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ISOLATION AND IDENTIFICATION OF BIOFLOCCULANT PRODUCING BACTERIA FROM SOIL SAMPLES OF TAY NGUYEN, VIETNAM

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ABSTRACT

This paper described isolation and identification of the bioflocculant producing bacteria from soil samples of Tay Nguyen region, Vietnam. A total of 117 bacteria were isolated, among of which 26 isolates showed flocculating activity more than 50%. In addition, the influence of carbon and nitrogen sources, as well as cultural conditions such as temperature, pH and incubation time on flocculating activity of the 3 selected flocculant producing isolates were investigated. Obtained results revealed that that selected isolates preferred organic nitrogen source and different carbon source for maximum flocculating activity. The suitable culture temperature and pH for their activity ranged from 30^0C-35^0C and from light acidity to light alkalinity, respectively. The incubation time for highest flocculating activity of the isolates varied from 48 h (Bx-vk22) to 72 h (Bx-f9 and Bx-vk9). The selected isolates were identified based on 16S rRNA gene sequence and the obtained results showed that the isolates Bx-f9, Bx-vk22 and Bx-vk9 belonged to three different genera, including *Bacillus, Lysinibacillus,* and *Enterobacter* respectively.

INTRODUCTION

Flocculants are chemicals that stimulate flocculation by aggregation of colloids and other suspended particles, forming a floc (Fujita *et al.*, 2000; Hubbard, 2004). A variety of flocculants, comprising of inorganic (polyaluminium chloride, aluminum sulfate), organic synthetic high-polymer (polyacrylamide, polyethylene amine) and natural flocculants or bioflocculants (gelatin, chitosan, guargum and microbial flocculants) have found widespread applications over the last decades (Salehizadeh and Shojaosadati, 2001). These flocculating agents are applied in a wide range of industrial processes, including tap water production, wastewater treatment, dredging, downstream processing, beverages as well as food and fermentation processes (Butterfield, 1935; Mckinney, 1956; Tenny and Stumm, 1965; Tenny and Verhoff, 1973; Fujita *et al.*, 2000; Gutcho, 1977; Morgan *et al*., 1990; Dube, 1992; Tong *et al.,* 1999; Nakata and Kurane, 1999; Salehizadeh and Shojaosadati, 2001; Zouboulis *et al.*, 2004, Crini, 2006; Mabinya *et al*., 2011).

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Although chemical flocculants have been popularly used because of their effective flocculating activity, low cost and versatile tailor ability (Saliehizadeh *et al.,* 2002), some synthetic flocculants are known to be hazardous to the ecosystem and environment (Master *et al.,* 1985; Dearfield and Abermathy, 1988; Kowall *et al.*, 1989). Most of high molecular weight is recalcitrant. Monomers of polyacralamide are potent carcinogen and neurotoxic to humans and other animals.

They have detrimental effect both on flora and fauna (Vanhoric and Moens, 1983; Dearfield and Ambermathy, 1988) while aluminium has been shown to cause Alzheimer's disease (Master *et al.,* 1985; Kowall *et al.*, 1989). Because of these concerns, the utilization of natural flocculants and bioflocculants is expected to develop (Li *et al.,* 2009; Liu *et al.,* 2010; Ugbenyen and Okoh, 2014). Bioflocculants are biodegradable and their products are harmless to the environment. Some of the microbial flocculants can be produced economically in large-scale culture. Furthermore, the extracellular bioflocculants can be produced at high rates and are easily recovered from the fermentation broth (Salehizadeh and Shojaosadati, 2001). Currently, microbial flocculants are considering as a promising substitute for chemical flocculants

(Ugbenyen and Okoh, 2014). Many flocculants produced by microorganisms have since been isolated from soil, activated sludge and wastewater, including algae (Kaplan *et al.,* 1987), bacteria (Butterfield, 1935; Kurane *et al.,*1966; Kakii *et al.,*1986; Huang, 1990; Fumio, 1991; Kurek *et. al.,* 1991; Hantula and Bamford, 1991a,b; Kim, 1993; Seo, 1993; Misra,1993; Cumming *et al.*, 1996; Yokoi *et al.,* 1996a; Suh *et al.,* 1997; Nakata and Kurane, 1999; Salehizadeh *et al.,* 2000, 2002; Fujita *et al.,* 2000; Mabinya *et al.,* 2011; Ugbenyen and Okoh, 2014), cyanobacteria (Fattom and Shilo, 1984; Bar-Or and Shilo,1987; Bender *et al.,* 1994), and fungi (Nakamura *et al.*, 1976a; Guirand, 1992; Sousa *et al.,* 1992). In this study, we isolated and identified the bioflocculant producing bacteria from soil samples of Tay Nguyen, Vietnam for further application in wastewater treatment and other applications.

MATERIALS AND METHODS

Isolation of bacteria from soil samples

Four soil samples were collected from various provinces of Tay Nguyen, Vietnam, and then 10g of each soil sample was put in 250mL flask and 90 mL sterile distilled water was added. The flasks were incubated on rotary shaker at 160 rpm for 15 min. 1ml sample was serially diluted up to 10^{-7}, and then 0.1 mL of diluted sample was spread on sterile production agar medium containing (per litter sterile distilled water): 10g glucose, 5g peptone, 0.3g $MgSO_4.7H_2O$, 5g K_2HPO_4, 2g KH_2PO_4, 15g agar, pH 7.0), and incubated for 2 days at 30°C. Single colonies were picked up and streaked on fresh agar plates to get pure culture and observe the morphological characterization.

Screening for bioflocculant producing bacteria

Each isolate was inoculated into a McCartney bottle containing 5 mL of liquid production medium and incubated on a shaker at 160 rpm for 2 days at 30°C. Then, the fermentation broth was centrifuged at 6000 rpm for 10 min to separate the cells, and the cell free culture supernatant was assayed for flocculating activity. Flocculating activity was determined according to the method of Kurane *et al.* (1994) as modified by Zhang *et al.* (2007). 3.0 mL of 1% $CaCl_2$ and 2.0 mL of the cell-free supernatant were added into 95 mL of kaolin suspension (4.0 g/L) in 250 mL flask. The mixture was vigorously stirred and allowed to stand for 5 min at room temperature. The optical density (OD_{550nm}) of the clarifying solution was measured at 550 nm. A control experiment was prepared in the same way but the cell-free supernatant was replaced with the un-inoculated production medium. The flocculating activity was estimated by the formula:

$$\text{Flocculating activity} = \{(A - B)/A\} \times 100\%$$

where A and B were optical densities of the control and samples respectively at 550 nm.

Effects of carbon and nitrogen sources on the flocculating activity

Effect of nutritional factors on flocculating activity, such as carbon and nitrogen sources was investigated. The bacteria were cultivated in production medium supplemented with 1% different carbon sources like Glucose, Galactose, Lactose, Mannitol, Sucrose and 0.5% different nitrogen sources such Soya bean, beef extract, yeast extract, peptone and tryptone. After 24 hours incubation at 30°C and shaking 200 rpm, the flocculating activity of isolates was determined.

Effects of cultural conditions on the flocculating activity

Influence of cultural conditions (pH, temperature, incubation time) on flocculating activity was determined by "one factor at a time" method. The bacteria were incubated in various pH (6.0; 6.5; 7.0; 7.5; 8.0), temperatures (from 20°C to 40°C) and different incubation periods (from 24 h to 120 h), then the flocculating activity of isolates was measured.

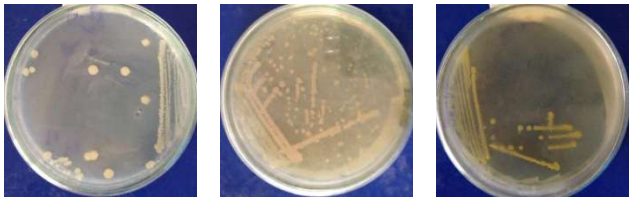
Identification of the flocculant producing bacteria by 16S rRNA analysis

The most potential flocculant producing isolates were identified using 16S rRNA gene sequence. The chromosomal DNA of strains was isolated according to Rainey *et al.* (1996) and the 16S rRNA gene was amplified with universal primers: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane *et al.*, 1985). The PCR cycling parameters: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 50 seconds, amplification at 72°C for 1.5 minutes and final extension at 72°C for 7 minutes. The 16S rRNA genes were sequenced by DNA Analyzer (ABI PRISM 3100, Applied Bioscience). 16S rRNA sequences were matched with sequences in the GenBank database using the Blast search programme (http://www.ncbi.nlm.nih.gov/). A phylogenetic tree based on the 16S rRNA sequences of isolates was generated using MegAlign via the Clustal W algorithm available in *DNA Star Lasergene v.8.0 sorfware* with *1000 bootstrap* replicates.

RESULTS AND DISCUSSIONS

Isolation of flocculant producing bacteria

A total of 117 isolates, with distinct colony characteristics were selected from the soil samples of Tay Nguyen. The assessment of flocculating activities showed that 26 isolates had the flocculating activity above 50%, of which Bx-f9, Bx-vk22 and Bx-vk9 had the highest flocculating capabilities with 79.1%, 77.13% and 75.16% respectively. The Bx-f9, Bx-vk22 and Bx-vk9 were selected for further studies. Morphology of the selected isolates was described in table 1 and figure 1.



Bx-f9 Bx-vk22 Bx-vk9

Figure 1. Colony of three selected isolates

Table 1. Morphology of three selected isolates

Isolates	Shape of colony	Colour of colony	Size of colony (mm)	Cell shape	Size of cell (µm)	Gram
Bx-f9	Circle, smooth	White	1.0 - 2.0	Long rod	3-4	+
Bx-vk22	Irregular, smooth	Orange	0.5-1.0	Short rod	11-13	+
Bx-vk9	Circle, smooth	Yellow	0.5-1.0	Short rod	10-11	-

Effect of carbon sources on flocculating activity

Obtained results showed that carbon sources significantly influenced on flocculating activity of isolates and this effect was dissimilar to isolates (Figure 2). Bx-f9 achieved the highest flocculating activity with sucrose as a carbon source, while glucose and lactose were the most favorite carbon sources for Bx-vk22 and Bx-vk9 respectively. Selecting the most favorite carbon source for bacterial flocculating activity was reported in previous studies. Bragadeeswaran *et al.* (2011) indicated that sucrose was the best carbon source for exopolysaccharides produced by *Bacillus cereus* GU 812900 while Jeganathan *et al.* (2014) used glucose as a carbon source for flocculant producing *Halobacillus trueperi* AJSK. Similarly, more than 90% of the flocculating activity of *Rhodococcus erythropolis* was released into medium when glucose was used as the carbon source (Kurane *et al.*, 1986a).

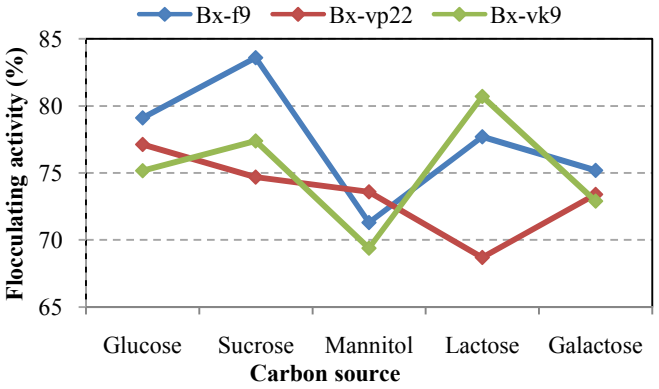


Figure 2. Effect of carbon sources on flocculating activity

Effect of nitrogen sources on flocculating activity

Nitrogen source was also a key factor effecting on flocculating activity. In this study, yeast extract was the best nitrogen source for enhancing flocculating activity of Bx-f9 and Bx-vk9, whereas Bx-vk22 reached the highest flocculating activity when culture medium was supplemented with peptone as a nitrogen source.

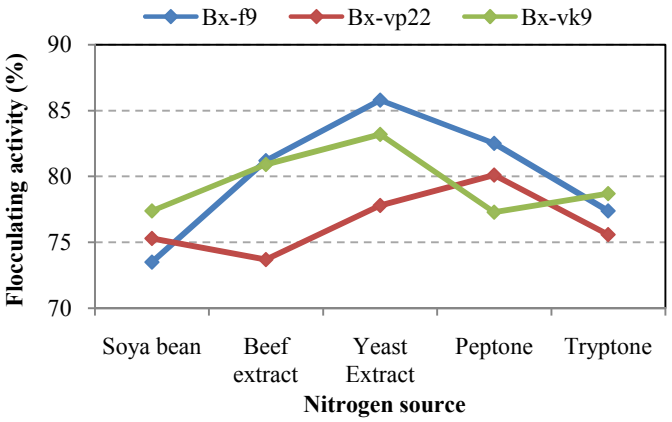


Figure 3. Effect of nitrogen sources on flocculating activity

Studies revealed that microorganisms required the specific favorite nitrogen source. Bragadeeswaran (2011) pointed out that ammonium sulphate is the best nitrogen source for exopolysaccharides produced by *Bacillus cereus* GU 812900 while the most favorite nitrogen source for bioflocculating activity produced by *Cobetia* sp. L222 was casein (Anthony *et al.*, 2012). Flocculating activity of *Bacillus* sp. was high when ammonium nitrate used as a nitrogen source (Ugbenyen and Okoh, 2013). In this study, our isolates preferred organic nitrogen sources.

Effect of temperature on flocculating activity

Experimental results revealed that cultural temperature had major influenced on flocculating activity of isolates. In this case, all isolates (Bx-f9, Bx-vk9, Bx-vk22) showed higher flocculating activity in a temperature range from 30^0C to 35^0C. Previous results indicated that temperature is physical factor influencing on flocculating activity and flocculant production. Cultural temperature for maximum extracellular polysaccharide of *Halobacillus trueperi* AJSK (Jeganathan *et al.,* 2014) and *Bacillus subtilis* (Sirajunnisa *et al.,* 2013) was 35^0C, while the optimal flocculant production temperature of bacteria such as *Flavobacterium* sp. (Endo *et al.*, 1976), *Rhodococcus erythropolis* (Kurane *et al.,*1986a), *Alcaligenes latus* (Kurane And Nohata, 1991), *Bacillus* sp. PY-90 (Yokoi *et al.,* 1995), and *Bacillus* sp. DP-152 (Suh *et al.,* 1997) have been reported at 30^0C. *Zoogloea* MP6 produced flocculants at 20^0C (Kakii *et al.*, 1996).

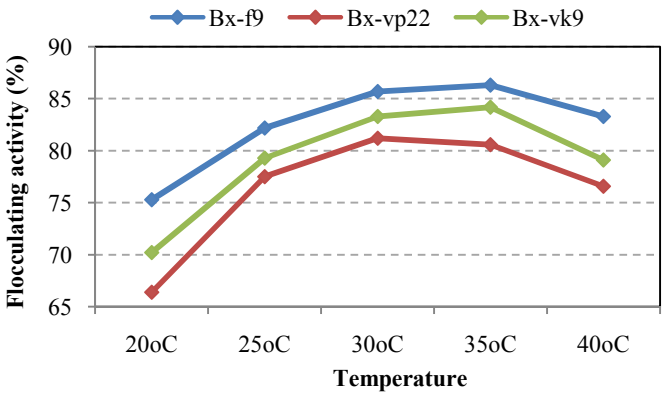


Figure 4. Effect of temperature on flocculating activity

Effect of pH on flocculating activity

Influence of various pH on flocculating activity of the isolates was investigated. Obtained results pointed out that Bx-f9 and Bx-vk9 achieved high flocculating activity in a range pH of 6.0 - 6.5 and of 7.0 - 7.5 respectively, while flocculating activity of Bx-vk22 was stable at a range pH of 7.0 - 8.0. The flocculating activity of flocculant producing bacteria varies with pH.

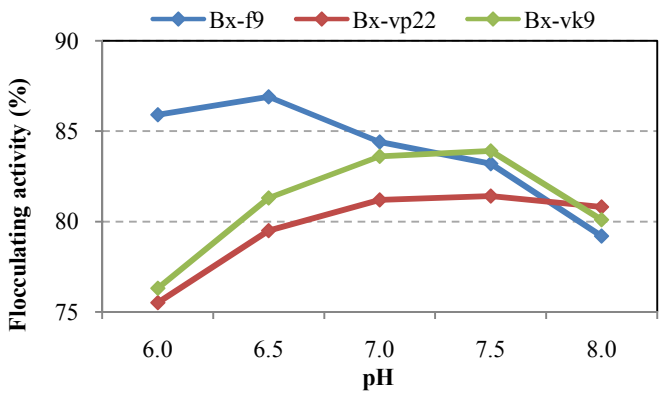


Figure 5. Effect of pH on flocculating activity

The flocculating activity of *Bacillus* sp. PY-90 was stimulated in a acidic pH range of 3.0-5.0 (Yokoi *et al.*, 1995) while the maximum flocculating activity of *Enterobacter* sp. BY-29 was observed at pH 3 and decreased with increasing pH (Yokoi *et al.*, 1996a). In the case of *Streptomyces griseus*, flocculant was active in acidic conditions ranging from pH 2 to 6 and the highest activity achieved at pH 4 (Shimofuruya *et al.*, 1996). The bioflocculant produced by *Rhodococcus erythropolis* is promoted at neutral pH (Kurane *et al.*, 1994b) whereas *Paceilomyces* sp. showed maximum flocculating activity in the pH range of 4.0-7.5 (Takagi and Kadowaki, 1985). The three screened isolates in this study gave high flocculating activity at pH near to neutral.

Effect of incubation time on flocculating activity

After different incubation time, the flocculating activity of the isolates was determined, and obtained results showed that flocculating activity of Bx-f9 and Bx-vk9 reached maximum after 72 h incubation, while flocculating activity of Bx-vk22 achieved maximum after 48 h. After reaching the maximum level, flocculating activity of the isolates decreased gradually. Depending on strains and incubation time, flocculating activity varied greatly. Incubation time for maximum extracellular polysaccharide of *Halobacillus trueperi* AJSK (Jeganathan *et al.,* 2014), *Bacillus subtilis* (Sirajunnisa *et al.,* 2013) and *Cobetia* sp. L222 (Anthony *et al.*, 2012) was 72 h. In the case of *Bacillus firmus*, bioflocculant peaked after 33 h culture (Salehizadeh and Shojaosadati, 2002), while bioflocculant produced by *Agrobacterium* sp. M-503 reached a peak after 48h

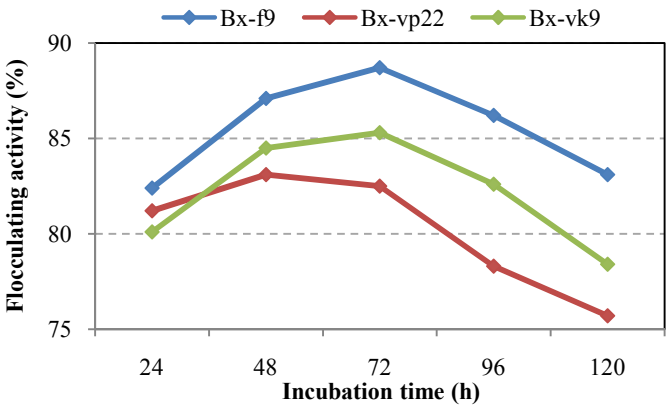


Figure 6. Effect of incubation time on flocculating activity

(Li *et al.,* 2010), and for *Vagococcus* sp. W31, flocculant production was maximum after 60 h (Gao *et al.,* 2006). Also, *Halomonas* sp. OKOH (Piyo *et al.,* 2011) and *Bacillus* sp. Gilbert (Mabinya *et al.,* 2011) attained maximum flocculating activities after 135 h and 240 h culture, respectively.

Identification of flocculant producing bacteria

The identification of 3 selected flocculant producing isolates was carried out, and the results showed that 16S rRNA sequence of isolate Bx-f9 exhibited 100% similarity with the 16S rRNA sequence of *Bacillus* sp. Bac50C (accession no. KF555237.1), while the isolate Bx-vk22 had 99% similarity of 16S rRNA gene sequence with those of *Lysinibacillus* sp. POUD (accession no. JQ007724.1). The isolate Bx-vk9 showed 99% similarity 16S rRNA gene with the 16S rRNA gene sequence of *Enterobacter sp.* CZBSD2 (accession no. KJ184880.1). Constructed phylogenetic tree also confirmed that Bx-f9, Bx-vk22, Bx-vk9 related to *Bacillus sp., Lysinbacillus sp. and Enterobacter sp.* respectively.

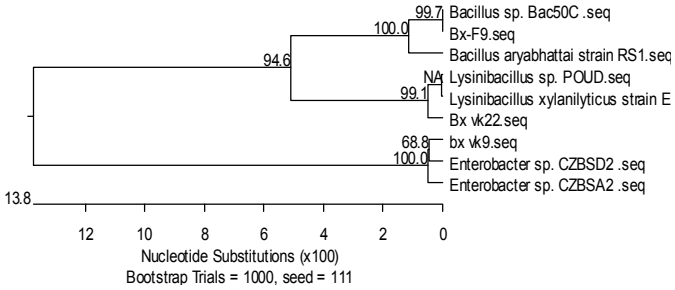


Figure 7. Phylogenetic tree of flocculant producing isolates

The previous studies showed that bioflocclants were produced by various bacteria, among of which *Bacillus* was the most popular, including *Bacillus* sp. DP-152 (Suh *et al.*, 1997), *Bacillus licheniformis* CCRC 12826 (Shih *et al.*, 2001), *Bacillus sp.* As-101 (Vossoughi *et al.*, 2001), *Bacillus firmus* (Salehizadeh *et al.,* 2001), *Bacillus subtilis* WD90, *Bacillus subtilis* SM29 (Kaewchai and Prasertsan, 2002), *Bacillus mucilaginosus* (Deng *et al.*, 2003), *Bacillus cereus* GU 812900 (Bragadeeswaran *et al*., 2011), *Bacillus subtilis* (Sirajunnisa *et al.,* 2013), *Bacillus* sp. (Ugbenyen and Okoh, 2014). Other flocculant producing bacteria such as *Flavobacterium* sp. 110 (Endo *et al.,* 1976), *Zoogloea* MP6 (Farrah and Unz, 1976), *Zoogloe aramigera* (Norberg and Enfors, 1982), *Pseudomonas* C-120 (Sakka *et al.,* 1981), *Nocardia amarae* YK1 (Takeda *et al.,*1992), and *Kluyveromyces cryocrescens* KA-103 (Kakii *et al.,* 1990), *Enterobacter* sp. (Yokoi *et al*., 1996a), and *Alcaligenes latus* B-16 (Kurane and Nohata, 1994), *Arcuadendron* sp. Ts-4 (*Lee et al.*, 1995) and *Aratrobacter* sp. (Wang *et al.*,1995), *Aureobasidium pullulans* ATCC (Lee *et al.*, 1999), *Enterobacter agglomerans* SM38 (Kaewchai and Prasertsan, 2002), *Halobacillus trueperi* AJSK (Jeganathan *et al*., 2014) have been isolated from soil and activated sludge samples. In this study, from soil samples of Tay Nguyen, three isolates with high flocculating activity were screened and they belonged to three different genera, including *Bacillus*, *Lysinbacillus* and *Enterobacter*.

Conclusion

In our research, flocculant producing bacteria were isolated from soil samples of Tay Nguyen, Vietnam, and three isolates with high activity were identified. 26 isolates with flocculating activity more than 50% were screened. Influence of carbon and nitrogen sources, as well as cultural conditions such as temperature, pH and incubation time on flocculating activity of isolates was carried out with 3 isolates possessing the highest flocculating activity. Obtained results revealed that selected isolates preferred organic nitrogen source and different carbon source for maximum flocculating activity. The suitable culture temperature and pH for their activity ranged from 30^0C-35^0C and from light acidity to light alkalinity, respectively. The incubation time for highest flocculating activity of the isolates varied from 48 h (Bx-vk22) to 72 h (Bx-f9 and Bx-vk9). The 16S rRNA gene analysis showed that Bx-f9 belonged to *Bacillus* sp., Bx-vk22 belonged to *Lysinibacillus* sp., and Bx-vk9 belonged to *Enterobacter* sp.

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