Full Length Research Paper

Optimization of the production of exopolysaccharides by *Bacillus thuringiensis 27* in sand biological soil crusts and its bioflocculant activity

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To improve the yield of exopolysaccharides (EPS) by *Bacillus thuringiensis* 27 from sand biological soil crusts in Gurban Tonggut Desert, Xinjiang, China and to analyze its bioflocculant activity, orthogonal matrix method was used and this method enabled us to obtain maximum EPS production. By studying the optimal medium condition of beef extract 3 g/l, peptone 10 g/l, maltose 40 g/l, and NaCl 4g/l we observed that the optimal medium condition was pH 6.0, incolum size 8%, liquid volume 40 ml in 200 ml flak and temperature 28°C. The maximum EPS production was 20.19 g/l which is about five times more than that at the basal condition. Furthermore, results obtained indicated that the flocculation activity of the extracellular polymer can be achieved at over 80.4% in kaolin suspension and this occurred at a concentration of 0.4 mg/l. This paper describes the optimum condition of exopolysaccharides production by *Bacillus thuringiensis* 27 and showed that exopolysaccharides had high bioflocculant activity. This work provides a scientific foundation to explore new exopolysaccharides and bioflocculation in sand biological soil crusts. Maximum production of exopolysacchaides under the optimal medium and condition can be achieved and exopolysaccharides have high bioflocculating activity in kaolin system.

Key words: Biological soil crusts, exopolysaccharides, flocculation, optimization, *Bacillus.*

INTRODUCTION

Extreme environments, once thought to be too hostile to permit survival of living organisms, are the natural habitat of certain microorganisms. It is now recognized that the microorganisms living in extreme environments have different genetic background and metabolic pathway when compare to general microbiology and their second-dary metabolites have special function (Kennedy et al., 2001). Among the secondary metabolites from extreme environments, polysaccharide for biotechnological applications has been reported in Literature (Nicolaus et al., 2004). Therefore a wide search for bacteria that are able to produce new polysaccharides with potentially useful properties has been undertaken. Sand biological soil crusts (BSCs) as extreme environment are a unique mini-nature landscape in desert district as well as the obvious sign of fixing mobile dune. They are composed of living microorganisms, their product of metabolism (mainly extracellular polysaccharides) and sand granule (Gundlapally and Garcia-Pichel, 2006). Exopolysaccharides (EPS) produced by microorganisms in BSCs are barely reported in the recent research.

Medium condition and other bacteria growth conditions are important factors for EPS production. There are large numbers of reports on optimization of EPS production by statistical optimization techniques (Xu et al., 2003; 2010; Kaditzky and Vogel, 2008; Hao et al., 2010) Orthogonal design as one of the important statistical methods has been successfully applied to improvement of the

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Abbreviation: EPS, Exopolysaccharide.

Table 1. Orthogonal matrix table of the L9 (3^4).

Factor	1	2	3
Beef extract (g/100ml)	0.2	0.3	0.4
Peptone (g/100ml)	0.5	1.0	1.5
Maltose (g/100ml)	2	3	4
NaCl (g/100ml)	0.4	0.5	0.6

production of primary and secondary metabolites in cultivation process (Xu et al., 2003).

This work is an attempt to isolated strain producing EPS from sand BSCs, analysize suitable media and culture condition for the production of EPS from *B. thuringiensis 27* and examines the bioflocculaton activity of EPS. To the best of our knowledge, the nutritional requirements and culture condition of EPS production form *B. thuringiensis 27* isolated from sand BSCs and its bioflocculation activity has not been demonstrated.

MATERIALS AND METHODS

Bacterial strain

Bacillus thuringiensis 27 was originally isolated from BSCs collected in the Gurban Tonggut Desert. It was maintained on agar slants containing (g/l): beef extract 3; peptone 10; NaCl 5 and agar 20 (pH 7.0-7.4). The slants were incubated at 35°C for 24 h and the fully grown slants were stored at 4°C.

Culture medium

Basal medium, beef extract 3 g, peptone 10 g, NaCl 5 g, water 1000 g, pH 7.0-7.4. Fermentation medium, beef extract 3 g, peptone 10 g, NaCl 5 g, water 1000 g, pH 7.0-7.4, temperature 35°C.

Single factor experiments of culture requirement

To find the optimal culture requirements, the following factors were investigated using the one-factor-at-a-time method, including carbon sources nitrogen sources inorganic ions, initial pH value, cultivation temperature, inoculum size, liquid volume triangle flask. All experiments were performed in triplicates (n = 3).

Orthogonal matrix method

The orthogonal L9 (3^4) was used to obtain the optimal medium after the test by the one-factor-at-a-time method. This enables us to determine which process variables affect the response. A logical next step is to determine the point in the important factors that leads to the best possible response (Di et al., 2003; Li et al., 2001). The levels of components of the culture medium are listed in Table 1. All experiments were performed in triplicate (n = 3). The software SPSS was used for experimental design, data analysis and model building. The optimal fermentation conditions for enhanced yield of EPS and cell growth were obtained by solving the regression

equation using the software Origin 8.0.

Determination of flocculating activity

A kaolin suspension was used to measure the flocculating activity of the EPS crude extracts. One milliliter of 90 mmol/l $CaCl_2$ and one milliliter were added into 50 ml 4.0 g/l kaolin suspension. The mixture was vigorously stirred for 0.5 min, and stand for 4 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 distilled water. The flocculating activity was calculated according to the following equation (Kurane et al., 1986):

$$\eta = (A-B) \times 100/A$$

where; A is the optical density of the control experiment at 550 nm and B is the optical density of the sample experiment at 550 nm. All experiments were performed in triplicates (n = 3).

Analytical methods

Samples collected at various culture conditions from shake flasks were centrifuged at 7000 g for 15 min. The 2 ml resulting supernatant was precipitated with threefold times 95% ethanol, stirred vigorously, and left overnight at 4°C. The precipitated polysaccharides were collected by centrifugation at 7000 g for 15 min, discarding the supernatants, and dissolved with distilled water, dialyzing for 24 h, adding distilled water to 20 ml. Phenol-Sulfuric Acid Method was used for determining EPS yield (Pazur et al., 1994), using glucose solution as a standard reference. The EPS yield was expressed as gram per liter.

RESULTS

Effect of carbon and nitrogen source on EPS yield

To find out the optimal carbon and nitrogen source for the EPS production of *B. thuringiensis 27* five 2% carbon sources (glucose, maltose, sucrose, lactose and glycerol) were separately provided in the basal medium and five 1% nitrogen sources (ammonium chloride, ammonium sulfate, peptone, beef extract and yeast extract) were separately instead of peptone employed in the basal medium. A high level of EPS was obtained when maltose and glycerol were used as the carbon source. Among the carbon sources tested, maximum EPS (10.45 g/l) was obtained in the maltose medium. Based on this study maltose is a good candidate. The maximum EPS
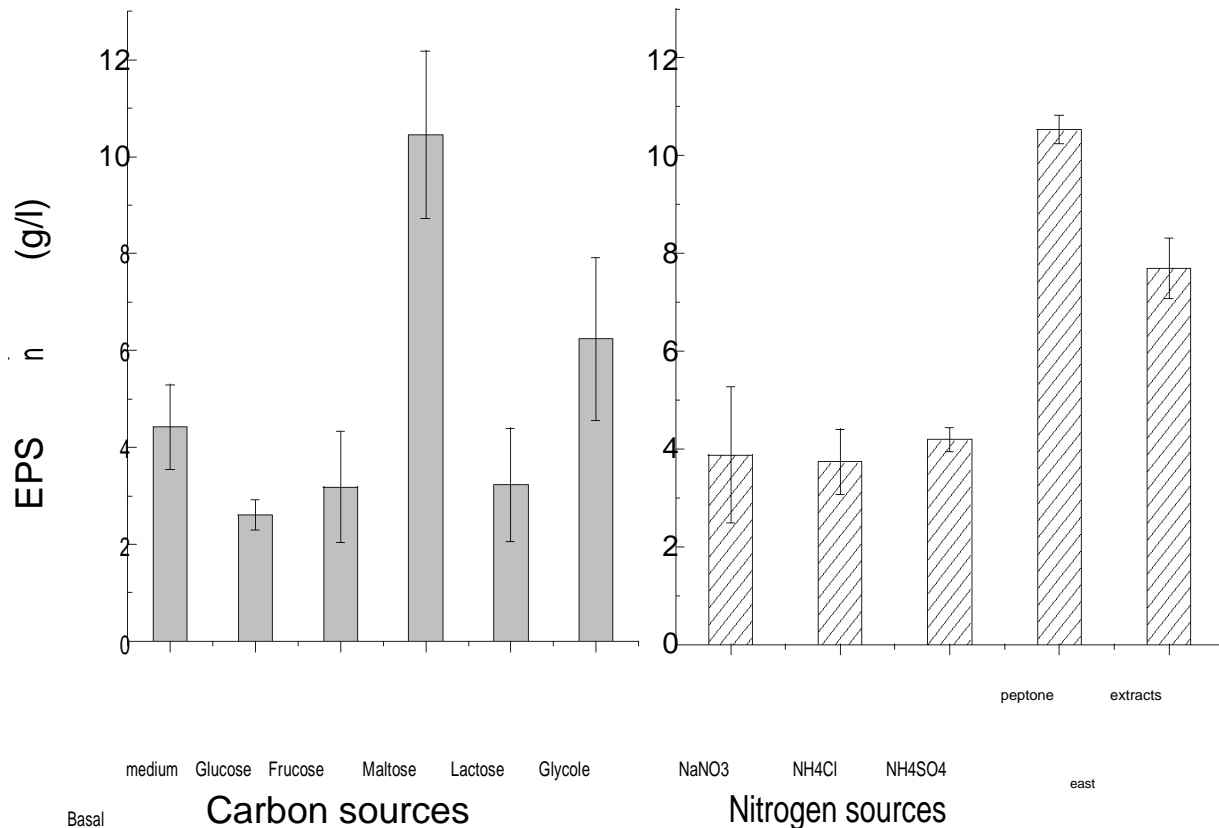
Figure 1. Effect of carbon and nitrogen sources on EPS production.

production (10.53 g/l) was obtained in the peptone (Figure 1). So we selected maltose peptone as our optimal nitrogen source.

Effect of concentration of carbon and nitrogen source on EPS yield

To find out the suitable concentration of maltose for the EPS yield *B. thuringiensis 27* five different concentration (1, 2, 3, 4 and 5%) maltose were separately instead of 2% maltose above. The maximum EPS production (10.25 g/l) was obtained when the concentration is 3% (Figure 2). To find out the suitable concentration of nitrogen source for the EPS yield five different concentration (0.1, 0.2, 0.5, 1.0 and 1.5%) peptone were separately instead of 1% peptone above. The maximum EPS production (10.74 g/l) was obtained when the concentration is 1% (Figure 2).

Effect of inorganic ions and its concentration on EPS yield

To find out the suitable mineral elements for the EPS yield of *B. thuringiensis 27* six different 0.5% mineral elements (sodium chloride, magnesium chloride, copper

sulfate, zinc chloride, ferric chloride and manganese chloride) were separately in basal medium. The maximum EPS production (15.36 g/l) was obtained in sodium chloride (Figure 3). Besides magnesium chloride, others depress the EPS production. Based on the result we selected sodium chloride as our selection.

Further, to find out the suitable concentration of NaCl for the EPS yield five different concentration (0.3, 0.4, 0.5, 0.6 and 0.7%) sodium chloride were separately instead of 0.5% sodium chloride above.

The maximum EPS production (15.36 g/l) was obtained when the concentration is 0.5% (Figure 3). The difference in EPS yield between the peptone concentrations of 0.5% and 0.6% was not significant because the cell could not absorb more mineral element. But considering the economic factor, we use the 0.5% NaCl as our optimal option.

Effect of initial pH and temperature on EPS yield

To find out the optimal temperature for and EPS production, *B. thuringiensis 27* was cultivated at various temperatures ranging from 23 to 45°C. The EPS (12.12 g/l) were observed at 28°C (Figure 4). *B. thuringiensis 27* was grown at initial pH 7.0 for polysaccharide production and optimum EPS production is 14.96 g/l) (Figure 4).
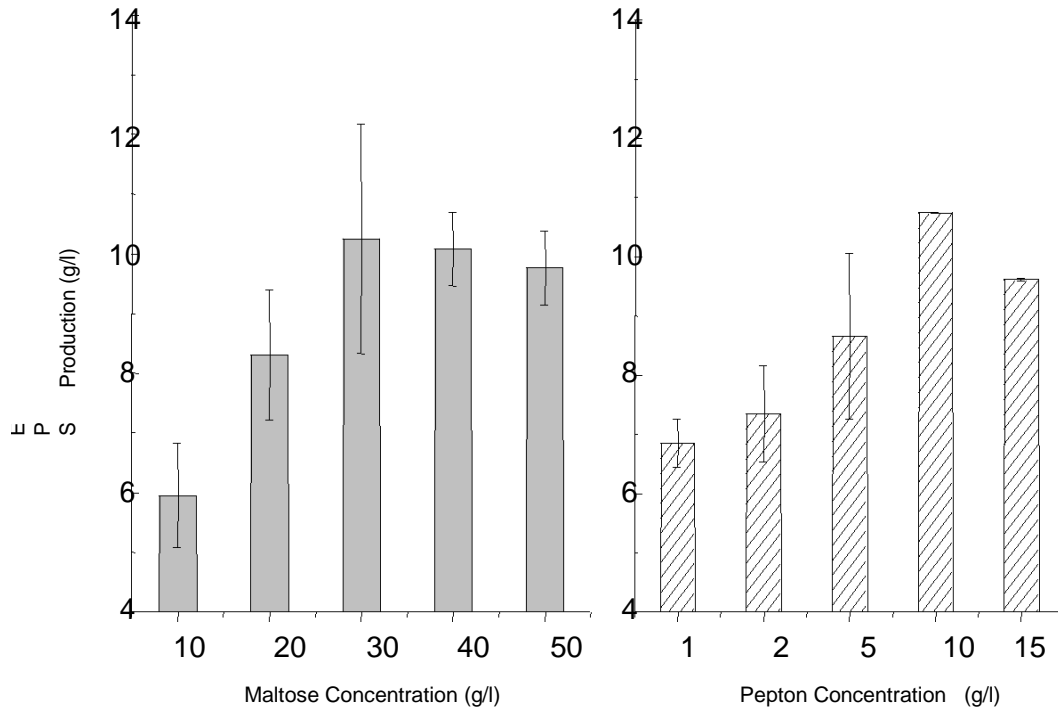
Figure 2. Effect of maltose and peptone concentration on EPS production.
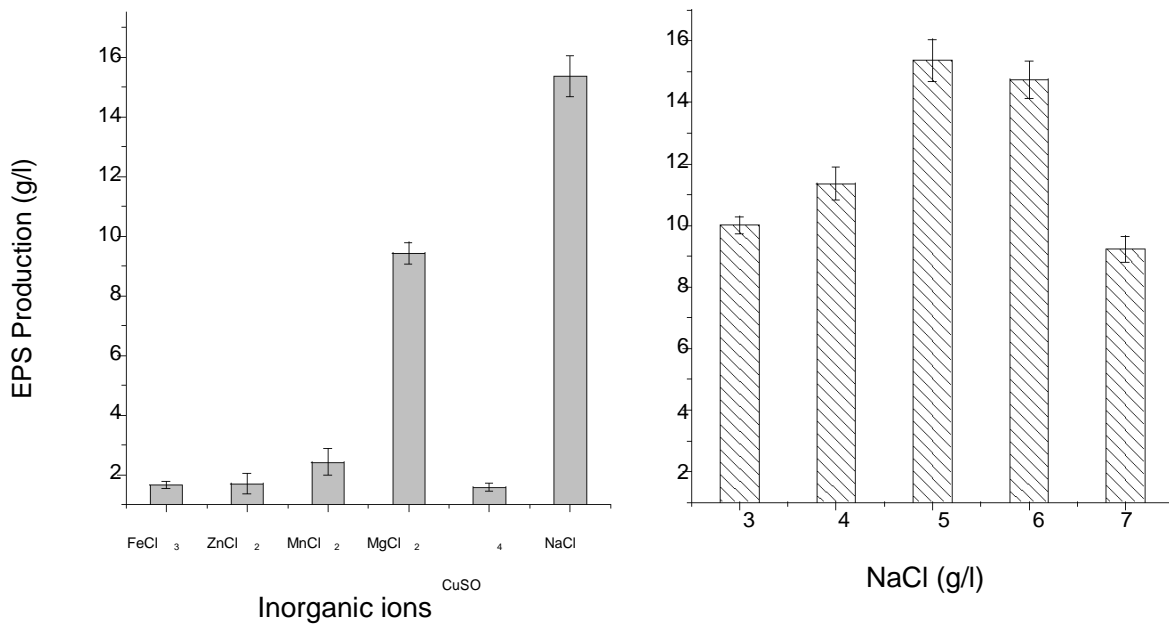

Figure 3. Effect of inorganic ions and NaCl concentration on EPS production.

Effect of inoculum size and liquid volume on EPS yield

To examine the effect of inoculum size, *B. thuringiensis* 27 was varying the inoculum size (2, 5, 8 and 11%) in basal medium. The results indicated 8% inoculums size

was fit for EPS production (Figure 5).

To find out the optimal oxygen rate four different culture medium volumes (25, 40, 55 and 70 ml) added in 200 ml flask were separately instead of 60 ml basal medium. The maximum EPS production (14.38 g/l) was obtained when the culture medium volume is 25 ml in 200 ml flask
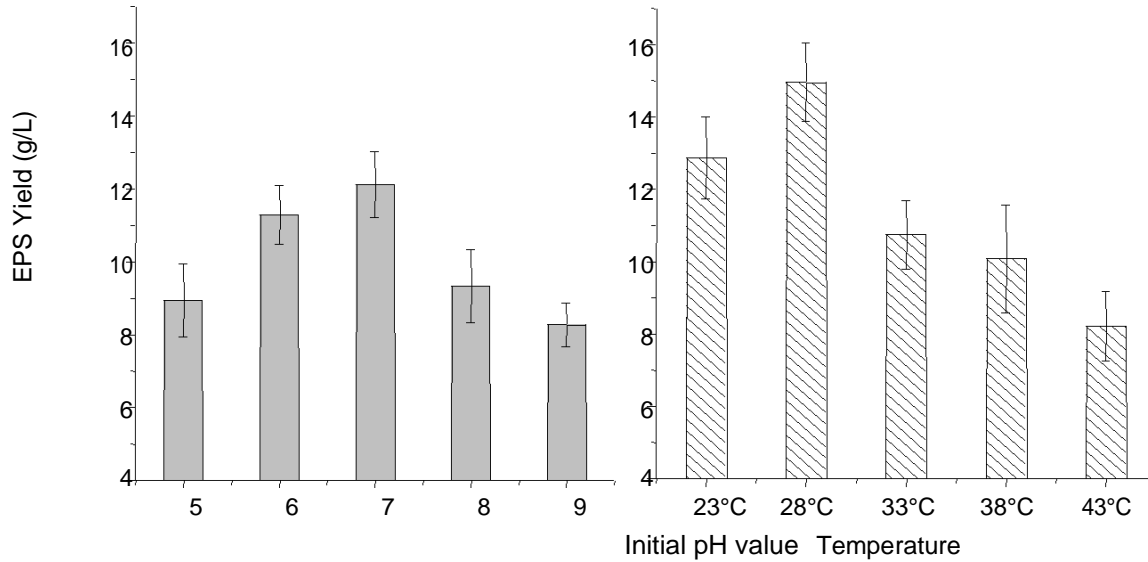
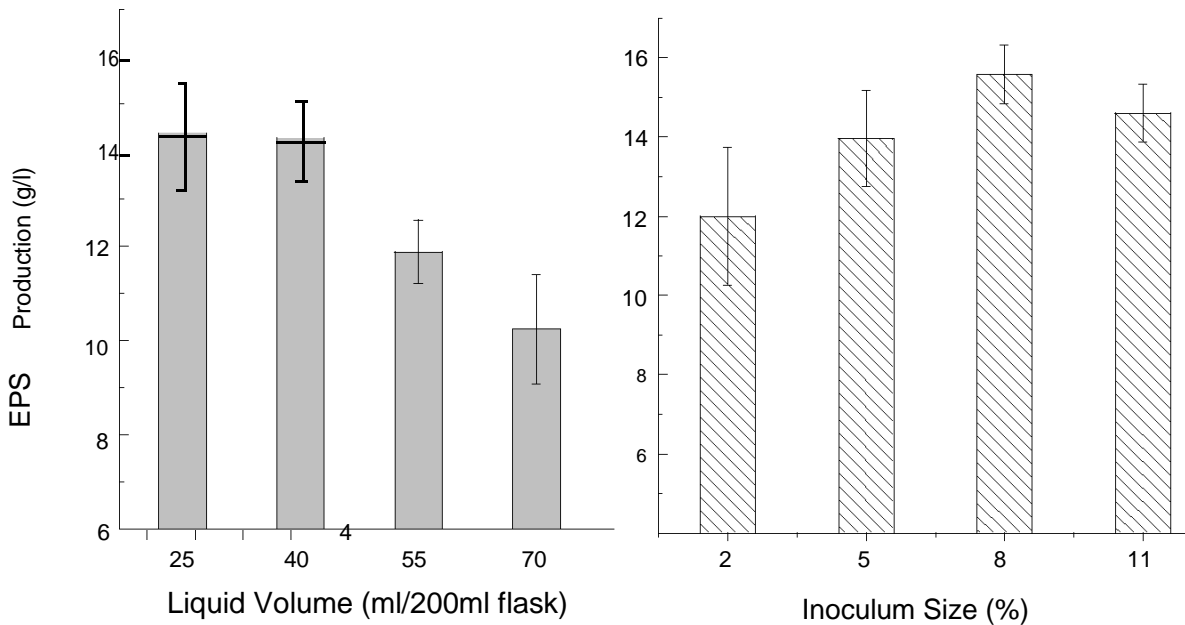**Figure 4.** Effect of initial pH value and temperature on EPS production.

Figure 5. Effect of liquid volume and inoculum size on EPS production.

(Figure 5).

Optimization by orthogonal matrix method

According to the orthogonal method, the effect of those medium on EPS production was analyzed and the results are shown in Table 2. Based on the magnitude order of R value (maximum difference), the order of effects of all factors on EPS production was maltose > peptone > NaCl > beef extract. In terms of the maximum K-value of

each column in Table 3, optimal level of each medium ingredient for EPS yield was 3 g/l beef extract glucose, 10 g/l peptone, 40 g/l maltose, 4 g/l NaCl.

Based on the magnitude order of R value (maximum difference), the order of effects of all factors on EPS yield was maltose >peptone >NaCl >beef extracts. According to ANOVA (Table 4), the factors, peptone and maltose had significant effect on EPS yield ($P<0.05$).

To validate the model, optimal culture medium (beef extract 3 g/l, peptone 10 g/l, maltose 40 g/l and NaCl 4 g/l), other condition (initial pH 7.0, temperature 28°C,

Table 2. Results of L9 (34) orthogonal test of exopolysaccharide production of *Bacillus* strain 27 strain in shake flask culture.

Run	1	2	3	4	
Factor	Beef extract (g/L)	Peptone (g/L)	Maltose (g/L)	NaCl (g/L)	Results (g/L)
1	2	5	20	4	5.63±0.2
2	2	10	30	5	15.98±1.07
3	2	15	40	6	13.24±0.73
4	3	5	30	6	5.83±0.31
5	3	10	40	4	18.08±2.26
6	3	15	20	5	5.59±0.52
7	4	05	40	5	10.54±2.21
8	4	10	20	6	8.57±0.22
9	4	15	30	4	13.48±0.2

Values are means ± SD of triple determinations.

Table 3. Analysis of media on exopolysaccharide production of Bacillus strain. 27 in shake flask culture with L9 (34) orthogonal test.

	Beef extract (g/l)	Peptone (g/l)	Maltose (g/l)	NaCl (g/l)
k1	11.61	7.33	6.59	12.39
k2	9.83	14.21	11.76	10.70
k3	10.86	10.77	13.953	9.21
R	1.78	6.87	7.356	3.18

Ki =Σ exopolysaccharide in thrice experiment at Xi. Values are means ±SD of triple determinations R = maximum Kix -minimum Kix. Values are means ± SD of triple determinations.

Table 4. Variance analysis of L9 (34) orthogonal experiment on EPS yield.

	Sum of squares	Degree of freedom	Mean square	Significance
Beef extract	4.809	2	1.000	
Peptone	70.933	2	14.750	*
Maltose	85.611	2	17.802	*
NaCl	15.221	2	3.165	
Error	4.81	2		

inoculum size 8%, liquid volume 40 ml in 200 ml flask) was carried on. Consequently, the maximal EPS production was 20.17 g/l. The EPS yield on optimal condition was about five times than that at the basal culture condition.

Bio-flocculating activity

The flocculating activity of the EPS was measure by using a kaolin suspension. In order to neutralize the charge, one milliliter of 90 mmol/L $CaCl_2$ were added into 50 ml 4.0 g/l kaolin suspension. The optical density (OD) of the clarifying solution was measured with a spectrophotometer at 550 nm. After calculation the flocculating activity of *B. thuringiensis 27* is 80.4%.

DISCUSSION

Carbon source plays a very important role in growth especially in polysaccharide production. A substantial change in the polysaccharide production was observed with different carbon sources. Maltose supported maximum EPS production which was difference with other bacillus (Lee et al., 1997a). The difference in EPS production among the maltose concentration of 3, 4 and 5% was not significant because the cell maybe not absorb more maltose. It also is report that see that the EPS production increased as the initial carbon resource concentration increased from 20 to 100 g/l (Lee et al., 1997b). Nitrogen sources in the form of proteins and nucleic acid play an important role in the cell mass. Peptone as organic nitrogen source is the best nitrogen

source in our work. Additionally, it was found in the study that organic nitrogen source are more suitable for the EPS production. It was reported that organic nitrogen sources were absorbed by the cells easier than the inorganic ones (Hwang et al., 2003; Gandhi et al., 1998). Inorganic ions affected EPS production by combined with enzyme. The result showed NaCl could provide the maximum EPS production, which did not match those of other researcher (Lung and Huang, 2010).

Optimum pH for polysaccharide production for bacteria ranges from 6.0 to 7.5 (Kumar et al., 2007), which exactly accorded with those of *B. thuringiensis 27*. Optimum EPS production (14.96 g/l) was obtained in the neutral pH range. The maximum EPS production by *B. thuringiensis* 27 were observed at 28°C, which is comparable to many kinds of bacillus that have relatively low temperature optimal (example 20 to 25°C) in their submerged cultures(Cerning et al., 1992). Among several bacteria physiological properties inoculum size may play an important role in biological development (Gancel and Novel, 1994). Inoculum size and liquid volume may play an important role in cell reproduction and EPS production (Chen et al., 2008). Result showed 8% inoculum size and 25 ml in 200 ml flasks was fit for EPS production.

From the single factors experiments the three critical factors affect the EPS yield were identified. Considered beef extract as an important nitrogen source it is selected to further optimize. To investigate the relationship among various factors the orthogonal experimental design technique as a mathematical method were used the orthogonal matrix method was obviously a serviceable experimental design to simultaneously investigate the relationship between the effect of medium components and their optimal concentrations (Gundlapally and Garcia-Pichel, 2006). In addition, the orthogonal matrix can comprehensively investigate central composite design, and thus facilitates economical benefit, experi-mental convenience. In our work by orthogonal method the EPS production arrived to 20.17 g/l, which is not only about five times than those of the basal medium but more than those of the maximum result of orthogonal design. That is means the orthogonal matrix method can be used for optimizing the EPS production in a submerged fermentation process. Furthermore it is applied to optimization of culture media for the production of primary and secondary metabolites in fermentation processes (Chen et al., 2008).

Flocculation technology as a kind of effective and quick method is used for wastewater treatment. And bioflocculation is a dynamic process resulting from the synthesis of extracellular polymers by living cells. In recent years, the use of microbial flocculants has been promoted as a solution to environmental problems because their intermediates are harmless and biodegradable. Thus searching and usage bioflocculation arouse the interesting of researcher in the world. The flocculating activity of 27 is 80.4% which is below the bioflocculant p-KG03 (Yim et al., 2007). To obtain new and better

bioflocculant, we need screen more strain to obtain functional EPS in BSCs. Otherwise new usage for microbiology in the desert and desert biological soil crusts is proved a scientific basis.

Conclusion

The traditional separation, screening and purification methods were used, 27 strains of producing EPS were isolated. Under optimal condition the strain could produce a large amount of EPS. And the EPS possess a certain degree of flocculation, the flocculation rate was 80.4%, which is lower than the previously reported strain isolated from the soil (flocculation rate can reach 97%). Further screening and optimization to increase the flocculation rate still carry on. It is well known that microbial flocculants as a kind of sewage treatment agent are non-toxic, harmless, no secondary pollution. In this subject, the discovery of new sources of microbial flocculant has brought new interest in dealing with the pollution of water resource. And new usage for the desert biological soil crusts is provided a scientific basis.

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