Bioflocculants Produced by Gram-positive *Bacillus* xn12 and *Streptomyces* xn17 for Swine Wastewater Application

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Strains xn12 and xn17 were isolated from activated sludge from a local hoggery, and preliminarily identified as Gram-positive *Bacillus* and *Streptomyces*, respectively. Their flocculating activities were mainly in the extracellular materials, and their components were mainly non-ketose polysaccharides, not proteins. Their flocculating efficiencies were over 95 % under the optimal conditions for production of xn12 and xn17 as follows: initial pH 5; inoculum volume 0.5 mL, 1.0 mL, respectively; cultivating temperature 30 °C; and cultivation time 4.5 days and 1.5 days, respectively. Each of the xn12 and xn17 had strong thermal stability under 30–80 °C. The optimal removal efficiency of turbidity for xn12 and xn17 in swine wastewater was 82 %, 87 %, respectively, their turbidity removal efficiencies were better than polyaluminium chloride alone, and that of COD was 42 %, 46 %, respectively.

Key words:
Bioflocculants, wastewater treatment, swine wastewater, flocculation

Introduction

A bioflocculant is a kind of metabolite produced by microorganisms during their growth. It is mainly composed of high polymers as extracellular polysaccharide, glycoprotein, protein, cellulose and nucleic acid.[1–3] Bioflocculants have gained much wider attention due to their biodegradability and safety.[4] Bioflocculants have gained wide attention and research interest. Most researchers have focused on microbial screening, production conditions, flocculating mechanisms, chemical structures, low-cost production.[1,2,5] In bioflocculant development for practical applications, low flocculating capability and large dosage requirements have been major problems.

In practical applications, bioflocculants have been used to treat dye solutions,[6] inorganic solid suspensions,[3,7] in downstream processing, for food and industry wastewater[2,8,9] and so on. Until now there were few reports about treating swine wastewater, which includes a high concentration of organic matter, inorganic nutrients and gaseous emissions, and exceeds the capacity for direct land disposal that would cause severe environmental pollution.[10,11] This research is aimed at flocculation processing of swine wastewater. Two bioflocculant-producing organisms named xn12 and xn17 were isolated from activated swine wastewater sludge. A series of experiments were performed to investigate the bioflocculant production and their application in swine wastewater treatment.

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Materials and methods

Bioflocculant-producing medium and culture conditions

The composition of the cultivation medium[12] was (in g L^{-1}): glucose, 20; yeast extract, 0.5; urea, 0.5; NaCl, 0.1; K_2HPO_4, 5; KH_2PO_4, 2; $(NH_4)_2SO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2, and the initial pH of medium was adjusted to 7.0–7.5. The composition of the seed medium (in g L^{-1}) was: peptone, 10; beef extract, 5; NaCl, 5, and the initial pH of medium was adjusted to 7. The medium for slant contained (in g L^{-1}): peeled potatoes, 200; sucrose, 20; agar, 18. After autoclaving, the seed liquid was inoculated into 250-mL flasks containing 50 mL culture medium and incubated for 3 days in a rotatory shaker at 35 °C, 150 rpm, and the flocculating efficiency was then examined. For seed culture preparation, the isolated strains were placed into 50 mL culture medium in a 250-mL flask and incubated for 3 days in a shaker at 30 °C, 150 rpm; finally, the cultured broth was used as seed liquid to prepare all experiments. In the experiments of cultivation conditions, the initial pH of culture medium, inoculum volume, cultivating temperature, and cultivation time were varied to investigate the culture conditions for the two strains.

Measurement of flocculating efficiency

Flocculating efficiency of the samples was measured using the kaolin clay suspension method.[12] One mL cultured broth and 2 mL $CaCl_2$ (1 wt %) was added to 100 mL kaolin (particle diameter 0.15–0.3 μm) suspension (5 g L^{-1}), after the pH was adjusted to 7.0–7.5 and stirred for 1 minute, held for 10 minutes, and supernatant taken from 1 cm below liquid level. The absorbance of the supernatant and the blank control without bioflocculant was measured in a spectrophotometer at 550 nm. The flocculating efficiency was calculated as follows:

$$\text{Flocculating efficiency (\%)} = \frac{OD_{blank} - OD_{550}}{OD_{blank}} \cdot 100. \quad (1)$$

where OD_{blank} is the absorbance of blank control and OD_{550} is the absorbance of supernatant.

Measurement of cell growth, composition analysis, and thermal stability of bioflocculants

Measuring the absorbance of culture broth at 660 nm (OD_{660}) is one of the most common methods used to determine the cell growth. The bioflocculant composition was analyzed by the color reaction of polysaccharides and proteins.[13] The polysaccharides were determined by the Anthrone reaction, Molish reaction and Resorcinol reaction. The proteins were determined by Ninhydrin reaction, Xanthoprotein reaction and biuret reaction. To examine the thermal stability of the bioflocculants, after 30 minutes of insulation at various temperatures (30–80 °C), the cultured broth was used to measure the flocculating efficiency at room temperature.

Extraction of flocculant material

Bioflocculant purification was achieved according to the modified method.[5] To purify the bioflocculant, the fermentation broth was centrifuged to remove cells (5000 rpm, 30 min). The supernatant was poured into two volumes of cold ethanol at 4 °C to precipitate the bioflocculant. After 12 h, the resulting precipitate was collected by centrifugation at 5000 rpm for 30 minutes and re-dissolved in water. After two such steps, the precipitate was dehydrated at 40 °C, and the crude bioflocculant was obtained.

Applications in swine wastewater treatment

Different doses of bioflocculant and 1 wt % $CaCl_2$ were added to 200 mL swine wastewater and the pH was adjusted to various pH values using 10 wt % NaOH solution, then the mixture was stirred at 150 rpm for 2 minutes, at a slow pace of 60 rpm, settled for 30 minutes, and the supernatant was taken to analyze the flocculating effects. In this study, the application of polyaluminum chloride (PACl) (10 wt %) alone was also tested in swine wastewater treatment.

The measurements of chemical oxygen demand (COD) and turbidity were performed according to the Standard Methods issued by the China National Environmental Protection Agency. The residual COD, turbidity, and pH were determined, and the removal efficiency of COD and turbidity was calculated based on the initial and final values after treatment.[9]

Results and discussion

Screening and identification of bioflocculant-producing strains

A total of 17 bioflocculant-production strains, whose flocculation-activities were over 90 %, were isolated from activated sludge from a local hoggery of Zhengzhou (China).[9,14] The flocculating efficiency of xn12 and xn17 were over 95 %. The surface characterizations of these two strains are shown in Table 1. According to Bergey's Manual of Systematic Bacteriology,[15] strains xn12 and xn17 were preliminarily identified as Gram-positive *Bacillus* and *Streptomyces* species, respectively.

Effect of initial pH, inoculum volume, and cultivating temperature on bioflocculant production

Some researchers have pointed out that the initial pH of the culture medium determines the electric charge of the cells and the oxidation-reduction potential, which can affect nutrient absorption and enzymatic reaction.[2] Different strains have different

Table 1 – *Surface characterization of strains*

Number	Surface characterization of colony			Morphological characterization of strains	Gram stain
	color	colonial morphology	surface morphology		
xn12	reddish gray	circular, irregular edges	flat, smooth, wet, viscous	rod-shaped	positive
xn17	French gray	circular, full edge	protuberance, dry, powder surface, black background color, earth tastes	spiral spore chain, circular conidium	positive

optimal initial culture medium pH values. The flocculant production by *R. erythropolis* was higher at alkaline pH values of 8.0–9.5 than at other pH values.[2] The optimal pH for the bioflocculant production by *Aspergillus parasiticus* was in the range of 5–6.[9] After cultivating in the liquid medium for 3 days, the effect of the initial pH on the bioflocculant production was examined. It was obvious that the alteration of the initial pH had no significant effect on flocculating efficiency (Table 2). The flocculating efficiencies of xn12 and xn17 were effective over a wide pH range, and the acidic condition was much better than the alkaline condition. The optimal initial pH range of bioflocculant production for xn12 and xn17 was 3–10, 3–9, respectively; when the pH was 5, the flocculating efficiency of xn12 and xn17 was all 97 %, which was similar to ZS-7 (99 %),[16] while higher than MBF7 (85 %) and MBF4-13 (86 %).[17,18] Moreover, comparing the final pH with initial pH, the final pH could maintain certain values in certain initial pH range. This was because the bacterial mixed-culture had buffer ability; the phenomenon was found in others.[5]

Table 2 – *Effect of initial pH of medium on flocculating efficiency*

xn12			xn17		
Initial pH	Final pH	Flocculating efficiency (%)	Initial pH	Final pH	Flocculating efficiency (%)
3.0	4.5	95	3.0	6.1	94
4.0	4.3	93	4.0	6.2	92
5.0	4.2	97	5.0	5.9	97
6.0	4.9	96	6.0	6.0	96
7.0	6.3	96	7.0	6.6	94
8.0	6.6	93	8.0	6.5	94
9.0	6.7	92	9.0	6.4	90
10.0	6.9	93	10.0	6.4	89
11.0	6.9	84	11.0	6.4	85

The effects of inoculum volume on bioflocculant production of strains were also investigated. From Table 3, the change of inoculum volume had a certain influence on the flocculating efficiency and the cell growth. The maximum flocculating efficiencies were obtained when the inoculum volume of xn12 and xn17 was 0.5 mL, 1.0 mL seed culture per 50 mL culture medium, respectively. However, any further increase with inoculum volume did not result in higher flocculating efficiency. It was also found that the maximum

Table 3 – *Effect of inoculum volume on flocculating efficiency*

xn12			xn17		
Inoculum volume (mL)	Cell growth (g L^{-1})	Flocculating efficiency (%)	Inoculum volume (mL)	Cell growth (OD_{660})	Flocculating efficiency (%)
0.1	1.915	91	0.1	1.88	89
0.5	1.727	94	0.5	3.18	95
1.0	1.868	96	1.0	4.95	93
3.0	1.725	95	3.0	5.50	93
5.0	1.798	91	5.0	3.80	94
8.0	1.859	87	10.0	3.88	88

Note: strains xn12 and xn17 seed culture contained approximately $1.4 \cdot 10^5$ and $2.5 \cdot 10^3$ cells mL^{-1}, respectively.

flocculating efficiency was not in agreement with the maximum cell growth, but rather the flocculating efficiencies of xn12 and xn17 were maintained within a certain range of inoculum volume. This was because the microbial growth was influenced by inoculum volume, too small inoculum volume prolonged the stagnant time, whereas a large inoculum volume made niche of strains overlap and restrained the bioflocculant production due to the limit of nutrient allocation.

Different cultivating temperatures were used to investigate the flocculating efficiencies of xn12 and xn17. The best production of bioflocculant by *A. sojae* was obtained at the temperature range of 30–34 °C.[2] The optimum temperature for bioflocculant production from *Enterobacter cloacae* WD7 was 30 °C.[19] After cultivating for 3 days, there was no significant difference in the flocculating efficiencies of xn12 and xn17 in the range of 20–40 °C with the maximum (all 95 %) at 30 °C (Fig. 1).

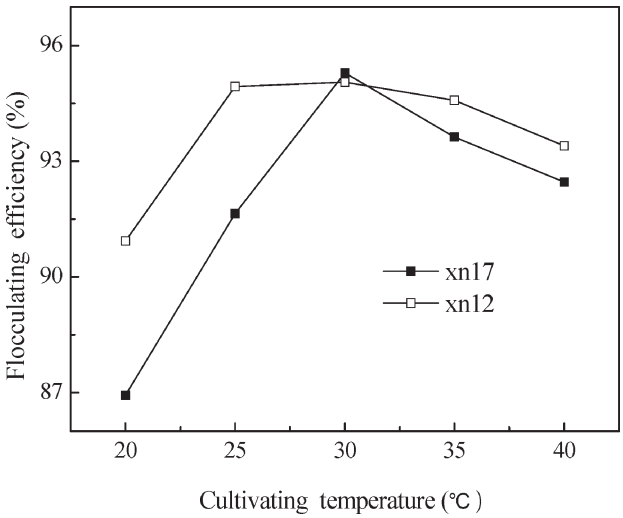

Fig. 1 – *Effect of cultivating temperature on flocculating efficiency*

Some researchers[19,20] have reported that all commercial polysaccharide-producing microorganisms were mesophiles.

Effect of cultivation time on bioflocculant production

The cultivation conditions were as follows: pH 5, temperature 30 °C, shaking speed 150 rpm, inoculum volume 0.5 mL, 1.0 mL (xn12 and xn17, respectively). Fig. 2 illustrates the growth, flocculating efficiency, and pH variation curves with time.

The production of bioflocculant by xn12 was not parallel to the cell growth during the initial growth phase, and a large amount of the bioflocculant was achieved at the logarithmic growth phase with flocculating efficiency of over 95 %, while the flocculating efficiency decreased at the stationary phase. The similar phenomenon was reported in the cultivation of *Alcaligenes latus* for the production of bioflocculant.[8] During the logarithmic phase, the production of xn17 was almost parallel to the cell growth, while xn17 reached its maximum flocculating efficiency at 4.5 days, and then decreased gradually. The similar phenomenon was reported in the cultivation of *Bacillus licheniformis* for the production of bioflocculant.[7] In addition, the results in Fig. 2 show that pH of the culture broth decreased with the increase in cultivation time, which suggests that some organic acids were produced and released into the medium by strains xn12 and xn17 during growth.[9,21]

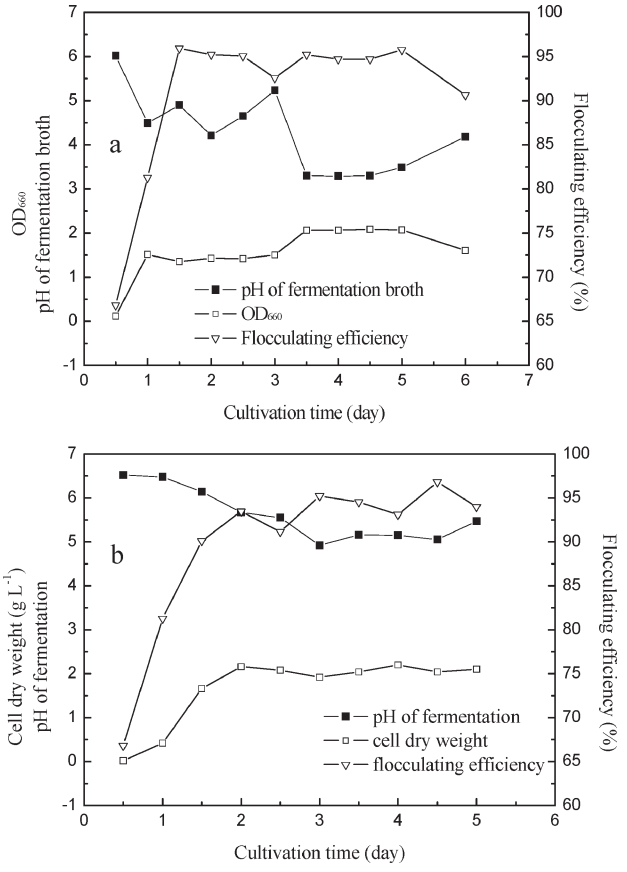

Fig. 2 – *(A) Growth, and flocculating efficiency of xn12; (B) Growth, and flocculating efficiency of xn17*

Distribution of flocculating activity in fermentation broth and analysis of bioflocculant compositions

The xn12 and xn17 distribution of flocculating activity in fermentation broth was studied. The supernatant was obtained by centrifugation at 5000 rpm for 30 minutes. After centrifugation, the precipitate (composition included such substrates as mycelium cells, cellulose, and so on) was collected and dissolved in distilled water. The flocculating efficiency of the fermentation broth (94, 95, respectively) and the supernatant (93, 90, respectively) were all high (over 90 %). Comparatively speaking, the residual precipitate (40, 44, respectively) has a very weak flocculating activity. This indicates that most of the flocculating activities are found mainly in the extracellular materials produced by mycelium cells. The extracted and dried raw flocculation material appeared as a white powder. For xn12 and xn17, the extraction amount of the extracellular polymer obtained under the optimal culture conditions ranged from 1.7 to 1.9 g L^{-1}, 1.4 to 1.7 g L^{-1} culture liquid, respectively.

The results of color reaction showed that the Anthrone reaction and Molish reaction were positive, whereas Resorcinol reaction, Ninhydrin reaction, Xanthoprotein reaction and biuret reaction were negative. Thus, we concluded that the main components of bioflocculant were non-ketose polysaccharides, not proteins.

Thermal stability of bioflocculant

There was a close relation between the thermal stability of the bioflocculant and its chemical composition. Some researches indicate that the heat resistance of bioflocculants is consistent with the general understanding that flocculants rich in polysaccharides have better thermal resistance than those of proteins and nucleic acids.[22,23] This was because the main components were polysaccharides which are insignificantly affected by temperature, while high temperatures might cause denaturization of proteins and the change of its spatial structure. From Fig. 3, the thermal stability tests of the bioflocculants within the range of 30–80 °C illustrate that no change in flocculating efficiencies was observed with the increase in temperature. The flocculating efficiencies were over 90 %, which we speculate is because the main backbone of the bioflocculants was polysaccharides.[21]

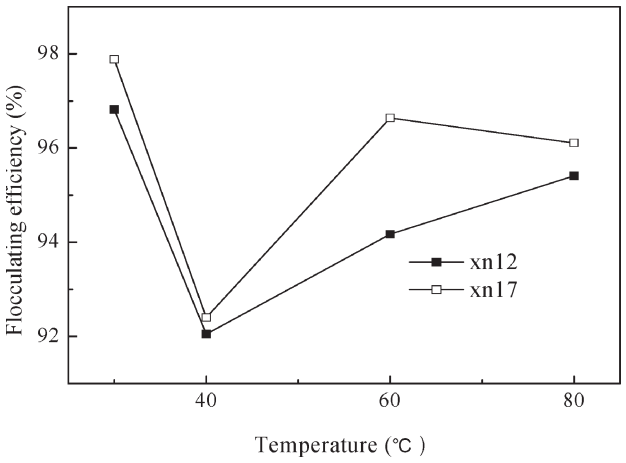

Fig. 3 – *Thermal stability of bioflocculants xn12 and xn17*

Applications in swine wastewater treatment

In this experiment, the COD, pH and turbidity of swine wastewater is 1372–3025 mg mL^{-1}, approximately 7.5, and 230–800 NTU, respectively. Bioflocculant properties under different conditions and the turbidity removal efficiency under optimal conditions are shown in Table 4. At first, bioflocculant properties increased as the dosage of bioflocculant and $CaCl_2$ increased, and the highest values were all reached at 2.0 mL, and then decreased. The influencing trend of pH on flocculating efficiencies was an increase as the pH increased. From this factor trend, pH is the most significant for the turbidity removal efficiency; the bioflocculant dose did not differ from the $CaCl_2$ dose. Though the flocculating efficiency reached 55 % and 61 %, respectively, at pH 9.5 without adding $CaCl_2$, a certain dosage of $CaCl_2$ was helpful in improving the turbidity removal efficiency in our experiments, so it can be preliminarily reasoned that the flocculation process of bioflocculants was a result of the interaction of both Ca^{2+} and bioflocculant under alkaline conditions.

The optimal turbidity removal efficiency for xn12 and xn17 in swine wastewater was 82 %, 87 %, respectively, the removal efficiency of COD was 42 %, 46 %, respectively, and the flocculation effect was better than PACl (81 %) alone.

Based on the above experimental results, the bioflocculant produced by strains xn12 and xn17 had good flocculating activity for both kaolin suspension and swine wastewater. The bridging and charge neutralization mechanism is most often used to explain the flocculation process of bioflocculants.[2,6] The effectiveness of the bridging mechanism depends on the molecular weight of the bioflocculant, the charge on the polymer and the particles, the ionic strength of the suspension, and the nature of mixing.[2] During the flocculation of kaolin suspension using the bioflocculant, many kaolin particles would adsorb on the active sites in the flocculant molecules and result in the formation of some flocs in which the flocculant molecules acted as a "bridge".[6] In the swine wastewater flocculation process, bridging and the charge neutralization mechanism could also explain the flocculation process of the bioflocculants. The components of xn12 and xn17 were polysaccharides, therefore their polysaccharide surface charge properties were negative under alkalinity conditions, and the colloidal particles of wastewater were negative. When Ca^{2+} was present, the negative charges of both polysaccharides and colloidal particles of wastewater could be neutralized, and the charge might be reversed from negative to positive. Then many colloidal particles could be adsorbed onto the bioflocculant chains, thereby bridging the two, and the aggregates entangle to form larger particles.

Table 4 – *Turbidity removal efficiency of swine wastewater under different pHs, dosages and $CaCl_2$ concentrations*

Removal efficiency (%)			Removal efficiency (%)			Removal efficiency (%)		
pH	xn12	xn17	bioflocculant dose (mL L^{-1})	xn12	xn17	1 wt % $CaCl_2$ (mL)	xn12	xn17
6.0	4.4	19	0.5	66	74	0.0	61	55
7.0	17	28	2.0	73	75	1.0	65	65
7.5	22	28	4.0	69	72	2.0	70	74
8.0	30	56	6.0	68	71	4.0	67	66
8.5	58	66	10.0	71	70	6.0	63	63
9.0	57	73	15.0	68	65	10.0	63	57
9.5	70	79						
10.0	73	81						
11.0	82	87						

Conclusions

Two novel bioflocculant-producing strains named xn12 and xn17 were isolated from activated sludge obtained from a local hoggery, and preliminarily identified as Gram-positive *Bacillus* and *Streptomyces*, respectively. Their flocculating activities mainly resided in the extracellular materials, and their effective components were mainly non-ketose polysaccharides, not proteins. Their flocculating efficiencies were over 95 % under optimal cultivation conditions, as follows: initial pH 5; inoculum volume 0.5 mL, 1.0 mL, respectively; cultivating temperature 30 °C; and cultivation time 4.5 days, 1.5 days, respectively. Each of the xn12 and xn17 could maintain effective flocculating efficiency under 30–80 °C. The optimal turbidity removal efficiency for xn12 and xn17 in swine wastewater was 82 %, 87 %, respectively, thus, their turbidity removal efficiencies were better than PACl alone, whereas that of COD was 42 %, 46 %, respectively. In general, the bioflocculants of xn12 and xn17 have good flocculating activities, but their industrial application would need further investigation.

List of abbreviations and symbols

COD – chemical oxygen demand
OD_{blank} – absorbance of blank control
OD_{550} – absorbance of supernatant
PACl – polyaluminum chloride

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