrum analysis of the EPS solution showed a maximum absorption peak at 234 nm as shown in Fig. 3.

Fourier Transform Infrared (FT-IR) spectrometry characterization: The FT-IR of EPS, obtained from *Nostoc carneum* reveals characteristic functional groups (Fig. 4) showing peaks at 3419, 2931, 2364, 2147, 1650, 1558, 1419, 1305, 1250, 1151, 1075, 1038, 921, 877, 674, 637 and 605 cm^{-1}.

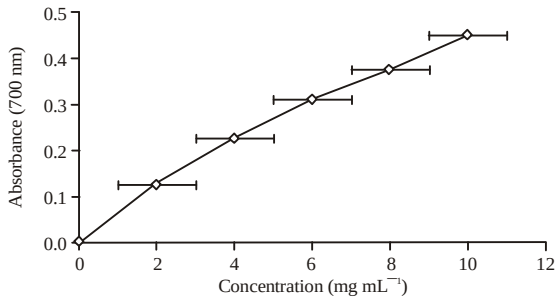

Fig. 1: Reducing capacity of extracted EPS

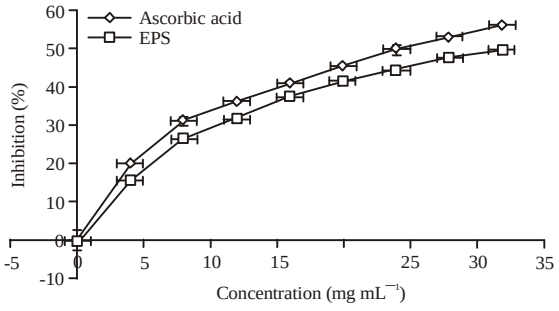

Fig. 2: DPPH free radical scavenging activity of *N. carneum* EPS

Table 1: Chemical analysis and monosaccharide composition of EPS (mg g^{-1} EPS)

				Monosaccharaides composition	
Protein content	Carbohydrate content	Uronic acids content	Sulfate content	Xylose	Glucose
101.46	543.28	217.61	139.36	215.2	105.5

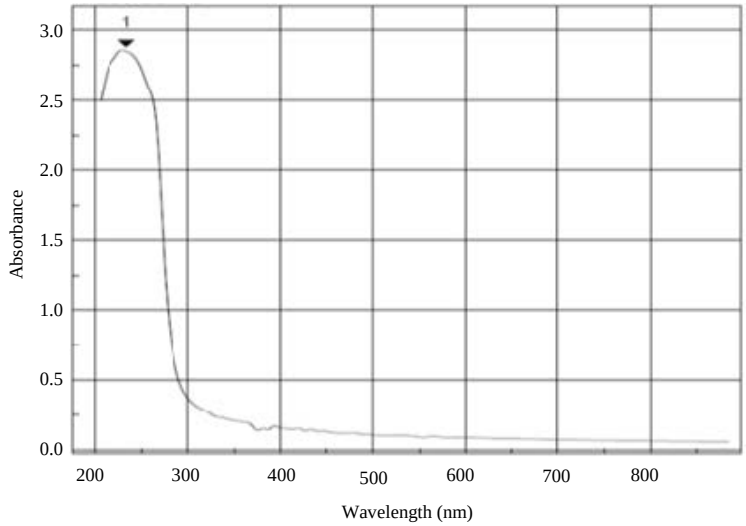

Fig. 3: UV-absorbance spectrum of aqueous solution of EPS

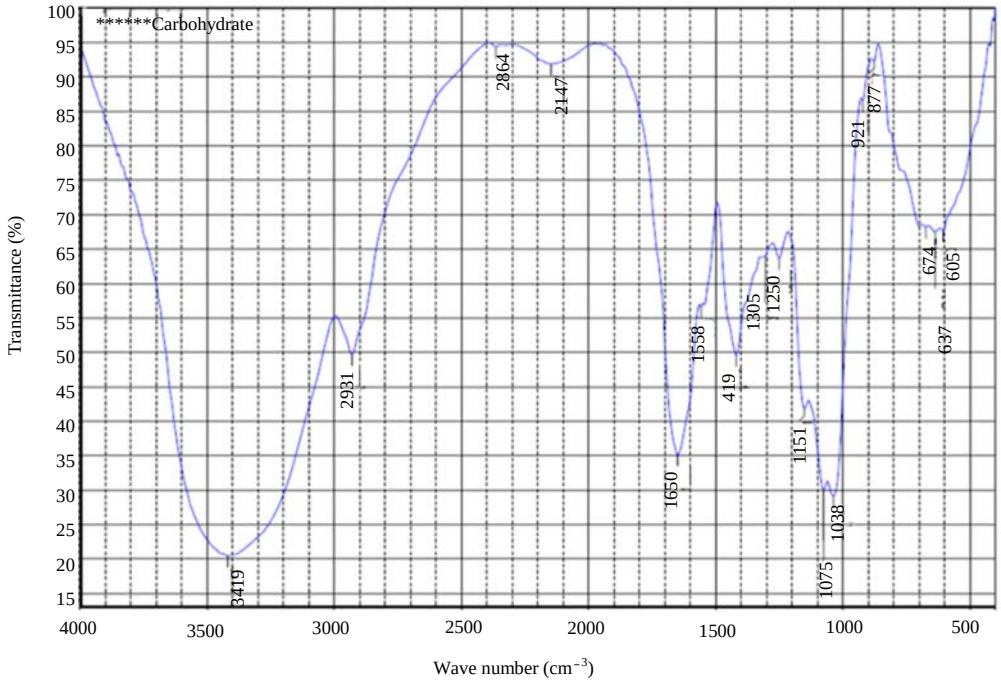

Fig. 4: FT-IR analysis of exopolysaccharide extracted from *Nostoc carneum*

Thermal characteristics of extracted polysaccharide

Thermogravimetric analysis: The thermo gram of the TGA analysis of *N. carneum* EPS showed that degradation occurred in three well-distinct steps (Fig. 5). In the first phase (phase I) the EPS powder showed a weight loss of 16% as the temperature raised from 25-155°C. At progressive increasing temperature, the weight remained relatively constant until the system reached 237°C, when the polysaccharide started to decompose (phase II) until approximately 378°C. At this point, the total weight loss reached about 39%. The third phase (phase III) occurred from 378-562°C with an additional 32% weight loss this decomposition continued with a further loss of mass about 0.739% at temperature 627-649°C.

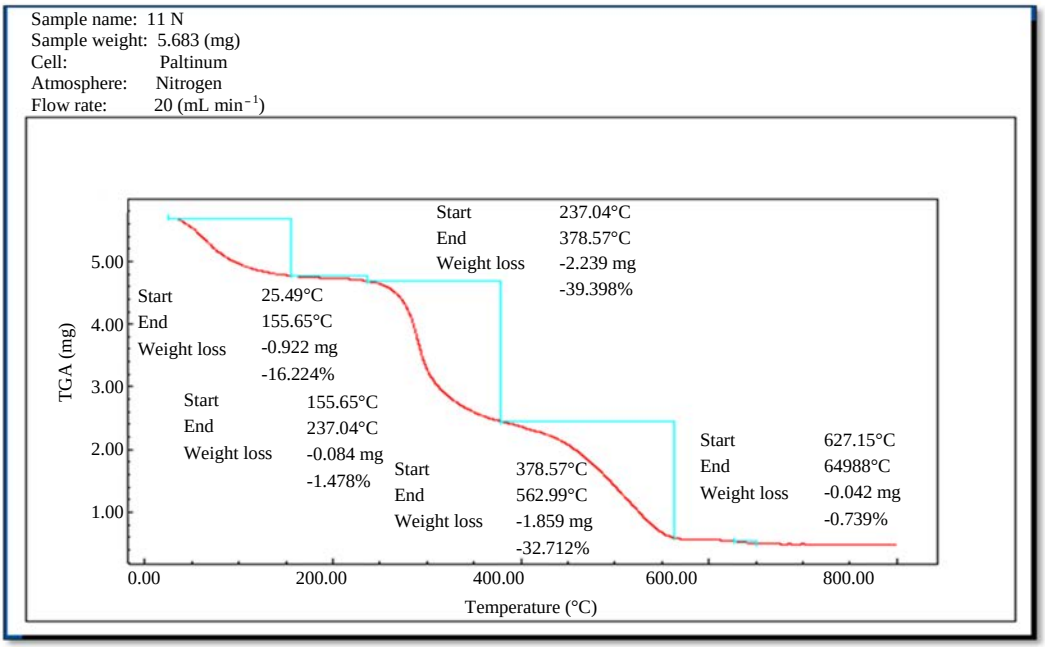

Fig. 5: Thermogravimetric analysis (TGA) of EPS

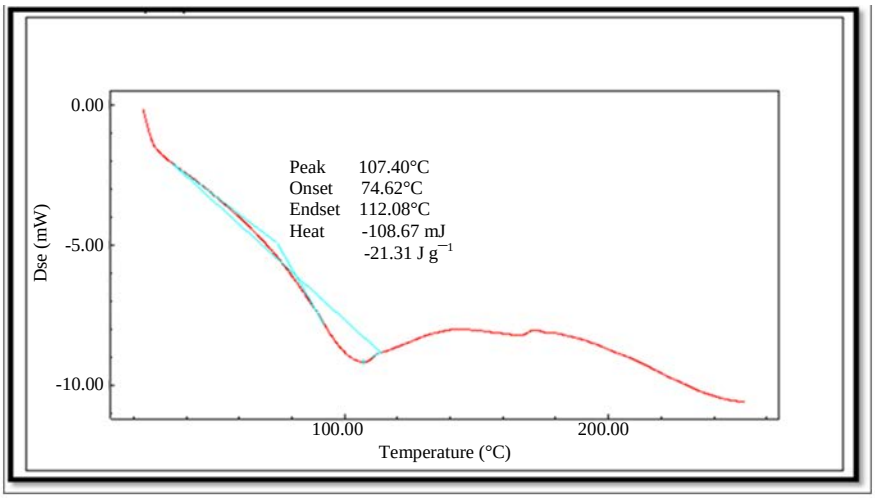

Fig. 6: Differential scanning calorimetry (DSC) analysis of EPS

Differential scanning calorimetry analysis: Differential scanning calorimetry was employed to recognize thermal transitions of EPS. The DSC thermogram exhibited the characteristic exothermic transition of the exopolymer with crystallization temperature (Tc) 107.4°C (on set temperature 74.62°C) and 108.67 mJ latent energy of crystallization as shown in Fig. 6.

Rheological property analysis of extracted EPS: Viscosity of *N. carneum* EPS solutions (5, 10 and 15 mg mL^{-1}) as a function of shear rate achieved maxima of 24.2, 70.1 and 140 cP viscosity, respectively at shear rate of 40 sec^{-1} (Fig. 7). While with increasing shear rate to 500 sec^{-1}, viscosity values decreases dramatically to 11.8, 25.1 and 28.9 cP, respectively. Figure 8 shows the flow curves (representation of the shear

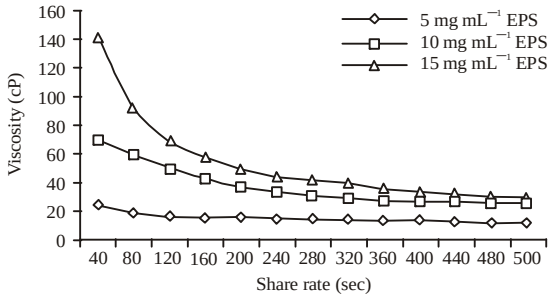

Fig. 7: Viscosity as a function of shear rate of aqueous solutions of *N. carneum* EPS at concentrations 5, 10 and 15 mg mL^{-1} EPS

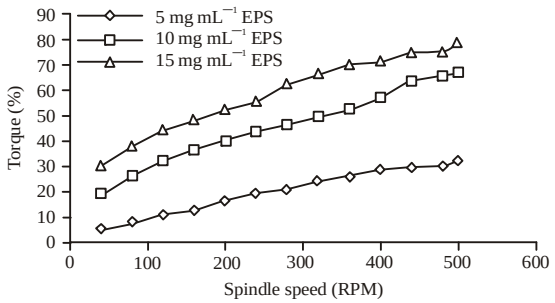

Fig. 10: Rheogram of the Torque vs. spindle speed of *N. carneum* EPS at concentrations 5, 10 and 15 mg mL^{-1} EPS

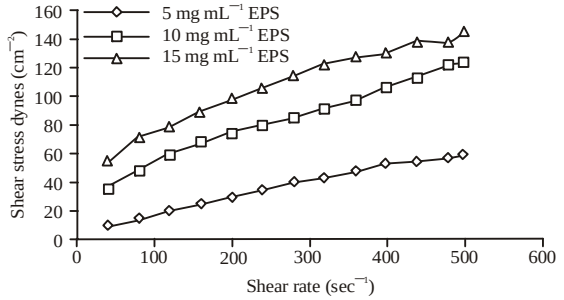

Fig. 8: Flow curve of the shear stress vs. shear rate of *N. carneum* EPS at concentrations 5, 10 and 15 mg mL^{-1} EPS

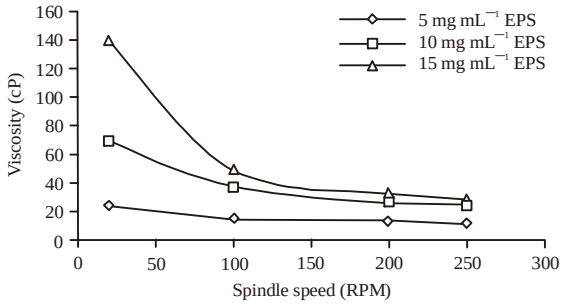

Fig. 11: Rheogram of the viscosity dependence of spindle speed (RPM) for aqueous *N. carneum* EPS concentrations 5, 10 and 15 mg mL^{-1} EPS

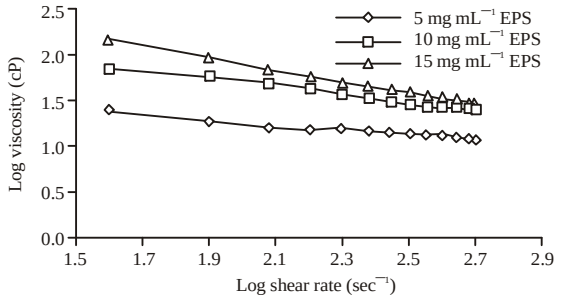

Fig. 9: Log-log plot of the viscosity vs. shear rate of 5, 10 and 15 mg mL^{-1} EPS

stress as a function of shear rate) for aqueous EPS solutions with different concentrations. For all EPS solutions, the shear stress tended to level off and approximate a restrictive stable value as a decrease in shear rate towards zero at low range of shear rates, demonstrating that these polymer systems exhibit a finite magnitude of yield stress. It is also revealed that the larger values of yield stress were obtained with increasing polymer concentration. As presented in Fig. 9 the EPS showed pseudoplastic or shear thinning property in aqueous solutions. The highest viscosities and the prominent shear thinning properties were recorded in the following descending manner 15, 10 and 5 mg mL^{-1} EPS. Rheogram of Fig. 10 showed that torque percent increased with increasing spindle speed (RPM). Figure 11 explained viscosity of *N. carneum* EPS aqueous solutions and spindle speed (RPM) relationship. It is obvious that with increasing spindle speed viscosity decreased.

DISCUSSION

Chemical analysis of EPS: The identification of two neutral sugars (xylose and glucose) and uronic acids in EPS of *N. carneum* confirmed earlier reports on complex composition of cyanobacterial EPS (De Philippis and Vincenzini, 1998).

The majority of the cyanobacterial EPSs are constituted of as a minimum one uronic acid, several numerous neutral sugars (ranging from 2-10) in combination with protein molecules (Otero and Vincenzini, 2003; Parikh and Madamwar, 2006). The HPLC analysis of *N. carneum* EPS revealed two monosaccharides(xylose and glucose) in molar ratio 4.3:2.1. Huang *et al*. (1998) reported that *Nostoc* sp. EPS composed of xylose and glucose but in different ratios. Parikh and Madamwar (2006) indicated the presence of xylose as well as ribose in *Nostoc* sp., *Nostoc commune* and *Nostoc carneum*. Challouf *et al*. (2011) and Ozturk *et al*. (2014) documented that cyanobacterial exopolysaccharides are distinguished with the existence of pentoses in addition to their anionic nature because of the occurrence of acidic sugars (glucuronic and/or galacturonic acids) and anionic organic (acetyl, pyruvil) and inorganic (phosphate and sulfate) substituents.

Antioxidant activity assays: Reducing capacity is an important assay in estimating antioxidant activity (Duh *et al*., 1999). In ferric reducing antioxidant power, the antioxidant activity established on the capability of the antioxidant fractions in the EPS solutions to reduce ferric (III) to ferrous (II) in a redox-linked colourimetric reaction (Li *et al*., 2006) that includes single electron transfer. The direct correlation between antioxidant activity and the reducing capacity had been documented by Qiao *et al*. (2009). The DPPH molecule that has a nitrogen free radical is readily destroyed by a free radical scavenger. This assay was used to investigate the potentiality of the oxidative compounds operating as proton radical scavengers or hydrogen donors (Singh and Rajini, 2004). In the DPPH assay, the *N. carneum* EPS reduce DPPH radical to the yellow coloured diphenyl picryl hydrazine.

Physical analysis of EPS: The ultraviolet scan spectrum analysis of the *N. carneum* EPS indicated the presence of proteins and nucleic acids due to absorption peak at wave length 234 nm according to Okajima-Kaneko *et al*. (2007). UV-vis spectroscopy analysis illustrated that the maximum wave length area of absorption spectra was 200-234 nm due to n-σ* and or π-π* transitions, which characterizes functional groups like amine, carboxyl, carbonyl and ester as suggested by Yun and Park (2003).

FT-IR characterization of EPS: The weak absorption band (Fig. 4) at 2931 cm^{-1} is characteristic band of the C-H stretching vibration of CH_2, the band at 1419 cm and 1305 cm^{-1} of the C-H bending of CH_2 or CH_3, all bands were typical of carbohydrates (Yee *et al*., 2004; Parikh and Madamwar, 2006; Khattar *et al*., 2010; Ozturk *et al*., 2014).

The peak at 3419 cm^{-1} can be assigned to the stretching vibration of the hydroxyl (–OH) or amine (–NH) groups (Parikh and Madamwar, 2006; Khattar *et al*., 2010; Mota *et al*., 2013; Ozturk *et al*., 2014). The amine and amide groups strengthens that the biopolymer is not only composed of polysaccharides but also some peptides and/or proteins as documented by Pagnanelli *et al*. (2000).

The peak at 1250 cm^{-1} can be related to the asymmetrical S = O stretching vibration (Mota *et al*., 2013). The bands 2364 and 2147 cm^{-1} can be attributed to the stretching vibration of C ≡ C and C ≡ N.

The presence of a carboxylic acid is detected by a band at 1650 which revealed the presence of (–COOH) groups (Parikh and Madamwar, 2006; Ozturk *et al*., 2014), Whereas, the band at 1151 is due to the asymmetrical and symmetrical C-O-S vibration (De Philippis and Vincenzini, 1998; Parikh and Madamwar, 2006). These data supported the presence of sulfate groups in the *Nostoc carneum* exopolysaccharide, as reported for other cyanobacterial exopolymers (Mahner *et al*., 2001; Yee *et al*., 2004; Zou *et al*., 2008). The peak at 1038 and 1075 cm^{-1} might be due to the contribution of C-O bond of polysaccharide. The bands lie in the range of 1000-1125 cm^{-1} is characteristic of uronic acids and O-acetyl ester linkage bond (Ozturk *et al*., 2014). Trabelsi *et al*. (2009) and Khattar *et al*. (2010) reported that the occurrence of numerous bands fewer than 1,000 cm^{-1} possibly due to several visible bands and/or to the presence of probable linkages between monosaccharides. These results suggested that EPS from *Nostoc carneum* contain uronic acids, sulfate groups and peptides in their composition which were further confirmed by chemical analysis of EPS.

Thermal characteristics of extracted EPS

Thermogravimetric analysis TGA: Thermogram of TGA illustrated that the decomposition of *N. carneum* EPS take place in three definite phases (Fig. 3). The present results are in agreement with results of EPS produced by *Cyanothece* sp. ATCC 51142 and *Nostoc* spp., (Parikh and Madamwar, 2006) and *cyanothece*. ccy 0110 (Mota *et al*., 2013). In phase I the *N. carneum* EPS exhibited a weight loss of 16% at rising temperature from 25-155°C, possibly because of desorption of physically absorbed water. Elevated level of carboxyl group in the EPS raised the decomposition of the 1st phase (30-120°C) as carboxyl group is bound to more water molecules as indicated by Kumar *et al*. (2004). With increasing temperature above 155°C, the EPS weight remained constant until temperature reached 237°C, then the polysaccharide began to degrade as a result of removal of structure water (phase II) until approximately 378°C with 39% weight loss. Temperature characteristic of the third phase (phase III) were recognized between 378-562°C with 32% EPS weight loss that can be referred to depolymerization accompanied by the rupture of C-O and C-C bonds in the ring units resulting in the evolution of CO, CO_2 and H_2O. Rising temperature from 627-649°C resulted in 0.739% weight loss due to formation of polynuclear aromatic and graphitic carbon structures. The high thermo stability of *N. carneum* EPS might be due to existence of sulfate groups and uronic acids that prevented complete decomposition of the polymer (Pooja and Chandra, 2009; Alves *et al*., 2010; Mota *et al*., 2013).

Differential scanning calorimetry: With increasing temperature, the amorphous solid will become less viscous and at a certain temperature the particles free enough to organize themselves into a crystalline state, known as the crystallization temperature (Dean, 1995; Mishra *et al*., 2011). This transition from amorphous solid to crystalline solid is an exothermic process and differential scanning calorimetric analysis exhibited an important thermal transition of EPSs as proposed by Mishra *et al*. (2011) and Singh *et al*. (2011).

Rheological properties of EPS: Rheological characterization of the *N. carneum* EPS (Fig. 7-11) showed decrease in viscosity with increasing shear rate while viscosity increased with increasing exopolymer concentration. This is expressive of a typical non-Newtonian pseudoplastic behavior or shear thinning property in aqueous solutions (Picout and Ross-Murphy, 2003). This rheological pattern was also illustrated by exopolysaccharides of a number of other cyanobacteria and algae (De Philippis and Vincenzini, 1998; Tuinier *et al*., 1999; Moreno *et al*., 2000; Bhatnagar *et al*., 2012). The viscous performance of exopolymer is reliant on its

structure and mass as indicated by Freitas *et al*. (2009). Khattar *et al*. (2010) suggested that the shear thinning behavior of EPS was a result of hydrodynamic forces produced during the shear breakdown of EPS structural units. This previous character is essential for different treats, such as mixing, pouring and pumping where various operative shear rates are applied. In addition, it was reported in 2011 that polysaccharides with pseudoplastic, non-Newtonian and shear thinning behavior are appropriate for food industries.

CONCLUSION

The physico-chemical analyses of *N. carneum* exopolysaccharide revealed its heteropolymeric nature with the presence of protein moieties. These extracted exopolymer had a complex composition that constituted of two neutral sugars (xylose and glucose) and uronic acid. FT-IR confirmed that EPS was a type of polyanionic polysaccharide that contained carboxyl, carbonyl and sulfate groups. Rheological investigations revealed the non-Newtonian, pseudoplastic shear thinning properties which recommends their significant applications in pharmaceutical and food industries.

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