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Properties and Role of Exopolysaccharides Produced by *Paenibacillus alvei* NRC14 for Cell Protection

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Abstract

The interest in polysaccharides produced by microorganisms has increased considerably in recent years, as they are candidates for many commercial applications in different industrial sectors. In this study, exopolysaccharides produced by the bacterial strain *Paenibacillus alvei* NRC14, under normal and abiotic stress conditions, was investigated. The strain was found to produce exopolysaccharides (EPSs) possess different characteristics such as the sugar content and flocculating properties. The EPS production was 3.2 g/L under stress conditions. The chemical analyses indicated that it is a glycoprotein composed of polysaccharide (85%) and protein (12%).The molecular weight of the EPSs from strain NRC14 were 7.6 x 10^5 and 5.4 x 10^6 Da under the normal and acidic pH, respectively. The flocculation activity of EPSs ranged from 95 to 97.8 % in less than 48 hrs of growth. Under normal conditions, the produced EPSs have shown to consist of aminosugars and has good flocculating activity, whereas the EPSs were greatly affected with the abiotic stress conditions. FTIR spectrometry of the EPSs indicated presence of carboxyl, hydroxyl, and amino functional groups which are the principles for the flocculating properties.

Keywords: Exopolysaccharides, *Paenibacillus alvei*, Abiotic stress, Biofilm, Flocculation, Aminosugars

1. Introduction

Exopolysaccharides are carbohydrate polymers of highly variable composition and structure, found outside bacterial cells. Bacterial EPSs are usually responsible for attachment to solid surfaces and to other bacteria, forming cell aggregates which called “biofilms”, surrounded by a complex matrix consists mainly of an EPS. Inside the biofilm communities, cells of microorganisms grow and connect by a network of open-water channels (Stoodley *et al*. 2002). Presence of the matrix confers a series of selective advantages, such as protection against environmental variations, nutrient and ions retention, resistance to desiccation and mechanical protection (Sutherland 2001). Some of EPSs exhibit flocculating activity properties and such natural bioflocculants could be successfully applied in the food, cosmotics, and pharmaceutical fields. Use of synthetic chemical flocculants, namely, inorganic coagulants such as aluminium (aluminum sulfate and poly-aluminum chloride), and synthetic organic flocculants such as polyacrylamide derivatives and polyethylene imine, have resulted in some health and environmental problems. Aluminum has been found

to induce Alzheimer's disease (Campbell, 2002; Nwodo *et al*., 2014), moreover, acrylamide, which still remains in the flocculant products, is not only neurotoxic and carcinogenic but also non-biodegradable in nature (Nwodo *et al*., 2014). Contrary, flocculants of microbial origin "bioflocculants" are environmentally friendly and attractive alternatives to the existing chemical flocculants. Extensive studies revealed that microbial bioflocculants are nontoxic, harmless, biodegradability, and lack of secondary pollution (Azmi *et al*., 2015). Exopolysaccharides with flocculating properties are widely applied in treatment of water and wastewater, downstream processing, food fermentation processes, and removal of heavy metals and dyes (Nwodo *et al*, 2014). Exopolysaccharides from different *Paenibacillus* spp. (production, isolation, structure, and bioactivities) are widely reported (Liang *et al*. 2015). However, studies on EPS production by *Paenibacillus alvei* is rare. The present study was performed to determine variation in EPSs produced by the *Paenibacillus alvei* under normal and abiotic stress condition, which is reported for the first time.

2. Materials and Methods

2.1 Microorganism

Bacillus alvei NRC-14, a bacterium isolated from Egyptian soil, was relocated as *Paenibacillus alvei* according to the 16S rRNA gene which has been usually used as a trustworthy molecular marker for phylogentic identification of organisms (Kumar *et al*. 2012). The strain produces a variety of carbohydrate-active enzymes, bioflocculants, exopolysaccharides, lytic enzymes, and proteases. The bacterium was maintained on nutrient agar slants and kept at 4^{o}C for further studies.

2.2 Growth medium and culture conditions

Production of EPSs by the strain was performed in 250 ml Erlenmeyer flasks containing 100 ml of medium. Fungal-biomass (*Mucor rouxii*) was used as a low-cost carbon source, and ammonium sulphate (0.2 %, w/v) as a nitrogen source (Abdel-Aziz *et al*. 2011). The pH of media was adjusted to 3.0 a temperature degree, 40^{o}C. Flasks were inoculated with 4% (v/v), of actively grown culture (OD_{600}=2.5) and incubated with shaking at 130 rpm for 5 days. Samples were withdrawn at different time intervals and monitored for cell growth, viscosity, EPS production, and measuring of flocculating activity. Culture broth was centrifuged at 7,000 x g for 20 min for separation of cells and cell- debris. In some detections, the production of EPSs was expressed by estimation of flocculating activity.

2.3 Extraction of exopolysaccharide

The viscous culture broth (400 ml) was mixed with three volumes of cold distilled water and centrifuged at 8000xg for 20 min. The resultant supernatant was poured into three volumes of cold ethanol (4°C) to precipitate the EPS, and left to stand at 4°C overnight (Shih *et al*. 2001). The precipitate was collected by

centrifugation at 8000xg for 20 min. After repeating the purification process twice, the EPS was dialyzed against distilled water at 4°C overnight. The precipitate was recovered and kept at 4 °C to be used to determine the flocculating-activity properties and for chemical analysis.

2.4 Enzymatic Hydrolysis of Exopolysaccharides

Degradation of the produced EPS by enzymes for determination of its stability was evaluated. Liberated reducing sugars were detected by the Elson-Morgan method (Chaplen and Kennedy 1986). Enzymes used for degradation of the EPS include chitinase, chitosanase, B-1-3, glucanase, and protease. Activity of these enzymes were 0.08, 1.7, 2.5, and 1.3 U/ml, respectively.

2.5 Analytical methods

Protein content was assayed using the Folin-Lowry method, using bovine serum albumin as standard (Lowry *et al*. 1951). Amino-sugars were determined by the Elson-Morgan method (Chaplin & Kennedy 1986). Total sugar was determined by the phenol–sulphuric acid method using glucose as the standard solution (Dubois *et al*., 1956). The average molecular weight of the EPSs in relation to the viscosity was calculated according to the method of Il'ina *et al.* (2001). The flocculating activity was determined as described by the method of Kurane *et al*. (1986).

2.6 Fourier-Transform Infrared Spectroscopy

Major functional groups was analyzed and detected using FT-IR-FT Raman (Nexus 670, Nicolet-Madison-WI-USA). The spectrum of the sample was recorded on the spectrophotometer over a wave number range 4000-400 cm^{-1}.

3. Results and Discussion

3.1 Effect of pH values

Productivity studies of most polysaccharide synthesized by microorganisms revealed the formation of EPS under normal culture conditions that permit growth and high yield. Growth of bacteria under abnormal conditions (extreme pH values and temperatures) is, however, rare despite of such conditions trigger the production of EPSs as a form of self-protection. EPSs are the main component in formation of biofilm matrix. Most microorganisms produced EPS bioflocculants during the exponential growth phase. In the present study, Fig. 1 (A and B) shows the relationship between the EPS production and cell growth over a cultivation time of 120 hrs. Under acid pH-stress condition, cell growth was slowly initiated during an acclimation period, followed by an obvious increase in cell growth accompanied with increasing both viscosity and EPS formation (Fig. 1A). Under normal condition (pH 6.0), cell growth increased gradually,

reached a maximum after 48 hrs. EPS formation increased in parallel with cell growth and viscosity (Fig. 1B). The pH range from 4-5 and 7-9 resulted in slight EPS formation (data not shown), indicating that acidic shock trigger the synthesis of EPS rather than alkali conditions. Initial pH of a medium for production of EPS bioflocuulants may determine the electric charge of the cells and the oxidation-reduction potential, which can affect absorption of nutrients and enzymatic reaction (Salehizadeh and Shojaosadati, 2001). The strain *Streptomyces griseus* (Shimofuruya *et al*., 1996) and *Streptomyces xn*17 (Zhang *et al*., 2013) produced EPS bioflocculants under acidic conditions. In contrast, however, it is reported that *Nocardiopsis aegyptia* sp. nov. EPS bioflocculant was produced at a pH range of 4–11, with the highest flocculating at neutral pH. The flocculation efficiency was low at acidulous, but active in alkalescent conditions. Similarly, *Arthrobacter* sp.; *Streptomyces* sp. Gansen and *Brachybacterium* sp. produced bioflocculant optimally under neutral pH (Su *et al*., 2011, Mabinya *et al*., 2012, Nwodo *et al*., 2012, 2013). The correlation between cell growth and EPS production by strain NRC14 revealed association of cell growth with the EPS formation, which indicates that the EPS was produced by biosynthesis during growth and not by cell autolysis.

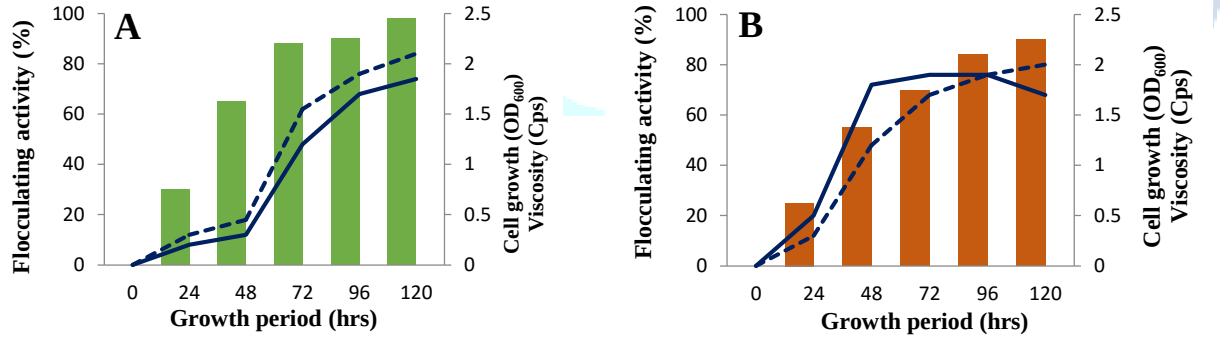

Fig. 1. Cell-growth behavior (*solid line*), viscosity (*dashed line*), and EPS production (*columns*) by strain *P. alvei* NRC14, under **A**; Extreme conditions (pH 3.0/40°C) or **B**; Normal conditions (pH 6.0/30°C) under shaking cultures.

Of interest is that, the EPS bioflocculants in this study was found to contain the main groups found in chitosan molecular chain. Moreover, the structure of EPSs from strain NRC14 is similar to that of chitosan. In fact, during the twenty-year history of working with the strain NRC14 under laboratory conditions, no cell lyses was observed and no contamination at slants or flasks was occurred. Moreover, the EPS is a fairly stable biopolymer and it may have a protective function for the cells of the strain. Persistence of the strain NRC14 is probably due to the presence of these highly stable EPSs. In addition, the EPS formation and

flocculating activity increased up to 120 hrs, indicating the stability of the EPS flocculant and that no deflocculating enzymes or toxic metabolites influenced this EPS. In contrast, it was reported that EPSs production and flocculating activity from the strains *Bacillus subtilis* DYU1 and *Bacillus* sp. AEMREG7 decreased steadily, and it might be due to deflocculating enzymatic activities or accumulation of toxic metabolic wastes affecting the produced bioflocculant (Wu and Ye 2007, Okaiyeto *et al*. 2015).

3.2 Effect of temperature

Being the most important environmental factors for microbial life, the temperature effect on cell grow behavior was evaluated due to the directly influence on functional properties of the cellular components. Growth of the strain with fugal biomass at 30°C and 40°C was obviously differed (Fig. 2). Under normal conditions (pH6 and 30°C), cell growth increased rapidly through 48 hrs (Fig. 2 A), with formation of an EPS bioflocculant, after which the growth was gradually decreased (Fig. 2 A). On other hand, cell growth under heat shock (pH6 and 40°C) resulted in lowering cell growth in the period of cell acclimation, rapid higher viscosity after 72 hrs, and increased (97.8%) flocculating activity (Fig. 2 B).

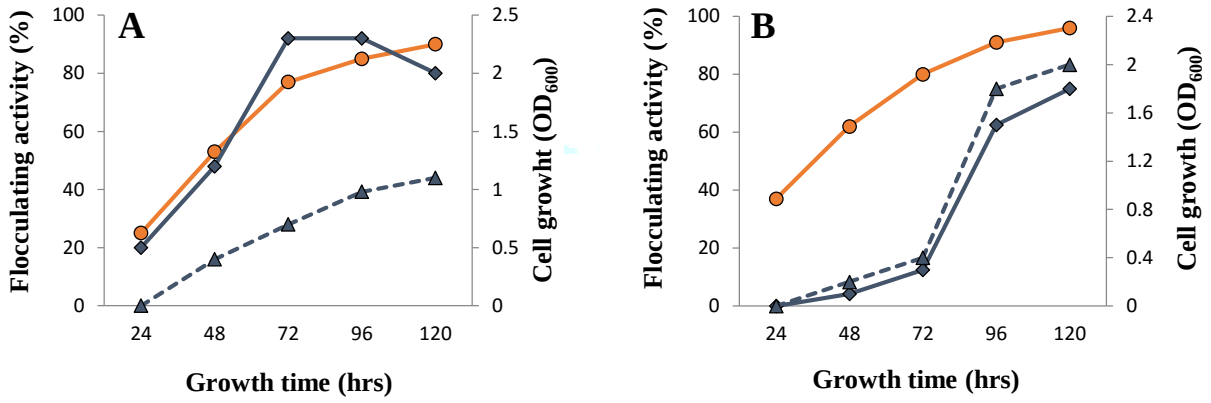

Fig. 2. Cell growth behavior of the strain *P. alvei* NRC14 at 30°C (**A**) and 40°C (**B**) (under culture shaking conditions. Symbols: Cell growth (*Blue solid line*), viscosity (*Blue dashed line*), and the flocculating activity (*Red solid line*).

These results may suggest that, after temperature elevation, the strain may adapt and respond by formation of a biofilm as a form of self-protection. Generally, sudden exposure to an abiotic stress lead to expressing a specific set of genes. The heat-shock response comprises the expression of protein chaperones and proteases (Paramita and Irvin 2011). Bacteria encounter stresses in their natural environments, these stresses elicit a variety of specific and highly regulated adaptive responses that not only protect bacteria from the offending stress but also manifest changes in the cell (Poole *et al*. 2012). Thus, exposure to nutrient starvation/limitation (nutrient stress), membrane damage (envelope stress), elevated temperature (heat

stress), pH variation, or ribosome disruption (ribosomal stress), all impact bacterial growth through their initiation of stress responses that positively induce resistance determinants or promote physiological changes compromise antimicrobial activity (Poole *et al*. 2012).

3.3 Effect of carbon source

Production of EPSs is well known to be affected by cultivation conditions and nutrition as critical factors. The importance of carbon and nitrogen sources has been reported to have a crucial effect on production of EPSs bioflocculant, which may differ according to the producing microorganisms (Okaiyeto *et al*. 2015). Glucose has been reported as a preferred carbon source in previous studies for bioflocculant production by various microorganisms (Cosa *et al*. 2013, Nwodo *et al*. 2012, Okaiyeto *et al*. 2015). However, our data suggest that strain NRC14 could grow and utilize a relatively wide range of tested carbon sources for the EPSs bioflocculant production, with flocculating efficiency ranging from 92-98% after 48-96 hrs of cultivation (Abdel-Aziz *et al*. 2011, Abdel-Aziz *et al*. 2013). Additionally, in our previous study, it was reported that, use of chitosan or chitosan-containing substrates is essential for the formation of an EPS bioflocculant similar to that of chitosan. In the present study, using *M. rouxii* biomass (containing chitosan in the cell wall) as a carbon source resulted in formation of an EPS bioflocculant with the main functional groups as chitosan. Biosynthesis of such EPSs, in fact, suggests a relationship between the presence of chitosan and production of the EPSs. We concluded that, degradation of chitosan by enzyme(s) secreted by the strain may probably result in an accumulation of aminosugars, *e.g*. glucosamine or galactosamine, which may be polymerized to form the EPS bioflocculant (Abdel-Aziz *et al*. 2011).

3.4 Flocculating properties

Using fungal biomass as a carbon source, the MW of the EPSs from strain NRC14 were found to be $6.9x10^4$, 7.6×10^5 and 5.4×10^6 Da under normal, acidic pH, and high temperature conditions, respectively. The MW and functional groups at the molecular chains are the most important factors in the flocculating activity of a bioflocculant. A large molecular- weight bioflocculant is usually long enough and has a sufficient number of free functional groups by which strong and large flocs are formed. Free functional groups can act as bridges to bring many suspended particles together (Michaels, 1954). The mechanism of flocculation in a biological system is not entirely clear, highly complex, and depends on many interacting variables such as temperature, pH, microbial species and medium components (Deng *et. al*. 2003). Flocculation occurs when a chemical or biological additive is mixed with solid-containing slurry causing agglomeration of the solid particles, formation of flocs, and rapid settling of the flocs out of the solution. Regarding the EPS bioflocculant from strain NRC14, one mechanism could be occurred, when the flocculant forms molecular "bridges" between the suspended solid particles creating large solid aggregates. Another mechanism for

flocculation, i.e., charge neutralization, occurs when the chemical additive interacts with only one (or a few) particles electrostatically. The resulting particle becomes charge neutral and losses much of its surface salvation water (Todd et al. 2010). Worthy mention is that, addition of metal ions to the EPS bioflocculant produced by strain NRC14 showed no positive effects on enhancing the flocculating activity, indicating its cation-independent nature (Abdel-Aziz and Mouafi 2005). Thus, such a mechanism may not, however, be occurred with the EPS bioflocculant produced by strain NRC14 (Abdel-Aziz and Mouafi 2005, Okaiyeto *et al*. 2015).

Fig. 3. Efficacy of the partially purified exopolysaccharide flocculants produced by the strain *P. alvei* NRC-14 under normal condition (*left image*) or abiotic stress condition (*right image*); in aggregation and precipitation of soil, starch, and charcoal particles after 3 min of addition at the concentration, 0.1% (v/v).

3.5 Stability of Exopolysaccharides

It is an interested feature to study the stability of the partially purified EPSs against degradation by enzymes. A variety of purified enzymes (chitinase, chitosanase, cellulase, and *B*, 1-3 glucanase) produced by the NRC-14 were tested for degradation of the EPSs, individually, or in combination. Results revealed that, the EPS was stable: firstly, individual use of chitinase, chitosanase or *B*-,1-3 glucanase resulted in slight degradation of the EPS as indicated by detection of reducing sugars (Fig. 4). Secondly, to some extent, liberation of reducing sugars was increased using a mixture of these enzymes, but however, after a long period (Fig. 4). Besides, negligible amounts of reducing sugars were detected when *B*, 1-4 glucanase, CM-cellulose, xylanase, or protease were used for degradation of the EPS. These results verified complexity of the EPS produced by strain NRC14, which is hardly degraded by a mixture of enzymes, indicating a fully cell-protection. Increasing the flocculating activity up to 120 hrs of growth may confirm our suggestion; no autolysis occurred by the enzymes that may be formed, though a long period (Fig. 2A and B).

On other hand, the heat-stability of the EPS revealed that it is a heat-stable polysaccharide; it remained about 99 % of its flocculating activity after heating at 100^{o}C for 20 min (data not shown). Such stable EPS bioflocculants are increasingly employed in water and waste water treatments for solid-liquid separation because they are biodegradable, environmentally safe and non-toxic. To maximize industrial application of

bioflocculants, optimization of several culture parameters is required to ensure high production of efficient bioflocculants.

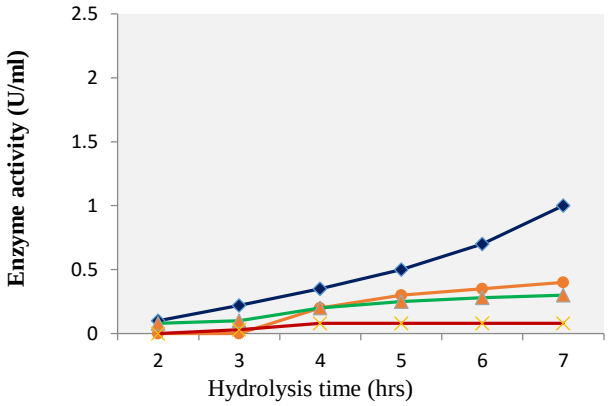

Fig.4. Degradation of the purified exopolysaccharide by: chitinase (*red line*); chitosanase (*orange line*); *B*, 1-3 glucanase (*green line*), or a mixture of all (*blue line*).

A comparative between production conditions for strains of *Paenibacillus* spp., culture conditions, and EPS yields that have been reported in the literatures is provided in the Table. EPSs yield varied with the bacterial species and culture conditions. In particular, our studies demonstrated that EPSs from strain NRC14 were produced using low cost carbon source. Production of inexpensive EPS is an important factor in the utilization of biomass wastes. In the present study, fungal biomass is verified to be a good inexpensive material for production of EPSs with a yield of 3.2 g/L (under stress conditions; pH 3.0 and 40ºC), with high flocculating activity properties.

Table: EPS production and yield by some *Paenibacillus* spp.

Bacterial Source	**Temperature (°C)**	**pH**	**Culture Vessel**	**Period (Days)**	**yield (g/L)**	**Reference**
P. jamilae CP-38	30	7.0	2 L bioreactor at 150 rpm	3	3.44	Aguilera *et al.* 2008
P. macerans TKU029	30	7.2	100 mL in a 250 mL flask at 150 rpm	4	3.46	Liang *et al*. 2014
P. polymyxa SQR-21	30	6.5	250 mL in a 1L flask	4	4.2	Raza *et al*. 2011
Paenibacillus sp. TKU023	37	7.2	50 mL in a 250 mL flask at 150 rpm	5	4.55	Wang *et al*. 2011
P. polymyxa ATCC 21830	50	7.0	400 mL in a 1 L flask at 150 rpm	4	6.89	Rafigh *et al*. 2014
P. polymyxa JB115	30		1 L medium at 180 rpm	3	10.0	Jung *et al*. 2007
P. polymyxa NRC14	40	3.0	100 mL in a 250 mL flask at 120 rpm	3	3.2	The present study

3.6 IR-spectra

The functional moieties in molecular chain of the EPS were identified with FTIR spectrophotometry (Fig. 5). The spectrum displaced an intense broad stretching peak at 3435 and 3435 cm^{-1}, which indicated the presence of a hydroxyl or amide group (Okaiyeto *et al*. 2015). The water solubility of the bioflocculant was attributed to the presence of hydroxyl group forming a hydrogen bond with a water molecule. A minor band observed at 2924cm^{-1} (Fig. 5) is well known to be typical of carbohydrates, indicated C-H asymmetrical stretching vibration. Furthermore, an asymmetric stretching peak was at 1568 and 1643 cm_{-1}, showed the presence of carbonyl group stretching vibration in the peptide (Yin *et al*. 2014). The bands detected at 1423 and 1432 cm^{-1} could be ascribed to the symmetric stretching of the –COO− group (Nwodo *et al*. 2012, Okaiyeto *et al*. 2015). The presence of carboxyl groups provides more adsorption sites for particle attachment, so many particles can be adsorbed to the long molecular chain of the bioflocculant (Luo *et al*. 2014, Okaiyeto *et al*. 2015). The small absorption peaks around 899 and 1000 cm^{-1} are known to be characteristic for all sugar derivatives and *B*-glycosidic linkages between the sugar monomers. The absorption peaks ranging from 1031 cm^{-1} were designated to C–O–C and C–O, which indicated the presence of polysaccharides [Freitas *et al*. 2009, Nie *et al*. 2014, Okaiyeto *et al*. 2015). The peak at and 1076 and 1118 cm^{-1} indicated presence of methoxyl groups (Zheng 2008).

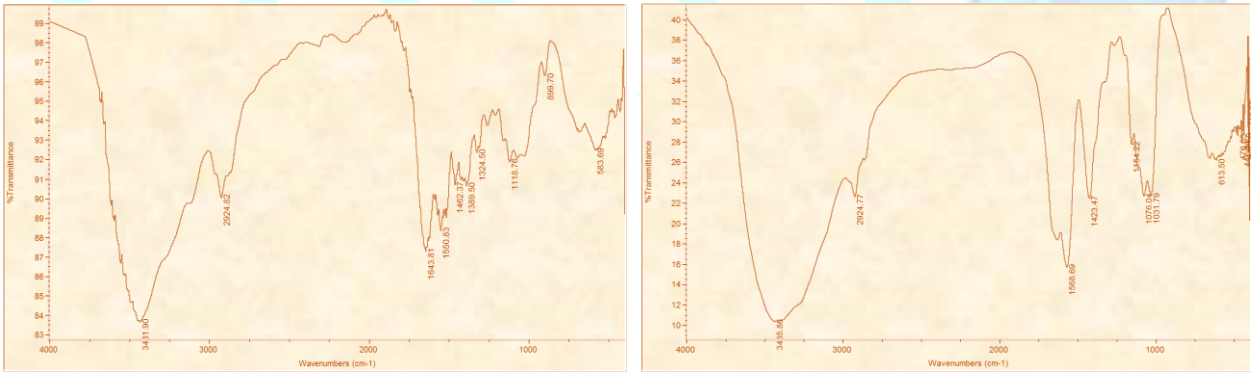

Fig. 5: FT-IR spectra of the partially purified biopolymers produced by the strain *P. alvei* NRC14 under normal conditions (*left image*) and a pH-stress condition (*right image*).

The characteristics of the FTIR spectrum showed the presence of hydroxyl, amide, carboxyl and methoxyl groups in the EPS bioflocculant structure as the main functional groups preferred for flocculation (Wan et *al*. 2013, Okaiyeto *et al*. 2015). The chemical analyses indicated that it is a glycoprotein composed of polysaccharide (85%) and protein (12%), indicating that the flocculation process might involve multiple-functional groups from both polysaccharide and protein. Multiple-functional groups imply many adsorption

sites for the particles precipitation, which led to the high flocculating efficiency observed with bioflocculants (Verma *et al*. 2015, Okaiyeto *et al*. 2015).

Conclusion

Industrial production of EPS bioflocculants is limited by their high costs and poor yields. *Paenibacillus alvei* NRC-14 produced EPS bioflocculants differed widely according to the carbon source, pH value, and temperature, using low-cost nutritional medium. EPSs from the strain gave high yields and showed a great flocculating activity. Addition of metal ions had no positive effects on enhancing the flocculating activity, indicating that the bioflocculant is cation-independent, which means avoiding of second pollution and reducing costs. Functional groups characteristic of hydroxyl, carboxyl, and methoxyl groups were obtained from the bioflocculant, suggesting that the polymer is including carbohydrates. EPSs from strain NRC14 may probably have a significant role for cell protection, adhesion of bacteria to solid surfaces, and participating in cell-to-cell interactions. The high bioflocculant yield and flocculating-activity properties of the bioflocculant portend industrial applicability.

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