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Isolation, screening and molecular identification of bioflocculants–producing bacteria

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Abstract. *Al Khafaji AM, Almansoory AF, Alyousif NA. 2023. Isolation, screening and molecular identification of bioflocculants–producing bacteria. Biodiversitas 24: 4410-4417.* Bioflocculants are biological compounds produced by different microorganisms with many applications for wastewater treatment as such become an important product in biotechnology and a consequence to be used in industries. The current study aimed to isolate, identify, and screen bioflocculant-producing bacteria from different sites in Basrah City in Iraq. The production of bioflocculants was enhanced by optimization of various cultural conditions such as (carbon source, nitrogen supply, pH, and inoculum sizes) which were estimated in terms of flocculating activity test. Four wastewater samples and oil-contaminated soil samples were collected. Twenty-one different bacteria were isolated from wastewater and soil. Eleven bacterial isolates showed flocculating activity values of more than 50 %. The results showed that two bacterial isolates were reported as the best bioflocculants-producing isolates with a flocculating activity value of 87.80 % and 81.38 % respectively, these two isolates belonged to *Aeromonas simiae* and *Exiguobacterium profundum* which identified by 16S rDNA gene sequencing. Four bacterial isolates were discovered and recorded as new strains in NCBI GenBank with the accession numbers OQ848055 (*Escherichia coli* strain ANABASR1), OQ848056 (*Stutzerimonas balearica* strain ANABASR2), OQ848057 (*Bacillus jeotgali* strain ANABASR3) and OQ848058 (*Hydrogenophaga temperata* strain ANABASR4). The maximum flocculating activity of 84.49% and 88% was reported for *A. simiae* and *E. profundum* respectively under optimum conditions (glucose as carbon source, $(NH_4)_2SO_4$ as nitrogen source, pH= 7 and 5% inoculum size). The phylogenetic tree was created in the current study based on 16S rDNA gene sequences of bioflocculants – producing bacteria to assess their close relationship and evolution.

Keywords: 16S rDNA gene, bioflocculant producing-bacteria, flocculating activity, wastewater

INTRODUCTION

The rapid growth of population, urbanization, industrialization, and agricultural operations have raised the need for clean sources of water. However, rivers, lakes, and reservoirs still contain high levels of pollutants like organic compounds, heavy metals, and pathogenic bacteria, making them unfit for human consumption (Fitriani et al. 2020; Akhter et al. 2021). The release of wastewater into water sources without suitable treatment may have harmful impacts on the health of both people and the environment. Water contamination can be caused by agricultural practices, unlawful dumping, landfill leachate leaks, and the discharge of industrial and sewage effluent (Arifin et al. 2018; Hassimi et al. 2020).

Raw water and wastewater have been converted into clean water using both traditional and Advanced technology. The effectiveness of those technologies in terms of treatment results is very high (Ang et al. 2020; Kurniawan et al. 2020). Water and wastewater can be treated via physical-chemical processes including (coagulation/flocculation, precipitation, conventional and advanced chemical oxidation) and biological processes. Flocculation is a procedure used to separate solids from liquids in a variety of industrial processes including wastewater treatment, drinking water purification, food and fermentation processes (Selepe et al. 2022).

The flocculants must not have any harmful effect on the environment or human health (Shahadat et al. 2017). Flocculants can be divided into natural (chitosan, bioflocculant), inorganic ($Al_2(SO_4)_3$ and polyaluminum chloride) and organic synthetic (polyacrylic acid, polyacrylamide derivatives) flocculants. Many organic and inorganic flocculants are used widely because of their low cost and high activity. However, the monomers and derivatives of both flocculants cause serious diseases, like neurological, cancer and Alzheimer's disease (Kurniawan et al. 2020).

Bioflocculants can be defined as extracellular polymeric substances (EPS) extracted from different sources, such as plants, animals and many microorganisms including (bacteria, algae and fungi) (Ajao et al. 2018; Kurniawan et al. 2021). They are composed of proteins, polysaccharides, lipids, glycoproteins, nucleic acid and cellulose making them biodegradable and eco-friendly, preventing secondary pollution. The commercial availability of bioflocculants is still limited because of lower flocculation properties, minimum production and high substrate cost (Ajao et al. 2018; Ang and Mohammad 2020). It is important to have information about flocculants classification and flocculants must not have any harmful effect on the environment or human health before choosing suitable flocculants (Shahadat et al. 2017).

Many different environments including aquatic systems, rivers, soil, activated sludge, and effluent, can serve as sources for producers of bioflocculants. Several bioflocculant-producing bacteria from various taxonomic genera have been reported the production of bioflocculant from different environments, such as *Enterobacter* sp., *Citrobacter* sp., *Rhodococcus erythropolis*, *Bacillus* sp., *Arthrobacter* and *Cellulomonas* (Peng et al. 2014; Nkosi et al. 2021). *Terrabacter* sp. bacteria isolated from freshwater was reported a flocculating activity of >80% (Agunbiade et al. 2018). *Klebsiella* sp. Isolated from oil-contaminated soil and exhibited a flocculating activity of roughly 95% (Ma et al. 2017).

The bacteria produce bioflocculants under normal conditions in extremely small quantities, therefore it is important to explore methods to increase bioflocculants yield by the bacteria. The optimization of the medium composition and the cultivation conditions including (carbon, nitrogen sources, inoculum size, temperature, initial pH of the medium and cultivation time) that affects bioflocculants yield (Gouveia et al. 2019; Abbas et al. 2020; Alyousif et al. 2022). A bioflocculant-producing bacterium was isolated from the soil and wastewater in the current study. The 16S rDNA gene sequencing was used to identify the bacteria and to test them for their ability to produce bioflocculants. The production of bioflocculants was improved by optimizing the medium composition and culture conditions. The purpose of the current study is to isolate, identify and screen bacteria that produce bioflocculants from various sites in the city of Basrah in Iraq. Four samples of wastewater and oil- contaminated soil samples were collected.

MATERIALS AND METHODS

Samples collection

Five samples were collected as three from wastewater samples (A, B and C) in the Hamden wastewater treatment plant and sample (E) from the Al-Dair treatment plant and sample (D) from contaminated soil by oil in Rumelia area north of Basrah city as shown in Table 1, where water samples were collected using clean and sterile bottles, while soil samples were collected in special sterile bags and transferred to the laboratory to isolate bacteria from them.

Isolation of bioflocculants-producing bacteria

The wastewater samples were diluted to a dilution of 10^{-6}, then draw 0.1 mL of each dilution, spread over a petri dish containing a nutrient agar medium using L- shape, dried, and then transferred to the incubator at 35°C for 24 h (Mulamattathil et al. 2014). One gram of oil-contaminated soil was weighed into a tube containing 9 mL of distilled water and vortexed. Then a series of dilutions was performed from (10^{-1}-10^{-4}), 0.1 mL of each dilution was taken with a micropipette and spread on the nutrient agar medium using a sterile spreader and incubated at 35°C for 24 h.

Screening of bioflocculant-producing bacteria

All bacterial isolates were inoculated in 100 mL of minimal salt medium (MSM) made of g/L Glucose (20), K_2HPO_4 (5), KH_2PO_4 (2), Yeast extract (0.5) $MgSO_4 \cdot 7H_2O$ (0.2), $(NH_4)_2SO_4$ (0.2), Urea (0.5) and NaCl (0.1), the flasks incubated in a shaking incubator at 150 rpm at 37◦C for 3 days. The fermented broth was centrifuged for 15 min at 4000 rpm to separate the cells, and each isolate's cell-free supernatant was utilized to examine the flocculating activity (Mathias et al. 2017).

Flocculating activity

According to the method described by More et al. (2015), the flocculating activity was evaluated by adding 2 mL of the supernatants and 3 mL of 1% $CaCl_2$ to 95 mL of kaolin solution (3 g/L). The mixture was shaken by vortex for 1 min and the mixture was poured into the measuring cylinders and left to stand for 5 min at room temperature, the supernatant's top layer (2 mL) was collected and their optical density (OD) was read at 550 nm using a spectrophotometer.

Following the calculation of the percentage flocculating activity (% FA), equation:

$$\%FA = \frac{(A1-A2)}{A1} \times 100\ \%,$$

Where:

A1: where A represents the control's optical density (OD 550 nm) measured at 550 (nm).

A2: represents a sample's optical density as measured at 550 nm (OD 550 nm).

Identification of bacterial isolates by 16S rDNA

The isolated bacteria were characterized by adopting an analysis 16S rDNA gene. The PrestoTM Mini g DNA bacteria kit from the Geneaid company was used to isolate the bacterial DNA. The polymerase chain reaction was used to amplify the 16S rDNA gene using primers 27F (5-AGAGTTTGATCCTGGCTCAG3) and 1492R (5-GGTTACCTTGTTACGACTT-3). The PCR program for amplifying the target 16S rDNA gene was an initial denaturation of 96°C for 3 min, 27 cycles including 96°C for 30s, annealing 56ºC for 25 s and elongation temperature at 72°C for 15 s and final elongation at 72°C for 10 min (Miyoshi et al. 2005).

Table 1. Types and sites of collected samples

Type of sample	No. of samples	Site of samples	Samples code
Wastewater	3	Hamden wastewater treatment plant	A B C
Soil	1	The Rumelia	D
Wastewater	1	Al-Dair treatment plant	E

The purification and sequencing of PCR products were performed by Macrogen company (South Korea). The proofreading of the obtained 16S rDNA gene sequences was conducted by utilizing chromas, the sequences were compared with NCBI nucleotide sequences using BLAST tools to determine the sequence similarity. The phylogenetic tree was created by using MEGA X. The sequences were aligned using the Clustal W program in MEGA X software (Kumar et al. 2018).

Improvement of the production of bioflocculants by screening of cultural conditions

To improve and increase the yields of bioflocculants and flocculating activity, experiments were conducted to determine the impact of various cultural conditions (carbon source, nitrogen source, pH, and inoculum sizes) on the growth and capacity of the chosen bacteria to produce bioflocculants. The best source of carbon for the formation of bioflocculants was determined using a variety of carbon sources, including (glucose, maltose, lactose, and starch). Each of these sources was added to the production medium (20 g). The optimal source of carbon was determined and then employed in further experiments (Liu et al. 2010). Three nitrogen sources NH_3Cl, NH_4NO_3 and $(NH_4)_2SO_4$ were added to the production medium to find the best nitrogen source for the production of the bioflocculants. Each of these sources was added to the production medium. The optimal nitrogen source was determined and then employed in further experiments **(**Ugbenyen et al. 2018). Prior to sterilization and inoculation, the pH of the production medium was modified using (1M) NaOH and (1M) HCl, adjusted with several pH values (4, 7 and 9) to establish the bioflocculants production. The optimal pH values were determined and then employed in further experiments (Adebami et al. 2013)**.** Using broth cultures ranging from (1, 2, 3, 4 and 5%). The optimum inoculum size was determined. The inoculum size was determined and then employed in further experiments (Abu Tawila et al. 2018).

RESULTS AND DISCUSSION

Isolation of bioflocculants-producing bacteria

Five samples from different sources were used in the current study to isolate the bacteria, from the collected samples, 21 bacterial isolates were obtained using the enrichment culture method. The Gram staining of bacterial isolates was recorded 14 (66.66%) Gram-negative bacteria and 7 (33.33%) Gram-positive bacteria. The distribution of bacteria in each sample was reported as 5 bacterial isolates from the A sample (5 -ve), 3 from B sample (1 +ve and 2 -ve), 5 from C sample (3 +ve and 2-ve), 4 from D sample (2 +ve and 2 -ve) and 4 from E sample (3 –ve and 1 +ve) as shown in Table 2.

Screening of bioflocculants-producing bacteria

Twenty-one bacterial isolates were screened for bioflocculants production employing the method of kaolin suspension. As it was shown in Table 3, the flocculating activity of all isolates ranged from 5.86% to 87.80%. Eleven bacterial isolates including D4, A5, B1, C3, A2, A1, B2, E1, D3, D2 and C2 showed flocculating activity values more than 50 %, they were 87.80 %, 81.38 %, 80.13 %, 80 %, 78.50 %, 70.62 %, 70.1 %, 60.97 %, 60.95 %, 58.80 % and 52.53 % respectively.

Table 2. Morphological and Gram's staining characteristics of bacterial isolates

Samples	Isolates code	Gram's staining	Cell shape	Percentage of Gr+ve	Percentage of Gr-ve
A	A1	Gr–ve	Rod	0%	100%
	A2	Gr–ve	Short rod		
	A3	Gr-ve	Rod		
	A4	Gr-ve	Rod		
	A5	Gr-ve	Rod		
B	B1	Gr+ve	Rod	33.33%	66.66%
	B2	Gr-ve	Rod		
	B3	Gr-ve	Rod		
C	C1	Gr+ve	Short rod	60%	40%
	C2	Gr+ve	Rod		
	C3	Gr+ve	Rod		
	C4	Gr–ve	Short rod		
	C5	Gr–ve	Rod		
D	D1	Gr+ve	Rod	50%	50%
	D2	Gr–ve	Short rod		
	D3	Gr-ve	Rod		
	D4	Gr+ve	Short rod		
E	E1	Gr–ve	Short rod	25%	75%
	E2	Gr–ve	Short rod		
	E3	Gr–ve	Rod		
	E4	Gr+ve	Rod		

Table 3. Screening of bioflocculants-producing bacteria

Samples	Isolates	Flocculating activity %
A	A1	70.62%
	A2	78.50
	A3	17.89
	A4	40.72
	A5	81.38
B	B1	80.13
	B2	70.1
	B3	32
C	C1	11.73
	C2	52.53
	C3	80
	C4	25
	C5	17.66
D	D1	5.86
	D2	58.80
	D3	60.95
	D4	87.80
E	E1	60.97
	E2	9
	E3	28.53
	E4	32.26

D4 isolate was the most effective bioflocculants-producing bacteria with a flocculating activity value of 87.80% followed by A5 with flocculating activity value of 81.38%. Eight bacteria isolates including A4, E4, B4, E3, C4, A3, C5, C1, E2 and D1 showed flocculating activity values less than 50%, they were 40.72%, 32.26%, 32%, 28.53%, 25%, 17.89%, 17.66%, 11.73%, 9% and 5.86% respectively. D1 isolate was a less effective bioflocculants-producing bacteria with a flocculating activity value of 5.86 %.

Identification of bacterial isolates by 16S rDNA

The PCR results of the 16S rDNA gene of all bacterial isolates were seen by electrophoresis under a UV transilluminator at the position of nearly 1500 bp in comparison with the DNA ladder. The bacterial isolates were characterized by sequencing and analyzing the 16S rDNA gene. The 16S rDNA gene sequences of all the bacterial isolates showed that these isolates at genus level as shown in Table 4 belongs to species of *Bacillus* (3 isolates), *Escherichia* (3 isolates), *Stutzerimonas* (3 isolate), *Vibrio* (2 isolate), *Exiguobacterium* (2 isolate), *Cronobacter* (1 isolate), *Aeromonas* (1 isolate), *Mesobacillus* (1 isolate), *Pseudomonas* (1 isolate), *Hydrogenophaga* (1 isolate), *Alishewanella* (1 isolate) and *Arcobacter* (1 isolate).

Four bacterial isolates (A1 and A4 from A sample, C3 from C sample and E2 from E sample) were characterized and reported as new bacterial strains and their sequences were recorded at the National Center for Biotechnical Information (NCBI) with accession numbers as shown in Table 5.

The phylogenetic tree was created by using MEGA X based on partial 16S rDNA sequences of bacterial isolates got in the current study to determine the relationship and evolution among them. The analysis of the phylogenetic tree showed Gram-negative bacteria, which are isolated from different sites in several groups, as shown in Figure 1 according to the similarity and relationships among them. Group 1 contained strains belonging to *Escherichia coli,* 1 strain belonging to *Cronobacter malonaticus and* 1 strain belonging to *Aeromonas simiae.* Group 2 contained strains belonging to *Vibrio cholerae*. Group 3 contained strains belonging to *Pseudomonas luteola* and *Stutzerimonas balearica.* Another group contained strains belonging to *Arcobacter cloacae, Hydrogenophaga temperata* and *Alishewanella fetalis.* Gram-positive bacteria appeared in two groups. Group 1 contained 2 strains belonging to *Exiguobacterium profundum,* while group 2 contained strains belonging to *Bacillus jeotgali, Mesobacillus persicus, Bacillus foraminis and Bacillus halotolerans*.

Improvement of the production of bioflocculants by the screening of cultural conditions

In the current study, various variables were assessed to determine the ideal conditions for bioflocculant yields. Different carbon sources have been investigated for the production of bioflocculants. The results shown in Table 6 exhibited that glucose was the best carbon source for bioflocculant production by *A. simiae* and *E. profundum* isolates with flocculant activity at 81.5% and 88.21% respectively. Whereas starch proved to be the poorest carbon source for bioflocculant production with flocculant activity at 0%.

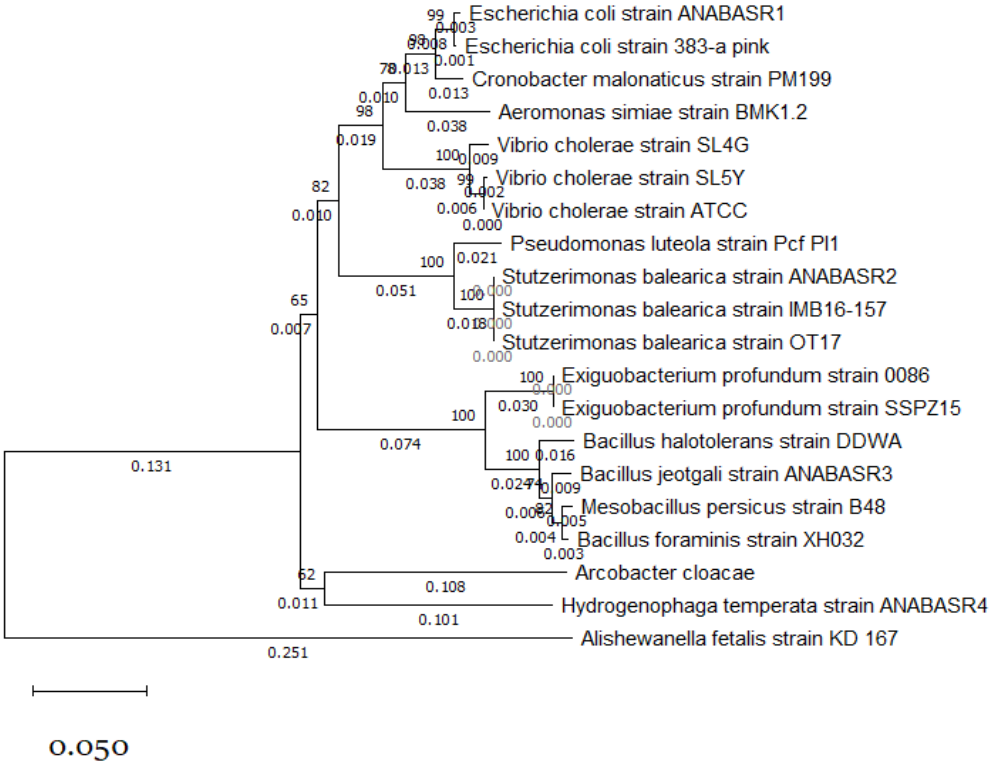

Figure 1. Neighbor-joining phylogenetic reconstruction tree showing evolutionary relationships of bioflocculants-producing bacteria

Table 4. Bacterial identification by 16S rDNA gene sequence, isolates code and the identical to the type strains of NCBI

Isolates code	Closet species	Accession number	Identity %
A1	*Escherichia coli* IRQBAS57	LC428294.1	99.86
A2	*Vibrio cholerae* strain SL4G	CP053796.1	100
A3	*Arcobacter cloacae* strain F26	HE565361.1	100
A4	*Stutzerimonas balearica* strain OT17	MH016374.1	99.91
A5	*Aeromonas simiae* strain BMK1.2	KU244021.1	100
B1	Fail		
B2	*Cronobacter malonaticus* strain PM199	JX307664.1	100
B3	*Stutzerimonas balearica* strain IMB16-157	MG190753.1	100
C1	*Exiguobacterium profundum* strain 0086	KP236222.1	100
C2	*Mesobacillus persicus* strain B48	NR_109140.1	100
C3	*Bacillus jeotgali* strain OdysseyP18	MT487594.1	99.9
C4	*Escherichia coli* strain 84BDN16	MH725681.1	99.25
C5	*Stutzerimonas balearica* strain OT17	MH016374.1	100
D1	*Bacillus foraminis* strain XH032	KF424730.1	100
D2	*Escherichia coli* strain 383-a pink	MN208212.1	100
D3	*Pseudomonas luteola* strain Pcf_Pl1	MT845202.1	100
D4	*Exiguobacterium profundum* strain SSPZ15	MT353657.1	100
E1	*Vibrio cholerae* strain SL5Y	CP053798.1	100
E2	*Hydrogenophaga temperata* strain TR7-01	NR_132598.1	99.28
E3	*Alishewanella fetalis* strain KD 167	MN809397.1	100
E4	*Bacillus halotolerans* strain DDWA	MK537363.1	100

Table 5. The bacterial isolates were recorded as new bacterial strains

Samples	Isolates code	New bacterial strains	Sequence identity (%)	Accession no. of new strain
A	A1	*Escherichia coli* strain ANABASR1	99.86%	OQ848055
A	A4	*Stutzerimonas balearica* strain ANABASR2	99.91%	OQ848056
C	C3	*Bacillus jeotgali* strain ANABASR3	99.9%	OQ848057
E	E2	*Hydrogenophaga temperata* strain ANABASR4	99.28%	OQ848058

Three nitrogen sources including $(NH_4)_2SO_4$, NH_4Cl_2 and $(NH_4)_2SO_4$ were investigated for bioflocculants production. The results as in Table 6 showed that $(NH_4)_2SO_4$ was the best nitrogen source for bioflocculant production with flocculant activity at 80.32% and 86.54% for *A. simiae* and *E. profundum* isolates respectively, whereas NH_4NO_3 was the poorest source of nitrogen for bioflocculants production by *A. simiae* isolate with value 37.27% and NH_3Cl was the poorest source of nitrogen for bioflocculants production by *E. profundum* isolate with value 35.24%.

Different pH values (4, 7 and 9) were investigated in the production of bioflocculant. The results shown in Table 6 exhibited that the optimum pH for bioflocculant production was 7 with flocculating activity values of 81.76% and 87.6% for *A. simiae* and *E. profundum* isolates respectively, whereas 9 was the poorest source of nitrogen for bioflocculants production with flocculating activity values of 0% and 0.44% for *A. simiae* and *E. profundum* isolates respectively.

The impact of inoculum size on the production of bioflocculant was investigated and introduced in Table 6. Maximum flocculating activity values were reported at an inoculum size of 5% with values of 88% and 84.49% for *A. simiae* and *E. profundum* isolates respectively.

Table 6. Effect of various parameters on bioflocculants production by *Aeromonas simiae* and *Exiguobacterium profundum*

Parameters	Flocculating activity (%)	
	***Aeromonas simiae* isolate**	***Exiguobacterium profundum* isolate**
Carbon sources		
Glucose	81.5	88.21
Lactose	57.61	69.87
Maltose	36.10	82.78
Starch	0	0
Nitrogen sources		
NH_3Cl	38.09	35.24
NH_4NO_3	37.27	74.79
$(NH_4)_2SO_4$	80.32	86.54
pH values		
4	19.64	40.06
7	81.76	87.6
9	0	0.44
Inoculum size (%)		
1	19.78	35.43
2	38.84	39.17
3	50	47.30
4	57.30	64.56
5	84.49	88

Discussion

The present study aimed to isolate bioflocculant producing bacteria from different samples including wastewater samples and petroleum-contaminated soil samples**.** Many environments such as rivers, marine systems, soil, activated sludge and effluent can be sources for Many microorganisms that produce bioflocculants, including bacteria, which have the capacity to produce extracellular polymers that are environmentally benign and function as bioflocculants (Salehizadeh et al. 2003).

The microbial bioflocculants are important substances that have a wide range of applications, including the removal of heavy metals and pollutants from industrial wastewater (Dih et al. 2019), suspended solids (Dlangamandla et al. 2018), dyes (Abbas et al. 2020) and turbidity (Buthelezi et al. 2009). Most of the bioflocculant demonstrated a notable increase in turbidity removal as compared to the control reactor, indicating the bioflocculant's positive contribution to turbidity removal (Agunbiade et al. 2017). The screening test used in the current study was flocculating activity a quick and simple way to screen and predict bioflocculants production. Where, as producers of bioflocculants, the bacterial isolates with the highest positive screening test results were chosen. Metal ions are also believed to affect the flocculating activity of bioflocculants, this is because the addition of cations decreases the negative ions in kaolin particles and biopolymer flocculants (Liu et al. 2023).

The 16S rDNA gene is used to characterize the isolates to species level and is considered a good tool for bacterial identification due to its presence in all bacteria, the function of the 16S rDNA gene has a consistent function over time and the 16S rDNA gene length is suitable (Al-Dhabaan 2019; Alyousif 2022). In the current study, four bacterial isolates were characterized as new bacterial strains and their sequences were recorded at the National Center for Biotechnical Information (NCBI). The emergence of new bacterial isolates is attributed to a mutation resulting from variables and chemical mutagens because bacteria exposed to altered environments lose the capacity to repair DNA damage, which becomes inherited (Ilmjärv et al. 2017).

Similar to bacterial species obtained in the current study reported to produce bioflocculants with high flocculating activity in previous studies (Li et al. 2007; Kasan et al. 2015). *Exiguobacterium profundum* isolate was the most effective bioflocculants-producing bacteria with a flocculating activity value of 87.80% followed by *A. simiae* with flocculating activity value of 81.38%. The production of bioflocculant from *Ochrobactrum oryzae* reported a yield of 3.768 g/L with the flocculating activity of 92% were obtained under optimum conditions when a (1% (v/v) inoculum size, starch as carbon source, yeast extract as nitrogen source, pH=7, 30◦C, and after 72 h of cultivation (Selepe et al. 2022).

The optimization of culture conditions of medium composition led to enhanced bioflocculant production by the bacterial isolates, these factors and conditions include carbon sources, nitrogen sources, temperature, inoculum size and initial pH. In the current study, flocculating activity values were reported 88% and 84.49% for *E. profundum A. simiae* isolates respectively under optimum conditions. Carbon sources are a significant material to support microbial growth and supply energy for growth, reproduction and bioflocculant production. In the current study, the highest flocculating activity was reported by glucose as a carbon source. The current study agrees with previous studies where glucose was reported as the best source for bioflocculant production by *Proteus mirabilis* and *Bacillus* sp. (Xia et al. 2008; Cosa et al. 2013).

The highest flocculating activity was reported with the utilization of $(NH_4)_2SO_4$ as a nitrogen source by test bacteria in the current study. Nitrogen sources provide the necessary material for synthesizing microbial enzymes (Luo et al. 2016). Nitrogen source requirement differs with different bacterial strains, some of them prefer organic sources and others prefer inorganic sources. Abdel-Aziz et al. (2011) reported that the bacteria *Bacillus alvei* NRC-14 prefers $(NH_4)_2SO_4$ as the best nitrogen source. The optimum pH for bioflocculant production was observed at the neutral pH (7). The pH of the medium affects the bacterial cells' electric charge and the oxidation-reduction potential, which can also have affected enzymatic reactions.

The electrostatic charge of the bioflocculant and suspended particles varies depending on the pH value, which affects how well kaolin clay particles bridge, thus affecting the bridging efficiency for kaolin clay particles (Okaiyeto et al. 2013). The flocculating activity is poor at the acidic value could be attributed to the excessive concentration of hydrogen ions that alters the electric charge (Agunbiade et al. 2018). The inoculum size is an important factor that improves the flocculating activity of bacteria. The maximum flocculating activity of 84.49% and 88% for *A. simiae* and *E. profundum* were reported at an inoculum size of 5% (v/v) (Table 6). The flocculating activity was low at 1% inoculum size due to that the isolates might have had an extended lag phase, consequently delaying the formation of the bioflocculant (Makapela et al. 2016).

In conclusions, many different environments can serve as sources for producers of bioflocculants, where 21 different bacteria are isolated from wastewater and soil. Eleven bacterial isolates showed flocculating activity values of more than 50%. Two bacterial isolates were reported as the best bioflocculants-producing isolates with a flocculating activity value of 87.80% and 81.38% respectively, these two isolates belonged to *A. simiae* and *E. profundum* which were identified by 16S rDNA gene sequencing. Four bacterial isolates were recorded as new strains in NCBI GenBank. The maximum flocculating activity of 84.49% and 88% were reported for *A. simiae* and *E. profundum* respectively under optimum cultural conditions (glucose as carbon source, $(NH_4)_2SO_4$ as nitrogen source, pH= 7 and 5% inoculum size).

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