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Characterization of bioflocculants produced by bacteria isolated from crude petroleum oil

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ABSTRACT: The aim of this study was to look for high efficient bioflocculant-producing microorganisms. Among 36 bacterial colonies isolated from a crude petroleum oil sample, three of them as *Bacillus subtilis and Pseudomonas* spp. exhibited flocculation activity exceeding 90 % after 3 days of cultivation. They were identified by 16 S rDNA sequence analysis as *Bacillus subtilis* and *Pseudomonas* sp. Spectroscopic analysis of the polymers by nuclear magnetic resonance and fourier-transform infrared revealed that the polymers were glycoproteins. These polymers were soluble in water and insoluble in any organic solvents tested. The effects of bioflocculant dosage, temperature and pH on the flocculation activity were evaluated. The maximum bioflocculation activities were observed at an optimum bioflocculant dosage of 3.5 mg/L (strains *Bacillus subtilis and Pseudomonas*) and 5.0 mg/L (strain CPO14), respectively. In addition, these biopolymers were able to flocculate kaolin suspension (5 g/L) over a wide range of pH (pH 3–9) and temperature (5–50 °C) tested in the presence of $CaCl_2$. The highest flocculation activities of strains CPO8, CPO13 and CPO14 were 96.03 %, 92.17 % and 97.59 %, respectively in the early stationary phase (at 24 h), while the cell production reached its maximum in the stationary phase (at 72 h). Their efficient flocculation capabilities suggest potential applications in industries.

Keywords: *Bioflocculant; Biopolymer; Flocculation activity; Optimization*

INTRODUCTION

Industrial process is a potential source of pollution and requires a specific treatment for the waste produced. Introduction of waste treatment increases the plant cost, therefore an attempt to improve its efficiency is valuable. This applies to all kinds of industries but is particularly important for low or medium added value industries with difficult wastes like tanneries and clay processing industry. Flocculation is the most widely used process for treating the wastewater in these kinds of industries (Rossini *et al.,* 1999). Separation by flocculation is effective and sufficient when the end objective is reduction in sludge volume and rapid separation of solids from liquids (Besra *et al.,* 2003).

The problem of removal of kaolinite turbidity from the effluents before discharging into the environment was studied by many researchers. Faust and Aly (1983) quote extensive application of kaolinite suspensions as a model for natural turbidity of raw surface water in Jar Test studies of coagulation and flocculation in the laboratory. Inorganic coagulants such as alum in combination with lime have been conventionally used for the removal of clay particles in the effluents from clay processing industry as well as from raw water. The sludge formed from such treatment poses disposal problems because of its aluminium content and tend to accumulate in the environment (Faust and Aly, 1983). Stauber *et al.* (1999) and Divakaran and Sivasankara, (2001) have reported that although not conclusively proved, increasing concern about the residual aluminium, which may be present in water as a result of alum treatment, is being expressed by the public in connection with Alzheimer's disease. Thus, it has become necessary to develop harmless, more efficient and environment friendly flocculants for removal of turbidity in surface waters and effluents.

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Bioflocculants have received considerable scientific and biotechnological attention in recent years due to their biodegradability, benign nature and lack of secondary pollution of their degradative intermediates (Salehizadeh and Shojaosadati, 2001). They reported that bioflocculants are microorganism-produced special natural organic macromolecule substances that can flocculate suspended solids, cells, colloidal solids, etc. In recent years, several kinds of microorganisms, which secrets flocculation biopolymer, have been screened and isolated from activated sludge, soil and wastewater. The species include bacteria, fungi, actinomyces and algae. Generally, soil and activated sludge samples are the major sources for isolating bioflocculant-producing microorganisms (Abd-El-Haleem *et al.,* 2008). However, isolation and characterization of bioflocculant-producing microorganisms from crude petroleum oil samples were not well documented in literatures. Therefore, present study aims to look for bioflocculant-producing microorganisms in crude petroleum oil sample collected on 28 May 2009 from a sews-gulf oil drilling field in Egypt. Screening, isolation and characterizations of both bioflocculant-producing microorganisms and their extracellular bioflocculants were performed.

MATERIALS AND METHODS

Media and cultivation conditions

Isolation medium

The Sup- plemented nutrient broth (SNB) medium used to isolate bacteria from the oil sample (collected from a sews-gulf oil drilling field in Egypt) was containing per liter of 500 mL of Nutrient Broth and 500 mL mineral salt solution. The 500 mL of salt solution was prepared by adding the various salts such as KH_2PO_4, 20 g; K_2HPO_4, 5.0 g, $(NH_4)_2 SO_4$, 30 g; NaCl, 0.1 g; $FeSO_4.7H_2O$, 0.01 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.01 g; $MnSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.01 g; and $MnSO_4.H_2O$, 0.002 g, glucose, 0.03 % (w/v); and yeast extract, 0.03 % (w/v) (Francy *et al.,* 1991). One-half SNB medium with 2 % Bacto Agar (SNA) was used to isolate heterotrophs.

Prescreening medium

The prescreening medium used to select for bioflocculant-producing bacteria contained (per liter) 1% glucose, 0.35% yeast extract, 0.5 % K_2HPO_4, 0.2 % KH_2PO_4, 0.05 % $MgSO_4$ $7H_2O$, 0.01% NaCl and agar 1.5 %. The broth of the prescreening medium was used as a seeding medium.

Fermentation medium

The fermentation medium was containing per liter; glucose 10 g, NH_4Cl 7 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, $FeCl_3$ 40 mg, $CaCl_2$ 150 mg and $MnSO_4$ 140 mg.

Cultivation conditions

The initial pH of all media was adjusted to 7.2 to 7.5 with NaOH (1 M) and HCl (0.5 M). All the media were prepared with distilled water and sterilized at 121 °C for 20 min. All cultivations were done at 30 °C.

Isolation, screening and identification of bioflocculant-producing bacteria

Screening process for bioflocculant-producing bacteria was performed in two experimental steps. First, all bacteria that might exist in the sample were isolated by adding 1 mL of oil sample to 99 mL of SNB medium in 250 mL flasks. The mixture was placed on a rotary shaker at 200 rpm for 2 h at 30 °C to produce a well-dispersed suspension. Subsequently, the suspension was diluted serially in SNB broth and plated in triplicate on one-half SNB with 2% Bacto Agar to isolate heterotrophs. The plates were incubated at 30 °C. Pure cultures of bacteria were isolated by cycles of re-plating on SNB agar plates. Thirty six pure bacteria with different colony morphologies were isolated and maintained on Nutrient Agar slants until used.

Secondly, to screen among all isolates for bioflocculant-producing bacteria, a loop of each isolate was suspended in sterile 1 mL distilled water and an aliquot ~ 100 µL was spread on the prescreening medium and incubated at 30 °C for 48 h (Dermlim *et al.,* 1999, Abd-El-Haleem *et al.,* 2008). Microorganisms were screened for their abilities to produce bioflocculant based on colony morphology (mucoid and ropy) (Murray *et al.,* 1994). The positive strains were grown in 50 mL fermentation medium in 250 mL flasks on a rotary shaker at 30 °C for 3 days. At the end of cultivation, the culture broths were measured for flocculation activities (described below).

The strains with the highest flocculating activities (CPO8, CPO13 and CPO14) were selected and identified based on 16 S rDNA sequencing. Their isolated genomic DNA was subjected to PCR amplification of 16 S rDNA using universal primers. Amplified products were gel purified using Qiagen gel extraction kit and purified products were sequenced (Kim *et al.,* 2006). Subsequently, comparative sequence analysis of 16 S

rDNA amplified genes was performed and a phylogenetic tree was generated according to the procedure described previously by Al-Thani *et al.* (2009).

Determination of flocculation activity

Flocculation activities were calculated and measured using a modified Kaolin clay suspension method (Kurane *et al.,* 1986). A mixture of 5 g/L synthetic clay suspension (Sigma–Aldrich, 0.1– 4 µm particle size) with 0.5 mL bioflocculant solution (culture broth, cell-free supernatant or extracted biopolymer) in the presence of $CaCl_2$ (as coagulant) was stirred with rapid mixing at 230 rpm for 2 min, followed by slow mixing at 80 rpm for 3 min using Laboratory Flocculator (Jar Tester Model CZ150) and left standing for 5 min. A sample for Optical densities (OD) measurement was withdrawn using automatic pipette from a height of 3 cm below the surface of clay suspension. Relying on the upper phase OD for clay suspension that was measured at 550 nm with a spectrophotometer (7230G, Shanghai, China) the flocculation efficiency of the different isolated strains was screened. A control was prepared using the same method but the sample was replaced by distilled water. The flocculation activity was calculated according to the equation:

$$Flocculationactivity(\%) = \left[\frac{A-B}{A}\right] \times 100$$

Where A and B are the supernatant OD of the control (clay suspension without any bioflocculant addition) and sample respectively, at 550 nm.

Time course of bioflocculant production

Time courses of the selected strains were carried out by cultivating in fermentation medium with the addition of 5 % seeding culture (24 h and viable cell count of 10^8 CFU/mL) at 30 °C on a rotary shaker at 200 rpm for 7 days. Samples were taken every 12 h to measure, optical density (OD 600 nm) and flocculation activity (as described above).

Partial purification of the bioflocculants

To produce bioflocculants, the strains were pre-cultured in the seed medium as described above and inoculated into fermentation medium with the addition of 2 % starter culture (24 h) on a rotary shaker (200 rpm) at 30 °C for another 24 h. To partially purify bioflocculants, culture broths were centrifuged to remove cells by centrifugal separation (4000 g, 30 min). Subsequently, cell-free supernatants were freeze-dried. The lyophilized precipitates were redissolved in an appropriate amount of distilled water then re-precipitated with four volumes of cold 95 % ethanol and left for four hours at 4 °C. After centrifugation at 7500 g, 4 °C for 20 min, above steps were repeated for two additional times and the final precipitates were freeze dried.

Characterization of the partially purified biopolymers

Infra-red spectra (IR) of the biopolymers were measured on a KBr disk with a Perkin-Elmer series 1600 FT-IR to determine the functional groups of the biopolymers. The ^1H NMR spectra of the bioflocculants were recorded on a 500 MHz Bruker a JOEL GSX 500 spectrometer (500 MHz). The bioflocculants were dissolved in $2H_2O$. The protein concentrations of the biopolymers were determined according to Bradford method (Bradford, 1976). Total sugars were determined by the phenol-sulfuric acid reaction using the procedure of Chaplin and Kennedy (1994). Additional analysis of the biopolymers was performed, which included the solubility test in distilled water and several solvents such as acetone, carbon tetrachloride, ethanol, isopropanol, hexane, methanol and nitrobenzene (Collins *et al.,* 1973). The effects of polymer concentrations (final concentration of 0–7 mg/L) on the flocculation activity were studied. Subsequently, the effects of pH (3–12) and temperature (5 - 100 °C) on the flocculation activity of the partially purified biopolymers were studied by measuring the flocculation activity of the reaction mixture containing the optimum concentration of biopolymers at the specified ranges of pH and incubated at different temperatures (Kwon *et al.,* 1996).

RESULTS AND DISCUSSION

Screening and identification of bioflocculant-producing bacteria

Despite, so many studies about bioflocculants have been done; flocculation activity and culture cost of bioflocculants are still the major limiting factors with regard to their application (Zhang *et al.,* 2007). Consequently, there is a need to identify new microorganisms especially from unusual environments (e.g. crude oil) with high bioflocculant-producing ability and improve upon the flocculation efficiency of the known bioflocculants. It is known

that crude oil or oil-contaminated sites are the main sources of biosurfactant-producing microorganisms (Yin *et al.,* 2009). However, no previous report exists on the isolation and characterization of bioflocculant-producing microorganisms from crude petroleum oil. In the present study, a total of 36 colonies were isolated from crude petroleum oil sample, and 10 pure culture strains with slimy or mucoid appearance were screened on the basis of kaolin suspension flocculation activity over 85 % (data not shown). Among them, three strains CPO8, CPO13 and CPO14 having kaolin at flocculation activity exceeding 90 % were selected for further studies.

According to their 16 S rDNA sequence analysis strains CPO8 and CPO13 were belonging to the species of *Bacillus subtilis*. While, strain CPO14 was identified as *Pseudomonas* sp., respectively. Their 16 S rDNA sequences have been deposited in the GenBank database with accession numbers HQ015371, HQ015373 and HQ015374. Phylogenetic tree based on 16 S rDNA sequences was constructed using the neighbor-joining method (Fig.1). Out of these results, two among three selected bioflocculant-producing bacteria are belonging to the genus *Bacillus*. This result was in the same direction with many previous studies which reported that several bacterial strains closely related to the genus *Bacillus* able to produce bioflocculants (Shih *et. al,* 2001; Deng *et al.,* 2003; Abd-El-Haleem *et al.,* 2008; Xiong *et al.,* 2010).

The effect of the time course on both growth and bioflocculants production of the three strains are given in Fig. 2a and 2b. The OD curve that may be represent the cell growth shows a sharp increment in the first 72 h then tends to be constant over the remaining studied culturing period. However, the flocculation activity during this period has been increased and reached its maximum value of 96.03 % (COP8), 92.17 % (CPO13) and 97.59 % (CPO14), respectively at 24 h, then decreased slightly until 132 h. Concerning both the bioflocculant production and the cell growth profiles,

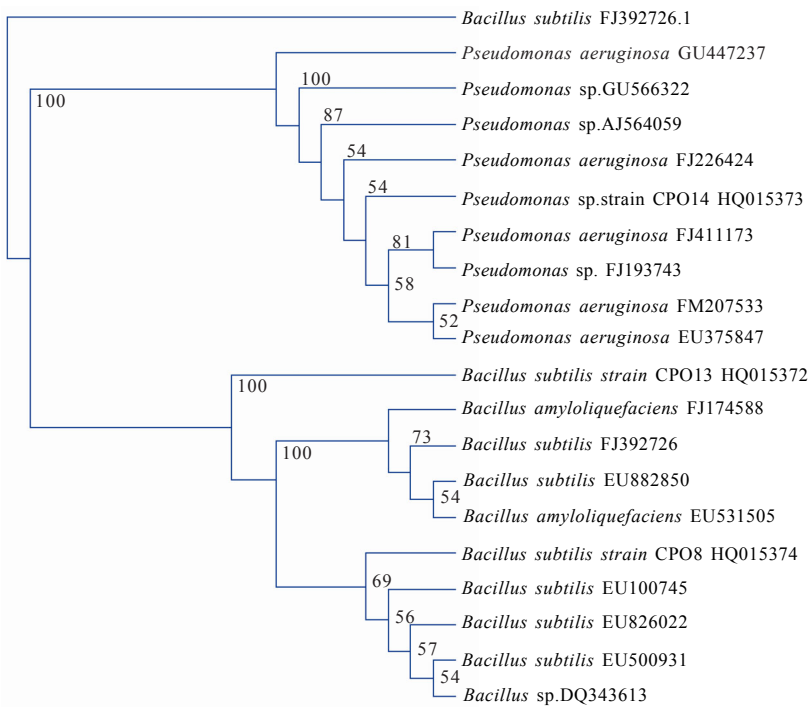

Fig. 1: Phylogenetic tree showing the relationships among the selected isolates (in boldface) and other closely related sequences collected from the Gene Bank. The dendrogram was generated by the neighbor-joining method. Bootstrap values per 100 bootstrap analysis presented for values greater than 52 %. The microorganism *Bacillus subtilis* FJ 392726 was used as an out-group

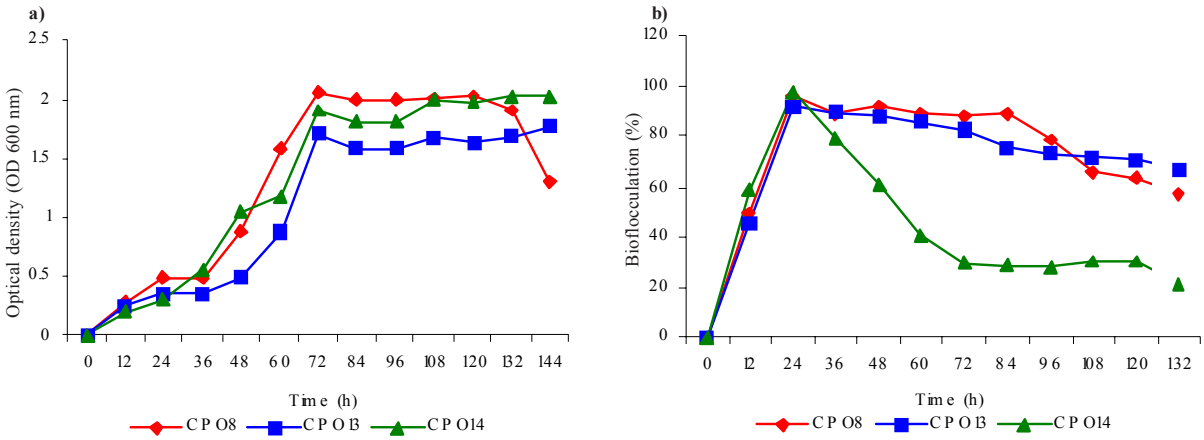

Fig. 2: Time course of growth (a) and bioflocculation activity (b) of strains CPO8, CPO13 and CPO14 in fermentation medium

it is evident that the bioflocculant production was almost in parallel with cell growth till 132 h studied (Liu *et al.,* 2010). Where the produced bioflocculant reached its maximum flocculating efficiency in the early stationary phase (at 24 h), while the cell production reached its maximum in the stationary phase (at 72 h), indicating that the bioflocculant was produced by biosynthesis during its growth, not by cell autolysis (Lu *et al.,* 2005). However, as the incubation time increased, the flocculating rate started to decrease in spite of the OD still recorded high values that may be related to the turbidity of the produced bioflocculant. This result indicated that all tested strains produce extracellular bioflocculants. Accordingly, in order to produce bioflocculant with high flocculating efficiency, 24 h culturing time has been chosen. Previously, the maximum flocculant production of *Alcaligenes latus* was achieved at the middle and late stage of the logarithmic growth phase (2–3 days), and flocculation activity began to decrease during the late stationary phase due to the activity of deflocculation enzymes (Kurane and Nohata, 1991). The growth curve of *P. mirabilis* TJ1 showed that the production of its bioflocculant was almost parallel with cell growth. The bioflocculant of *P. mirabilis* TJ1 reached its maximum flocculation rate (93.13 %) at stationary phase (Xia *et al.,* 2008). In the same way, production of the bioflocculant by *Enterobacter aerogenes* was in parallel with cell growth and reached its maximum flocculation rate in early stationary phase (Lu *et al.,* 2005). The flocculation rate of the bioflocculant from *Serratia ficaria* and *Bacillus* sp. F19 reached its maximum (94.3 % and 97 %, respectively) in early stationary phase (Gong *et al.,* 2008).

Characterization of bioflocculants

The phenol-sulfuric acid method showed that the partially purified biopolymers contained 74 %, 40 % and 47 % of total sugars, while protein contents detected by the Bradford method were 13 %, 17 % and 14 % for the strains CPO8, CPO13, and CPO14, respectively, indicating the composition of all bioflocculants are glycoproteins. The Fourier-transform Infra-red (FTIR) spectra (Fig. 3 a, b and c) of the three bioflocculants were analyzed according to Xiong *et al.* (2010). It clearly showed the presence of carboxyl, hydroxyl, amide and amino groups in its molecules, which are the preferred groups for the flocculation, similar to those observed in polyelectrolytes (Ganesh-Kumar *et al.,* 2004). The spectra showed a broad stretching intense peak displayed around 3440/cm that was characteristic of hydroxyl and amino groups (Liu *et al.,* 2009). The weak stretching band at ~ 2955/cm known to be typical of carbohydrates, indicated COH asymmetrical stretching vibration. An asymmetrical stretching peak observed at ~1,640/cm was characteristic of CAO stretching vibration in ONHCOCH3 (Xiong *et al.,* 2010). The weak band near 1405/cm indicates the bending vibration of CH_3 and the scissor vibration of CH_2. The weak peak at 1,261/cm in the bioflocculants produced by the *Bacillus* strains CPO8 and CPO13 may be assigned to be CAO symmetrical and asymmetrical stretching of a carboxylate group in the bioflocculant. However, this

peak was absent in the bioflocculant produced by strain CPO14. In addition, the strong absorption band at the range of 1049 - 1097/cm indicated asymmetrical stretching vibration of a C-O-C ester linkage. These infrared spectra showed characteristic peaks for carbohydrates and amides. Therefore, it can be inferred that the bioflocculant is a types of glycoproteins.

To confirm FTIR results, Proton nuclear magnetic resonance (^{1}HNMR) spectra analysis of the three bioflocculants were performed. As shown in Fig. 4 a, b and c, the first notable result of the ^{1}H NMR spectra was that the spectra were virtually identical. In addition, the presence of carbohydrates is recognized by the intense signals in the characteristic bulk region at 3.4-4.3 ppm. In the region 4.8-5.2 ppm severe overlap of the carbohydrate anomeric signals with those stemming from C-protons of the protein backbone is observed. Previously, de-Beer *et al.* (1994) reported that most cross peaks stemming from carbohydrates attached to an intact glycoprotein appear in a region (3.4-5.2 ppm) in the spectrum essentially devoid of cross peaks of the protein backbone. However, the strong water (HOD) signal at 4.8 ppm obscures the beta-anomeric proton signal at about 4.75 ppm. The signals for the methyl protons of the 6-deoxy sugars are seen at about at 1.44–1.48 ppm (Kumar *et al.*, 2004). The results obtained from the NMR analysis confirmed the FTIR results and indicated that all of selected bacterial strains produced

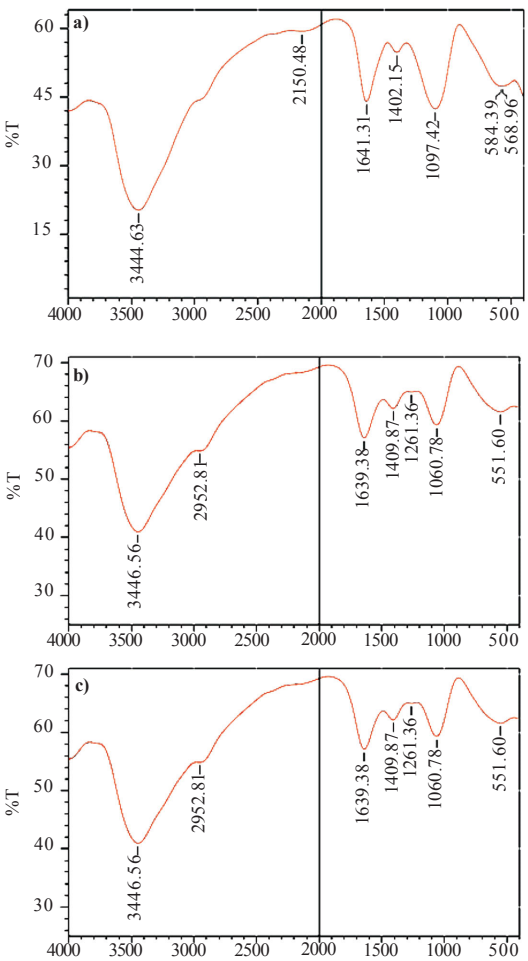

Fig. 3: FT-IR spectra of the partially purified biopolymers from strains CPO8 (a), CPO13 (b) and CPO14 (c)

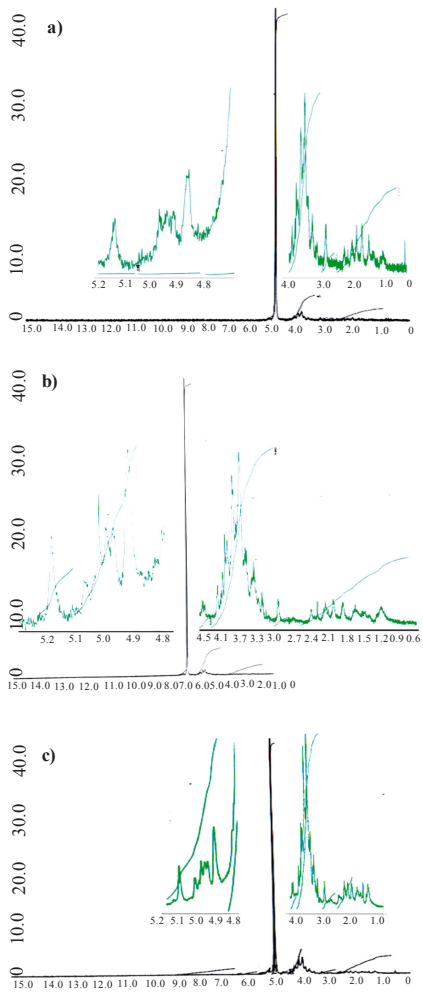

Fig. 4: ^{1}H NMR spectra of the partially purified polymers from strains CPO8 (a), CPO13 (b) and CPO14 (c)

glycoproteins. Out of these results, the protein and the carbohydrate contents of the purified biopolymers may explain their stability toward all studied organic solvents (acetone, methanol, ethanol, chloroform, dimethylsulphoxide). However, it has a tendency to be soluble in water as well as both acidic and alkaline media.

Effect of bioflocculants concentration pH and temperature on bioflocculation activity

Fig. 5 shows the relationship between the flocculants dosages and flocculation activities. When the culture broth in kaolin suspension (5.0 g/L) was tested in the dosage range of 0.5- 39 mg/L, it was apparent that the flocculation activity increased proportionally to the flocculant dosage of 0.5 to 7.0 mg/L and was highest at 3.5 mg/L of strains CPO8 and CPO13 and at 5.0 mg/L of strain CPO14, respectively. These results could be clarified as follows: a) The incomplete dispersion of excess polysaccharide, only the kaolin particles around the polysaccharides participated in the flocculation reaction, therefore, other kaolin particles did not participate in the reaction (Yokoi *et al.,* 1997) and b) The excess polysaccharide was oversaturated on many binding sites of the surface of kaolin particles, thus the attractive force of the other particles was reduced and the flocculation activity decreased (Kwon *et al.,* 1996). Thus, either the deficiency or excess amount of polysaccharide and kaolin clay decreased or even prevented the flocculation activity (Lee *et al.,* 1995).

The partially purified bioflocculants had the optimum pH for flocculation activity at pH 7 and were not affected by the pH range of 3–9 (Fig. 6a). At higher pH of 9–11, the flocculation activity decreased gradually with all strains except of strain CPO13 which still has high flocculation activity (96.4 %) up to pH 11. The pH range for flocculation reaction of these biopolymers were wider than those of the polyglutamate from *Bacillus subtilis* PY-90 (pH range of 3–5) (Yokoi *et al.,* 1995) and the cationic polysaccharide from *Paecilomyces sp.* I-1 (pH range of 4–8) (Takagi and Kadowaki, 1985). It is demonstrated that the biopolymer solution is suitable to be applied in neutral, weakly acid and weakly alkaline circumstances. However, its flocculation efficiency slightly reduces at highly acidic (pH<5) and highly alkaline (pH>9) circumstances. This may be returned to the biopolymer shows different electric states at different pH, in turn affects the bridging efficiency of the biopolymer for clay powder (Yong *et al.,* 2009).

However, the flocculation activity of the bioflocculants produced by strains CPO8 and CPO13 was not affected by increasing the reaction temperatures in the range of 5–70 °C (Fig. 6b). While, the flocculation activity of strain CPO14 was decreased gradually by increasing the reaction temperature above 50 °C. It is elucidated that flocculation efficiency increments as the clay suspension temperature increased from 5-70 ºC, but decreased as the clay suspension temperature increased above 70 ºC. This result indicates that the too high clay suspension temperature is unfavorable for the flocculation performance of the biopolymer. This can be explained by chemical kinetics. Both the rate of biopolymer

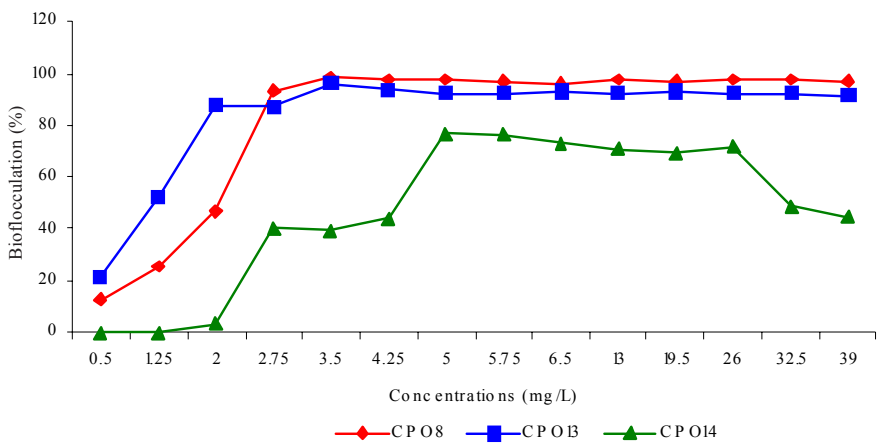

Fig. 5: The effect of dosage on the flocculation activity of the partially purified biopolymers from strains CPO8, CPO13 and CPO14

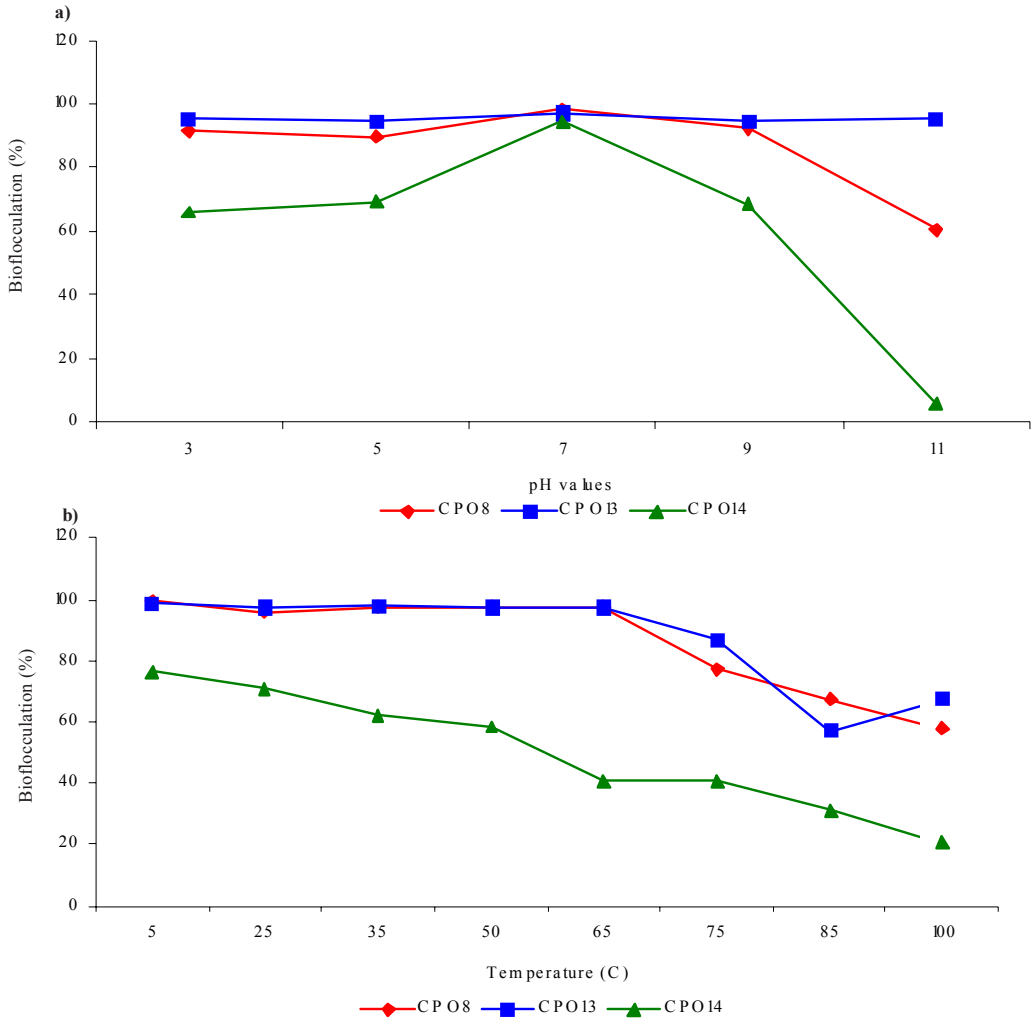

Fig. 6: The influence of initial pH (a) and temperature (b) of Kaolin clay suspensions on the flocculation activity of the partially purified biopolymers from strains CPO8, CPO13 and CPO14

diffusion and suspended particle collision frequency were improved at higher temperatures, this contributes to the increase in rate of reaction (Lu *et al.,* 2005; Gong *et al.,* 2008). The flocculating activity of strain CPO14 that decreased significantly when the temperature exceeded 50 °C. This can be explained by denaturalization of proteins in the bioflocculant and an increase in hot movement of kaolin particles (Liu *et al.,* 2009).

CONCLUSION

Three bioflocculant-producing strains were isolated from contaminated crude petroleum oil. Two of them CPO8 and CPO13 are belonging to the genus *Bacillus*, while strain CPO14 identified as *Pseudomonas*. Chemical analysis showed that the main compositions of their partially purified bioflocculants were carbohydrates containing some proteins. FTIR indicated the presence of carboxyl, hydroxyl, and methoxyl groups. 1HNMR analysis confirmed that the extracted bioflocculants were glycoproteins. The highest flocculation activities of strains CPO8, CPO13 and CPO14 were 96.03 %, 92.17 % and 97.59 %, respectively at 24 h of cultivation. The thermal and pH stabilities of the biopolymer solution indicated its thermal stability over the temperature range of (5-100 ºC). Moreover, the biopolymer solution was suitable to be applied in neutral, weakly acid and

weakly alkaline circumstances. The advantageous properties of these bioflocculants, such as stable thermal and pH characteristics and dosing rate comparable to those of chemical flocculants to flocculate suspended solids, suggest its potential industrial utility.

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