Scientia Iranica, Vol. 8, No. 3, pp 207-210
© Sharif University of Technology, July 2001

SCIENTIA IRANICA

Research Note

Characteristics of a Biopolymer Flocculant Produced by *Bacillus sp.As-101*

M. Vossoughi*, I. Alemzadeh[1] and H. Salehizadeh[1]

In this paper, a biopolymer flocculant-producing bacteria, strain As-101, isolated from activated sludge and identified as *Bacillus species* is considered. Study of the basic characteristics and comparison of the strain were carried out according to Bergey's manual of bacteriology and the bacterium was identified as *Bacillus Coagulants.* The activated sludge was treated by a commercial protease (trypsin) with an optimum concentration of 0.4 mg/ml for producing the maximum extent of deflocculation, which was near 16%. The production of bioflocculant by *Bacillus As-101* was not parallel to the cell growth and a large amount of the bioflocculant was released into the culture at the end of the stationary phase, with the ability to make flocculation of kaolin clay suspension. The effect of cation concentration rate was also evaluated. The flocculating of bioflocculant was stimulated by the addition of Ca^{2+}, with an optimum concentration of 0.068 mM. The cell flocs were deflocculated not only by trypsin, but also by EDTA which was after 10 hours by 1 mM EDTA.

INTRODUCTION

Various flocculants such as chemical flocculants are used in a wide range of industrial processes, including wastewater treatment [1]. Although organic high polymer flocculants such as polyacrylamide are frequently used because they are inexpensive and highly effective, some of them are not easily degraded in nature and some of the monomers derived from synthetic polymers have neurotoxic and carcinogenic effects and, therefore, are harmful to the human body [2,3].

In recent years where emphasis has been placed on solving environmental problems, utilization of microbial flocculants has been anticipated due to the biodegradability and harmlessness of their degradation intermediates to the environment.

The activity of microbial flocculating substances has been examined from various view points, such as coagulation of caolin clay and removal of microorganisms in the fermentation industry [2,3].

Microbial flacculants are obtained from various sources. Some bacteria are isolated from soil. Foaming activated sludge can produce a biopolymer having flocculating activity culture. It has, recently, been reported that some microorganisms such as *Rhodococus erythropolis*, *Aspergillus sojae* and *Streptomyces griseus*, produce certain kind of bioflocculants [2]. Among these reports, Nakamura et al. [3] have presented serial studies on a fungal, *Chlamydomonas mexicana*, generating flocculating agents which are polysaccharide. Kurane et al. [4] have investigated about *R.erythropolis* S-1 which produces a lipid bioflocculant, effective for various colloidal suspension and coloured pigments.

Several assumptions have been proposed concerning the floc-formation of pure bacterial cultures, including bridging, gelatinous matrix, electrokinetic forces, polymerization and ionic theories. Studies on pure bacterial cultures have revealed that extracellular polymers, such as cellulose, protein, lipid, mucopolysaccharide and DNA, are involved in flocculation. In this study, isolation of a microorganism producing a new bioflocculant was accomplished and characteristics of the bioflocculant in terms of flocculation rate were investigated.

*. *Corresponding Author, Department of Chemical Engineering, Biochemical and Bioenvironmental Research Center (BBRC), Sharif University of Technology, Tehran, I.R. Iran.*

1. *Department of Chemical Engineering, Biochemical and Bioenvironmental Research Center (BBRC), Sharif University of Technology, Tehran, I.R. Iran.*

MATERIAL AND METHODS

Trypsin Treatment of Activated Sludge Method

The activated sludge with 1.8 g/l MLSS was transferred into a 100 ml graduated cylinder. After 30 min of settling, 50 ml of supernatant was pipetted off. An appropriate amount of trypsin and distilled water, resulting in 100 ml final value, were added to the mixed liquor, then pH of the mixture was adjusted to 7.7.

The mixture was transferred into a 100 ml beaker and stirred gently (100 rpm) with a magnet stirrer for 4 h. The mixture was then returned to a 100 ml graduated cylinder and settled for 30 min. The turbidity of supernatant was measured at 660 nm. The settled sludge was centrifuged at 10,000 rpm for 10 min, then it was washed twice and dried at 90°C.

Bacterial Isolation

Bacteria were isolated using the supernatant cell suspension obtained through trypsin treatment and then centrifuged at 650 rpm for 2 min. The cell suspension was diluted and pipetted out. An agar medium containing pepton, 5 g; yeast extract, 2.5 g; glucose, 1 g and agar 15 g in 1 l distilled water was used as the solid medium in perti dishes. The initial pH was adjusted to 7.2.

Colonies which developed after 24 h incubation at 25°C were chosen and purified through repeated transfer onto fresh agar medium.

Cultivation

One loopful of bacteria grown on slant agar at 25°C for 24 h was suspended in 10 ml of sterilized distilled water. A sample (1 ml) of the cell suspension was inocculated into a 500 ml flask containing 300 ml culture medium using a reciprocal shaker for 30 h. The composition of the medium was as follows: pepton, 0.5 g; $(NH_4)_2SO_4$, 0.2 g; yeast extract, 0.1 g; $CaCl_2.2H_2O$, 0.07 g; NaCl, 0.01 g; $MgSO_4.7H_2O$, 0.02 g; K_2HPO_4, 0.1 g; glucose, 0.1 g; in 100 ml of deionized water.

The culture temperature and initial pH (before sterilization) were 30°C and 7.0. After cultivation, extent of flocculation, flocculation rate and nitrogen in the culture broth were measured. Amounts of inorganic and organic nitrogen were measured using the micro-kejldahl method [5,6].

Measurement of the Extent of Flocculation

For measuring the extent of flocculation, the cell suspension was centrifuged at 650 rpm for 2 min. The ratio in percentage of the dry weight of precipitate to the total dry weight of the cells was defined as the extent of flocculation [7].

Assay of Flocculation Rate

Flocculation rate was measured using kaolin clay (practical grade, Wako) as a suspended solid. 0.4 ml of bioflocculant solution and 6 ml of kaolin suspension 5 g/l were diluted to 10 ml by distilled water in a test tube, then pH of the mixture was adjusted by 0.1 N HCl or NaOH solution till the maximum flocculating activity was observed. The mixture was stirred with a vortex mixer and left standing for 3 min. 2 ml of supernatant was carefully removed from the upper layer and its absorbance was measured at 550 nm (OD_{sample}). A similar procedure was carried out for the blank using bioflocculant solution and absorbance was also measured at 550 nm (OD_{blank}). Flocculation rate (FR) was calculated using the following equation [2,8,9]:

$$FR\% = \frac{(OD_{blank} - OD_{sample})}{OD_{blank}} * 100.$$

RESULTS AND DISCUSSION

Previous studies [10] indicate that the isolated strain As-101 was identified as *Bacillus coagulants* according to Bergey's manual of systematic bacteriology [11]. The isolated strain As-101 is a Gram positive aerobic catalase-positive and sporogenous rod (Figure 1). Acid was produced from D-glucose but not from L-arabinose, D-xylose and D-mannital. Furthermore, casein and starch hydrolyzation as well as Voges-Proskaure tests were positive, while gelatin liquefaction was negative. Growth was observed at 30°C and 50°C but not at 65°C. The 24 h reaction in TSI environment (yellow surface/yellow depth = Acid/Acid) depicted lactose/glucose fermentation and indicated complete β-homolysis on sheep blood agar [11].

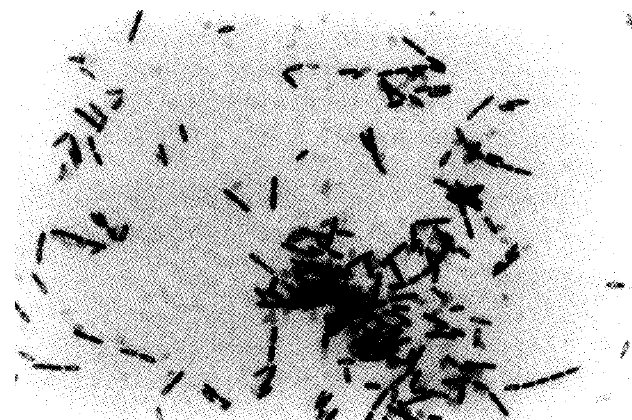

Figure 1. Scanning electron micrograph of strain As-101. The strain was cultured on slant agar medium.

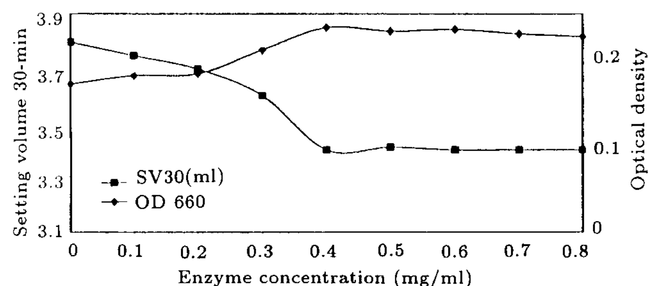

Figure 2. Treatment of activated sludge (MLSS =1.8 g/l) with proteolytic enzyme (trypsin).

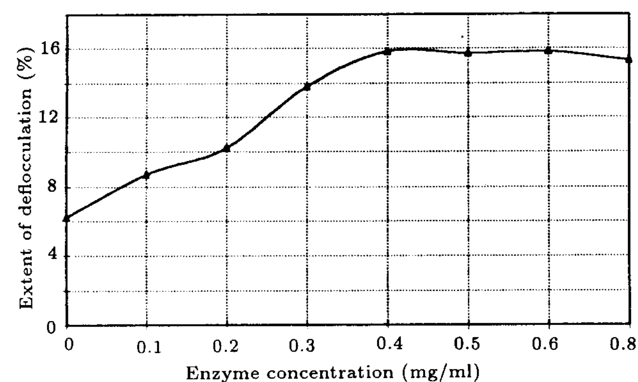

Figure 3. Effect of enzyme concentration on extent of deflocculation.

Trypsin Treatment of Activated Sludge

When activated sludge was treated with about 0.4 mg/ml of trypsin for 4 h at pH = 7.7, the 30-min settled volume of sludge (SV30) decreased from 38% to 34% as shown in Figure 2. The turbidity of supernant after 30 min of settling was about 0.23 at optimum concentration of proteolytic enzyme (0.4 mg/ml). In this case, the extent of deflocculation was 16%, indicating that only a small part of the sludge was deflocculated (Figure 3).

Effect of Growth Phase of Strain As-101 on Flocculation Rate

Figure 4 illustrates the variations of growth, flocculation rate and pH during fermentation time. As shown in this figure, maximum rate of flocculation was observed at the end of the stationary phase. Significant decrease in the flocculation rate after 50 h was probably due to the increase in proteolytic enzyme during cell lysis.

Concentration of inorganic and organic nitrogen sources and pH variations during the fermentation of strain As-101 are depicted in Figure 5. Increase in pH can be explained by organic nitrogen consumption and production of ammonia in the metabolic pathway.

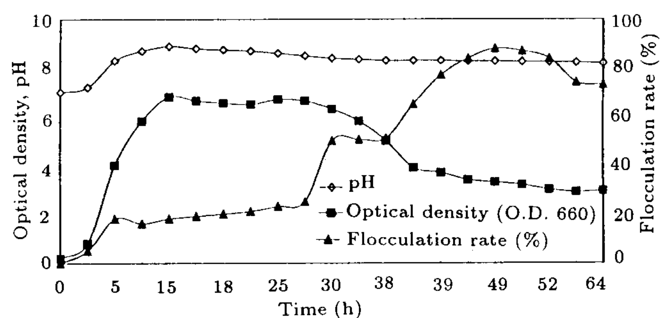

Figure 4. Growth, flocculation rate and pH variations of strain As-101 with time.

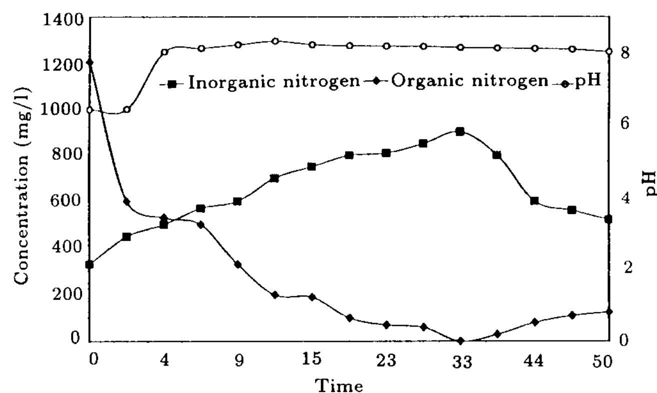

Figure 5. Relationship between inorganic, organic nitrogen concentration and acidity of medium with time.

Table 1. Effects of cation Ca^{2+} on flocculation rate.

Calcium Concentration (mM)	0.0	0.017	0.034	0.047	0.068	0.34	0.68	1.7
Extent of Flocculation (%)	43	57	63	87	95	89	89	88

Effect of Cation Ca^{2+} on Flocculation

In this section, the effect of addition of cation Ca^{2+} to the reaction mixture on flocculation rate is examined. Table 1 shows the extent of flocculation by *Bacillus As-101* for various concentration of Ca^{2+}. The optimum concentration of Ca^{2+} for maximum extent of flocculation was about 0.068 mM.

Effect of Trypsin on Cell Growth

Cell growths with and without trypsin were compared using dry weight basis. Two separate cultures (100 ml each) were started at the same time and trypsin (0.4 mg/ml) was added to one culture at the lag phase. As shown in Figure 6, trypsin stimulated growth as judged by dry weight.

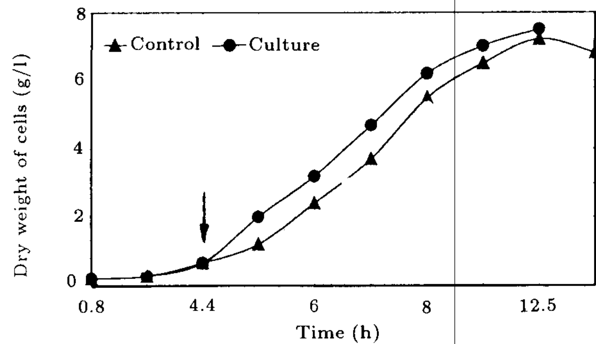

Figure 6. Effect of trypsin on cell growth. (Trypsin was added at the time indicated by an arrow to one culture but not to the control.)

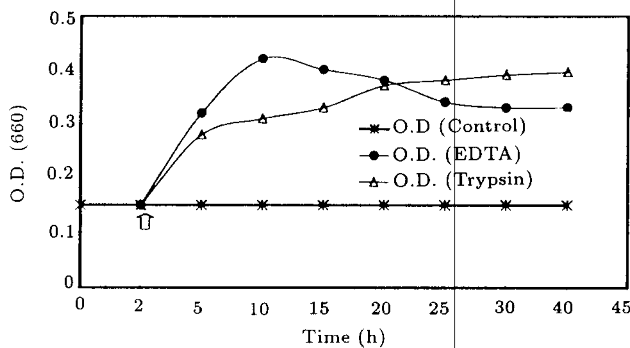

Figure 7. Deflocculation induced by EDTA and trypsin. (Trypsin or EDTA was added at the time indicated by an arrow.)

Deflocculation by EDTA and Trypsin

Washed flocculated cells of *Bacillus As-101* were suspended in 0.01 M Tris buffer (pH = 7.5) at O.D = 0.15 and were placed in 3 test tubes. EDTA (1 mM) or trypsin (0.4 mg/ml) was added to each tube and the time course of deflocculation was measured through optical density. As shown in Figure 7, deflocculation occurred up to 40 h and 10 h by trypsin and EDTA, respectively.

CONCLUSION

A bioflocculant-producing strain As-101, identified as *Bacillus coagulans* was isolated from activated sludge. The strains were deflocculated by commercial protease (trypsin) treatment. The flocculated cells were deflocculated not only by trypsin, but also by EDTA. An adequate amount of calcium ion in the medium increased the flocculating rate. The bioflocculant from strain As-101 demonstrated a higher flocculation activity than that of a polysaccharide flocculant from *Bacillus sp.* PY-90 [5], comparable with the activity of a protein flocculant from *R.erythropolis* but less than the bioflocculant from *Bacillus DP-152* [12-14].

REFERENCES

1. Gutcho, S. "Waste treatment with polyelectrolytes and other flocculants", Noyes Datad Corp, Park Ridge, New Jersey (1977).
2. Shimofuruya, H., Koide, A., Shirota, K., Tsuji, T., Nakmura, M. and Suzuki, J. "The production of flocculating substances by *Streptomyces griseus*", *J. Biosci. Biotech. Biochem.*, **60**(3), pp 498-500 (1996).
3. Nkamura, J., Migashiro, S. and Hirose, Y. "Screening, isolation and some properties of microbial cell flocculant", *J. Agr. Biol. Chem.*, **40**(2), pp 377-383 (1976).
4. Kurane, R., Hatamochi, K. and Kakuno, T. "Purification and characterization of lipid bioflocculant produced by *R. erythropolis*", *J. Biosci. Biotech.*, **58**(11), pp 1972-1982 (1994).
5. AOAC, *Official Methods of Analysis*, 15th Ed., Association of Official Analytical Chemists, INC. Washington DC, USA (1990).
6. Jayaraman, J., *Laboratory Mannual in Biochemistry*, Wiely Eastern Limited, Dehli, India, pp 75-76 (1981).
7. Endo, T., Nakaruma, K. and Takahashi, H. "Pronase susceptible floc-forming bacteria", *J. Agr. Biol. Chem.*, **40**(2), pp 2289-2295 (1976).
8. Yokoi, H., Natsuda, O., Hirose, J., Hayashi, S. and Talasaki, Y. "Characteristics of biopolymer flocculant produced by *Bacillus sp.* PY-90", *J. Ferment. Bioeng.*, **79**, pp 378-380 (1995).
9. Takadea, M., Koizumi, J., Matsuoka, H. and Hikuma, M. "Factors affecting the activity of protein bioflocculant produced by *Nacardia amamrae*", *J. Ferment. Bioeng.*, **74**(6), pp 408-409 (1992).
10. Vossoughi, M., Salehizadeh, H. and Alemzadeh, I. "Some investigation on bioflocculant producing bacteria", *Bioch. Eng. J.* (in pres).
11. Peter, H.A.S., Nicholas, S.M., Sharpe, M.E. and Holt, J.G., *Bergey's Manual of Systematic Bacteriology*, **2**, Williams and Wikin Co., Baltimore, pp 1120-1136 (1986).
12. Suh, H., Kwan, G., Lee, C., Kim, H., OH, H. and Yoon, B. "Characterization of bioflocculant produced by *bacillus sp.* DP-152", *J. Ferment. Bioeng.*, **84**(2), pp 108-112 (1997).
13. Yokoi, H., Yoshida, T.T, Mori, S., Hirose, J., Hayashi, S. and Takashi, Y. "Biopolymer Flocculant Produced by an *Enterobacter sp.*", *Biotechnol. Lett*, **66**(19), pp 569-573 (1997).
14. Takeda, M., Kurane, R., Koizumi, J. and Nakamuri, J. "Protein bioflocculant produced by *R. erythropolis*", *J. Agr. Biol, Chem.*, **55**, pp 2663-3664 (1991).