JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Isolation, Characterization and Optimization of Bioflocculant Producing Bacteria from the Aquaculture Ponds

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***Abstract*:** A bioflocculant producing slightly mucoid bacterium *Bacillus* species (BF9) has been isolated from shrimp pond water. This study represents the isolation of bioflocculant producing microorganisms from diverse sources. Three isolates BF6, BF8, and BF9 shows bioflocculant activity out of 20 studied samples, among them BF9 represented the highest bioflocculant activity and was selected for subsequent study. Isolate BF9's protein and carbohydrate composition has been determined. Various bioflocculant concerning parameters such as carbon, nitrogen, pH, temperature, and cation has been optimized by utilizing kaolin as suspended particle via flocculation assay. Glucose and ammonium chloride are confirmed to be a good sources of carbon and nitrogen. Among the other cations, ferric chloride had a significant influence, enhancing the flocculation rate of BF9 by 83%. At pH 8, isolate BF9 had an 86.24% flocculation activity. At a temperature of 30°C, the flocculation activity of BF9 was reported to be 78.8%. The optical density of flocculating activity has been measured using a Kaolin clay assay.

Keyword: Bioflocculant, Extracellular Polymeric Substances, Flocculation, Shrimp Pond Water.

I. Introduction

The rapid growth of industry with regular human activities has led to massive increases in the amount of waste and wastewater comprising organic and inorganic contaminants discharged into the environment (Agunbiade, Pohl, & Ashafa, 2016). The formation of floc initiates due to the aggregation of colloid particles facilitated by flocculants via the process of flocculation. Flocculant is categorized into three groups: Organic flocculants includes polyethylene imine and polyacrylamide derivatives, Inorganic flocculants like polyaluminium chloride and aluminium sulfate and naturally occurring flocculants such as sodium alginate, chitosan, and microbial flocculants. Although chemical flocculants are low cost with effective flocculation resulted in some environmental and health issues (Xia et al., 2008). Since chemical flocculants are harmful to the environment, therefore, the need for safer and eco-friendly alternative sources is necessary. Microbial flocculants could be utilized as an alternative to chemical flocculants.

Fungi, bacteria, and yeast are prominent sources of bioflocculant producers (Mathias, Hammantola, & Ishaku, 2017). Some of the reported bioflocculant producing microorganism are *Bacillus* sp., *Pseudomonas alcaligenes, Citrobactor* sp., *Enterobacter cloacae*, *Halomonas* sp. and *Klebsiella pneumonia* (Shahadat et al., 2017). In bioflocculation, flocculation activity is facilitated by the microorganism. Bioflocculants (microbial flocculants) produced during the cell growth of microorganisms comprise macromolecular polymers such as cellulose, proteins, glycoproteins, polysaccharides, and nucleic acids. Bioflocculant are advantageous for biota as they are devoid of toxins, biodegradable, cost-effective, and harmless (Azmi et al., 2015; Gao et al., 2006). Flocculation is a process of charge destabilization and bridging generated by polymeric substances of microorganisms. It has been considered as an essential parameter to eliminate suspended solids from industrial and domestic wastewater. Source of bioflocculant producing organisms are considered as crude oil (Zaki et al., 2011), activated sludge (Li, Xing, Ma, & Pan, 2015), soil (Jiang et al., 2015), as well as lignocellulosic biomass (Guo et al., 2017). To quantify flocculation activity colour removal, turbidity and chemical oxygen demand are significant parameters.

The organic and inorganic impurities released by industries such as dyeing, paints, papers, tanning, textiles, and pharmaceuticals are responsible for water contamination and deterioration of the environment. The release of untreated industrial effluent into aquatic systems enhances Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) ultimately adversely affecting the natural environment (Verma, Dash, & Bhunia, 2012). Therefore, the elimination of pollutants becomes an essential step before discharging the contaminated effluents into the water system. Treatment of industrial effluent can be achieved via several methods such as electrocoagulation, mixed ion exchange materials, modified bentonite, activated carbon, and

membrane filtration. To manage industrial effluents, these treatment technologies play a crucial role but the huge amount of sludge generation and cost associated with treatment technologies are drawbacks.

Optimized *Bacillus megaterium* isolated from pond water bio-floc utilizing ideal carbon and nitrogen source from glucose and beef extract, respectively for flocculation activity (Luo et al., 2016). This study represents bioflocculant-producing bacteria isolated from different sources such as shrimp pond water, fishpond water, dosa batter and meat. Isolate BF6, BF8, and BF9 out of the 20 exhibited optimum flocculant activity. Among three, isolate BF9 was selected for optimization study via different parameters like temperature, pH, cations, carbon-nitrogen sources respectively using kaolin clay assay. The flocculation activity was measured with the help of optical density.

II. Material and method

2.1 Collection of Samples

The sample collection ponds were selected from Kheda and Surat districts of central and southern Gujarat - India respectively. Sample of shrimp pond water (*Lipotenius vannamei*) and fishpond water was collected from Dumas and Masra, as shown in Figure 1. Autoclaved sterile bottles were used for storage of samples collected from different sites. In addition, meat, milk, dosa batter, and *Bacillus pumilus* (MTCC-8373) were also selected for measuring bioflocculant activity.

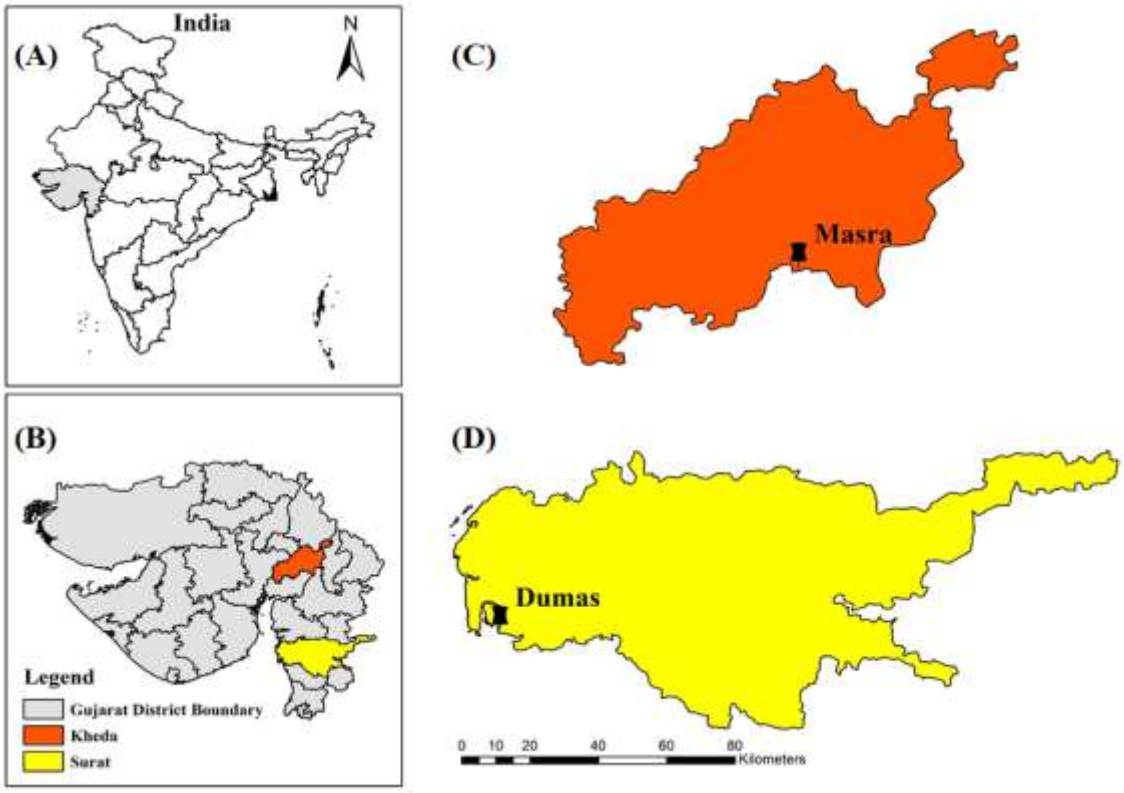

Fig 1. (A) Map of India, (B) Map of Gujarat with study sites, (C) Kheda, (D) Surat

2.2 Media and Cultivation Condition

Isolation and cultivation of flocculants producing bacteria were conducted using Marine agar. Marine agar contains (per liter) 5g of Peptone, 0.1g of Ferric citrate, 1g of Yeast extract, 8.8g of Magnesium chloride, 19.4g of Sodium chloride, 1.8g of Calcium chloride, 3.240g of Sodium sulfate, 0.16g of Sodium bicarbonate, 0.5g of Potassium chloride, 0.022g of Boric acid, 0.034g of Strontium chloride, 0.080g of Potassium bromide, 0.004g of Sodium silicate, 0.001g of Ammonium nitrate, 0.002g of Sodium flourate, 0.008g of Disodium phosphate, 15g of Agar, Final pH (at 25°C) 7.6 ± 0.2 (Zaki et al., 2011).

Bioflocculant producing bacteria screening was carried out using yeast peptone glucose (YPG) which contained 15g agar-agar powder, 20g poly-peptone, 20g D(+)- Glucose, and 10g yeast extract powder in 1L at pH 7 using 1M hydrochloric acid and 1M sodium hydroxide and incubated at 35°C for 48 hours (Harun et al., 2017).

The bacteria were cultured in an enrichment medium, prepared as a seeding medium which contains 5g of K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2g of Peptone, 0.2g of KH_2PO_4, 0.5 g urea, 10 g glucose, and 0.5g of yeast extract in 1 L of filtered seawater at pH 7 using 1 M hydrochloric and 1M sodium hydroxide incubated at 35°C for 48 h using an incubator shaker at 120 revolutions per minute (rpm) for three days Using a method derived from Zhang et al., (2012) and modified by Cosa et al., (2011). The cell-free supernatant was utilized for flocculation after centrifuging the culture broth at 4000 rpm for 30 minutes.

To compare flocculation activity with enrichment media (EM) the bioflocculant production broth (BPB) was used. Bioflocculant production broth containing 2g KH_2PO_4, and 0.5 g yeast extract, 10g glucose, 0.5g $CaCO_3$, 0.1g NaCl, and 0.2g $MgSO_4.7H_2O$ mixed in 1L deionized water with the 7.0 pH. The best suitable media resulted from the comparison study will be used for further investigation.

2.3 Screening and Enrichment of Bioflocculant Producing Bacteria

Loopful of sample streaked on marine agar plates via four flame method under sterile condition. Isolates based on colony morphology were screened on marine agar. Pure colonies were re-streaked onto yeast peptone glucose (YPG) agar plates for determination of colonies having mucoid morphological characteristics. Further, isolates with mucoid morphology were transferred to enrichment media and incubated for 3 days at 35ºC, 120rpm.

2.4 Purification of Bioflocculant Producing Isolate

The bioflocculant producing isolate was purified according to the procedure described (Adebami, Adebayo-Tayo, & Akinyugha, 2017) and modified as follows; isolates were grown in a 250ml Erlenmeyer flask which having enrichment media broth at 25ºC on the rotatory shaker (120rpm) for 24h. Centrifuge 10ml of broth after incubation at 4000 rpm for 30 minutes at 4ºC and supernatant was used to estimate flocculating activity using Kaolin assay.

2.5 Flocculating Activity Assay

For checking flocculation activity, (Kurane et al., 1994) and modified method (Gong et al., 2008) was applied. For the Kaolin clay assay, the stock solution was made by mixing kaolin clay with distilled water at a 5g/l at pH 7. Now mix 9 ml kaolin clay suspension, 0.1 ml culture supernatant and 0.25 ml 1% $CaCl_2$. For the reference tube, culture was replaced with distilled water. Make the final volume of all test tubes 10 ml with distilled water and mix the sample gently. Allow the solution to settle for 5 min at room temperature. Take optical density (OD) of upper phase at 550nm with a UV spectrophotometer and flocculation activity calculated as follows:

$$\text{Flocculating rate}(\%) = \left[\frac{b-a}{b}\right] \times 100$$

Here, a & b are optical densities at 550nm of the sample and control, respectively.

2.6 Chemical Analyses of Bioflocculant

The sugar content was determined by the phenol-sulfuric method; glucose was used as a standard solution for the reference (Chaplin and Kennedy, 1986). The total protein content of the sample was determined in which the reference solution of bovine serum albumin was used (Lowry et al., 1951).

2.7 Morphological and phenotypic Classification of Bioflocculant Producing Bacteria

Microscopic examinations employing the Gram staining method revealed the morphological properties of bioflocculant producing bacteria. To determine the taxonomy of isolated bioflocculant producing microbes, morphological characterization was performed in accordance with Bergey's Manual of Systematic Bacteriology that included descriptions and pictures of species as well as tests to differentiate between genera and species (Sneath et al., 1986).

2.8 Optimization of Cultural Parameters for Bioflocculant Production

Enrichment medium inoculated with freshly cultured isolate and incubated for 24 h at 120rpm with different concerning parameters. Enrichment media supplemented with different like carbon, nitrogen, cation sources, and temperature - pH ranges were applied to check bioflocculant activity.

III. Results and discussion

3.1 Collection of Samples for Bioflocculant Producers

For isolating flocculant-producing microorganisms water, aquaculture, activated sludge, and soil samples were noted to be the noble source (Ugbenyen, Simonis, & Basson, 2018). Shrimp pond water, fishpond water, meat, milk, dosa batter, and *Bacillus pumilus* (MTCC-8373) were used to isolate bioflocculant-producing bacteria. As shown in Table 1 out of 20 bacterial isolates, 10 (50%) isolates isolated from shrimp pond water, 3 (15%) isolates from fish pond water, 3 (15%) isolated from milk, 2 (10%) isolated from meat, 1 (5%) isolated from dosa batter and 1(5%) obtained from MTCC (Microbial Type Culture Collection and Gene Bank).

Table 1 Bacterial isolates with their respective sources

Source	Isolates	Numbers of isolates
Shrimp pond water	BF6, BF7, BF8, BF9, BF14, BF15, BF16, BF17, BF18, BF19	10
Fishpond water	BF1, BF2, BF3	3
Bacillus pumilus (MTCC-8373)	BF4	1
Meat	BF5, BF10	2
Dosa batter	BF13	1
Milk	BF11, BF12, BF20	3

3.2 Checking of Flocculating Activity of Bacterial Isolates

The flocculant activity of bacterial isolates was assessed by comparing two different media to see which one had the best flocculant activity. Only one medium was chosen for further investigation. The growth of flocculant-producing bacteria is aided by EM (enrichment media) that comprises glucose as the sole carbon source, salts such as $MgSO_47H_2O$, K_2HPO_4, KH_2PO_4, and peptone as the nitrogen source. BPB (Bioflocculant producing broth) on the other hand, comprises glucose and yeast extract as carbon and nitrogen sources, as well as salts such as $MgSO_47H_2O$, NaCl, and $CaCO_3$. In comparison to bioflocculant production broth, isolates BF6, BF8, and BF9 demonstrated better bioflocculant activity in enrichment media as shown in Table 2. The isolate BF9 that had the maximum flocculant activity in enrichment media was chosen for further research.

Table 2 Effect of different media on flocculation activity (%)

Isolates	Enrichment media (%)	Bioflocculant production broth (%)
BF1	68.4	-
BF2	60.6	-
BF3	60	-
BF4	62.8	-
BF5	61	-
BF6	69	-
BF7	51.8	34.6
BF8	80	25.4
BF9	83	44.2
BF10	-	-
BF11	-	5.4
BF12	-	10.4
BF13	-	-
BF14	23.8	12
BF15	25.2	-
BF16	-	-
BF17	17.8	-
BF18	-	-
BF19	31	9
BF20	11.8	-

3.3 Chemical Analyses of Bioflocculant

3.3.1 *Protein Estimation by Lowry's Method*

Folin-Lowry method was used to calculate the protein content of BF9. The protein content was calculated using bovine serum albumin as a standard and the bioflocculant BF9 had a protein concentration of 2.81 mg/ml, as shown in Figure 2(A) and Figure 2(B) respectively. Any intracellular and extracellular proteins involved in bioflocculant action have charged anions with weak carboxylic groups on their surfaces (Hess & Van Der Vegt, 2009).

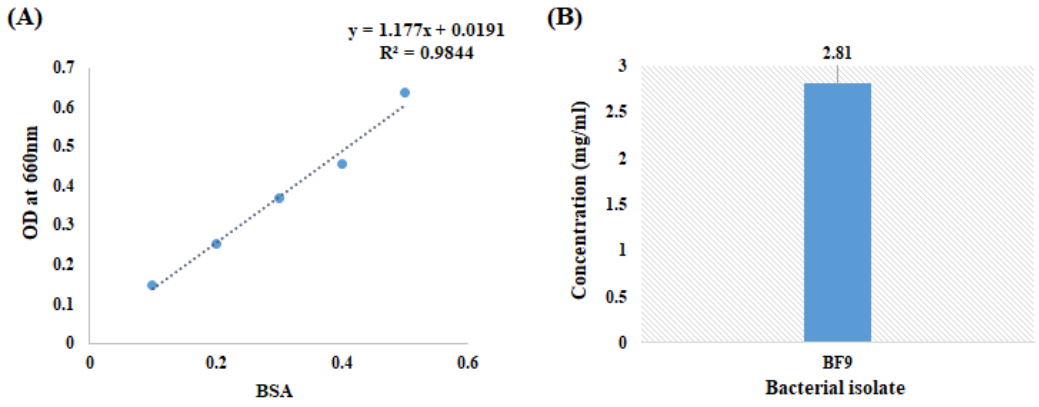

Fig 2. (A) Standard graph of Bovine serum albumin and (B) Protein concertation of isolate BF9

3.3.2 Phenol Sulphuric Acid Method for Total Carbohydrate

The phenol sulphuric acid method was used to determine the total carbohydrate concentration of isolate BF9. As shown in Figure 3(A), the amount of total carbohydrate concentration was calculated using glucose as the standard. The findings of the phenol sulphuric acid technique revealed that BF9 has a carbohydrate concentration of 1.86 mg/ml as shown in Figure

3(B). Several flocculants have been observed to include a large amount of polysaccharide backbone, such as the bioflocculant synthesized by *Enterobacter aerogenes*, the bioflocculant produced by *Virgibacillus* sp. and the bioflocculant MBF-5 produced by *Klebsiella pneumonia* (Lu et al., 2005; Zhao, Liu, & Zhou, 2013).

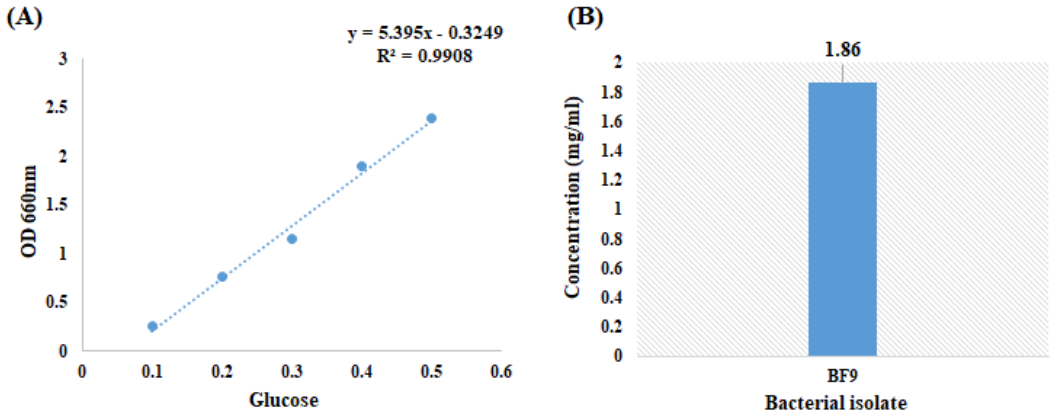

Fig 3. (A) Standard graph of Glucose and (B) Total carbohydrate concentration of isolate BF9

3.3.3 Morphological and Phenotypic Classification of Bioflocculant Producing Bacteria

To identify the isolate, morphological, phenotypic categorization, and several biochemical tests were used. BF9 was confirmed as a Gram-positive *Bacillus* sp. at the Genus level as shown in Table 3 and Table 4. *Bacillus licheniformis, a Bacillus* sp. isolated from contaminated LB medium, were identified using 16S rRNA gene sequencing and biochemical/physiological data (Xiong et al., 2010).

Table 3 Colony and morphological characteristics of isolate BF9

Characteristics	BF9
Size	Large
Shape	Round
Pigmentation	White
Margin	Entire
Elevation	Convex
Surface	Smooth
Optical appearance	Opaque
Motility	Motile
Morphology	Rod shaped
Gram's nature	Gram-positive

Table 4 Biochemical Analysis of isolate BF9

Tests	BF9
Indole production	+
Methyl red (MR)	-
Voges-Proskauer	-
Citrate utilization	+
Triple sugar iron agar (TSI)	+
Carbohydrate fermentation 1.Glucose 2.Sucrose 3.Fructose 4.Galactose 5.Lactose	 + + + + +
Ammonia production	-
Catalase	-
Casein hydrolysis	+
Starch hydrolysis	+
Hydrogen sulfide production	-

Gelatine hydrolysis	+
Phenylalanine deamination	-
Urea hydrolysis	-
Dehydrogenase	+
Motility	Motile
Grams staining	+
Genus	*Bacillus* species

Here: Positive (+) and Negative (-).

3.4 Optimization of Culture Conditions for Bioflocculant

3.4.1 Effect of Various Carbon Sources on Bioflocculant Activity for Selected Isolates

Microbial cells use a variety of carbon sources to provide energy for reproduction, microbial growth, and motility. To test the effect of the carbon source, similar quantities of dextrose, sucrose, starch, and fructose were substituted in the enrichment media, while the other components remained unaltered. For glucose as a carbon source, isolate BF9 was able to produce an appropriate level of bioflocculant. In comparison to other carbon sources, glucose was shown to be acceptable for bioflocculant generation, with flocculating efficiency exceeding 86 % for bacterial isolate BF9 (Figure 4). It is reported that when glucose is the only carbon source, *B. licheniformis* forms extracellular polysaccharides (P. Liu, Chen, Yang, Li, & He, 2017). In the case of *Rhodococcus erythropolis*, cell elongation and bioflocculant production were caused by glucose and fructose (Cosa, Ugbenyen, Mabinya, & Okoh, 2013).

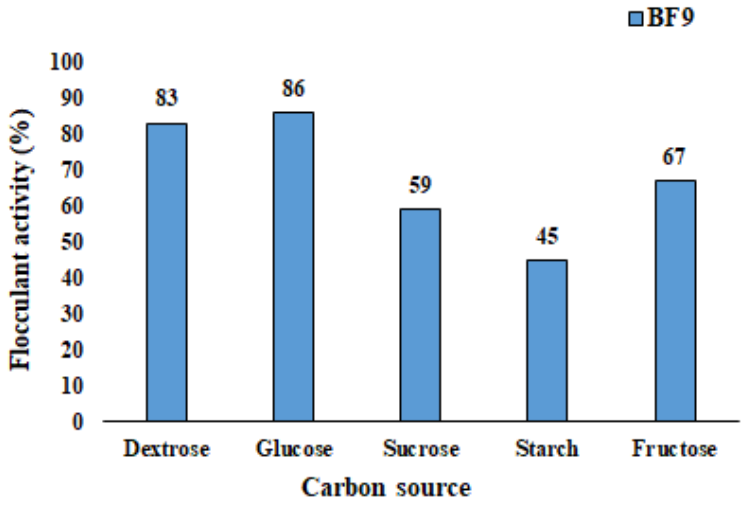

Fig 4. Effect of carbon sources on bioflocculant activity of isolate BF9

3.4.2 Effect of Various Nitrogen Sources on Bioflocculant Activity for Selected Isolates

The impact of organic and inorganic nitrogen sources on the formation of flocculants was studied. Ammonium chloride was revealed to be the best nitrogen source with the highest flocculating activity, 84% isolate BF9, while tryptone had the lowest flocculation activity of all the nitrogen sources tested (Figure 5). It is reported that the flocculating activity in an experiment with a novel intracellular bioflocculant (called MBF-W6) produced by *Chryseobacterium daeguense* W6, an yeast extract was found to be a good nitrogen source (W. J. Liu, Wang, Li, Yuan, & Yang, 2010).

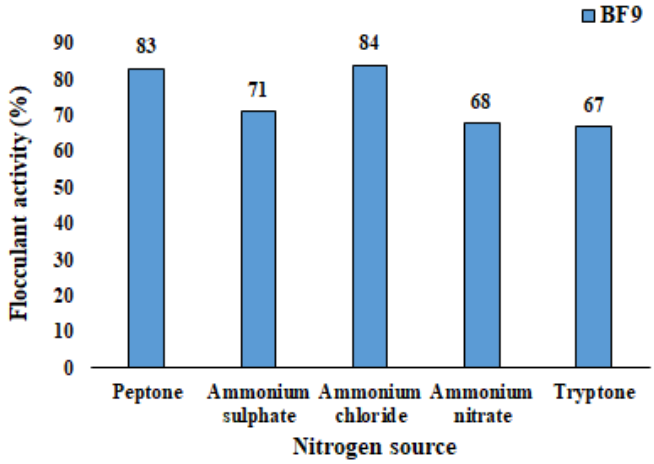

Fig 5. Effect of nitrogen source on bioflocculant activity of isolate BF9

3.4.3 Effect of Various Cations on Bioflocculant Activity for Selected Isolates

Cation improves the flocculating activity of culture broth by neutralizing and stabilizing the residual negative charge of functional groups and forming bridges between particles. The flocculating activity of isolate BF9 was studied using various cations (Figure 6). Amongst the cations tested, ferric chloride enhanced flocculating rate with the highest activity 83%. Monovalent cations did not affect bioflocculant activity when compared to divalent cations, but they did reduce floc size, density, and resistance. Divalent cations, notably Ca^{2+} and Mg^{2+}, were found to be the best cation source for enhancing flocculation by the UPMB13 bioflocculant, with the flocculating activity of 85% and above, a considerable increase of around 15% above the control (+) biofloc treatment (Zulkeflee, Aris, Shamsuddin, & Yusoff, 2012).

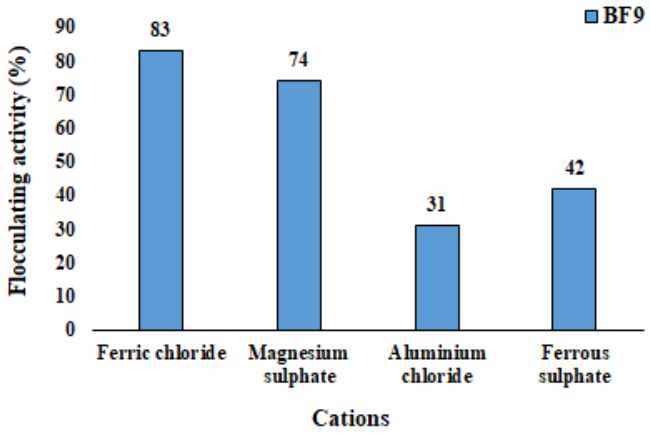

Fig 6. Effect of different cations on bioflocculant activity of isolate BF9

3.4.4 Effect of Different pH on Flocculant Activity

The initial pH of the culture can alter the electric charge of the cells, as well as the enzymatic response, nutrition assimilation, and oxidation-reduction potential of bioflocculant producers (Salehizadeh & Yan, 2014). At a pH range of 2-10, the influence of pH on bioflocculant production in Enrichment media was investigated, and maximum bioflocculant production was seen at pH 8 of isolate BF9, with flocculant activity of 86.24 %. (Figure 7). *Halomonas* sp. V3a' demonstrated 80% flocculant activity at pH 3-11, and at pH 7, maximal flocculant activity of 97% was recorded. *Bacillus megaterium* showed the highest flocculant activity at pH 9, ranging from acidic to an alkaline pH of 12 (He et al., 2010; Zheng, Ye, Fang, Li, & Cai, 2008)

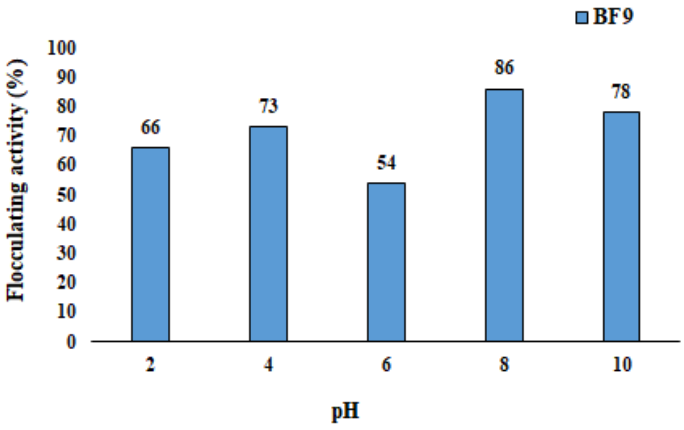

Fig 7. Effect of different pH on bioflocculant activity of isolate BF9.

3.4.5 Effect of Different Temperatures on Flocculant Activity

At various temperatures, the flocculating efficiency and temperature relationship of a bioflocculant producing isolate were examined (4º, 14º, 30º & 50ºC). At 30ºC, isolate BF9 shows 78.8% bioflocculant activity (Figure 8). After heating at 100°C for 25 minutes, the pure bioflocculant from a consortium of *Cobetia* sp. and *Bacillus* sp. retained 87% of its flocculating activity, indicating that the bioflocculant is thermally stable (Cosa & Okoh, 2014).

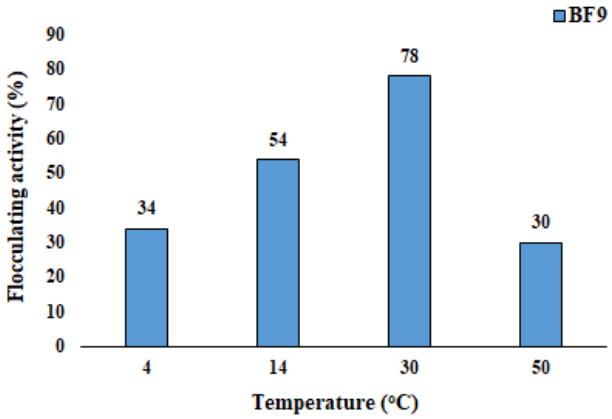

Fig 8. Effect of different temperatures on flocculation activity of isolate BF9

IV. Conclusion

In summary out of 20, isolate BF9 demonstrated the highest bioflocculant activity in enrichment media and screened for bioflocculant activity. BF9 isolate was chosen for further investigation due to its high bioflocculant activity. Biochemical analysis of BF9 revealed that the isolate belongs to *Bacillus* sp. at the Genus level using Bergey's manual of systematic bacteriology. The highest flocculation activity of the BF9 isolate was observed with glucose and ammonium chloride as a source of carbon and nitrogen. Ferric chloride represents the strongest action among other cations and enhances the flocculation rate of BF9 by 83%. At pH 8, isolate BF9 showed flocculation activity of 86.24%. The flocculation activity of BF9 was measured as 78.8% at 30ºC temperature. Isolated BF9 bacteria exhibited high flocculation activity and could be considered as better microbial flocculant in comparison to chemical flocculant.

V. Acknowledgment

Authors are thankful to Dr. P. C. Mankodi, Professor of Zoology department, at The Maharaja Sayajirao University of Baroda, Vadodara – Gujarat. The authors would like to thank the In-charge head at ARIBAS College, New Vallabh Vidhyanagar, Anand – Gujarat for providing a laboratory facility.

VI. Conflicts of Interest

The authors have no conflict of interest to declare.

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