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Bioflocculants Produced by Bacterial Isolates from Egyptian soil 1-Characterization and Application of Extracellular Bioflocculants and Nanoparticles for Treatment of River Nile Water

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Abstract: *Bioflocculants are essentially polymers produced by microorganisms during their growth . In the present study, screening of twenty eight bacterial isolates producing flocculating substances were carried out .They were isolated from cultivated soil from El-Qanater El-Khayria, Egypt and the raw water samples tested [River Nile water] have been collected from Ismailia Canal (about 13Km^2 from EL-Marge station for water treatment plant). From all isolates , two isolates that secret a largest amount of bioflocculant were selected . Based on16S rRNA gene sequencing and its morphological, physiobiochemical, characteristics, the isolates were identified as* ***Bacillus cereus*** *and* ***Bacillus thuringiensis*** *respectively . Maximum bioflocculant producing activity percent in water samples was affected by pH between 7-8 and at temperature rang 30- 40^{o}C and during growth period of strains from 72 to 96 h .The biopolymer flocculants named FQ-B1 and FQ-B2, produced by* ***Bacillus cereus*** *and* ***Bacillus thuringiensis*** *were precipitated chemical elemental analyses and UV scan were achieved for investigating the purified bioflocculant. In two isolates bioflocculants , total carbohydrate content were 0.524 µg/ 1m (16.99%) and 0.321 µg/ 1ml (15.27%) respectively , while the total protein content were 2.56 µg/ 1ml (83.01%) and 1.78 µg/ 1ml (84.73%) respectively. There are 18 types of mino acid were determined in both bioflocculants and the highest amino acid were Aspartic acid (14.9 µg/ 1ml), and Leucine (25.88 µg/ 1ml) . The present results anticipated that FQ-B1 and FQ-B2 had flocculating activity range from 75% to 76.3% and potential of application in raw water treatment, owing to their effective bioflocculation effective and harmlessness towards humans and the environment . Application of Silver 30 nm & Gold 60 nm nanoparticles alone and in conjugation with bacterial bioflocculants exhibited efficient flocculation capabilities in the treatment of River Nile water .Using Gold and silver nanoparticles alone in raw water treatment was approximately similar to that obtained two bioflocculants FQ-B1and FQ-B2 and the conjugation of nanoparticles with bioflocculants ranged from (75 to 80%) .Transmission electron microscopy(TEM) observed images of the nanoparticles irregular circular in group or in linear shape,when conjugation with bioflocculants, nanoparticles trapping and converted to a large sponge structure , this refered to highly flocculating efficiency without addtion nanoparticles .*

Key words: ***Bacillus cereus*** *and* ***Bacillus thuringiensis****. , Bioflocculant, Flocculating activity, Water treatment, Gold and silver nanoparticles* **.**

I. Introduction

The human attentions to the quality of drinking water go more than five thousand years. The purpose and limited extent have been used during the historical periods for treatment processes were boiling, filtration and sedimentation, and add some salts. In the eigheenth and nineteenth century a lot of serious attempts in the countries of Europe and Russia to advance the technology of water treatment. Famous salts that used in water and wastes sedimentation were aluminum sulfate and ferric chloride, and some assistance such as certain organic polymers can used to remove many organic compounds that cause a change in the taste and odor of water. The high turbidity observed with the use of aluminum sulfate as a coagulant can be due to the production of aluminum hydroxide precipitate in water. Besides being voluminous, the alum sludges are gelatinous, acidic , and difficult to dewater and dispose in the environment **(Buthelezi *et al.*, 2009) .** Flocculants have been widely used in a variety of industrial processes, such as waste water treatment, the food and fermentation industries, drinking water purification, and industrial downstream processes **(Shih *et al.*, 2001 and Wu & Ye, 2007)**. Flocculating agents are generally classified into three groups: (a) inorganic flocculants, such as aluminum sulfate and poly-aluminum chloride; (b) organic synthetic flocculants, such as poly-acrylamide derivatives and

poly-ethylene-amine; (c) naturally occurring flocculants, such as chitosan, sodium alginate and bioflocculant **(Salehizadeh & Shojaosadati , 2001 and Zhang *et al.*, 2007)**. Despite the effective flocculation performance and low cost of the synthetic chemical flocculants, their use has resulted in some health and environmental problems. For example, aluminum has been found to induce Alzheimer's disease **(Arezoo, 2002)**. Furthermore, the acrylamide monomer is not only neurotoxic and carcinogenic, but also non-biodegradable in the nature. On the contrary, bioflocculants produced by microorganisms during their growth are safe and biodegradable polymers **(Deng *et al*., 2003).** Many bioflocculant-producing micro-organisms including bacteria, fungi and actinomyces have been reported to produce extracellular polymeric substances, such as polysaccharides, functional proteins and glycoprotein's, which function as bioflocculant **(Kumar *et al*., 2004).** Nanotechnology is an emerging and fast-growing technology. Currently, there are more than 1,317 nanotechnology-based products on the market. Silver nanoparticles account for more than 23% of all nano-products . The extensive application of the silver nanoparticle (AgNP) results in their inevitable release into the environment . Silver nanoparticles are known as excellent antimicrobial agents, and therefore they could be used as alternative disinfectant agents . On the other hand, released silver nanoparticles could pose a threat to naturally occurring microorganisms (**Zhang, 2013**)**.** So that this study deals with the screening and isolation of bioflocculant producing bacteria from cultivated clay soil . Identification for the selected isolates by morphological, biochemical, physiological methods as well as 16s rRNA was carried out. Characterization of the produced bioflocculants was done. Application of silver/gold nanoparticles for water treatment alone and in conjugation with tested bioflocculants was assessed taken in consideration aluminum sulfate in our comparison .

II. Materials and Methods

1- Sample collection:

Twenty eight bacterial strains were isolated from cultivated clay soil from El-Qanater El-Khayria, Egypt. Water samples collected from Ismailia Canal (about 13Km2 from EL-Marge station for water treatment plant) for detection of bio-flocculating activity. All containers used for sampling were been cleaned and rinsed following trace metal protocols and were sterile.

2- Screening and identification of highly bioflocculant-producing bacteria: Isolation of bioflocculant-producing bacteria was carried out using nutrient agar medium with composition of glucose 10g; yeast extracts 5g; peptone 5g; NaCl 1g and agar 15g / l of distilled water at pH 7+ 0.2 and incubated at 37°C for 24h **(Chen and Zhao, 2003)**. Bioflocculant producing bacteria were originally screened based on colony morphology (mucoid ,ropy and their color). Purified isolates were inoculated into 50ml of bioflocculant production medium with composition of starch 20g, yeast extracts 3g, $CaCO_3$ 5g; K_2HPO_4 0.5g; $MgSO_4.7H_2O$ 0.2g; NaCl 0.2g;$CaSO_4$ 0.1g; $MnSO_4.7H_2O$ traces; $FeSO_4.7H_2O$ 0.01g; $Na_2MoCu.2H_2O$ traces and 15 g of agar, completed to 1000 ml with distilled water **(Desouky *et al.*, 2008)**, pH was adjusted at 7.2 and incubated at 37°C for 3 days. After incubation period cultures broth was centrifuged at (8000xg, 15min.) to separate the cells and tested for flocculating activity every 72h. Finally, the isolates with high and stable flocculating activities were selected for further studies.

3 - Determination of flocculating activity by Jar or clarifier testing (Gregory, 2006).

Jar or clarifier test is a major method of simulating a full-scale water treatment process, providing system operators a reasonable idea of the way treatment chemical will behave and operate with a particular type of raw water. The absorbance of supernatant and blank control without bioflocculant was measured at 550 nm (as OD_{550} and OD_{blank}, respectively) with spectrophotometer. The flocculating activity (ή) was defined and calculated as follows: $(ή) = (OD_{blank} - OD_{550}) / OD_{blank} \times 100$ **(Gregory, 2006).**

4- Separation and purification of Bioflocculant (Peiyong et al., 2004):

Purified isolates were inoculated into 50ml of bioflocculant production medium. After incubation period (3 days), the culture broth was diluted with two volumes of distilled water and centrifuged at 4,000xg for 15min. The supernatant was poured into three volumes of acetone (1:3) and added three times to precipitate the biopolymer flocculant. The precipitate was collected by centrifugation at 8,000 x g for 20 min. then washed by ether. The crude obtained was dialyzed at 4°C overnight in de-ionized water and vacuum-dried over night in a desiccator to obtain pure bioflocculants named FQ-B1 and FQ-B2 and re-dissolved in distilled water**.**

5 -Determination & characterization of bioflocculants FQ-B1 and FQ-B2:

Elemental analyses were achieved with UV Scan for measuring total carbohydrate contents. Total soluble carbohydrate concentration can be determined by Ultraviolet-Visible Range Spectroscopy (UV-Vis) at wavelength 485 and expressed as the glucose equivalent. This method is simple, fast, accurate, and specific to carbohydrate methodology by **Ammar (2013)**. Protein concentration was determined by the using bovine serum

albumin as a standard protein, the color was read at 750 nm using spectrophotometer according to Bradford method **Bradford (1976).** Amino acid moiety in the flocculant proteins were determined by amino acid autoanalyzer. This experiment was done in National Center for Radiation and Technology, Cairo, Egypt .

6 -Optimization of cultural parameters on bioflocculant activity:

Several factors affecting on bioflocculant activity ,within the intervals of 1 day up to 12 days, were studied as follows:.**1**- Effect of different incubation temperature range of (25- 60°C). **2-** The effect of different pH values were adjusted at different pH between (6.0 to10.5). **3**- Effect of different incubation period from (12h.-192h). **4-**Effect of various concentrations of mineral salt $CaCO_3$, $FeCl_3$, $FeCl_2$, $MnSO_4$, $CuCl_2$, $MgCl_2$,and $(NH4)_2SO_4$. **5-** Effect of different nitrogen sources as yeast extract , urea, peptone beef extract and $(NH_4)_2SO_4$ were used. **6**- Effect of different carbon sources as glucose, sucrose, fructose, lactose and starch .

7- Testing bioflocculants activity in comparison with aluminum sulfate:

The comparison between tested water treated by the investigated bioflocculants and those treated with aluminum sulfate were estimated by Jar test. Different factors have been tested in this experiment, such as: turbidity, pH, total dissolved salt (TDS), ammonia, nitrite, nitrate, total hardness, amount of Ca^{+2}, Mn^{+2}, Fe^{+3}, Fe^{+2} , Mg^{+2} , Al, total count of algae, total bacteria and total coliform .

8 -Identification of bioflocculant producing bacteria:

8-a)-The morphological, biochemical, physiological characteristics of the selected strains using Cowan and Steel's, 1977 methodology and were identified according to Bergey's Manual of Systematic Bacteriology **(Sneath, 1986 and Hotle *et al*., 1994).**

8-b)-16s sequence determination and phylogenetic characters:

Molecular techniques including genomic DNA extraction by **Sambrook et al.(1989),** PCR mediated amplification of the 16S ribosomal DNA, purification of PCR products and sequencing of the PCR products for the isolates under study were performed in VACSERA, El-Agoza, Egypt.

8-c)-Amplification and sequencing of the 16S rRNA gene:

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using eubacterial universal primers. That was 104F with the sequence
5'-GGACGGGTGAGTAACACGTG-3' and R1492 with the sequence

5'-CACGTGTTACTCACCCGTCC-3' **(Rainey *et al*., 1996).** The PCR mixture consisted of 30 picomoles of each primer, 10µg of chromosomal DNA, 200 µM dNTPs and 2.5 units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles at 94 ^{0}C for 1 min, 55^{0}C for 1 min and 72 ^{0}C for 2 min. After completion, PCR product was purified using PCR purification kit (Qiagen, Germany). phylogenetic analysis **(Yoon *et al*., 2000) by** DNA sequences were obtained using an ABI PRISM 3700 DNA sequencer and ABI PRISM Big Dye Terminator Cycle Sequencing.

8-d)-Cluster Analyses for Bacillus cereus, Bacillus thuringiensis by DNA sequence similarities and phylogenetic analysis:

Sequence data were analyzed in the GenBank database by using the BLAST program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) . The unknown sequences were compared to all of the sequences in the database to assess the DNA similarities **(Claudio, *et al*., 2002) .** For studying phylogenetic relationships of the investigated isolates with other similar reference strains depending on morphological biochemical , physiological characteristics and phylogenetic analysis viz. *Bacillus cereus, Bacillus thuringiensis* TS2. Bacillus spRsr, Bacillus spBB61, *Bacillus cereus* strain DBT3SC1, *Bacillus cereus* strain SS07. *Bacillus cereus* strain KVP109, *Bacillus cereus* strain KVP105, *Bacillus anthracis* strain KS-1 and Bacillus sp GS18 and *Bacillus thuringiensis* B4 (1) were evaluated by using statistical cluster analysis with joining (tree clustering) being the clustering method . The phenotypic characters were amalgamated by un-weighted pair-group average method analysis (UPGAMA). However, complete linkage was the method for studying character profiles using statistics for windows, release 4.5f, and state Soft, Inc. **1993** software. Euclidean distances (similarity matrix) were used as the distance metric in both as well as dice coefficient as the calculation method.

9) preparation and characterization of Silver and Gold Nano-particles (Pradeep and Anshup, 2009):

9-a) Determination using Ultraviolet-Visible Range Spectroscopy (UV-Vis) (Ammar, (2013):

The wavelength of nano-particles can be determined by Ultraviolet-Visible Range Spectroscopy (UV-Vis) at wavelength **421w.v.** for silver and **527 w.v**. for gold nano-particles.

9-b)Transmission electron microscopy (TEM) images of silver and gold nanoparticles :

This method was used for magnified of bacterial bioflocculant, silver and gold nanoparticles. The method was done according to **John (2010)** .

9-c) Application of Silver & Gold Nanoparticles with Bacterial bioflocculants FQ-B1 and FQ-B2, for water treatment (Farland, *et al.*, 2004) and (Gang , 2013):

This experiment was carried out to investigate the application of nanoparticles with biofloccul-ants for water treatment. Each one of the tested bioflocculants (FQB1, FQB2) was dissolved in 1ml phosphate buffer saline and mixed with 1ml of nano-particle solutions and then added to raw water in Jar test for determination the effect of silver and gold nanoparticles that conjugated with bacterial bioflocculants for water treatment, also for determine the activity of gold and silver nanoparticles alone by using Jar or Clarifier test. **(Gang 2013)**

10)-Statistical analysis:

Results were analyzed statistically using student's t-test. Values of $p<0.05$ were considered statically significant. All data in the text and tables are expressed as a percentage of dark control standard error (SEM) of at least three samples; experiments were repeated 3 times. The statistical analysis was carried out by Graphpad® prism soft ware (USA).

III. Results

1-Screening of bioflocculant with slimy or mucoid appearance producing bacteria:

A total of twenty eight bacterial isolates with slimy or mucoid appearance were isolated from cultivated clay soil from El-Qanater El-Khayria, Egypt . These isolates were purified and screened for their flocculating activity using water samples collected from Ismailia Canal (about 13Km^2 EL-Marge station for water treatment plant) based on the decreasing turbidity. Among them, two strains causing the lowest turbidity after 72h (**Table 1**) were selected for further studies . These selected strains were designated as B1 & B2 on preliminary test and exhibited turbidity values 3.21, 4.3 respectively compared to control (raw water turbidity) 7.8 NTU (National Turbidity Unit) . The bioflocculants produced by the selected isolates **B1 & B2** were named **FQ-B1** and **FQ-B2** respectively .

(Table 1) : Preliminary test for determination the flocculants activity produced by different bacterial isolates on raw water using jar test .

Organism	Turbidity	Organism	Turbidity
Bacterial isolates from soil	**mean**	**Bacterial isolates from soil**	**mean**
1	7	**15 (B1)**	**3.21**
2	6.4	**16 (B2)**	**4.3**
3	6.5	17	6.9
4	4.9	18	7
5	4.81	19	6.9
6	4.9	20	7.36
7	4.8	21	7.56
8	5.3	22	6.97
9	5	23	7.3
10	7.8	24	7.8
11	7.2	25	7.49
12	5.6	26	7.8
13	7.6	27	7.06
14	7.8	28	7.7

Turbidity of raw water (Control): is 7.8 NTU (National Turbidity Unit)

2-Effect of different cultural parameters on flocculants FQ-B1 and FQ-B2 Production:

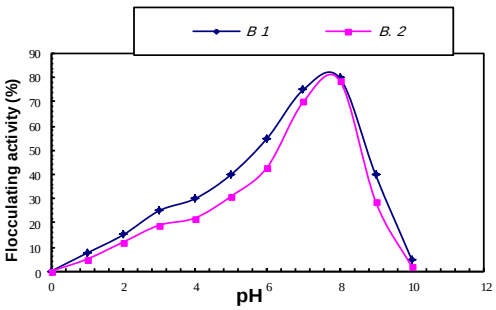
Maximum flocculating activity was affected by different cultural parameters **(Figs. 1, 2, 3, 4, 5,6,7and 8) .**The maximum values of **FQ-B1** and **FQ-B2** were between 7-8 of both investi-gated strains .The favorable temperature ranging from 30-45^{o}C decreased obviously beyond 55^{o}C for both studied strains .The production of flocculants FQ-B1 and FQ-B2 from two strains also affected by the presence of some cations . It was maximum in the presence of Ca^{+2}, Mg^{+3}, Fe^{+3} and Mn^{+2}. The flocculating activity of two strains reached its peak in at the

end of growth phase and at the beginning of stationary phase (72h.), the data obtained indicated also that , flocculating rate and the growth of the two strains increased with increasing cultivation time till forth day. Among the organic nitrogen source tested , urea, peptone and yeast extract were appear favorable for both cell growth and flocculating production of the two tested strains . Also glucose , fructose and starch were appeared favorable for both tested strains. On the other hand the best concentration of **B1** bioflocculant on turbidity of raw water was 2.56 µg / 1m and 1.78 µg/ 1ml for **B2** Effect of bioflocculant dosege : **Figs 7 and 8** showed the relationship between the concentration of the bioflocculants and thier activities. The maximum flocculating activity (80%) was achieved of bioflocculant dosage of 2m/L for **B1** isolate and it was around 76.2% when the flocculants concentration was adjusted to 4ml/L. for **B2** isolate .

3-Identification of strains:

The morphological character of **B1** colony strain was circle, mucoid, ropy and brown in color while **B2** was circle, mucoid, ropy and white in color. From these characteristics and physiological, biochemical characteristics listed in (**Table 2, Figs. 9 -a and -b),** the investigated bacterial isolates B1 and B2 resemble ***Bacillus cereus*** **and** ***Bacillus thuringiensis*** respectively according to Bergey's Manual of Systematic Bacteriology **(Sneath, 1986 and Hotle et al., 1994) .** At the same time the 16s rDNA of these strains were sequenced following PCR amplification .The strain B2 showed obvious similarity within the cluster of Bacillus sp. So according to morphological, physiobiochemical characterastics and 16S rDNA and in comparison with those in other database in GenBank the strains **B1** shared approximately 50% identity with *Bacillus cereus* and **B2** approximately 100% with *Bacillus thuringiensis*.

(Fig 1): Effect of different pH values on B1and B2 bioflocculants production



(Fig 2) Effect of temperature on activity of both isolates B1&B2 bioflocculants production

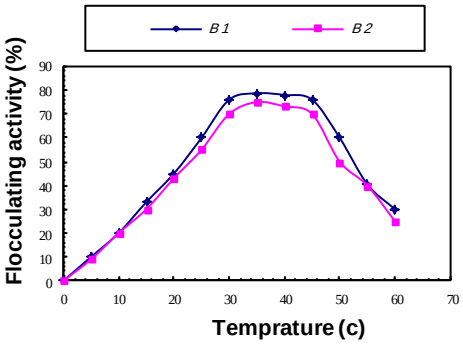
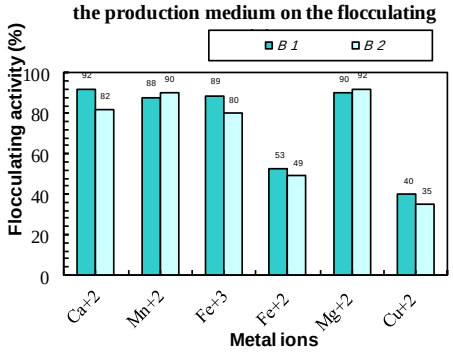
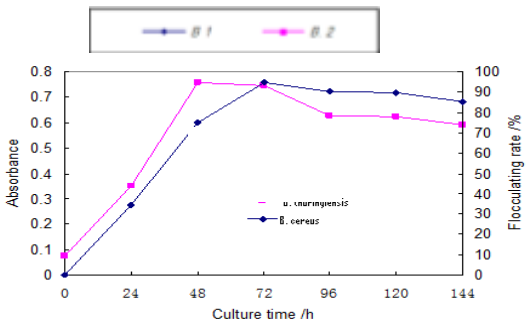


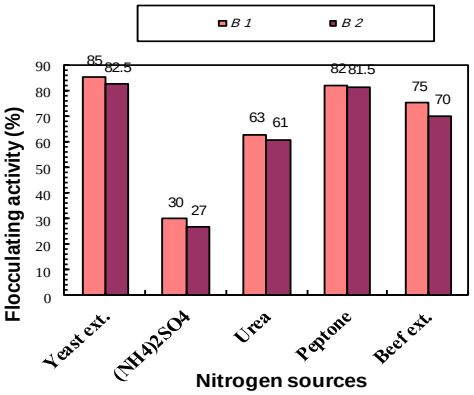
Fig (3) Effect of the presence of metal ions in the production medium on the flocculating



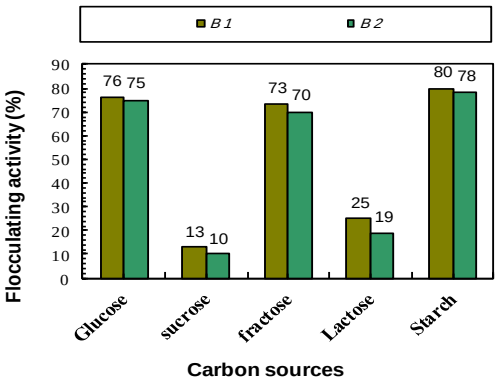
(Fig 4) Activity percent of flocculant isolats at different incubation periods



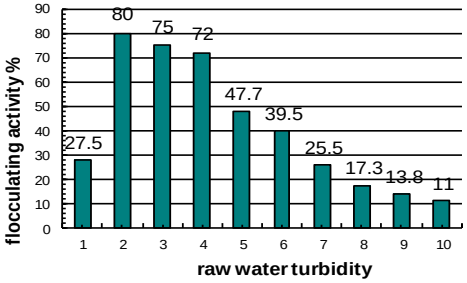
(Fig 5): Effect of different nitrogen sources on isolates B1&B2 flocculent productions.



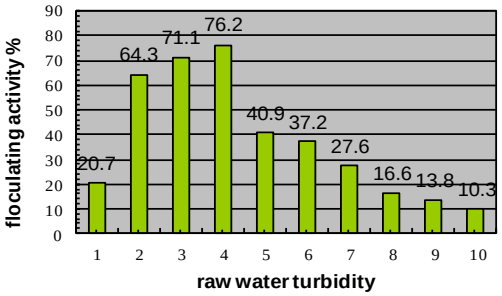
(Fig 6): Effect of different carbon on both isolates B1&B2 flocculent production



(**Fig 7**): **Effect of different concentrations of B1 in bioflocculant** FQ-B1 **on turbidity of raw water .**



(Fig 8): **Effect of different concentrations of B2 bioflocculant** FQ-B2 **on turbidity of raw water .**



(Table2): A-physiobiochemical characteristics of *Bacillus cereus* & *Bacillus thuringiensis:*

physiobiochemical test	**Bacillus cereus**	**Bacillus thuringiensis**
Motility	Motile	Motile
Growth areation	Facultative anaerobic	Facultative anaerobic
Gram's reaction	+ ve	+ ve
KOH	+ ve	+ ve
Colony colour	**circle, mucoid, ropy and brown**	**circle, mucoid, ropy and white in color**
Shape	Rod shape	Rod shape
Lengh um	**3-5**	**>1**
Endospores producer	**+ ve**	**Formed outside**
Lactate	**+ ve**	**- ve**
Growth at pH 5.7 **6.8**	+ ve + ve	+ ve + ve
Strick anaeorobic	- ve	- ve
Sufate activity reduced to sulfid	- ve	- ve
Oxidase	**- ve**	**+ ve**
succinate	+ ve	- ve
Temperature range 30°C **40°C** **65°C**	**+ ve** **- ve** **- ve**	**+ ve** **+ ve** **- ve**
Utiling of citrat	+ ve	+ ve
Casein hydrolysis	+ ve	+ ve
Gelatin hydrolysis	+ ve	+ ve
Starch hydrolysis	+ ve	+ ve
Catalase test	+ ve	+ ve
Indole test	- ve	- ve
Vogas-preskaure test	+ ve	+ve
Urease test	+ ve	+ ve
Nitrate reduction	+ ve	+ ve

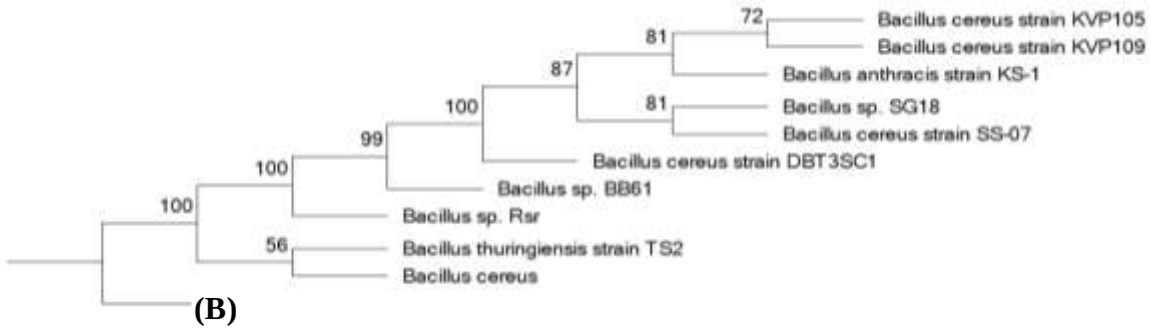
Carbohydrate fermentation		
Glucose	+ ve	+ ve
Sucrose	+ ve	+ ve
Fructose	+ ve	+ ve
Sucrose	+ ve	+ ve
Maltose	+ ve	+ ve
Xylose	- ve	- ve
Manitole	- ve	- ve
glycerol	**+ ve**	**- ve**

4-Characteristics & composition analysis of bioflocculant:

Chemical elemental analyses were achieved with UV Scan for analyses of the purified bioflocculant from broth culture (Glycoprotein's), total carbohydrate content of *Bacillus cereus* and *Bacillus thuringiensis* were 0.524 µg/ 1m (16.99%) and 0.321 µg/ 1ml (15.27%) respectively. While the total protein content in both isolate 2.56 µg/ 1m (83.01) and 1.78 µg/ 1ml (84.73) respectively. Amino acid analysis was determined that there are 18 types of amino acids found in bioflocculants in both isolates. In *Bacillus cereus* and *Bacillus thuringiensis* bioflocculant soluble protein found that Leucine (25.88 µg/ 1ml) and Aspartic acid (14.9 µg/ 1ml) were highly amino acid concentration respectively as shown in Table **(3)** and Fig. **(10).**

(3-a) Phylogenetic tree showing the relationships among the selected isolates and published 16S rDNA sequences (a) for B1 and (b) for B2

(Fig. 9 - a): Phylogenetic tree of *B. cereus:*



(Fig. 9-b): Phylogenetic tree of *B. thuringiensis*

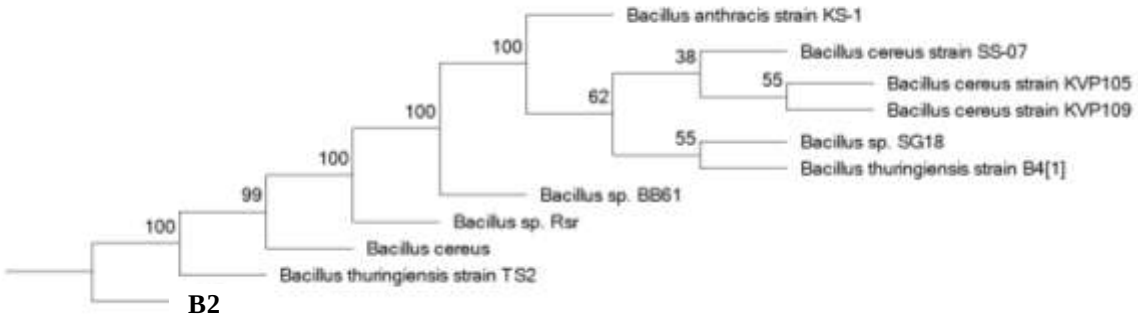


Table (3): Measuring total carbohydrates and total protein in the culture broth of two strains

Sample Name	**Total Protein / 1ml**	**Total Carbohydrates/ 1ml**
Bacillus cereus	2.56 µg	0.524 µg
Bacillus thuringiensis	1.78 µg	0.321 µg

Fig (10): Amino acid compositions of *B. cereus and B. thuringiensis* bioflocculant soluble protein by µg / ml with different concentration .

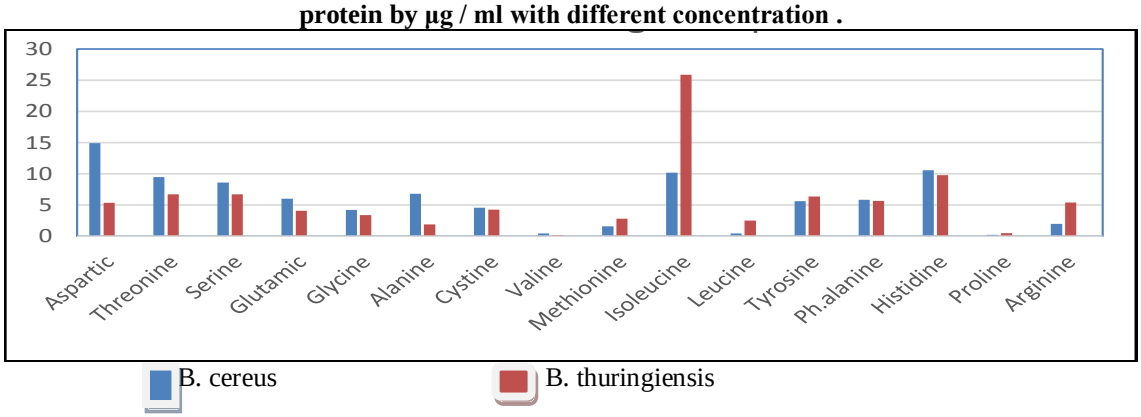


Table. (4): Comparison between water treated with Aluminum sulfate and water treated with investigated bacterial bioflocculants:

Type of analysis	Raw water	Water treated with $Al_2(SO_4)_3$	Water treated with bioflocculant of *Bacillus cereus*	Water treated with bioflocculant of *Bacillus thuringiensis*	Maximum limits in Tap water
Turbidity% Flocculating activity %	**13.5 (100%) 0 %**	**1.7 12.6 % 87.4 %**	**3.2 23.7 % 76.3 %**	**3.4 25.0% 75%**	**1.0 NT 7.4% 92.6%**
Temperature◦C	21	22.5	21.4	21.4	22 ◦C
pH	8.25	7.2	7.2	7.2	6.5-8.5
TDS	259	269	2 62	264	1000 mg/L
Ammonia	0.05	UDL	UDL	UDL	0.5 mg/L
Nitrate	0.001	UDL	UDL	UDL	45 mg/L
Nitrite	UDL	UDL	UDL	UDL	0.2 mg/L
Total alkalinity	142	120	120	118	mg/L
Total hardness	126	128	126	126	500 mg/L
Calcium	30	30	30	30	350 mg/L
Magnesium	12	12	12	12	150 mg/L
Chloride	27	31	27	28	250 mg/L
Sulfate	24	35	25	25	250 mg/L
Silica	1.7	1.9	1.7	1.7	mg/L
Phosphate	0.005	UDL	UDL	UDL	mg/L
Fluoride	0.3	0.21	0.20	0.20	0.8 mg/L
Ferrous	0.15	UDL	UDL	UDL	0.3 mg/L
Manganese	UDL	UDL	UDL	UDL	0.4 mg/L
Aluminum	0.24	0.19	0.15	0.18	0.2 mg/L
Total Alga	7000	18	27	27	Unit/ 1ml
Total Coliform bacteria	+1600	1>	1>	1>	1> unit/ 100 ml
Total bacterial count	2500	50>	50>	50>	50> unit/ 100 ml
Microscopic examination	Warms & life protozoa	Free from Warms & life protozoa	Free from Warms & life protozoa	Free from Warms & life protozoa	Free from Warms & life protozoa

NTU: National Turbidity Unit, TDS: Total dissolved salts, UDL: undetected limit .

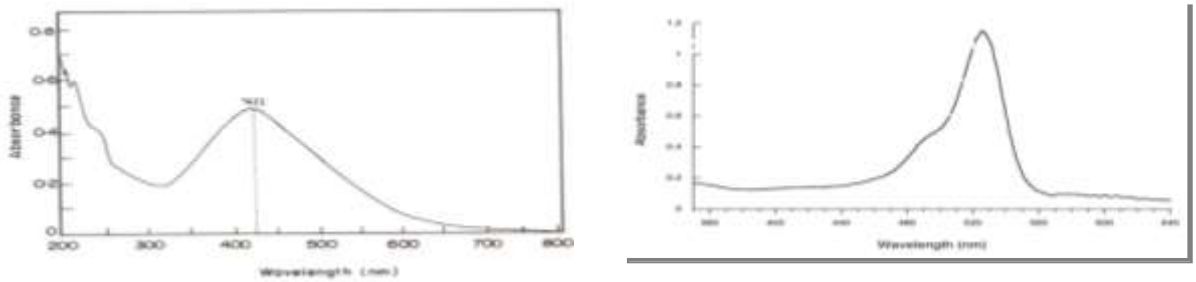
5) Comparison between water treated with Aluminum sulfate and water treated with bacterial bioflocculants

The ability of *B. cereus* & *B. thuringiensis* bioflocculant to flocculate various suspended particles in raw water was investigated and compared with the data obtained by using $Al_2(SO_4)_3$. It is clear from the data summarized from table (4) that the tested bioflocculants had obvious flocculating activity against all suspended particles beside their ability to minimize and remove the microbial and algal contaminations. $Al_2(SO_4)_3$. floccul-ating activity about 87. 4% ,while two bacterial bioflocculants 75% and 76.3 % .

6-Application of gold and silver nanoparticles conjugated with bacterial bioflocculants in water treatment:

(6-a) Characterization of silver and gold nanoparticles : In recent of first twenty century the use of

Fig. 11): UV-Vis spectrum of silver Nanoparticles show the w.v. of silver at 421nm and gold at 527 nm



nanotechnology in the treatment of raw water was popularized, so in this research silver and gold nanoparticles were prepared in order to test its effectiveness in reducing the turbidity of raw water, also it has been uploaded to the bacterial bioflocculants

Fig (12 : Transmission electron microscopy (TEM) : (magnification power 10.000 xs.)

(A)- images of silver nanoparticles with diameters of 30 nm .

(B) images of gold nanoparticles: with diameters of 60 nm .

(C) Images of *Bacillus cereus* bioflocculants with Silver nanoparticles trapping .

(D) Images *of Bacillus thuringiensis* bioflocculants with Gold nanoparticles trapping .

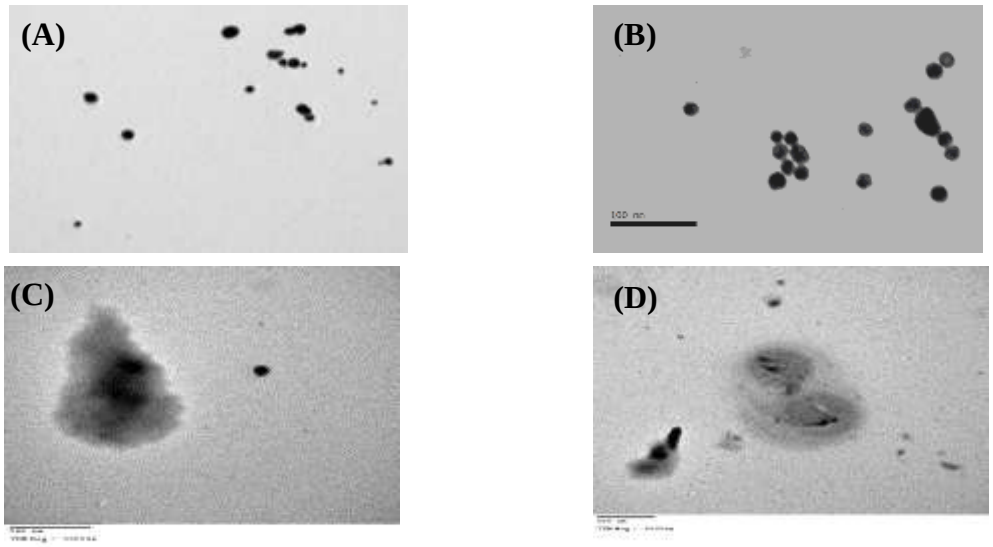


Table (5): Silver Nan-particles with bioflocculant of *B. cereus & B. thuringiensis* and its effectiveness on raw water. 1ml contains 2.56µg /ml flocculants.

Concentrations of silver nanoparticles SNP ml/l	**Turbidity of H_2O**	**Results of SNP (bioflocculants activity %)**						**Statistical analysis**	
		SNP alone		**SNP+ *B. cereus***		**SNP+*B. thuringiensis***		**Std.D.**	**+Std.E.**
		Turbidity+ %		**Turbidity+ %**		**Turbidity+ %**			
1 ml	14.5	11	24.1*	9	38*	9.1	37.2*	3.550	+ 1.123
2		8	45**	7.1	51**	7.2	50.3**	1.11	+ 0.011
3		**3**	**79.3*** **	**2.7**	**80.7*** **	**2.9**	**80*** **	1.21	+ 0.025
4		5.8	60***	5.3	63.4***	5.8	60***	1.7	+ 0.02
5		7.3	49.6**	7	51.7**	7	51.7**	2.01	+ 0.21
6		7.9	45.5**	7.2	50.3**	7.4	48.9**	2.51	+ 1.13

* Non-significant change in comparing with control raw water (P>0.05).

** Significant change in comparing with raw water (P<0.05).

***Highly significant change in comparing with control raw water (P<0.0001)

under study in order to increase its influence in reducing turbidity and show which the best treatment process for raw water as shown in Tables **(5 and 6) and Figs (11& 12**). Using nanoparticles as raw water treatment was approximately similar to that obtained two bioflocculants FQ-B_1 and FQ-B_2 and the conjugation of nanoparticles with bioflocculants ranged from (75.5% -80%) . while $(Al_2(SO_4)_3$ precipitation was 87.4%. Also, the conjugation of nano-particles with bioflocculants about 80% . Generally transmission electron microscopy TEM images of the nanoparticles irregular circular group or irrgular circular in linear shape , when conjugation with bioflocculants nanoparticles trapping and converted to a large sponge structure as a result of the interaction between bioflocculant and nano particles in both of two organisms cause unaffected of nanoparticles and become similar any metales treaed in raw water treatments by bioflocculant .This refered to high flocculating efficiency without addtion nanoparticles .

Table (6): **Gold Nan-particles with bioflocculant of *B. cereus & B. thuringiensis*** and **its effectiveness on raw water. 1ml contains 2.56µg/ml flocculants .**

Concentrations of Gold nanoparticles GNP ml/l	**Turbidity of H_2O**	**Results of GNP (bioflocculants activity %)**						**Statistical analysis**	
		GNP alone		**GNP+ *B. cereus***		**GNP+B. thuringiensis**		**Std.D.**	**+Std.E.**
		Turbidity+ %		**Turbidity+ %**		**Turbidity+ %**			
1 ml	14.5	11.5	20.7*	9.4	35.2*	9.25	36.2*	2.41	+ 1.12
2		8.7	40**	7.3	49.6**	7.21	50.2**	1	+ 0.012
3		**3.5**	**75.9*** **	**3**	**79.3*** **	**3**	**79.3*** **	**1.10**	**+ 0.02**
4		6.9	52.4***	6	58.6***	6	58.2***	1.56	+ 0.026
5		7.7	46.9**	7.1	51**	7.3	49**	2.11	+ 0.81
6		8.2	43.4**	7.7	46.9**	7.8	46.2**	2.43	+ 1.10

* Non-significant change in comparing with control raw water (P>0.05).
** Significant change in comparing with raw water (P<0.05).
***Highly significant change in comparing with control raw water (P<0.0001).

IV. Discussion

Bioflocculants are essentially polymers produced by microorganisms during their growth .Bioflocculants have special advantages such as safety, strong effect, biodegradable and harmlessness towards humans and the environment (eco-friendly), so they may potentially be applied in drinking and waste water treatment. The organic flocculants, widely used in industrial fields, have been shown to be harmful to the environment and to be dangerous sources of pollution . This research aims to screening of twenty eight bacterial isolates flocculating producing substances. Two isolates were classified as *Bacillus cereus* and *Bacillus thuringiensis* based on 16S rRNA gene sequencing and its morphological , physiobiochemical characteristics and highly bioflocculant production . Maximum bioflocculant producing activity percent in water samples was affected by pH between (7- 8) and at temperature rang 30- 40 ^oC and during growth period of strains from 72 to 96 hours . The results are in accordance with **Zhang *et al.* (2007) and Xia *et al.* (2008)** ,they reported that, the bioflocculant production is affected by many factors, such as the constituents of the culture medium and culture conditions. The effects of the key factors, like culture time, initial pH of the production medium, carbon source, nitrogen source, C/N ratio, metal ion, ionic strength , culture temperature, shaking speed and inoculum size , on the flocculating activity of the bioflocculant were investigated with an aim to identify the cost-optimal culture conditions **.** The production of bioflocculant is influenced by metal ions in the culture medium, flocculating activity increased in the presence of K^+, Na^+ or Ca^{2+}. The flocculant production of Flavobacterium sp. was stimulated in the presence of Ca^{2+}, $Mn^{2+,}$ and $Ba^{2+,}$ but was inhibited by Mg^{2+} **(Buthelezi *et al.*, 2009)**. Bioflocculants are polymers produced by microorganisms during their growth . It has been reported that all types of microbe's i.e; bacteria, fungi and algae produce flocculants with various properties . Different microorganisms are reported to produce variety of bioflocculants, e.g. R. erythropolis S1 and Nocardia armarae YK reported for protein bioflocculant. Glycoprotein bioflocculant production by B. muciligenous **Shih *et al.* (2001) and Zhang *et al.* (2007) .** Turbidity removal rate of raw water were observed a lower with increase in bioflocculant concentrations this is may be due to when the optimum concentration is exceeded, the aggregated particles can redisperse and this disturbs particle-settling ..Or this has been attributed to an increase in the repulsive energy between the flocculants and the microorganisms, which causes hindrance in floc . In general it is known that the genus of Bacillus includes a variety of industrially important species and has a history of safe use in both food and industry , these suggestions agreement with **(Salehizadeh. & Shojaosadati, 2003; Buthelezi *et al.*, 2009 and Sekelwa *et al.*, 2011).** A bioflocculants named FQ-B1and FQ-B2, produced by *Bacillus cereus* and *Bacillus thuringiensis* respectively were precipitated by acetone, and then washed by ether, and the crude dialyzed at 4^oC over night then vacuum-dried to obtain pure biofloccular. The highest flocculating rates were achieved at bioflocculants dosage of 2.560 mg /l. However, the depression of

flocculating activity at high concentration of all bioflocculants is largely due to incomplete desperation of excess bioflocculant **(Gao *et al.*, 2006). Zhang, *et al.* (2007) and Xia *et al.* (2008)** found approximately the same results effect on a bioflocculant TJ-F1 with high flocculating activity, produced by Proteus mirabilis TJ-1 strain . It's known that Bacillus isolates made use of soluble starch and other nutrients and produces a polysaccharide or glycoprotein bioflocculant. Despite of that the starch can be modified into a flocculant through chemical reaction **(Khalil and Ali, 2001)** .Generally *Bacillus cereus* and *Bacillus thuringiensis* produced an excellent biopolymer flocculant (glycoprotein) . Chemical elemental analyses were achieved with UV Scan for analyses of the purified bioflocculant from broth culture (Glycoprotein's) and total carbohydrate content of bioflocculant soluble protein . Amino acid analysis was determined that there are 18 types of amino acids and Aspartic acid and Leucine were highly amino acid concentration respectively, this agreement with investrgation of **Deng *et al.* (2003) and Sekelwa *et al.* (2011)** . However, if the major component of the bioflocculant is a glyco-protein its stability will depend on the relative components of protein and polysaccharide. For protein bioflocculant the amino and carboxyl groups are the effective group bioflocculation . A major condition for flocculation is the molecules of flocculants could adsorb onto the surface of particles (suspended solids in a River Nile). The charge of particles in water is negative. When bioflocculants were approaching particles in water, an attractive force must exceed the electrostatic repulsion force . Glycoprotein is a positive charge stimulates flocculating activity by neutralizing and stabilizing the residual charge of functional groups as binding distance is shortened **Desouky *et al.* (2008)** .

In comparison between water treated with Aluminum sulfate{$Al_2(SO_4)_3$}and with bacterial bioflocculants found three samples were free from Warms & life protozoa , total alga and total coliform bacteria and flocculating activity of $Al_2(SO_4)_3$ was 87.4% , but being voluminous, the alum. sludge's are gelatinous, acidic, and difficult to water and dispose in the environment. However, medical reports indicated that aluminum might induce Alzheimer's disease.While two bacterial bioflocculants 75% and 76.3% and thier potential of application in raw water treatment, owing to their effective bioflocculation effective and harmlessness towards humans and the environment . Two bacterial bioflocculants were able to reduce both turbidity and bacterial load from the contaminated river water to a varying degree, with higher bacterial load removal rate observed with increasing concentrations of the bioflocculants . While **Faust and Aly (1998)** showed that aluminum sulfate . was not effective in removing bacteria within the range of 5–10 ppm, with a removal of 99.7% achieved with 50 ppm, which is closer to the observation in most of studies . The high turbidity observed with the use of alum . as coagulant can be due to the production of aluminum hydroxide precipitate in water. **Arezoo, 2002; Gao *et al.* (2006); Sekelwa *et al.* (2011) and Buthelezi *et al.* (2009)** found that **,** compared to alum. which resulted in an acidic pH of 4-.14, this makes bacterial bioflocculant preferable in the practical terms as no further chemical addition is necessary in order to correct the pH of the finished water . Application of Silver 30 nm & Gold 60 nm nanoparticles alone and in conjugation with bacterial bioflocculants (FQ-B1 and FQ-B2), exhibited efficient flocculation capabilities in the treatment of River Nile water. Using Gold and silver nanoparticles alone in raw water treatment was approximately similar to that obtained two bioflocculants FQ-B1and FQ-B2 and the conjugation of nanoparticles with bioflocculants ranged from (75 to 80%). Transmission electron microscopy (TEM) observed images of the nanoparticles irregular circular in group or in linear shape,when conjugation with bioflocculants, nanoparticles trapping and converted to a large sponge structure , as a result of the interaction between bioflocculant and nano particles in both of two organisms cause unaffected of nanoparticles and become similar any metales treated in raw water treatments by bioflocculant , this refered to high flocculating efficiency without addtion nanoparticles .**Desouky *et al.* (2008) and Hongyin (2013)** obtained that silver nanoparticles in surface water, ground water, and brackish water are stable. However, in seawater conditions, AgNP tend to aggregate. Also showed that the antimicrobial activity of AgNP can be impaired by the presence of a humic substance and high concentrations of divalent cations . These results are helpful in explaining how discharged AgNP behave in natural aquatic systems as well as their environmental toxicological effects on naturally occurring microorganisms.

V. Conclusion

A bioflocculant-producing by strains *Bacillus cereus* and *Bacillus thuringiensis* an excellent biopolymer flocculant (glycoprotein's) with total protein was more than that of carbohydrate content. Also, bacterial bioflocculants FQ-B1and FQ-B2 had potential application about 76.5 % in waste water treatment similar to gold and silver nanoparticles . When conjugation of bioflocculants with nanoparticles trapping and converted to a large sponge structure cause unaffected of nanoparticles this refered to high flocculating efficiency without addtion nanoparticles . At the same time, aluminum sulfate had highest flocculants activity 87.4% , while residual aluminum concentrations in treated water can also impose health problems apart from the production of large amounts of sludge. Therefore, the use of high concentrations of alum . in the treatment of river water must be avoided . Thus making bioflocculant a better , wider and alternative in wastewater treatment and drinking water processing industry by *B.cereus* and *B. thuringiensis* as excellent biopolymer

flocculant (glycoprotein) , because of its effective flocculation and harmlessness towards humans and the environment .

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