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Bioflocculant production by marine psychrotolerant *Psychrobacter cibarius* H41A KF207755 with its special role in silver nanoparticles production

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ABSTRACT

The present study screened 23 previously isolated and identified marine psychrotolerant bacterial strains for the production of bioflocculant. Factors affecting the production process as well as the bioflocculant activity, in addition to the biosynthesis and the characterization of silver AgNPs were carried out. *Psychrobacter cibarius* H41 AKF207755 exhibited high bioflocculation activity at 75.3%. The effect of different physical and nutritional factors on the bioflocculant production process by *P. cibarius* H41Awas determined. The bioflocculating activity was increased to 82.1% after incubation for 60 h and to 84.7% at pH 6.5. Also, sucrose and urea improved the activity. The type of cations, pH level, and temperature were examined on the produced bioflocculant, which reached to 90.3% at pH 9 and to 92.1% at 70ºC. The addition of $FeSO_4$ raised the bioflocculant activity up to 94.0%. The concentrations of total protein, total carbohydrate, and total lipid were estimated for the partially purified bioflocculant as; 20.285, 31.215, and 12.2 gL^{-1}, respectively. It was found that the AgNPs can be synthesized using the bioflocculant, which remains stable for up to 16 months. Characterization of these AgNPs by UV-spectroscopy, FTIR, and TEM analyses takes place. The pH 11 supported the maximum synthesis of AgNPs at 2 mM $AgNO_3$. TEM revealed that the size of the produced AgNPs ranged from 1.31 nm to17.45 nm and no agglomeration was observed. Upon the applicable level, the antimicrobial property of AgNPs synthesized by the bioflocculant of *P. cibarius* H41A KF207755 was confirmed. The concentration dosage that gave the optimum flocculating activity (92.5%) was 4.0 $mgmL^{-1}$.

INTRODUCTION

Bioflocculation is a process, in which separation of solid liquid mixture is achieved by the whole microorganisms or their by-products so-called bioflocculants (Gao *et al.*,2006). Microbial bioflocculant is an extracellular polymer produced by several microorganisms during their growth, resulting in the formation of stable aggregates of flocs. These bioflocculants has different polymeric substances such as,

exopolysaccharide, polysaccharides, glycoproteins, protein, nucleic acid and cellulose (Jie *et al.*, 2006). Bioflocculants have been established to be produced by various microbes such as bacteria, actinomycetes, fungi, and algae (Deng *et al*., 2003; Xia *et al*., 2008). Early, there was a great interest in studying bioflocculants to replace the hazardous synthetic flocculants because of their benefits as biodegradable, safe, and eco-friendly biopolymers (Deng *et al.*, 2003; Bhunia *et al.*, 2012).

Many studies have been reported on bioflocculants in the industrial sector. They are effectively applied in the drinking and wastewater treatment, downstream processing, and fermentation processes, reduction of water pollution and in aquaculture treatment (Shahadat *et al.*, 2017; Hashim *et al*., 2019). They are used in the field of wastewater treatment for removing suspended solids and metal ions, at which colloids come out of suspension in the form of flocs or flakes (Li *et al*., 2003). For instance, it was found that that the bioflocculants produced by *Alcaligenes latus* had the ability to absorb water 1000 times of its weight and 5 times stronger than that of synthetic absorber polymers (Kurane and Nohata, 1997). The production of bioflocculants has been studied and then applied in the batch fermentation like many biological products such as amino acids, vitamins and enzymes. However, there have been few reports on the biosynthesis of flocculants on a pilot scale (Patrick and Finn, 2008; Abd El-Salam *et al*., 2017).

On the other side, many microorganisms including bacteria (Mathias *et al*., 2017), fungi (Shahverdi *et al*., 2007), actinomycetes (Silambarasan and Abraham, 2013), and algae (Landage and Wasif, 2012) can aggregate inorganic materials and used for green synthesis of AgNPs intracellularly or extracellularly. Indeed, microbial polysaccharides such as bioflocculants are a promising alternative for the synthesis and stabilization of nanoparticles (Sathiyanarayanan *et al*., 2013). In particular, this is an interesting area of research where both biosynthetic AgNPs and bioflocculants are economically important (Zaki *et al*., 2014).

The present study investigated the production and optimization of bioflocculant by marine psychrotolerant *Psychrobacter cibarius* H41A KF207755. In addition, the produced bioflocculant was characterized via standard tools. Studying some valuable applications such as production of highly stable bactericidal AgNPs was accomplished.

MATERIALS AND METHODS

Media and culture conditions

The pre-culture and flocculants production media used during this study were mentioned in the work of Luo *et al*. (2016). The enrichment medium included (gL^{-1}); beef extract, 3; peptone,10; and NaCl, 5; and it was amended with 1.8% agar. ii) The fermentation medium included (gL^{-1}): glucose, 20; KH_2PO_4, 1; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.5; $(NH_4)_2SO_4$, 0.2; NaCl, 0.1; urea, 0.5 and yeast extract, 0.5.

Screening of bioflocculant production

Twenty three different strains of bacteria that previously isolated and identified by NIOF stuff members (Abdelnaby *et al*., 2019**)** were screened. Selection of bioflocculant producers were based on the mucoid morphology of appeared and obvious microbial colonies. Subsequently, selected colonies were cultured in broth medium at 30ºC in a rotary shaker at 120 rpm for 24 h. Then, 2 mL inoculum of the pre-cultures with optical density (OD_{550}) nm was adjusted at 1.0 were transferred into new flasks containing 50 ml of fermentation medium and re-incubated as described above for 3 days. After the cultivation period, the fermented broth was centrifuged (5000 rpm, 30 min, 4ºC) to separate the bacterial cells and the cell-free supernatant was assessed for flocculating activity.

Determination of bioflocculation efficiency

A protocol of Kurane *et al.* (1994) is used to determine the bioflocculating activity, a mixture of 2 ml of the culture broth of selected strains, 5 mL of $CaCl_2$ (1%, w/v), and 93 mL of kaolin (5 gL^{-1} distilled water) suspension were mixed in a 200-mL beaker. A control experiment without bioflocculant was carried out. The mixture was stirred at 180 rpm min^{-1} for 1.5 min and at 80 rpm $min^{-1}$for 3 min with a vortex mixer (QL-861, Shanghai Jingmi Instrument Co., Ltd., China) and then kept still for 10 min. A sample for optical densities (OD) measurement was withdrawn using automatic pipette from a height of 1 cm below the surface of clay suspension. The bioflocculation activity of the different isolated strains was screened relying on the upper phase OD for clay suspension that was measured at 550 nm with a spectrophotometer. The flocculating efficiency was calculated according to the following equation:

Bioflocculation activity = (B-A)/B×100, where; A and B are ODs of the culture sample and the control, respectively.

Factors affecting bioflocculant production process

The effect of the incubation period on the bioflocculant production of the most promising strains was assessed for different time intervals (12, 24, 36, 48, 60, and 72 h). However, the production medium was adjusted at pH 7and 30°C. To evaluate the effect of a gradual change in pH levels on the bioflocculant production, pH was adjusting by using 0.1 M HCl and 0.1 M NaOH at the pH range of 5-9 (Zheng *et al*., 2008). In addition, different carbon sources (sucrose, dextrose, xylose, starch, and glucose) were examined to determine the most suitable. All flasks were incubated in a rotary shaker at 120 rpm at 30°C with the optimum incubation period. To elucidate the effects of nitrogen source, urea was replaced by peptone, ammonium chloride, and potassium nitrate. These different nitrogen sources were employed with the most suitable carbon source then examined to determine which maximize the production of bioflocculant. All flasks were

incubated at the optimum conditions. Cells were removed by centrifugation then the bioflocculant activity was measured in the supernatants.

Extraction and purification of bioflocculant

Adopting the method of Kurane *et al.* (1994), the extraction of the bioflocculant was performed. Briefly, after 60 h of fermentation, the culture broth was centrifuged (5000 rpm, 30 min) to remove the bacterial cells. Two volumes of ethanol were later added to the supernatant, stirred and left to stand for 12 h at 4°C. The supernatant was discarded and the pellets were centrifuged at 5000 rpm, 30 min. In addition, the precipitate obtained was vacuum-dried to obtain crude bioflocculant. Finally, two volumes of ethanol were added to recover the precipitate and then lyophilized.

Effect of pH, temperature, and cations on bioflocculant activity

To study the effect of the different pH levels on the flocculating activity of bioflocculant produced, the pH of the kaolin clay suspension was gradually changed from 2 to 11 using 0.1 M HCl and 0.1 M NaOH (Zheng *et al*., 2008). Also, heat stability was evaluated by incubating the bioflocculant solutions in water bath at a temperature range of 30, 40, 50, 60 ,70, and 80°C for 25 min. Afterwards, the residual flocculating activity was determined using the protocol of Kurane *et al.* (1994). Additionally, the effect of different cations on bioflocculant production was assessed by replacing $CaCl_2$ in the production medium with Na^+, K^+, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Fe^{3+} using the method of Agunbiade *et al.* (2017).However, different salts used were: $MgSO_4$, $FeSO_4$, $FeCl_3$, $MnSO_4{\cdot}7H_2O$, KCl, and NaCl.

Characterization of purified bioflocculant

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). Total lipids were determined according to Frings *et al.* (1971). Also, sample was metalized with a thin gold film using sputtering device (JFC-1100 E JOEL, USA) for 12 min. Scanning electron microscopy was performed with JSM 5300 JOEL, USA Scanning Electron Microscope at 20 kV in the Centre Laboratory, City of Scientific Research and Technological Applications-Alexandria, Egypt. In addition, the purified bioflocculant was measured on a KBr disk with a Perkin-Elmer series1600 to fourier transmission infrared spectroscopy (FTIR) in Attenuated Total Reflection(ATR) mode (500-4000 cm^{-1}) to determine the functional groups of such biopolymer. In addition, its scanning electron microscopic images were taken at 5 kV with a FE-SEM (Zeiss, P-Sigma, Germany).

Biosynthesis of silver nanoparticles

Fifty milliliters of aqueous solution of 0.5, 1.0, and 2.0 mM $AgNO_3$ were separately treated with 50 mL of *P. cibarius* H41A supernatant solution in a 250 mL Erlenmeyer flask. The whole mixture was put into a shaker at 30°C (120 rpm) for 1 day and maintained in the dark conditions. The control was maintained without addition of $AgNO_3$ with the experimental flask containing bioflocculant. The extracellular synthesis of AgNPs was monitored by visual inspection of flasks for the change in color of the bioflocculant from a clear light yellow to brown (Ahmad*et al*., 2003). Synthesis of AgNPs was optimized with different pHs.

Characterization of AgNPs

The synthesized AgNPs were first characterized by UV-visible spectrophotometer (Perkin-Elmer, Germany) between 200-800 nm. In addition micrographs of synthesized AgNPs were obtained using Hitachi H-7500 transmission electron microscope and Jeol JSM-5300 scanning electron microscope (Sathiyanarayanan *et al*., 2013).

Antimicrobial application of AgNPs

The well-cut diffusion technique was used to evaluate the antimicrobial activity of the produced AgNPS against the microbial pathogens (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Klebsilla pneumonia* ATCC 13883, and *Candida albicans* ATCC 10231), separately. All plates were incubated at 37°C for 24 h. An inhibition zone (mm) was recorded as positive (Amer and Hassan, 2019).

Effect of storage conditions

The produced AgNPs was tested after storage at cold condition (stored in a 4°C fridge). The antibacterial effect was tested every 45 days where the sample was relatively less frequently exposed to the air (Korshed *et al.*, 2018). Also, the stability of the produced AgNPS was confirmed after 16 months of storage at the fridge by the UV-vis spectrophotometry.

Jar test determination of bioflocculant dosage

Different concentrations (0.3 to 5.0 $mgmL^{-1}$) of the partially purified bioflocculant were prepared and their flocculating activities measured against 5 gL^{-1} kaolin clay suspension as previously mentioned by Kurane *et al.* (1994).

RESULTS

Screening of bioflocculant production

Twenty-three marine bacterial strains, previously isolated and identified, were screened for bioflocculant property (Table 1). The selection of the bioflocculant producers was based on the mucoid morphology of appeared and obvious microbial colonies. Visually, eight isolates characterized by the mucoid appearance, where 3 isolates showed high bioflocculation activity (*P. cibarius* H41A KF207755, *P. pacificensis* H62, and *P. vallis* H65). However, these three isolates were selected for

further investigation. The results in Table (2) found that the bioflocculating activity ranged between 61.5 and 75.3%. Also, the results showed that the isolate *P. cibarius* H41A KF207755 (Fig. 1) was the most active (75.3%), followed by *P. pacificensis* H62 (70.4%). So, *P. cibarius* H41A was chosen for further investigations. The marine psychrotolerant *Psychrobacter cibarius* H41A KF207755 was applied in the current study to produce a potent bioflocculant, which had unique characteristics qualifying it to valuable applications. Our results were in the same direction with many previous studies which reported that several bacterial strains able to produce bioflocculants (Elkady *et al*., 2011; Zaki *et al*., 2011; Abd El-Salam *et al*., 2017).

Table 1. Mucoid appearance (MA) for the tested marine bacterial strains.

Isolates codes	MA	Isolates codes	MA
Pseudomonas sp. H20	-	*Pseudomonas* sp. H20S	-
Pseudomonas sp. H26S	-	*Pseudomonas* sp. H69A	+
Pseudomonas sp. H44	+	*Psychrobacter cibarius* H41A KF207755	+
Pseudomonas sp. H45A	-	*Psychrobacter* sp. H62	+
Pseudomonas sp. H49	-	*Psychrobacter*sp. H65	+
Pseudomonas sp. H50	-	*Stenotrophomonas* sp. H43S	+
Pseudomonas sp. H60	-	*Halomonas* sp. H68	-
Pseudomonas sp. H64	-	*Paracoccus* sp. H26A	+
Pseudomonas sp. H73	-	*Planomicrobium* sp. H44A	-
Pseudomonas sp. H67S	+	*Planomicrobium* sp. H71	-
Pseudomonas sp. H67	-	*Pseudoalteromonas* sp. H43	-

Table 2. bioflocculating activity of the most potent producers.

Bacterial strain	Bioflocculating activity* (%)
Psychrobacter cibarius H41A KF207755	75.3
Psychrobacter pacificensis H62	70.4
*Psychrobacter vallis*H65	61.5

Factors affecting bioflocculant production by *P.cibarius* H41A KF207755

The effect of different physical factors such as different incubation periods and pH on the bioflocculant production process by *P. cibarius* H41A KF207755 was determined. Also, the effect of different carbon and nitrogen sources on bioflocculant production by *P. cibarius* H41A KF207755 was estimated. The results in Fig. (1A) and Fig. (1B) exhibited that the bioflocculating activity was increased to 82.1 and 84.7% after incubation for 60 h at pH 6.5, respectively. Additionally, sucrose improved the bioflocculating activity to 88.2% followed by glucose and dextrose (Fig. 1C). The data presented in Fig. (1D) demonstrated that, none of the tested nitrogen sources was comparable to urea with respect to bioflocculating activity.

Bioflocculants can be distinguished into either primary or secondary metabolites depending on the period of secretion in the culture broth (Salehizadeh and Yan, 2014). Maximum production in our results achieved after 60 h and that agreed with Gong *et al.* (2008) who reported that a bioflocculant produced by *Serratiaficaria* reached its maximum flocculating activity in the early stationary phase (72 h) and subsequently

observed a slow decrease after 84 h which was attributed to autolysis and enzymatic activity. The majority of reported studies in the literature revealed that bioflocculants are produced during active growth phase of microorganisms (Salehizadeh and Yan, 2014). On the other hand, the bioflocculant produced by *Chryseobacter iumdaeguense* W6 was associated with cell autolysis and not cell growth (Liu *et al*., 2010), running parallel to the profile of flocculating activity. It is known that, pH dependence for bioflocculant production differed with various microorganisms because the nutrient uptake and enzymatic activity of microorganisms could be influenced by initial pH of the medium that affected the electric charge of microbial cells and also the oxido-reduction potential (Xia *et al.,* 2008).

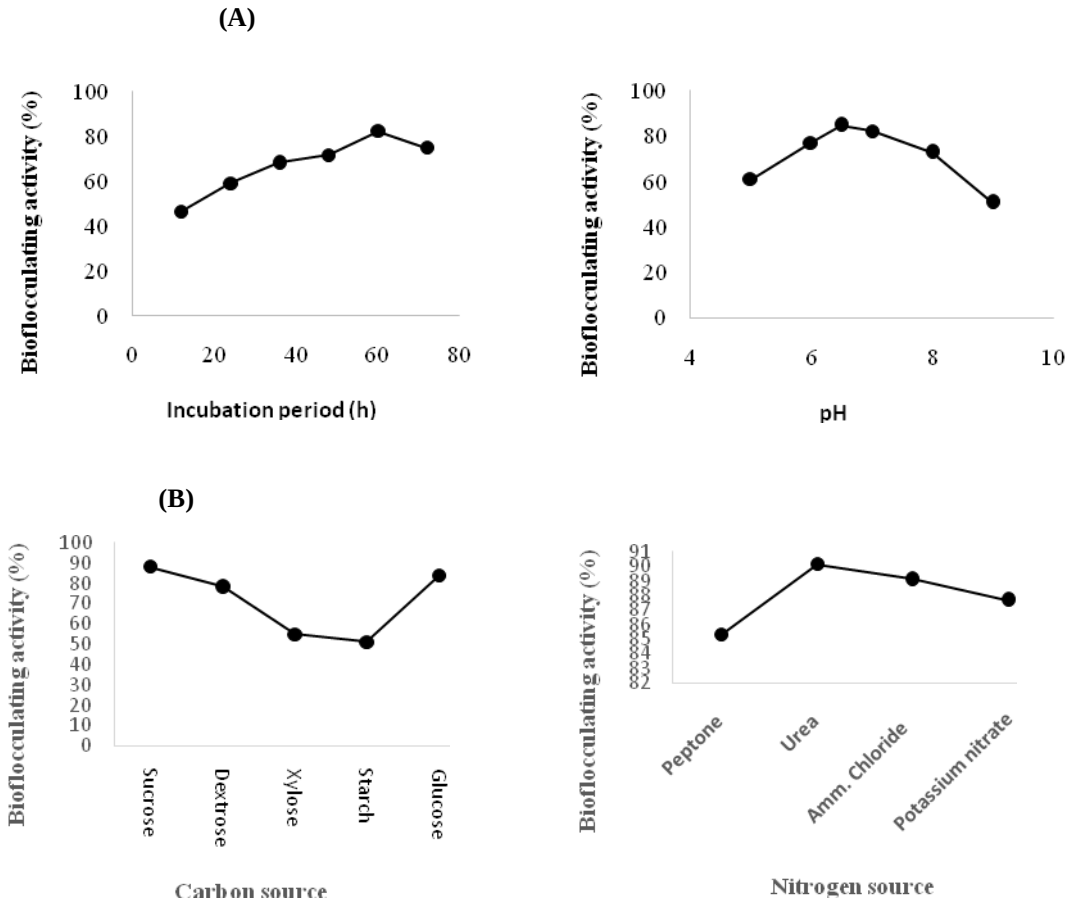


Fig. 1. Effect of different incubation period and pH (A) & carbon source and nitrogen source (B) on the production of bioflocculant by *P. cibarius* H41A KF207755.

The highest bioflocculant activity record was at pH 6.5. Abd El-Salam *et al*. (2017) statedthat the pH values among 7-9 were the optimum, while the acidic and/or alkalinic pH caused reduction in flocculating activity. The same authors revealed that different carbon sources were noticed to influence the bioflocculant PSK1 production. They observed that the most favorable carbon sources enhancing the bioflocculant production were glucose and maltose. Moreover, they detected that among examined nitrogen

sources, the organic in particular yeast extract were more suitable than inorganic nitrogen sources for the bioflocculant PSK1 production. These results disagreed with our results which showed that the *P. cibarius* H41A KF207755 gave the highest activity in culture medium amended with urea (90.1%). In addition, nitrogen sources such as $NaNO_3$, NH_4Cl, and urea stimulate the growth of *Klebsiella* sp. ZZ-3, allowing the production of glycoprotein with flocculating activity ranging from 90.4% to 94.5% (Yin *et al*., 2014).

The pH of reaction mixtures is a key factor influencing the flocculation process (Zaki *et al*., 2013) by affecting the stability of suspended particles and floc formation (Ugbenyen *et al.* 2014). In particular, our findings demonstrates that acidic and basic pH media support flocculating efficiency, with the most pronounced activity observed in the basic medium at pH 9. This observation corroborates the fact that bioflocculants exhibit varying degree of electrical states at different pH which impact on the flocculating activity of the bioflocculant for kaolin particles (Pan *et al.*, 2009). These results were found to be similar to the observations reported by Ugbenyen *et al.* (2014), in which the flocculating activity of the bioflocculant produced by a consortium of *Cobetia* and *Bacillus* species was over 70% across a wide pH range of 3-11 with the highest flocculating activity attained at pH 8.

In addition, thermal stability in the bioflocculating activity up to 70°C were detected, however, 18.3% of bioflocculating activity was lost at 80°C after heating for 25 min. These results suggest that the main backbone of the bioflocculant is a polysaccharide. Likewise, the results of the biochemical analysis of the purified bioflocculant confirmed that 31.21% of polysaccharide was detected. This is consistent with the reported findings that flocculants rich in polysaccharide are more heat-stable than those composed of mainly protein and nucleic acids (Gao*et al.*, 2009; Wang *et al.*, 2013). Liu *et al*. (2010) confirmed that the flocculating activity was slightly decreased by 2.9% after heating the bioflocculant at 80ºC for 45 min. This decline in activity may be due to that the polysaccharide chain of the bioflocculant could be degraded at high temperature which reduced the ability of bioflocculant to form bridges with suspended particles. Also, Tang *et al.* (2014) stated that high temperature could denature the protein fraction of the biopolymer which reduced the flocculating efficiency. The results obtained by Abd El-Salam *et al.* (2017) elucidated that the bioflocculant PSK1 was thermally stable when heated at the temperature range of 40-80ºC of various time periods. The flocculating activity showed an increase of 1% more than that of the control after heating the bioflocculant at 70ºC for 25 min. This result may be due to the release of poly-substances when the biopolymer was heated at higher temperature (Gong *et al.*, 2008).

Factors affecting the activity of produced bioflocculant

In general, there were three factors investigated on the bioflocculant activity. They were pH level, temperature and type of cations. However, the data are shown in Fig. (2). The bioflocculant activity increased to 90.3% at pH 9 and to 92.1% at 70ºC. Also, the addition of $FeSO_4$, as cation, raised the bioflocculant activity up to 94.0%.

Basically, it seems that the monovalent and divalent cations help to neutralize negative charges on bioflocculant and the suspended kaolin particles, shortening the distance between them, increasing the initial adsorption ofbioflocculant onto the kaolin particle and thus leading to floc formation and sedimentation (Yim *et al.*, 2007). There have been several reports on divalent cations stimulating the flocculating activity of bioflocculants (Nwodo *et al*. 2012), on the other hand, very few bioflocculants have been reported to have a high flocculation rate without cations' aid (Zhao *et al*., 2013). Ugbenyen and Okoh (2014) also reported that the divalent cations tested (Ca^{2+}, Mg^{2+}, and Mn^{2+}) were the best metal ions that enhanced the activity of the bioflocculant produced by the consortium of the *Cobetia*a and *Bacillus* species which in turn was completely inhibited by Li^{2+} and K^{2+}. We can say that high percentage of the results of the previously mentioned authors agreed with our results such as enhancing the flocculating activity in presence of Ca^{2+} and $Mn^{2+}$and reduction of the activity in presence of K^{2+} and Fe^{3+}.

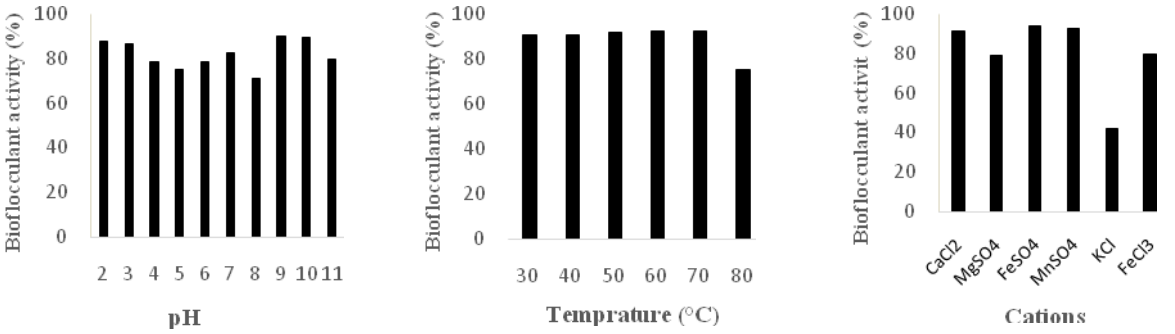


Fig. 2. Effect of different pH, temperature and cations on the activity of the produced bioflocculant by *P. cibarius* H41A KF207755.

Characterization of *P. cibarius* H41AKF207755 bioflocculant

The concentrations of total protein, total carbohydrate, and total lipid were estimated for the bioflocculant produced by *P. cibarius* H41A KF207755. The values of them were; 20.285, 31.215, and 12.2 gL^{-1}, respectively. Other means such as; characterization of the partially purified bioflocculant of *P. cibarius* H41A KF207755 was determined by taking SEM images as shown in Fig. (3). The purified bioflocculant appeared as whitish flakes structure (Fig. 3A), the Kaolin clay particles appeared in Fig. 3B. The interaction of the purified bioflocculant and the kaolin particles resulted into compacted flocs formation (Fig. 3C), because of the adsorption of the kaolin particles on the binding sites of the purified bioflocculant and the subsequent interactions resulted into agglomeration of larger flocs.

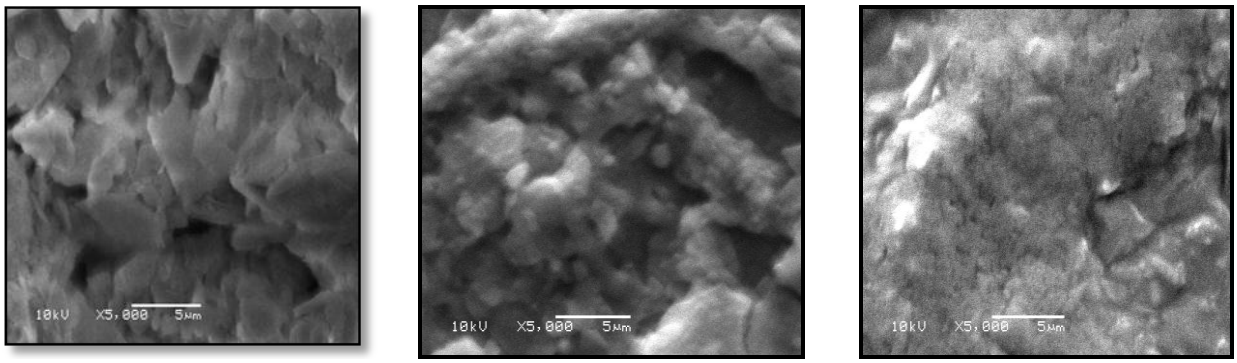


Fig. 3. SEM macrograph showing the features of: (A) purified bioflocculant produced by *P. cibarius* H41A KF207755: (B) kaolin powder suspension only, and (C) the partially purified bioflocculant flocculating kaolin suspension.

Also, FTIR spectroscopy analyses were performed to elucidate the chemical structure and the essential functional groups of the partially purified *P. cibarius* H41A KF207755 bioflocculant (Fig. 4). The FTIR spectroscopic analyses showed the presence of different and many chemical groups in the bioflocculant as shown in Fig. (5). The strong peaks at 1049.31 cm^{-1} and 3417.01 cm^{-1} are characteristic of –COO− group and (amides or amines) respectively. The weak peaks appearing at 1401.33 & 1406.15 and 2457.39 cm^{-1} correspond to O-H group characteristic for carboxylic acid. The spectral signals at 1520.92, 1573.97 cm^{-1} refers to (C–N or N–H bending) and the strong peaks at 1644.37 & 1648.23 correspond to (C=O stretching). The spectral signals at 2457.39 cm^{-1} corresponding to CH_3 stretching, also a weak peak observed at 2366.74 cm^{-1}. Another weak C-H stretching vibration band was observed at 2927.08 and 2956.97 cm^{-1} representing alkane.

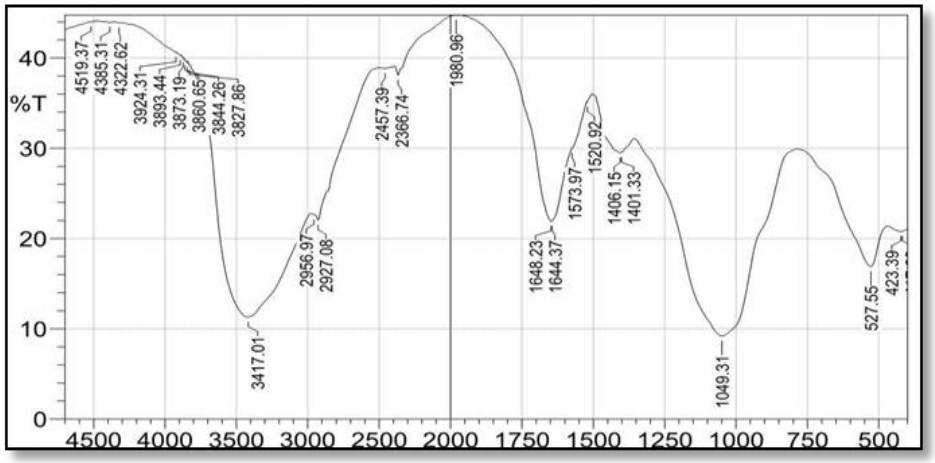


Fig. 4. FTIR of the partially purified bioflocculant produced by *P. cibarius* H41A KF207755.

The chemical composition analyses of *P. cibarius* H41A KF207755 bioflocculant were carried out and the results indicated that protein and carbohydrates contents were higher than lipids, which indicated that the bioflocculant was mostly glycoproteinalso Okaiyeto *et al*. (2015) revealed that their bioflocculant; MBF-UFH was composed of both

polysaccharide and protein; this showed that the flocculation process might involve multiple functional moieties from both polysaccharide and protein. Multiple functional moieties imply many adsorption sites for the kaolin particles, which led to the high flocculating efficiency observed with bioflocculant (Verma *et al*., 2012).

The SEM images of *P.cibarius* H41A KF207755 bioflocculant were taken and showed an amorphous structure of a compact nature also reported by Okaiyeto*et al.* (2015). Its configuration might be accountable for its high flocculation efficiency. They also stated that before the flocculation process, the kaolin clay particles appeared to be fine and scattered, and after the flocculation process, the functional moieties in the molecular chain of bioflocculantwere used for attachment on the kaolin clay particle. Consequently, the interaction between the bioflocculant and kaolin clay particle resulted in the formation of flocs that later aggregated to larger sized flocs, which precipitated out of the suspension as the result of gravity. This observation showed that bridging played a vital role in the flocculation process (Ugbenyen and Okoh, 2014).

The presence of COO^- group which referred to the peaks at 1401.33& 1406.15 cm^{-1} 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).Furthermore, the presence of sugar moieties were detected (Sathiyanarayanan *et al*., 2013). The presence of amide group belongs to the broad peak 3417.01 matches with the result obtained by He *et al.* (2010) and Kavita *et al.* (2013). A weak peak observed at 2366.74 cm^{-1} was either CO_2 adsorption or may be from the amine group (Ahluwalia and Goyal,2005), Presence of signals at 1648 cm^{-1}, 1520.92 cm^{-1} and 1573.97 cm^{-1} were indication to the presence of C=O and N–H i.e. amines and amides, these results coincided with Alhazmi **(**2019). The characteristics of the FTIR spectrum showed the presence of amines, amides, carboxyl and CH_3 groups were found in the *P. cibarius* H41A KF207755 bioflocculant structure as the main functional moieties preferred for flocculation as reported by Wan et al. (2013). Many other previous researches reported the presence of hydroxyl and amino groups in the microbial bioflocculants such as that produced by *Pseudomonas*sp. 38A (Farag *et al*., 2014) and *B. velezensis* 40B (Zaki *et al.*, 2013). In addition, Abd El-Salam *et al.* (2017) indicated a broad stretching intense peak at 3425 cm^{-1} demonstrated the presence of hydroxyl and amino groups. The weak stretching band detected at 2927 cm^{-1} revealed the presence of aliphatic CAH stretching. The broad peak at 2460 cm^{-1} was characteristic of NAH group.

Biosynthesis and evaluation of AgNPs

The flasks containing both the bioflocculant and $AgNO_3$ mixtures showed yellowish to brown color after 24 h of incubation. Typical reaction mixtures contained equal volumes of supernatant and 0.5, 1.0, and 2.0 mM silver nitrate solution. The appearance of the yellowish brown color was an indication of the formation of colloidal AgNPs in the medium and there is no color change in the control flask incubated in the same environment. In the present study, pH 11 supported the maximum synthesis of AgNPs.

On the other hand $AgNO_3$ solution with bioflocculant, which incubated at different pH (7-9) showed no synthesis of AgNPs. Concentration of 2.0 mM silver nitrate solution at pH 11 gave the deepest brown color indicating the maximum synthesis of silver nanoparticles (Fig. 5).

The synthesis of well-dispersed, single silver nanoparticles was accomplished via the treatment with the bioflocculant produced by *P. cibarius* H41A KF207755 as a reducing and stabilizing agent. The appearance of the brown color is due to excitation of surface plasmon resonances (SPR) in metal as reported by Hamedi *et al.* (2012) and Mondal *et al.* (2012). In agreement with our result, Zaki *et al.* (2014) reported that UV-vis showed a peak at ~420 nm corresponding to the plasmon absorbance of nanosilver. Also, absorbance intensity provided indication on the reduction, productivity, and amount of Ag^+ion (Hamedi *et al*., 2012). This may be due to the availability of more reducing biomolecules and concentrations of AgNPs.

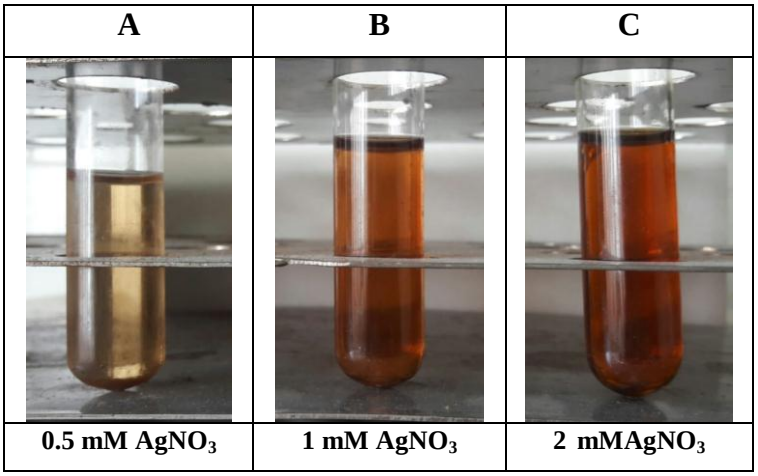


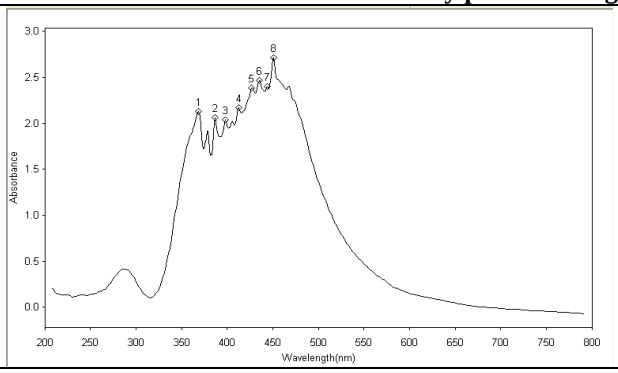
Fig. 5. Culture tubes containing cell filtrate and different concentration of$AgNO_3$ solution with a ratio of 1:1 at pH 11. All of them were maintained under the same conditions for 24 h of reaction in dark at 30ºC**.**

Characterization of AgNP

The synthesis of AgNPs by *P. cibarius* H41A KF207755 was further confirmed by observing the solution in the UV-vis spectrophotometry. UV-vis absorption spectrum of the (bioflocculant and 2.0 mM $AgNO_3$ solution) showed the surface Plasmon resonance derived from the AgNPs at around 369-451 nm (Fig. 6A). The stability of the produced AgNPS extended to 16 months which confirmed by the UV vis spectrophotometry (Fig, 6B), it was observed that AgNPs peaks remained close between 360 nm to 449. In addition, the purified AgNPs were then characterized by the bright field of TEM micrograph to determine the size and morphology of AgNPs. Results in Fig. 7 revealed that size of particles was ranged from 1.31 to 17.45 nm and they are relatively polydispersed-spherical and prismal shape and agglomerated particles were not observed in TEM analysis. The TEM profile has been employed to characterize the size, shape, and

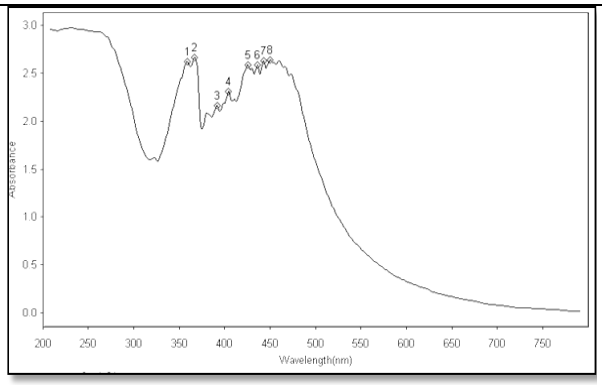
morphologies of the formed AgNPs. Particles with high density will appear darker in the TEM (Lei, 2007). These nanoparticles are polydisperse and varying in size which ranges from 1.31 to 17.45 nm and shape, which mainly includes nanospheres and nanoprisms; such variation in shape and size of nanoparticles synthesized by biological systems is common (Bubey *et al*. 2009). After 16 months,the silver nanoparticles peaks remained close to the first result which indicates that the particles were well dispersed in the bioflocculant solution and there was not much aggregation, whereas previous studies stay stable for 5 months (Sathiyanarayanan *et al.*, 2013).

Freshly produced AgNPs



Peak no.	Wavelength	Abs
1	369	2.127
2	387	2.063
3	398	2.036
4	412	2.161
5	427	2.386
6	435	2.464
7	444	2.401
8	451	2.713

After 16 months later



Peak no.	Wavelength	Abs
1	360	2.612
2	368	2.658
3	391	2.158
4	405	2.305
5	426	2.589
6	436	2.587
7	443	2.631
8	449	2.629

Fig. 6. UV absorbance of partially purified biofloculant produced by *P. cibarius* H41A KF207755: (A) freshly produced and (B) after 16 months of storage.

Antimicrobial activity of AgNP by *P. cibarius* H41A KF207755 bioflocculant

As shown in Table 3 the antimicrobial activity was evaluated against six bacterial pathogens besides one yeast strain as mentioned before. After incubation period, inhibition zones (mm) were detected against all pathogens except *E. faecalis* ATCC 29212. In particular, the activity ranged from 21 mm (*C. albicans* ATCC 10231) to 32 mm against (*P. aeruginosa* ATCC 9027). However, these findings confirmed the wide range of antimicrobial property of the nano-silver synthesized by the bioflocculant of *P. cibarius* H41A KF207755.The bactericidal activity of AgNPs was highly comparable with the results of Kim *et al.* (2008) and AgNPs shown a broad spectrum activity and

which was similar with the results obtained from other workers who synthesized AgNPs using plant based polysaccharides (Santos *et al*., 2017; Xia *et al.*, 2017).

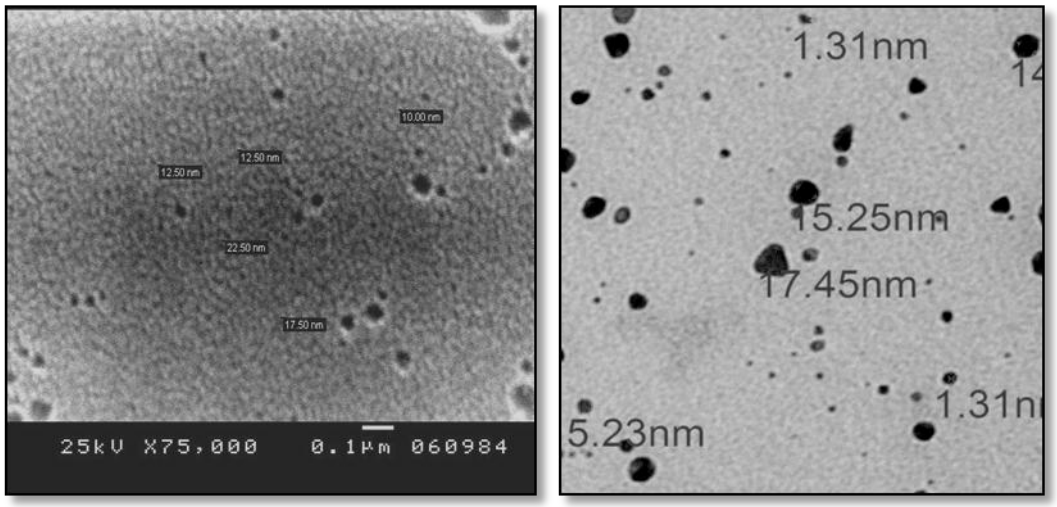


Fig. 7. TEM images recorded from the drop-coated film of the silver nanoparticles synthesized by the silver nitrate solution with *P. cibarius* H41A KF207755 partially purified bioflocculant.

Table 3. Antimicrobial activity of AgNPs by *P .cibarius* H41A KF207755 bioflocculant.

Microbial pathogen	Inhibition zone (mm)
S. aureus ATCC 25923	30
P. aeruginosa ATCC 9027	32
B. subtilis ATCC 6633	25
E. faecalis ATCC 29212	No activity
K. pneumonia ATCC 13883	28
C. albicans ATCC 10231	21

Effect of storage conditions

Antibacterial effect of AgNPs was tested every 45 days until 16 months later, and nearly similar results were detected which revealed the stability of the compound. Also UV–vis spectrophotometry results after 16 months confirmed the same results (Fig. 6).

Jar test determination of bioflocculant dosage

The effect of biofloculant dosage on flocculation efficiency for kaolin clay suspension was examined in an attempt to determine the most cost-effective dose for flocculation process. A range of concentrations from 0.3 to 5.0 $mgmL^{-1}$ were prepared for the purified bioflocculant and then their flocculating activities were estimated against 5 gL^{-1} kaolin clay suspension. The concentration dosage that gave the optimum flocculating activity (92.5%) was 4.0 $mgmL^{-1}$. By increasing the concentration of the biofloculant, no increase in the activity has been achieved. Data in Fig. 8 exhibited the efficiency of the purified bioflocculant in precipitating kaolin clay.

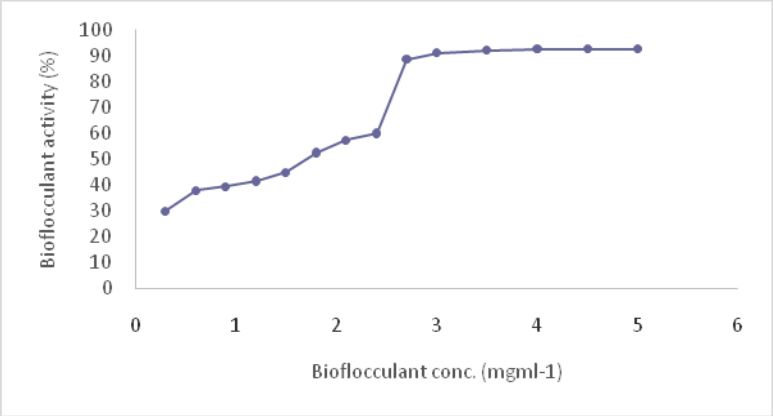


Fig. 9. Bioflocculant dosage in relation to kaolin clay precipitation.

Under optimal conditions, the maximum flocculating activity is usually attained at the optimal bioflocculant dosage (Salehizadeh and Yan, 2014). Okaiyeto *et al.* (2015) investigated the flocculating activity of purified MBF-UFH in a range of 0.01-0.5 $mgmL^{-1}$. The highest flocculating activity of 92.6% attained at 0.3 $mgmL^{-1}$. The decrease in flocculating activity of MBF-UFH observed at 0.5 $mgmL^{-1}$ might be due to the over addition of the negatively-charged MBF-UFH, generating strong repulsive forces between the kaolin clay particles and the bioflocculant. These processes restabilized the suspended particles, increasing the viscosity of the suspension, blocking the adsorption sites and noticeably reduced floc formation (Yuan *et al*., 2011). The results of the above mentioned authors coincided with our results where the optimum dosage in this work was 0.4 $mgmL^{-1}$ (92.5%) and at higher concentration, no increase for the flocculating activity was achieved.

CONCLUSION

A bioflocculant produced by marine strain; *Psychrobacter cibarius* H41A KF207755 showed high flocculating activity with high thermal stability. Different factors affectting the production of bioflocculant were examined and resulted in increasing the bioflocculant efficiency up to 94%. Characterization of our partially purified bioflocculant revealed that it was a glycoprotein bioflocculant which had different functional groups with charged moieties which lead to strong bioflocculation process enable us to use it to recover suspended solid materials in aquaculture, waste water and various industrial processes to create a clear environment. The produced AgNPs characterized by their high stability and very small particle size with broad spectrum antimicrobial activity. The bioflocculant obtained from *Psychrobacter cibarius* H41A KF207755 can be used as a multifunctional material in different areas as aquaculture industry, drinking and wastewater treatment, downstream processing, and fermentation processes .Also production of bacteriocidal AgNPs.

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Conflict of Interest

There is no conflict of interest.

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