Extracellular Metabolites Produced by a Novel Strain, *Bacillus alvei* NRC-14: 3. Synthesis of a Bioflocculant that has Chitosan-Like Structure

Shadia M. Abdel-Aziz*[1], Hoda A. Hamed[1], Foukia E. Mouafi[2,] and Nayera A. M. Abdelwahed[3]

Microbial Chemistry Dept.[1], Microbial Biotechnology Dept.[2], Chemistry of Natural and Microbial Products Dept.[3], National Research Centre Dokki, Cairo, Egypt.
*abdelaziz.sm@gmail.com

Abstract: An extracellular biopolymer flocculant (BPF) was produced by a novel strain, *Bacillus alvei* NRC-14, cultivated in low nutritional medium. Effect of carbon and nitrogen sources as well as the initial pH and medium components, on production of the BPF were studied. Production of this BPF is induced in presence of chitosan or chitosan-containing substrate as carbon source, suggesting a correlation between the activities of several key enzymes involved in the pathway with the yield of the BPF. Therefore, biosynthetic pathway of this BPF may starts with an aminosugar as a precursor which was then, presumably, polymerized and converted to a bioflocculant. The highest production was achieved in low nutrition medium containing: dried mycelium of the fungus *Mucor rouxii* (10g/L), $(NH_4)_2SO_4$ (1.5g/L) and $MgSO_4.7H_2O$ (0.5g/L). The flocculating activity reached 98% in less than 48 hr of growth. The produced bioflocculant is a polysaccharide consisting of aminosugars and has good flocculating activity in charcoal or kaolin clay suspension, without any cation addition. IR-spectra elucidate that, the BPF has chitosan-like structure with a molecular weight of 6.9×10^4 Da. Surprisingly, shelf-life studies of this BPF revealed that it retained 94% of its flocculating activity after keeping at room temperature for up to 6 months period, indicating its higher stability. This is the first report about a bioflocculant that has chitosan–like structure produced by a novel strain, *Bacillus alvei* NRC-14.

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Key words: Extracellular metabolites, *Bacillus alvei* NRC-14, biopolymer flocculant, *Mucor rouxii*, chitosan.

1. Introduction

Bioflocculation is a dynamic process resulting from the synthesis of extracellular polymers by microorganisms during their growth. Flocculation was first reported, in 1876, by Louis Pasteur (Salehizadeh and Shojaosadati**, 2001**) and has been extensively investigated update. A correlation was established between the accumulation of extracellular bioflocculants and cell aggregation (Tenny and Verhoff, 1973). The term flocculation is used to describe the aggregation of microbial cells to form flocs with other sediments in the culture. Bioflocculants are useful in aggregation of colloids, cells, cell debris, sediments, etc. The predominant components of bioflocculant are extracellular polymeric substances such as polysaccharides, proteins, glycoproteins, or nucleic acids **(**Lazarova and Manem 1995; Gao et al. 2006; Leonard et al. 2011). Flocculating agents can be classified into three groups: 1) inorganic flocculants such as aluminium sulfate and polyaluminium, 2) Organic synthetic high-polymer flocculants such as polyacrylamide and polyetheleneamine, and 3) naturally occuring flocculants such as chitosan, and the microbial flocculants (Shih et al. 2001). Organic synthetic flocculants are widely applied in industrial fields nowdays because they are highly effective, however, their use is environmentally harmful and some of their degraded monomer such as acrylamide, are neurotoxic and even strong human carcinogens (Kwon et al. 1996) and can induce Alzheimer's disease (Arezoo 2002) caused by aluminum salts. Moreover, when iron coagulants (such as ferric chloride) are used excessively they may cause corrosion and staining of appurtenances and give rise to unpleasant aesthetic concerns such as odor, color, and metallic taste (Lijun et al.2009). The usage of synthetic flocculants has been restricted mainly in European countries because of their hazardous effects (Ho et al. 2010). Compared with synthetic flocculants, bioflocculants have special advantages such as safety, strong effects, biodegradable, and harmlessness to the environment and human, so they may potentially applied in drinking and wastewater treatment, downstream processing as well as fermentation processes (Mao et al. 2011). Furthermore, flocculation is an industrially accepted practice used in many food processing and pharmaceutical operations, demonstrating that many of the polymers are generally regarded as safe when

the product is intended for human or animal consumption (Cartier et al.1997, Wang et al. 2011). However, a major bottleneck for the commercialization of bioflocculants is the higher production cost compared with inorganic and synthetic flocculants.

In the present study, attention was focused on the bioflocculant produced by a novel strain, *B. alvei* NRC-14. During a study for production of chitosanase by this strain, using mycelium of the fungus *Mucor rouxii* as a carbon source, it was noticed that a culture broth of the strain was highly viscous and displayed high flocculating properties, i.e. the particles of the mycelium were extremely aggregated to the cells of the strain and other sediments. The progress and extent of flocculation were followed by an obvious decrease in turbidity of the culture broth, and the medium seemed very clear. Therefore, in the present study attention was turned for isolating this biopolymer flocculant (BPF). Optimization of the culture conditions, nutritional requirements for maximum production, and some properties of the produced BPF are reported herein. To our knowledge, production of a bioflocculant by a newly isolated, *B.alvei* NRC-14, is reported for the first time.

2. Materials and methods

Microorganism and growth conditions

The strain *B. alvei* NRC-14, used in this study, is a local bacterial strain isolated from the Egyptian soil. Identification and taxonomic studies on the isolate were carried out by methods described in *Bergey's Manual of Systematic Bacteriology* (Juni, 1986). The strain was maintained on nutrient agar slants, stored at 4^{o}C, and subcultured at intervals. To study the effect of growth conditions on production of the bioflocculant, the organism was grown in a broth medium (Tabata and Terui, 1962), containing (g/L): 10, flaked chitosan; 2, K_2HPO_4; 1, KH_2PO_4; 0.5, $MgSO_4.7H_2O$; 0.1, CaCl; 0.5, yeast extract; and 0.1, NaCl. The medium was supplemented with different carbon and nitrogen sources, and the pH was adjusted to different pH values. Flasks were incubated for 5d at 30^{o}C, under shaking conditions (130 rpm).

Production of the bioflocculant

To study the effect of medium components on production of the BPF, the organism was grown in a minimal medium (MM) containing (g/L):10.0, fungal mycelium (FM) of *Mucor rouxii*; 1.5, $(NH_4)_2SO_4$ and 0.5, $MgSO_4.7H_2O$. For comparison, an enriched medium (EM) was used, which contained (g/L): 10.0, FM; 1.5, $(NH_4)_2SO_4$; 2.0, K_2HPO_4; 1.0, KH_2PO_4; 0.1, NaCl; 0.1, CaCl; 0.02, $FeSO_4$; 0.05, $MnSO_4$; and 0.5, $MgSO_4.7H_2O$. The pH value of both medium was adjusted to 5.5. Experiments were done in 1L conical flasks containing 400 ml of each medium. Flasks were inoculated with a pre-culture (4%, v/v) and incubated at 30^{o}C for 5d under shaking conditions (130rpm). Samples were taken at different time intervals and monitored for cell growth, pH, and detection of flocculating activity (FA). Cell-free supernatant was used as the test BPF to determine the flocculating activity (FA).

Preparation of fungul mycelium

The fungus, *M. rouxii*, was grown in potato-dextrose broth and incubated at 30^{o}C for 7d. The pellets were harvested, washed with distilled water, and dried at 50^{o}C. The dried mycelium was homogenized, and used as a carbon source for the production of the BPF.

Calculation of molecular weight

The molecular weight (MW) of the produced BPF in relation to viscosity was calculated according to the method of Il'ina et al. (2001).

Determination of flocculating activity

The FA was measured according to the method of Kurane et al. (1986) using a suspension of charcoal (5g/L, in distilled water) as a test material. To 9.5 ml of charcoal suspension in a test tube, 0.5 ml of cell-free supernatant was added. The reaction mixture was stirred with a vortex mixture and allowed to stand for 5 min. The absorbance of the upper phase was measured at 550 nm , using spectrophotometer. A control sample without the bioflocculant was used. The FA was calculated according to the following equation: Flocculating activity (%) = [(A- B)/ B)] x100%, where A and B are the optical densities of the control and the sample, respectively.

Extraction and purification of the crude bioflocculant

The BPF of strain NRC-14 was purified by the method of Shih et al. (2001) as follows: the viscous culture broth (400 ml) was mixed with three volumes of cold distilled water and centrifuged at 7000xg for 20 min. The resultant supernatant was poured into two volumes of cold ethanol to precipitate the bioflocculant. The precipitate was collected by centrifugation at 7000xg for 20 min, and kept at 4^{o}C.

Characterization and some properties of the bioflocculant

The culture supernatant together with the purified BPF was used as test bioflocculants to estimate the effect of flocculant dosage, pH, and temperature on the FA. The culture broth and the purified flocculant were obtained at the same time of cultivation. A control sample was prepared and the FA was measured and calculated as described above. To estimate the influence of the pH value on the FA, a reaction mixture containing charcoal suspension and the bioflocculant was adjusted to pre-determined pH values using HCL or NaOH, and then the FA was measured. The temperature dependence of both flocculants was determined at different degrees of temperature followed by measuring the residual FA of charcoal suspension (Gao et al. 2006).

Analytical methods

Protein content was assayed by the method of Lowry, using bovine serum albumin as standard (Lowry et al. 1951). Amino-sugars were determined by the Elson-Morgan method (Chaplin & Kennedy 1986).

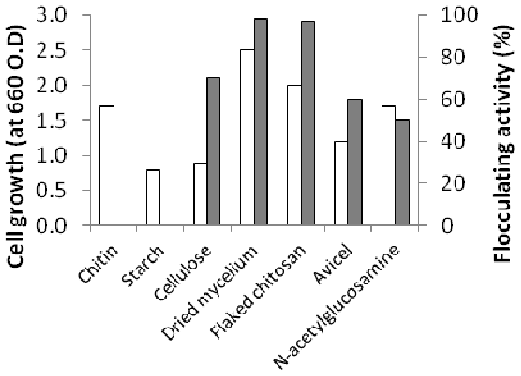

Fig. 1. Effect of carbon source on cell growth (□) and flocculating activity (■). The culture time was 48 h.

IR-spectra of the bioflocculant

The viscous product was analyzed by infrared using a 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}.

Shelf-life of the bioflocculant

The shelf-life stability of the BPF was studied at room temperature, at 4°C, and under refrigerator conditions. Samples were tested for the FA at different time intervals for up to 12 months.

3. Results

Effect of carbon and nitrogen sources

Strain *B.alvei* NRC-14 grew well with all the carbon sources tested, however, production of the BPF was efficiently produced with FM of *M .rouxii* or flacked chitosan; the FA reached 98% and 97%, respectively (Fig.1). To study the effect of different nitrogen sources on growth and production of the BPF, 0.15% (w/v) each of $NaNO_3$, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, as well as yeast extract and peptone were used.

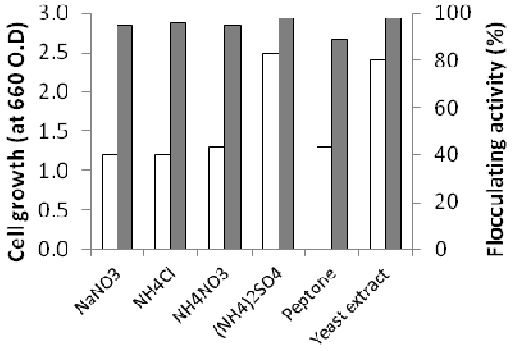

Fig. 2. Effect of nitrogen sources on cell growth (□) and flocculating activity (■). The culture time was 48 h.

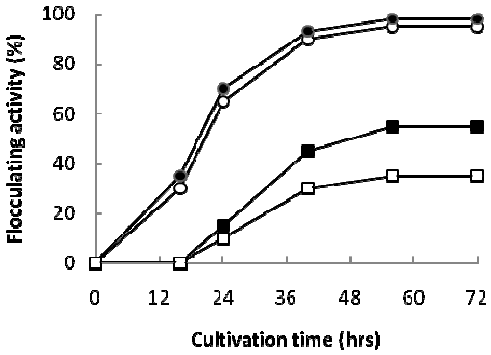

Fig. 3. Effect of initial pH of medium on the bioflocculant production by strain NRC-14. pH values, 4.5 (●), 5.5 (○), 6.5 (■), and 7.5 (□).

As shown in Fig. 2, yeast extract or $(NH_4)_2SO_4$ were the most favorable nitrogen source for both the growth and production of the BPF, using FM as carbon source.

Effect of pH

The pH of growth medium greatly affected production of the BPF by strain NRC-14. Highest production was occurred at pH values ranged from 4.5-5.5. At these pH values, a rapid increase in production was observed after less than 48h of

growth (Fig. 3), whereas, higher pH values (6.5-7.5) does not favored the BPF production.

Effect of medium components.

Effect of medium component on maximum production of the BPF was also studied using minimal medium (MM) and enriched medium (EM). Despite of the differences in composition between the two media, the organism had nearly similar growth curve and maximum production throughout the growth period (Fig. 4, A and B). During the cultivation period, the FA increased with culture time and reached its maximum (98%) in early stationary phase. Production of the BPF was in parallel with cell growth curve up to 40 hr, after which the cells aggregated to the fungal mycelium particles and the medium seemed clear (Fig. 5). The FA increased with time, indicating that the BPF is produced during the growth of the strain, not by cell autolysis (Shih et al. 2001; Xia et al. 2007). The pH increased by time probably, due to the liberation and release of alkaline metabolites such as -NH_2 from chitosan degradation. Growth of the strain in a minimal medium with maximum production of the BPF elucidate that this strain is not exigent, i.e. the growth of the strain for efficient production of the BPF recommended no growth factors, no stimulants, and no metals cation.

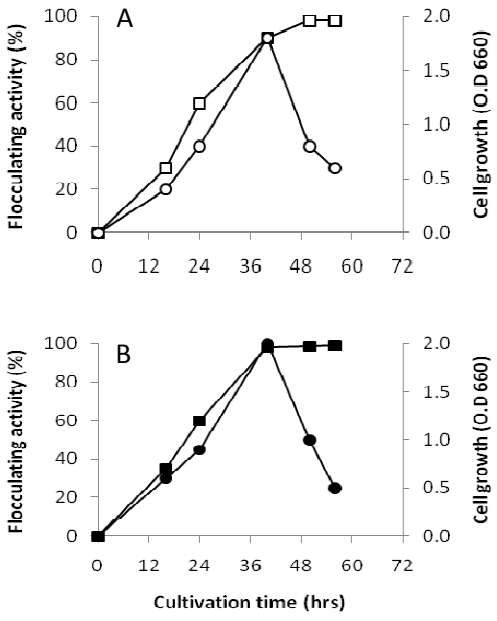

Fig. 4. Effect of medium components on production of the bioflocculant by strain NRC-14. Growth curve (○,●), and flocculating activity (□,■) determined for (A) the minimal (MM), and (B) the enriched (EM) growth media

Recovery of the bioflocculant

From 400 ml of culture supernatant under optimize culture conditions (MM medium, pH 5.5, 30°C, 48 hrs growth), 100 ml of the BPF were recovered by ethanol precipitation as a white, mucoid, ropy bioflocculant. After recovery of the BPF by ethanol, the resultant precipitate was found to be soluble in water, but not in solvents.

Characterization of the bioflocculant

Effect of dosage on the flocculating activity

Optimum dosage of the purified and crude bioflocculant was 3 and 15 ml/L for maximum flocculating activity (Fig. 6). Excess dosage of a bioflocculant may cause re-suspension of charcoal particles and leading to a reduction in the FA.

Effect of pH

Flocculating activity of the BPF of strain NRC-14 varied widely with the pH values. Using citrate buffer, the FA of the crude flocculant increased at pH values of 4~7, whereas FA of the purified flocculant increased at pH range of 8.0-9.5 (Fig. 7). The purified flocculant remained 92% of its FA at pH 9.5.

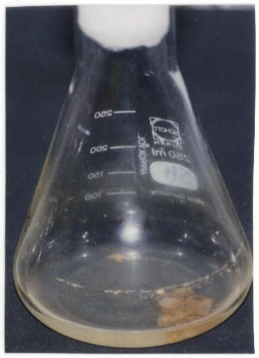

Fig. 5. Aggregation of dried mycelium of the fungus *M. rouxii* and other sediments by the bioflocculant produced by strain NRC-14 after 48 hrs of growth in a minimal medium.

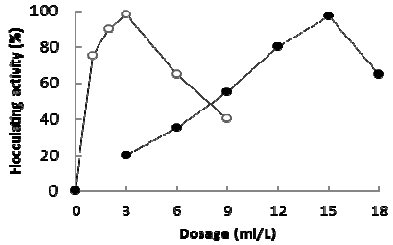

Fig. 6. Relationship between dosage of the crude (●), and purified (○) bioflocculant and its flocculating activity.

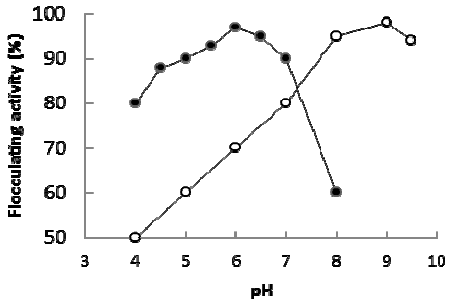

Fig. 7. Effect of pH values on the flocculating activity of the crude (●), and purified (○) bioflocculants.

Effect of temperature.

The temperature dependence of the BPF revealed that, the FA of both bioflocculants could effectively occur at temperatures ranging from 30-90°C. Below temperatures less than 20°C, the purified bioflocculant acquired a mucoid and ropy property; therefore, recovery steps of the BPF were performed with cold distilled water and ethanol. The BPF was stable after heating at 100°C; the residual activity of the crude and purified flocculant reached about 97 and 98%, respectively, after heating at 100°C for 40 min. The main backbone of the BPF from strain NRC-14 is a polysaccharide. In this respect, complete hydrolysis of the viscous bioflocculant was carried out with 2N HCl at 100°C, and it was found that the majority of sugar constituents in the HCl hydrolysate were found to be aminosugars.

IR-spectra of the flocculant

Fig. 8, showed a transmission pattern of the BPF; surprisingly, it is similar to that of authentic chitosan, in which a hydroxyl band at 3420-3450 cm^{-1}, and an amide band at 1550-1655 cm^{-1}, and an amine band at 1550-1630 cm^{-1} were measured as characteristic bands for chitosan (Yokoi et al 1995, Muzzarelli et al 2004) The absorption band at 3428 cm^{-1} suggested the presence of -OH, and the bands at 1587 cm^{-1} and 1414 cm^{-1} may be assigned to the C=O asymmetrical and symmetrical stretching in carboxylate, respectively (Dermlim et al 1999). The absorption peaks observed at 989 cm^{-1} and 1273 cm^{-1} are generally known to be typical characteristics of aminosugar derivatives (Suh et al 1997). The infrared spectrum of this bioflocculant, thus, shows the presence of carboxyl, hydroxyl, and amino groups which are known to contribute to the flocculation property of a bioflocculant(Zajic and knetting 1970).

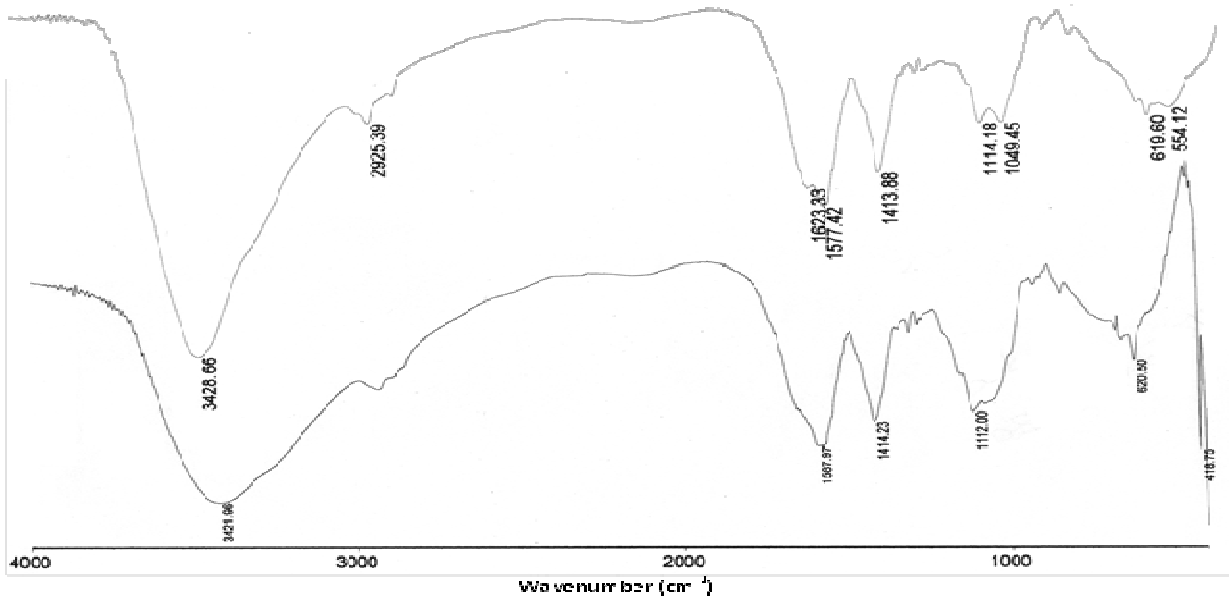

Fig. 8. IR-spectra of the original chitosan (upper curve) and the produced bioflocculant (lower curve) by *B. alvei* NRC-14.

Shelf- life stability

The shelf-life stability of the purified BPF from strain NRC-14 was studied at refrigerator conditions (0°C), 4°C, and at room temperature (20-38°C) up to 12 months. The stability of the purified BPF is wonderful; it kept all of its activity at refrigerator and at 4°C for up to 12 months, and retained 94% of its FA for up to 6 months at room temperature (Fig. 9). Worthy mention is that, the FA of the crude BPF showed similarities to the flocculating efficacy of the purified one; the efficacy of precipitating charcoal particles by the crude BPF reached 90% after 6 months of keeping at room temperature (data not shown).The bioflocculant produced by strain NRC-14 did not require any metals to show maximum FA, some other flocculants need addition of metals to achieve high flocculating rates.

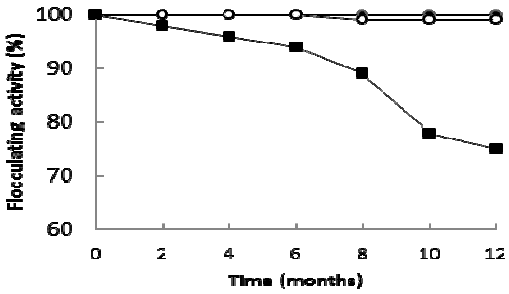

Fig. 9. Shelf-life stability of the purified bioflocculant produced by strain NRC-14. The flocculating activity was determined during 12-month period kept at refrigerator (●), 4 °C (○), and room temperature (■).

4. Discussion

Most bioflocculants are produced by microorganisms during their growth periods. Bacteria can utilize the nutrients in the culture medium to synthesize high molecular-weight polymers internally within the cells under the action of specific enzymes, and these polymers can be exerted and exist in the medium or on the surface of the bacterium as capsules. Therefore, by the action of such bacteria, the simple substances in their environment can be converted into complex polymers (Tenny and Verhoff, 1973). In the present study, it could be concluded that chitosan or chitosan-containing substrate are essential for the formation of the flocculant by the strain. Biosynthesis of this BPF, in fact, suggests a relationship between the presence of chitosan and the production of the flocculant. In previous studies (Abdel-Aziz, 1999; Abdel-Aziz et al. 2008) for production of chitosanase by strain NRC-14 using flacked chitosan, a bioflocculant was frequently produced by the strain. We concluded that, degradation of chitosan by enzyme(s) secreted by the strain may probably result in accumulation of aminosugars. e.g. glucosamine which may polymerize to form the bioflocculant. The structure of the BPF which is extremely similar to that of chitosan would provide evidence that a monomer such as glucosamine may be an important precursor for the synthesis of the bioflocculant by the strain. Of interest is that, during the twenty-year history usage of strain NRC-14 under laboratory conditions, no variability or cell lyses was observed. Moreover, the BPF is a fairly stable polymer and it may have a protective function for the cells of the strain. The genetic stability of the strain is probably due to the presence of the BPF, which is highly stable (Pirog et al. 1997).

Optimization of medium composition in the present study revealed the significant effect of medium components. Low nutritional requirements have shown that strain NRC-14 is not an exigent organism, i.e. high BPF is produced under low nutritional medium, using low-cost substrate. Production of the bioflocculant is induced only in presence of chitosan as a determinant carbon source, while the best nitrogen sources were yeast extract or $(NH_4)_2SO_4$. Many reported strains can use organic nitrogen sources or organic combined with inorganic nitrogen sources to produce bioflocculants. For example, peptone (organic nitrogen source) and sodium nitrate (inorganic nitrogen source) provided the best nitrogen source for *Aspergillus parasiticus* for production of the bioflocculant, whereas with $(NH_4)_2SO_4$ no flocculant was produced (Deng et al. 2005). Compared with inorganic sources, beef or yeast extract were also more favorable for bioflocculant production by strain X-14 (Li et al. 2009). However, a complex of nitrogen source consisting of beef extract and urea was better than solely inorganic or organic nitrogen source (Gong et al. 2008). Strain TJ-1 was able to use beef extract, yeast extract, or peptone as an organic nitrogen source for production of a bioflocculant (Xia et al. 2008). In our study, strain NRC-14 can effectively use both inorganic or organic nitrogen sources to produce a bioflocculant. This result is in accordance with that reported for strain, *B. licheniformis* X-14.

During the logarithmic phase, the production of the BPF by strain NRC-14 almost paralleled the cell growth up to 40 hrs of growth, while maximum production of the BPF reached about 98% after 48 hrs of growth. Many reported bioflocculants are collected in the late logarithmic growth phase and the early stationary phase because the FA began to decrease due to the activity of deflocculating enzymes (Kurane et al. 1986). However, the FA of the bioflocculant from strain NRC-14 did not

decrease when the strain entered the decline phase (at 60 hrs), suggesting that this strain did not secrete deflocculating enzymes.

The purified bioflocculant was found to be dissolved in aqueous solutions but not in organic solvents. Since the bioflocculant contained hydroxyl groups, it had the possibility of hydrogen bonding to one or more water molecules. The abundance of hydroxyl groups build up strong forces of attraction between the bioflocculant molecules, resulting in relatively hard crystalline form. These forces are too great to be broken by organic solvents, so the purified bioflocculant is insoluble in organic solvents (James et al., 1986).

The relationship between the bioflocculant dosage and the FA revealed that, the activity initially increased with increasing the flocculant dosage, but then decreased. This is because, the adsorption of excess bioflocculant restabilize the charcoal particles; only the particles around bioflocculant precipitated in the flocculating reaction in a moment, and other particles did not precipitate, subsequently the FA decreased (Suh et al. 1997).

The MW of the BPF was found to be $6.9x10^4$ Da. The MW and functional groups at the molecular chains are the most important factors in the FA of a bioflocculant. Currently, the components and structures of bioflocculants are complex, and different flocculants produced by microbes can have different properties. However, 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 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, Todd et al. 2010). 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. One mechanism that flocculation can occur, when the flocculant forms molecular “bridges” between the suspended solid particles creating larger solid aggregates. A second mechanism for flocculation, 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). In our study, the bioflocculant was a cation-independent, whose flocculating capability need no cation addition. This may, probably, be due to its flocculation which may depends primarily on bridging rather than on the mechanism of charge neutralization (Zheng et al.2008).

The IR-spectrum of the bioflocculant is extremely similar to that of chitosan. Spaink et al. (1994) have reported that, low molecular-weight chitin oligosaccharide has produced by *Rhizobium leguminasarum*. Fujita et al. (2000) have reported for the first time the bacterial production of a biopolymer flocculant by *Citrobacter* sp., which has a structure similar to that of chitin. Within the range of our research, this is the first report on a bioflocculant has chitosan-like structure, produced by the novel strain *Bacillus alvei* NRC-14.

Conclusion

Industrial production of bioflocculants is limited by their high costs and poor yields. The bioflocculant, produced by the newly isolated *Bacillus alvei* NRC-14, is induced by chitosan or chitosan-containing substrate, using low-cost nutritional medium. Studies on the effect of carbon and nitrogen sources revealed that the production of the BPF is affected mainly by the carbon source used and the pH value, but not by the nitrogen source used . The BPF is an exopolysaccharide, heat stable, with relatively low dosage requirement. Addition of metal ions had no positive effects on the flocculating activity, indicating that the bioflocculant is cation-independent, which means that it could avoid second pollution and reduce costs. The IR-spectra of the flocculant is extremely similar to that of chitosan. The shelf-life stability of the crude and purified bioflocculants is wonderful; both flocculants remained most of its activity after 6 months period of keeping at room temperature. The practical application of the bioflocculant in industry would be studied in further progress.

Corresponding author
Shadia M. Abdel-Aziz
Microbial Chemistry Dept.,
National Research Centre, Dokki, Cairo, Egypt.
abdelaziz.sm@gmail.com

References

Abdel-Aziz, SH. M. **(1999).** Production and some properties of two chitosanases from *Bacillus alvei*. J. Basic Microbiol.,39, 79-87.

Abdel-Aziz, SH., Moustafa, Y., and Mouafi, F. **(2008).** Partial purification and some properties of the chitosanases produced by *Bacillus alvei* NRC-14. J. Appl. Sci. Res.,4,1285-1290.

Arezoo,C. **(2002).**The potential role of aluminium in Alzheimer's disease. Nephrol.Dial.Transplant,17, 17-20.

Cartier, S., Tatoud, L., Theoleyre, M., and Decloux, M. **(1997).** Sugar refining process by coupling flocculation and crossflow filtration. J. Food Eng., 32, 155-166.

Chaplin,M. and Kennedy, J. **(1986).** *Carbohydrate analysis* . IRL Press, Washington, DC, pp. 2-5.

Deng,S., Bai,R.,Hu,x. and Luo,Q. **(2003).** Characteristics of a bioflocculant produced by *Bacillus mucilaginosus* and its use

in starch wastewater treatment. Appl. Microbiol. Biotechnol., 60, 588-593.

Deng, S., Yu, G. and Ting, Y**. (2005).** Production of a bioflocculant by *Aspergillus parasiticus* and its application in dye removal. Colloids and surfactant. Biointerf. 44,179-186.

Dermlim,W., Prasertsan,P. and Doelle,H. **(1999).** Screening and characterization of bioflocculant produced by isolated *Klebsiella* sp. Appl. Microbiol. Biotechnol., 52, 698-703.

Fujita, M., Ike, M., Tachibana, S., Kitada,G., Kim, S., and Inoue, Z. **(2000).** Characterization of a bioflocculant produced by *Citrobacter* sp.TKF04 from acetic and propionic acids . J. Biosci. Bioeng., 89, 40-46.

Gao,J., Bao, H.,Xin,M., Liu,Y., Li,Q. and Zhang,Y. **(2006).** Characterization of a bioflocculant from a newly isolated *Vagococcus* sp.W31. J. zhejiang Univ. Sci.,3, 186-192.

Gong, W., Wang,S., Sun, X., Liu, X., Yue, Q., and Gao, B. (**2008).** Bioflocculant production by culture of *Serratia ficaria* and its application in wastewater treatment. Biores. Technol., 99,4668-4674.

Ho,Y., Norli,i.I., Alkarkhi,A., and Morad,N. **(2010).** Characterization of biopolymeric flocculant (pectin) and organic synthetic flocculant (PAM): a comparative study on treatment and optimization in kaolin suspension. Bior. Technol., 101,1166-1174.

Il'ina,A.,Varlamov,V., Melent,A. and Aktuganov, G. **(2001).** Depolymerization of chitosan with the chitinolytic complex from bacteria of the genus *Bacillus* sp.739. Prikl. Biokhim. Microbiol., 37, 160-163.

James, K**. (1986).** *Drugs and the Pharmaceutical Science*. In: Solubility and Related Properties, Vol., 28, Marcel Dokker New York.

Juni, E. **(1986).** *Bergey's Manual of Systematic Bacteriology*, In: Genus *Bacillus*, p. 1115-1139, J. G. Holt, (ed.), Williams and Wilkins, London.

Kurane,R., Takeda,K., and Suzuki,T**.,(1986).** Screening for and characteristics of microbial flocculants. Agr. Biol. Chem., 50, 2301-2307.

Kwon,G., Moon,S.,Hong,S.,Lee,H., Kim,H., Oh,H. and Yoon,B**.(1996).** A novel flocculant biopolymer produced by *Pestalotiopsis* sp. KCTC-8637.Biotechnol. Letters, 18, 1459-1464.

Lazarova,V. and Manem,J. **(1995).** Biofilm characterization and activity analysis in water and wastewater treatment. Water Res., 29, 2227-2245.

Leonard, V., Cosa, S., Noxolo, M. and Okoh, A. **(2011).** *Halomonas* sp. OKOH-A marine bacterium isolated from the bottom sediment of Algoa Bay—produces a polysaccharide bioflocculant: partial characterization and biochemical analysis of its properties. Molecules, 16, 4358-4370.

Li, Z., Zhong, S., Lei, H.,Chen, R., Yu, Q., and Li, H. **(2009).** Production of a novel bioflocculant by *Bacillus licheniformis* X14 and its application to low temperature drinking water treatment. Biores. Technol., 100, 3650-3656.

Lijun,Y., Lu,F., Li,D., Qiao,Z., and Yin,Y. **(2009).** Preparation and flocculation properties of cationic starch/chitosan crosslinking-copolymer. J. Hazard. Materials,172,38-45.

Lowry, O., rosebrough, N., Farr, A., and Randall, R. **(1951).** Protein measurement with the folin phenol reagent. J. Biological Chem., 193, 265-275.

Mao, Y., Tian,C., Zhu, J.,Zhang, T. and Tong, L. **(2011).** Production of a novel biopolymer by culture of *Bacillus cereus* B-11 using molasses wastewater and its use for dye removal. Adv. Materials Res., 230, 1119-1122.

Michaels, A. (**1954).** Aggregation of suspensions by polyelectrolytes. Industrial Eng. Chem., 46, 1485.

Muzzarelli, G., Tosi, G., Oriano, F., and Muzzarelli, R. **(2004).** Alkaline chitosan solutions. Carbohyd. Res., 338, 2247-2255.

Pirog, T., Grenbirg, T., and Malashinko, Y. **(1997).** Isolation of microorganisms-producing enzymes that degrade *Acinetobacter* sp. exopolysaccharides. Prikl. Biokhim. Microbiol., 33, 491-495.

Salehizadeh,.and shojaosadati,S. **(2001).** Extracelullar biopolymeric flocculants. Resent trends and biotechnological importance. Biotechnol. Adv., 19, 371-385.

Shih,L.,Van,Y.,Ych,L.,Lin,H. and Chang,Y. **(2001).** Production of biopolymer flocculant from *Bacillus licheniformis* and its flocculation properties Biores. Technol., 78, 267-272.

Spaink, H., Wijfjes, A., Drift, V., Haverkamp, J. et al. **(1994).** Structure identification of metabolites produced by the NodB and NodC proteins of *Rhizobium leguminsarum*. Mol. Microbiol., 13, 821-831.

Suh,H., Kwon,G., Lee,C., Kim,H., and Oh,H. **(1997).** Characterization of bioflocculant produced by *Bacillus sp.* SDp-152. Ferm. Bioeng.,84,108-112.

Tabata,S. and Terui,G. **(1962).** Studies on microbial enzymes active in hydrolyzing yeast cell walls:Isolation of a strain and culture conditions for enzyme formation. J. Ferm. Technol., 40, 366-373.

Tenny, M. and Verhoff, F. **(1973).** Chemical and auto flocculation of microorganisms in biological waste treatment. Biotechnol. Bioeng.,15, 1045-1053.

Todd, M. **(2010).** Polyelectrolyte flocculation of grain stillage for improved clarification and water recovery within bioethanol production facilities. Biores. Technol., 101, 2280-2286.

Wang, J., Wei, J. and Ma, H. **(2011).** Screening of a flocculant-producing strain and optimal conditions for production of bioflocculant. Adv. Materials Res.,183,829-833.

Xia, S., Zhang, Z., Wang, S., Yang, A., Chen,L., Zhao,J., Leonard,D. and Jaffrezic-Renault,N. **(2007).** Production and characterization of a bioflocculant by *Proteus mirabilis* TJ-1. Biores. Technol., 99, 6520-6527.

Yokoi, H.,Osamu, N., Jun, H., Sachio, H., and Takasaki,Y. **(1995).** haracteristics of a biopolymer flocculant produced by *Bacillus* sp.PY-90. J. Ferm. Bioeng., 79, 378-380.

Zajic,J. and Knettig,E. **(1970).** Flocculants from para-finic hydrocarbons: *Development in industrial microbiology*, p.87-98.American Institute of biological science,Washington DC.

Zheng, Y., Ye, Z., Fang, X., Li,Y. and Cai, W. **(2008).** Production and characteristics of a bioflocculant produced by *Bacillus* sp. F19. Biores. Technol., 99, 7686-7691.

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