

## Screening and Characterization of a Bioflocculant Produced Strain *B. amyloliquefaciens* B-15

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(Received: 28 September 2012; accepted: 12 November 2012)

A novel bioflocculant-producing bacteria was isolated from the soil of Shandong Province in China. The flocculant activity of this strain cultured in fermentation medium was 90.98% after 72h cultivation. It was identified as *Bacillus amyloliquefaciens* by 16S rRNA gene sequencing and its biochemistry, physiological characteristics. The accession number was JX848636 in GenBank. The flocculant characterization of *B. amyloliquefaciens* was also investigated in this paper. The *B. amyloliquefaciens* strain produced an extracellular bioflocculant when grown in a fermentation medium containing sucrose as sole carbon source, inorganic ammonium compound as sole nitrogen source. The best pH and culture time of this strain produced bioflocculant were 6 and 84h respectively. Infrared spectrometry of purified bioflocculant indicated the presence of carboxyl, hydroxyl and amino groups, typical for heteropolysaccharide. The composition of bioflocculant was heteropolysaccharide containing little protein.

**Key words:** Bioflocculant, *Bacillus amyloliquefaciens*, Characterization, Composition.

Bioflocculant has been recognized for their harmlessness, biodegradability and lack of secondary pollution from their degradative intermediates<sup>1</sup> compared with chemical flocculants in wastewater treatment, drinking-water treatment, and industrial downstream processing. Bioflocculant were special macromolecules secreted by microorganisms. So far, microorganisms including algae, bacteria, actinomyces, and fungi have been reported to produce bioflocculant<sup>2</sup>. The majority of bioflocculant produced by different

microorganisms are usually high molecular weight polymers such as polysaccharides, proteins, glycoproteins and nucleic acids<sup>3</sup>. A number of microorganisms have been screened for their bioflocculant-producing capabilities<sup>4,5,6,7</sup>, but very little has been accomplished on a commercial scale<sup>8</sup>. The high cost of production coupled with low yield seem to be the major deterring factors in the advancement of research in developing bioflocculant for both scientific and commercial applications<sup>9</sup>. In recent years, utilization of microbial flocculants has been promoted due to their biodegradability and their environmentally inert nature<sup>10,11</sup>. Screening high flocculating activity microorganisms has become a subject of urgent investigations. A flocculants producing bacteria belonging to the *Bacillus* genus was

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isolated from the soil of Shandong province in China. The bacteria was identified as *B. amyloliquefaciens* with 16S rRNA gene and its biochemistry, physiological characteristics. Fermentation conditions on flocculant activity of this strain, the composition of the bioflocculant produced by the strain were also studied in this paper.

## MATERIALS AND METHODS

### Screening the bioflocculant-producing microorganisms

More than 40 samples of soil, sediment and activated sludge were collected in Shandong, Hebei, Liaoning province and Beijing city. A medium used to enrichment bioflocculant producing strains as following: dimethyl phthalate 5 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5 g, K<sub>2</sub>HPO<sub>4</sub> 0.2 g, KH<sub>2</sub>PO<sub>4</sub> 0.2 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.02 g, NaCl 0.01 g, per litre of deionized water at pH 7.5. After enrichment cultivation, solid beef extract peptone medium was selected as screening medium. The medium that consists of sucrose 20 g, urea 0.5 g, yeast extract 0.5 g, K<sub>2</sub>HPO<sub>4</sub> 5 g, KH<sub>2</sub>PO<sub>4</sub> 2 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.2 g, NaCl 0.1 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g in per litre of deionized water at pH 7.0 was selected as bioflocculant fermentation medium.

The flocculating activity was measured as described elsewhere<sup>12,13,14</sup> with modification. Five milliliters of 10% (w/v) CaCl<sub>2</sub> and 2.0 ml culture fermentation medium of B-15 were added into 100 ml of kaolin suspension (4.0 g/l) in a beaker, and the pH value was adjusted to 7.5 using NaOH or HCl. The mixture was vigorously stirred (700 r/min) for 1 min and slowly stirred (80 r/min) for 5 min, and then kept still for 5 min. The optical density (OD) of the clarifying solution was measured with a spectrophotometer at 550 nm. A control experiment was conducted in the same manner by replacing culture broth with fresh culture medium. The flocculating activity was calculated according to the following equation:

$$\text{Flocculating activity} = \frac{B - A}{B}$$

where A is the OD of the sample experiment at 550 nm, and B is the OD of the control experiment at 550 nm.

### SEM analysis of flocculate grains and kaolin grains

Five milliliters of 10% (w/v) CaCl<sub>2</sub> and 2.0

ml culture fermentation medium of B-15 were added into 100 ml of kaolin suspension (4.0 g/l) in a beaker, and the pH value was adjusted to 7.5 using NaOH or HCl. The mixture was vigorously stirred (700 r/min) for 1 min and slowly stirred (80 r/min) for 5 min, and then kept still for 5 min. A control experiment was conducted in the same manner by replacing culture broth with fresh culture medium. The sediments were air dried, and the dried sediments were observed with scanning electron microscopy (SEM, JSM-6700F).

### Identification of the microorganism physiology and biochemistry identification

Morphologic observation, glucose fermentation test, methylred reaction test (M.R. test), Voges-Proskauer test (VP test), indol test, catalase test, and gelatin liquefaction test according to<sup>15</sup> were carried out to roughly identify the strain B-15.

### 16S RNA molecular organism identification

Strain B-15 was incubated in 250-ml flasks containing 50 ml fresh LB medium for 24 h at 37°C with shaking at 150 rpm. The genomic DNA of the strain was then extracted using microbial DNA isolation kit (Shenzhen Bi'ansheng. Science & Technology Development Co., Ltd). PCR amplification was carried out to amplify the partial 16S rRNA gene. The PCR program was 30 cycles of 94°C (1 min), 55°C (1 min), and 72°C (2 min). The PCR primers were 5-AGAGTTTGATCCTGGCTCAG-3 (forward) and 5-AAGGAGCTGATCCAGCCGCA-3 (reverse). Purification of the PCR products and the determination of sequences were performed by GeneCore BioTechnologies Co., Ltd. (Shanghai, China). The 16S rRNA gene sequence of strain B-15 obtained was compared with the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### Effect of fermentation culture conditions on the flocculating activity of B-15

Flasks of 250 ml capacity, containing 100 ml fermentation medium with sole culture condition changes were selected. The sole carbon (glucose, sucrose, maltose and starch, nitrogen (urea, ammonium sulfate, yeast extract, peptone and beef extract) sources and pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0) were varied in the culture medium to study the effect of carbon, nitrogen and pH on the flocculating activity of B-15. The time of fermentation was 72 h, and the shaker speed was

180 r/min. The effect of culture time on the flocculating activity of B-15 was carried out as following: 100 ml fermentation medium in 250 ml flasks cultured with different time (24 h, 48 h, 60 h, 72 h, 84 h, 96 h) to exam the flocculating activity variety with the change of culture time.

#### Purification of the bioflocculant

The bioflocculant produced by strain *B. amyloliquefaciens* B-15 was purified according to the following method<sup>2,6</sup>. The fermentation broth was centrifuged at 5,000 r·min<sup>-1</sup> for 30 min to remove bacterial cells. The supernatant was then mixed with 2 volumes of chilled ethanol and left to stand at 4 °C for 12 h. The resultant precipitate was collected by centrifugation at 5,000 r·min<sup>-1</sup> for 30 min, and the crude bioflocculant was obtained. The crude bioflocculant was redissolved in distilled water, followed by the addition of 2 % hexadecyl trimethyl ammonium bromide (HTAB) with stirring at 100 rpm. After 3 h, the HTAB suspension was separated by centrifugation (5,000 r·min<sup>-1</sup>; 15 min) and redissolved in NaCl (0.5 M). Two volumes of cold ethanol were added then. The resulting precipitate was then washed three times with ethanol and finally vacuum dried to obtain to collect the purified bioflocculant.

#### Analysis of Purified Bioflocculant

The total protein content of the flocculant was investigated by the Bradford method with bo-vine serum albumin as standard<sup>16</sup>. The total

sugar content of bioflocculant was determined by a phenol-sulphuric acid method using glucose as a standard solution<sup>17</sup>. The dried product was analyzed using a Fourier transform infrared (FT-IR) spectrophotometer (Nicolet 5700, USA). The spectrum of the sample was recorded on the spectrophotometer over a wavenumber range of 4000~400 cm<sup>-1</sup>.

## RESULTS AND DISCUSSION

### Screening the flocculating bacterium

68 strains that have flocculating capability were screened from the soil samples. Strain B-15 showed the highest flocculating activity in kaolin suspension, and thus it was chosen for further research. The flocculating activity of this strain cultured in fermentation medium was 90.98%. The SEM flocculate grains and kaolin grains before flocculating were showed in Fig 1. Kaolin only combined with inorganic salt without bioflocculant added, and the combination was loose and irregular. Kaolin grains were little, and they suspended stable in solution. Bioflocculant combined with kaolin and inorganic salt when the bioflocculant was added into the kaolin suspension, and the combination was compact and stable. Each flocculate molecule can combine with a lot of kaolin grains, and each kaolin grain also can combine a lot of flocculants molecules at the same time. The combination

**Table 1.** Effects of source, pH in medium and the culture time on the flocculant activity of B-15

Carbon source	Glucose	Sucrose	Maltose	Starch		
Flocculating activity (%)	76.87	89.67	75.93	34.78		
Nitrogen source	Peptone	Ammonium sulphate	Urea	beef extract	yeast extract	
Flocculating activity (%)	43.52	90.41	87.83	60.88	27.09	
pH	4	5	6	7	8	9
Flocculating activity (%)	52.39	55.09	93.98	89.96	74.07	39.12
Culture time(h)	24	48	60	72	84	96 108
Flocculating activity (%)	31.25	51.39	73.15	89.85	93.06	79.16
62.31						

between kaolin grains and flocculate molecules formatted bridging function which led to big grains, and the big grains subsided rapidly in suspension solution.

#### Identification of B-15

The bacteria colonies of B-15 were viscous, ivory-white color and opaque with unsmooth edges. The Strain B-15 was a long, rod-shaped, Gram-positive that formed spores. Some of the biochemical and physiological characteristics of the strain were as follows: xylose (positive), sucrose (positive), glucose (positive), urea (positive), starch (positive), indole test (negative), M.R. test(negative), VP test(positive), catalase test(positive), and gelatin liquefaction test (negative). B-15 was identified as *Bacillus*

according to Bergey's Manual of Systematic Bacteriology<sup>18</sup>.

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene of the bacteria produced an amplicon of expected size (approx. 1.5 kb). BLAST analysis of 16S rRNA gene nucleotide sequence of B-15 was 99% similarity to some of *Bacillus*. A phylogenetic tree was constructed between it and similar sequences found in GenBank (Fig. 2). Strain B-15 was identified as *B. amyloliquefaciens* by both its physiological, biochemical and phylogenetic characteristics. The GenBank accession number of B-15 is JX848636.

*Bacillus* is a genus of Gram-positive, rod-shaped bacteria and a member of the phylum

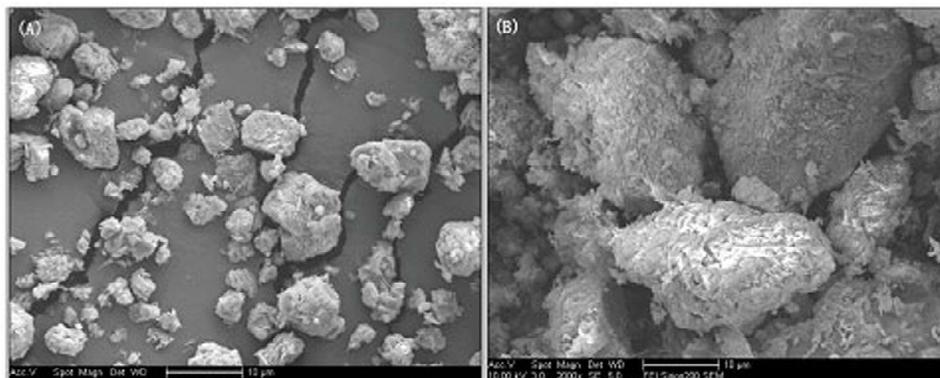


Fig. 1. SEM imaging of kaolin grains 1(A) and the grains after flocculating 1(B)

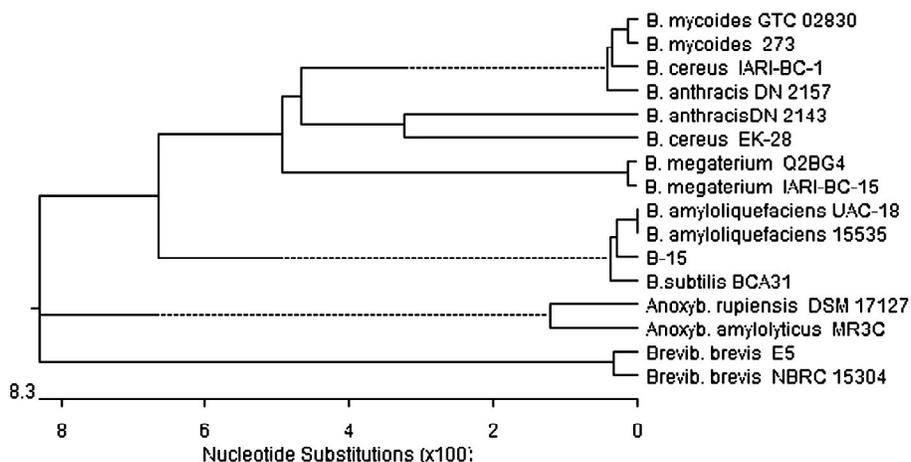


Fig. 2. Phylogenetic tree based on the nucleotide sequences of the 16S rRNA gene

Firmicutes. *Bacillus* species can be obligate aerobes or facultative anaerobes, and test positive for the enzyme catalase. *Bacillus* are ubiquitous in nature which includes both free-living and pathogenic species. Under stressful environmental conditions, the organisms produce oval endospores to over the stressful environmental conditions. This characteristic make *Bacillus* can not only grown in nourishment abundance environment but also survive in poor environmental condition. They can be found nearly

in all kinds of environment, and the genus includes a variety of industrially important species with a history of safe use in both food and industry. For example, *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. amyloliquefaciens*, and *B. clausii* offer several advantages in industrial applications<sup>19</sup>. There are also several *Bacillus* that can be used to produce microbial pesticide, fungicides or fertilizers in agricultural biotechnology<sup>20</sup>. *B. amyloliquefaciens* is well known as the source of a natural antibiotic

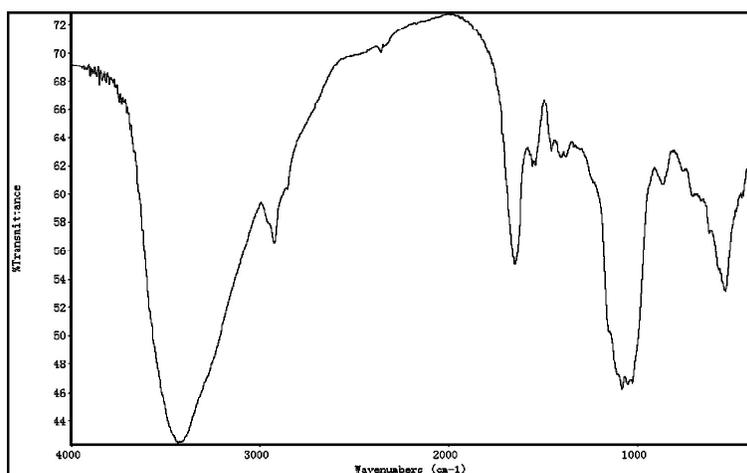


Fig. 3. FT-IR spectra of purified bioflocculant produced by *B. amyloliquefaciens* B-15

protein—barnase (a ribonuclease) and is also reported for much of the world production of Q-amylase and protease<sup>21</sup>. *Bacillus* species also can also produced a lot of active compound, such as *thermophilic alkaliphile*<sup>22</sup>, *biosurfactant—lychenisin*<sup>23</sup> lychenisin A<sup>24,25</sup> and so on. However, reports regarding their implication in the production of bioflocculant are very limited<sup>26</sup>. This research can enrich the reports in this field.

#### Effect of culture conditions on flocculating activity

Bioflocculant production was affected by various factors, such as carbon and nitrogen sources, initial pH of the culture medium and so on<sup>7,26,27</sup>. Effects of carbon and nitrogen sources, initial pH of fermentation media and culture time are showed in Table 1. Sucrose was found to be the best carbon source with a flocculating activity of 89.67%, while ammonium sulphated was the best nitrogen source (90.41% flocculating activity) in this research condition. Carbon and nitrogen are

very important for bioflocculant production<sup>7,26,27</sup>. Different microorganisms showed different requirement of these sources. Glucose and fructose boosted for *Rhodococcus erythropolis* to produce bioflocculant<sup>28</sup>. The best carbon source of *Serratia ficaria* for the production of the bioflocculant was lactose<sup>29</sup>. *B. amyloliquefaciens* B-15 preferred sucrose and inorganic Ammonium compound for bioflocculant production in our study. This phenomena was similar with bacterium *Bacillus* sp. Gilbert and *Bacillus* sp. F19<sup>26,30</sup>. There are also report that *B. licheniformis* X14 preferred sucrose, starch and ethanol as favorable carbon sources for the production of ZS-7 bioflocculant<sup>31</sup>. Different strain of *Bacillus* also showed different requirement of these sources for bioflocculant production.

The initial pH of the medium is one of the factors affecting the production and flocculating activity of the bioflocculant<sup>26,32</sup>. The effect of initial pH of the medium on flocculating activity

of *B. amyloliquefaciens* B-15 was investigated at pH from 4 to 9, and the flocculating activity was found to be distinctly higher (93.98%) in subacidity pH conditions (pH 6.0) than in other pH conditions (Table 1). Initial pH of culture media have been shown to variously affect the production bioflocculant of different bacterium strains. The bioflocculant produced by *Rhodococcus erythropolis* was reported to be active at neutral pH<sup>33</sup>. *Virgibacillus sp. Rob* preferred alkaline conditions to produce bioflocculant<sup>7</sup>. There are also reports about that bacteria produced bioflocculant optimally at strong acidic initial pH<sup>26,34</sup>. Initial pH of media influence the bioflocculant production. Also, change of pH might vary the charge status of the bioflocculant and surface characteristics of suspended materials, hence resulting in variation of flocculating activity<sup>26</sup>, but in our research, the suspension of cultured fermentation medium of B-15 and kaolin was adjusted to 7.5 which would discharge this aspect effect.

The flocculating activity of *B. amyloliquefaciens* B-15 increased rapidly with the prolong of culture time, and the flocculating activity reached a peak activity of 93.6% after 84h of culture in fermentation media. The flocculating activity decreased with the increase of culture time after 84h. A similar phenomenon was reported in the study of *Bacillus sp. Gilbert*<sup>26</sup> and *Streptomyces griseus*<sup>7</sup>. however, several studies reported different culture times for maximum bioflocculant production by different bacteria. For example, in *B. firmus*, bioflocculant production peaked after 33 h<sup>6</sup>. In *Agrobacterium sp. M-503*, production peaked after 48 h<sup>35</sup>, while, for *Vagococcus sp. W31*, production peaked after 60 h<sup>36</sup>. The consequent decrease of flocculating activity may possibly be as a result of cell autolysis and enzymatic activity<sup>26,29</sup>. This trend suggests that the bioflocculant decline after 84h could be due to autolysis or bioflocculant-degrading enzyme presence.

#### Composition of the purified bioflocculant of B-15

Phenol-sulfuric acid method showed that the flocculant contained 92.61% of total sugar, and 4.16% of protein content being detected by Bradford method. The result showed that the main composition of the bioflocculant was polysaccharide. The FT-IR spectrum (Fig.3) of the bioflocculant was analyzed in this research.

The strong absorption peak near 3,400cm<sup>-1</sup> was characteristic of OH. The peaks in the range from 2,900 to 2,800 cm<sup>-1</sup> were an indication of aliphatic C-H stretching. The absorption peak around 1,620 cm<sup>-1</sup> were characteristics of C=O stretching vibration in NHCOCH<sub>3</sub>. The strong absorption peaks observed in the range from 1,000 to 1,200 cm<sup>-1</sup> are generally known to be typical characteristics of all sugar derivatives<sup>37</sup>. The small absorption peak about 895 cm<sup>-1</sup> could be associated with  $\beta$ -glycosidic linkages between the sugar monomers<sup>2,38</sup>. The infrared spectrum showed characteristic peaks for carbohydrates and little amides. The FT-IR spectrum also indicated that the bioflocculant was heteropolysaccharide containing little protein.

## CONCLUSIONS

A novel bioflocculant-producing strain B-15 was isolated from the soil of Shandong province in China of which flocculating activity was 90.98% cultured in fermentation medium, and it was identified as *B. amyloliquefaciens*. Sucrose and inorganic Ammonium compound were the best carbon and nitrogen sources to produce bioflocculant of *B. Amyloliquefaciens* B-15. The optimal conditions for bioflocculant production were an initial pH of 6.0 in fermentation medium and a fermentation period of 84h. The composition of the purified bioflocculant of B-15 were heteropolysaccharide containing little protein. These results suggest that *B. Amyloliquefaciens* B-15 has a promising application in wastewater treatment.

## ACKNOWLEDGMENTS

The present study was supported by Major Science and Technology Program for Water Pollution Control and Treatment (2012ZX07 201002), Shandong Natural Science Foundation (Y2008B67) and International Cooperation Training Program, Shandong Education Department (SED).

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