

Screening of flocculant-producing strains by NTG mutagenesis

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Abstract: Screening of new microorganism being able to produce efficiently flocculants was carried out. A new model for screening efficient flocculant-producing strains was designed and tested. The results showed that this model for screening efficient flocculant-producing strains is very reliable and can greatly shorten the screening period. 13 flocculant-producing strains were isolated from activated sludge by conventional method. A strain, designated as HHE6, produced the bioflocculant with the turbidity removal 98% for kaolin suspension. Six of 13 strains selected as the original strains were treated with NTG as mutagen, and five mutant strains (HHE-P7, HHE-A8, HHE-P21, HHE-P24, HHE-A26) with high flocculation efficiency was obtained by selection, which exhibited the flocculation rate for kaolin suspension above 90%. Strains HHE6, HHE-P7, and HHE-P24 were classified as *Penicillium purpurogenum*, HHE-P21 as *Penicillium cyclopium*, HHE-A26 as *Aspergillus versicolor* and HHE-A8 as *Aspergillus fumigatus*, and it is hitherto unreported for bioflocculant-producing strains of *Penicillium*. The growth of the six strains (HHE6, HHE-P7, HHE-A8, HHE-P21, HHE-P24, HHE-A26) had similar curves, i.e. firstly increasing rapidly, keeping relatively constant then and finally decreasing gradually with cultivation time. The production of bioflocculants by strains showed the similar pattern to strain growth.

Keywords: bioflocculant; mutation breeding; bioflocculant-producing strains; *Penicillium*; *Aspergillus*

Introduction

Various flocculants such as inorganic flocculants, organic high-polymer flocculants and naturally occurring flocculants have been widely used in industrial fields. Although chemical flocculants have been used widely due to their effective flocculation efficiency and lower cost, they have neurotoxic and carcinogenic monomers and their usage is restricted (Kwon, 1996). Naturally occurring materials including chitosan and gelatin are safe and biodegradable, but they show only weak flocculation efficiency in applications (Takagi, 1985). Thus, the development of a new biodegradable bioflocculant with strong flocculation efficiency is attracting wide research interest.

Bioflocculant is a kind of safe and biodegradable macromolecular flocculant secreted by microorganisms. The bioflocculants have been already investigated and reported include PHB (Crabtree, 1965), DNA (Watanabe, 1998), protein flocculants (Shih, 2001; Takeda, 1991; 1992) and polysaccharide flocculants (Kobayashi, 2002; Yokoi, 1996).

Recently, many microorganisms, including bacteria, fungi, yeast, and algae, which could produce flocculating substance have been isolated from soil and wastewater with conventional method (Salehizadeh, 1998; 2000; 2001; 2003; Kobayashi, 2002). Although all the bioflocculants had been identified, none of them has been practically applied in industry because of the low productivity and high cost. Screening new microorganisms which could produce flocculants with high flocculation efficiency and optimizing the fermentation process to improve productivity have, therefore, become another research focus in recent years.

In this study the screening of new flocculant-producing strain by combining conventional method and mutation breeding technology was carried out. The biological characteristics of the flocculant-producing strains were also discussed in detail.

1 Materials and methods

1.1 Strains source

Activated sludge was from Guangzhou Wastewater Treatment Plant, which is situated in Guangzhou, China.

1.2 Media preparation

Composition of the screening medium was as follows: 20 g glucose, 2 g KH_2PO_4 , 5 g K_2HPO_4 , 0.2 g $(\text{NH}_4)_2\text{SO}_4$, 0.1 g NaCl, 0.5 g urea, 0.5 g yeast extract, and 1 L tap water. pH was adjusted to 7.5—8.5.

1.3 Screening methods

1.3.1 Conventional method

An activated sludge was used as a strain source in the above 5 types of culture media for primary screening. After enriched culturing of samples, the single colony could be obtained by streaking the purified strains. After cultivating the strains on a shaker at 30°C for 72 h, initial screening of every culture solution was carried out through jar test. According to the flocculation rate, we noted the results as “++++” (excellent, above 90%), “+++” (good, 80%—90%), “++” (ordinary, 70%—80%), “+” (slight, 60%—70%), “-” (ineffective, below 60%). Cultivate the obtained strains in a parallel way with the screening culture medium, test the flocculation rate of the culture broth quantitatively and screen again. Preserve the flocculant-producing strains obtained from the secondary screening and classify them.

1.3.2 Mutation breeding

The flocculant-producing strains obtained from conventional screening method, as original strains, were treated with nitrosoguanidine (NTG) and the mutant strains with high flocculation efficiency were obtained. It was proved that flocculation efficiency of the mutant strains was stable by pass-generation tests. The method of the primary screening and secondary screening of the mutated strains was the same as the conventional one.

1.4 Mutating methods (Zhang, 1984)

1.4.1 Microbes suspension preparation

The original strains were inoculated from the corresponding agar slant into 250 ml conical flasks containing 50 ml screening culture broth. The culture was incubated at 30°C at the shaking speed of 150 r/min. The culture broth in the logarithm growing period for microbe (3—5 d after fungus cultivation) was as the samples. The samples were centrifugalized for 15 min at a speed of 3000 r/min, then the cell precipitated were washed three times with phosphoric acid buffer solution and were collected and modulated into

cell suspending liquid with phosphoric acid buffer solution. The concentration of the liquid was: bacteria 10^8 /ml and mycete 10^6 /ml.

1.4.2 Treatment of mutagenic agent

One milliliter bacteria suspending liquid was put in a 20 ml test tube, mixing with 1 ml NTG of a concentration of 1 mg/ml. Thus the NTG concentration was 0.5 mg/ml. The sample was shaken in a water bath at 37°C , with a shaker rotating at the speed of 150 r/min for 30 min. After that, it is centrifugalized for 15 min at a speed of 3000 r/min. The mutated strains were collected then. After being washed three times with phosphoric acid buffer solution, the strains were transferred into a 250 ml conical flask with 50 ml liquid culture medium in beforehand.

1.4.3 Primary and repeated screening of the mutated strains

The mutated strains were cultivated for 1—2 d, then the strains liquid was sampled and diluted. 0.1 ml diluted liquid was spread on a solid culture medium flat plate. After 96 h cultivation, the strains were selected and purified with streaking separately, cultivated in a 50 ml liquid culture medium, respectively. Thereafter their flocculation rate was tested and preliminary screening was proceeded. The initially screened strains were fermented in a parallel way, then their flocculation rate was quantitatively measured and screened repeatedly. After the repeated cultivation, according the stability of the flocculation efficiency, the perfect strain obtained from the repeated screening was ascertained.

1.5 Flocculation rate measurement

Eighty milliliter distilled water, 0.4 g kaolin, 5 ml 1% CaCl_2 , 2 ml fermented liquor were put into a 100 ml cylinder, then graduated with distilled water, pH was adjusted to 7.5, and then the liquid was transferred to a 150 ml beaker. There after the liquid was agitated for 2 min on a magnetic stirring apparatus, and then left to stand still for 5 min. Instead of fermented liquor, distilled water was used as the control solution. By measuring the decrease of turbidity in upper phases, flocculation efficiency was expressed as flocculation rate (FR).

The flocculation rate (FR) can be calculated by the following equation:

$$FR(\%) = \frac{A - B}{A} \times 100$$

Where A and B are optical densities of the control and sample, respectively at 550 nm.

1.6 Identification of the flocculant-producing strains

Referring to document methods (China microorganism fungus preservation and Management Committee, 1992; Song, 1993; Zhang, 1984; Qiu, 1998), the eventually obtained efficient flocculant-producing strains were inoculated on Czapek's plate separately, then the colony morphology and microbial configuration was observed and identified.

(1) The high-efficiency flocculant-producing strains were inoculated and cultivated on Czapek's plate, one colony each Czapek's plate with three replicates, observing at $28 \pm 1^\circ\text{C}$.

(2) 3—5 d after growth of the colony on Czapek's culture media, the texture, color, growth speed of the surface of the colony were carefully observed by using (5—10) \times magnifying glass, then the shape and color of conidiophore head were observed by using (50—100) \times dissecting glass.

(3) 1—2 colony pieces were put at the edge of the

single colony which grows for 3—5 d on Czapek's culture media on carrier glass for temporary observation. Then, the shape and color of the conidiophore head, spore-producing morphology and conidiophore were carefully observed.

(4) The single colony was cultivated on Czapek's culture media for 20—30 d at $28 \pm 1^\circ\text{C}$, the color and pigment production of the surface of the colony (the back side) were observed.

(5) Preliminary conclusion could be made based on the above observation. Results could be further confirmed by cultivating colony with yeast culture media, and observing colony characteristics, conidiophore head, spore-producing morphology and conidiophore.

1.7 Periodicity of the flocculant-producing strains' activity

Put 50 ml the screening medium in a 250 ml conical flask, cultivate in a shaker at 30°C , take a sample at intervals, measure pH of the nutrient culture medium, strains increment (test OD_{660} or cell dry weight) and the flocculation rate.

2 Results

2.1 Screening and mutation breeding of the flocculant-producing strains

2.1.1 Separation and screening results

Total 13 strains which had the ability to flocculate kaolin clay were separated from activated sludge using conventional streaking method. The result of the screening is shown in Table 1. The microorganisms were distributed in bacteria and fungi. Through secondary screening and test of the flocculation rate, the flocculation rate of three strains (V_2 , V_3 , HHE6) was found to be above 85% for kaolin suspension. The turbidity removal of the bioflocculant produced by HHE6 for kaolin suspension reached 98%.

2.1.2 Mutation breeding of the strains

Six of the 13 flocculant-producing strains mentioned above were selected as the original strains. Nine mutant strains were obtained and 5 of them showed high efficient (the flocculation rate for kaolin suspension was above 90%). The mutation screening results are listed in Table 2. After incubation for five generations in a shaker, the stability of the flocculants obtained from mutation was quite desirable.

So twenty-two flocculant-producing strains were obtained from the combination of the two screening methods. Six strains, named HHE6, HHE-P7, HHE-A8, HHE-P21, HHE-P24 and HHE-A26 (Fig. 1), had high flocculation efficiency with flocculation rate of over 90% for kaolin suspension. Further studies were proceeded on the biological characteristics of the six flocculant producing strains of high flocculation efficiency. In addition, the flocculant-producing strains were identified.

Through observation of the colony morphology and microbial configuration of the six strains, it was found that the colony morphology on Czapek's agar plates was the same as those of the mycetes (dry colony; front and back colors as well as central and edge colors were not the same, big colony diameter, etc.). HHE-A26 and HHE-A8 had the feature of typical aspergillus sporodochium. Its conidiophore head enlarged and formed top cyst. Pedicel grew from top cyst directly and conidiospore grew from pedicel top. The others had the typical features of blue mold, with brush conidiophore head.

Table 1 Conventional screening of flocculant-producing strains

Number	Cultivation character on solid culture medium	Description of liquid cultivation	Flocculation efficiency	Classification
V8	Dry, fluffy, and hairy surface of 3.5 mm in diameter, with golden yellow center and colorless edge, three obvious loops on the back, no water soluble pigment	Yellow, bacteria loop and much white floccule, turbid on surface	++	Fungi
V9	The same color on two sides, wet, light yellow, edge in good order, with four circles of 8 mm in diameter each	Brown, turbid, many white floccule	+	Bacteria
V10	Yellow, wet, 3.5 mm in diameter, wavy and opaque edge	Yellow, turbid, brown thick	+	Bacteria
V11	Dry, thick, diameter: 6 mm, brown and black, black back, depressed center, prominent surface	Gray, green, many gray and green global floccule	++	Fungi
V13	Wet, yellow on both sides, 3.0 mm in diameter, smooth, globe-shaped surface, edge in good order	Yellow, turbid	++	Bacteria
V14	Wet, red on two sides, diameter of 5 mm, globe-shaped surface, edge in good order	Red, turbid	++	Bacteria
V2	Wet and round surface, prominent center, yellow on both sides, diameter: 2.0 mm	Turbid, air bubble and many 1—2 mm floc-materials	+++	Bacteria
V3	Wet, opalescent, edge in good order, prominent center, diameter: 2.5 mm	Turbid, bacteria loop and air bubble on surface	+++	Bacteria
V7	Wet, red, diameter of 1 mm, prominent surface, smooth edge	Red, turbid	+ ++	Bacteria
V5	Wet, smooth, colorless, globe-shaped, 0.6 mm in diameter	Turbid, many white floccule	++	
HHE4	White, hairy and loose	Turbid, many white floccule	++	Fungi
HHE5	Flocculent, 1 cm in diameter, prominent and yellow center, red back	Turbid	++	Fungi
HHE6	Green center, almost round, fluffy	Slightly turbid, much white global floccule	+++	Fungi

Table 2 Part of the results of mutation and screening of the flocculant-producing strains

Number	The cultivating character of the solid nutrient medium	Liquid cultivating condition	Flocculation efficiency	Classification
HHE1	Deep gray, red center, flocs white surface, red back, diameter: 1.8 cm	Loops, and many 5 mm small globe, thick liquid	+++	Fungi
HHE-P7	Fine, hair-like texture; blue green surface firstly, gray green later, red brown back	Yellow, turbid, many globe solid	++++	Fungi
HHE-A8	Fine hair-like, blue gray, dark gray or gray with green color at the edge at the early stage of growth, and turning to be brown gray or dark gray later, back side of the colony is dark gray	Bacteria loops on bottle, grows well, with solid	++++	Fungi
HHE18	Diameter: 3.5 cm, with blue gray center, the edge is white flocs and thickness	No loops, small solids.	+++	Fungi
HHE-P21	Diameter: 1.1 cm, with loop, blue gray center, 0.8 mm in diameter, white out, assistant with the nutrient medium, blight yellow aerial mycelium in the younger zone	With loops and solid turbidly	++++	Fungi
HHE23	Flocs-shape, blue and gray center, and thickness, white edge, blood red back	With loops and solid turbidly	++	Fungi
HHE-P24	Fine, hair-like texture; blue green surface firstly, gray green later, red brown back	Turbidly, with solid material	++++	Fungi
HHE25	White center, red water-dissolved color, diameter: 1.5 cm	Turbidly, with solid material	++	Fungi
HHE-A26	compact fine hair-like or vermicelli-like and rather thick texture; gray white at early stage and turning to gray brown or bright brown gradually, with the middle part showing dark olive or gray green color; the back side of the colony is light yellow brown	Yellow, with loops	++++	Fungi

HHE-A8: after cultivating on Czapek's culture media at $28 \pm 1^\circ\text{C}$ for 8 d, the diameter of the colony reached 20—35 mm. Aerial mycelium was fine hair-like or vermicelli-like, being blue gray, dark gray or gray with green color at the edge at the early stage of growth, then turning to be brown gray or dark gray after 15 d. The back side of the colony was dark gray. Hypha body was colorless, transparent, small and separated. The conidiophore head was firstly spherical or semi-spherical, and being compacted sticky cylindrical shape when matured, with a size of 120—300 μm ; flask-like top cyst, single-layer spore-producing structure; spherical or approximately spherical.

HHE-A26: after cultivating on Czapek's culture media at $28 \pm 1^\circ\text{C}$ for 7—10 d, the diameter of the colony reached 20—35 mm with compacted fine hair-like or vermicelli-like and rather thick texture. Colony was gray white at early stage and turning to gray brown or bright brown gradually, with the middle part showing dark olive or gray green color. The back side of the colony was light yellow or pink. The conidiophore head was spherical at early stage, tending to be radiant shape, with a diameter of 60—120 μm ; semi-spherical top cyst, two-layer spore-producing structure; spherical conidiophore.

HHE6, HHE-P7, HHE-P24: colony grew rather fast,

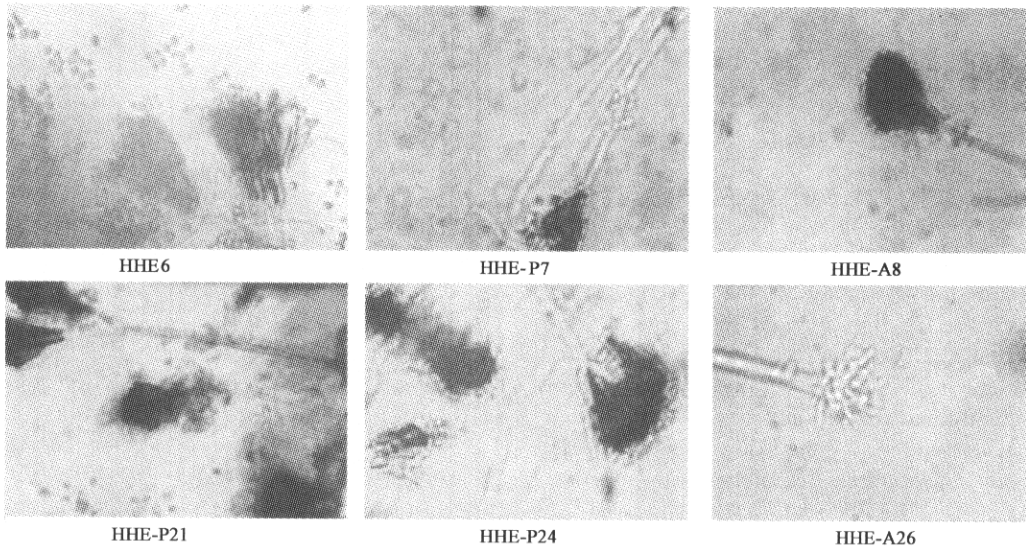


Fig.1 Microphotos of flocculant-producing strains

reaching a diameter of 30—45 mm after cultivating on Czapek's culture media at $28 \pm 1^\circ\text{C}$ for 7—10 d, with fine hair-like texture. The colony had blue green surface at first, and turned to gray green later, and red brown back with pigment penetrating into culture media. The pedicels of the conidiophore grew from the culture media; brush conidiophore head was typically double-wheel symmetrical, and being compacted; small pedicels were thin and long, needle-like, with 3—5 pedicels forming a compacted and paralleled cluster; ovum spherical elliptic conidiophore.

HHE-P21: colony grew rather fast, reaching a diameter of 30—45 mm after cultivating on Czapek's culture media at

28°C for 7—10 d, being fine hair-like or vermicelli-like, and obviously garrulous in the younger zone; colony was gray green or blue green, with 1—2 mm white belts at the edge and slight yellow aerial mycelium in the younger zone on the surface, and brown at the back side only not penetrating into culture media. Non-symmetrical and compacted brush conidiophore head, 3—6 verticillate pedicels; spherical or ovum-spherical conidiophore. According to the ecological features and relevant literature, HHE-A26 could be identified as fungi imperfecti subjected to *Aspergillus versicolor*, and HHE-A8 belonged to *Aspergillus fumigatus*, HHE6, HHE-P7, and HHE-P24 were fungi imperfecti subjected to

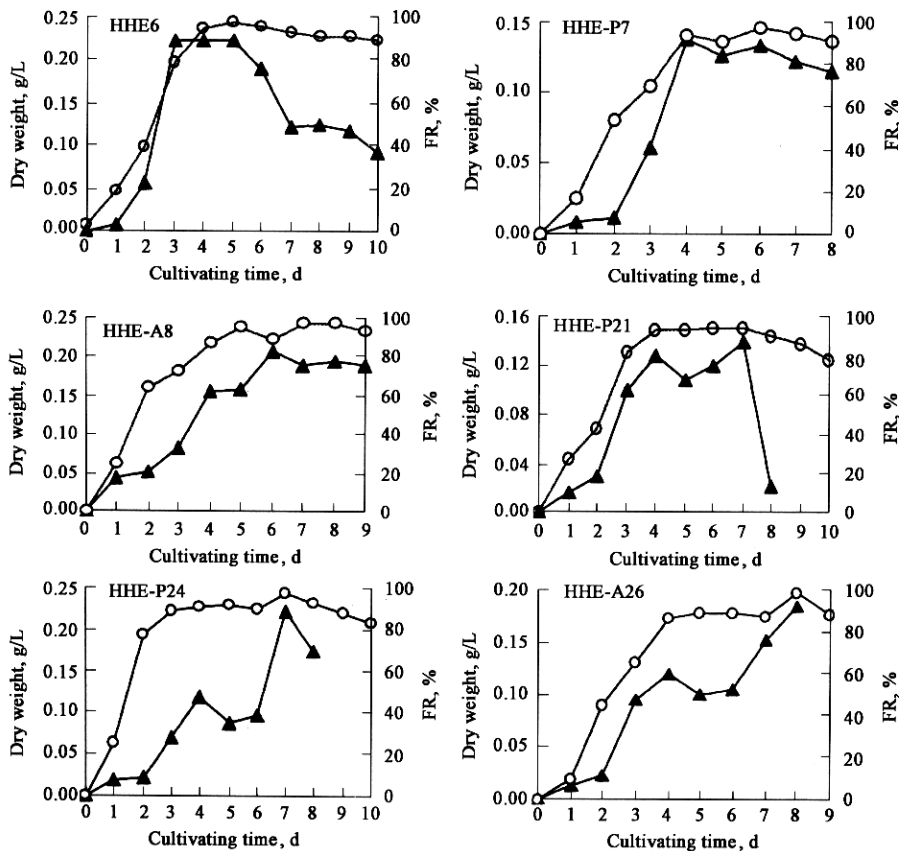


Fig.2 Pattern of flocculation rate and growth of flocculant-producing strains
 - - - ○ - - - flocculation rate curves - - - ▲ - - - growth curves

Penicillium purpurogenum. HHE-P21 belonged to *Penicillium cyclopium*.

2.2 The growth curve and flocculation rate of the flocculant-producing strains

Fig. 2 and Fig. 3 are the growth curves and pH changing curves of flocculant-producing strains: HHE6, HHEP-7, HHE-A8, HHE-P21, HHE-P24, HHE-A26.

It could be seen from Fig. 2 that both flocculation rate and bacteria growth increased synchronously for HHE6 cultivating liquid. In the late phase of the bacteria growth (about the day 5), the flocculation rate reached the maximum; For bacteria HHE-P7, it reached the stable stage in the day 4 and the flocculation rate became steadily high in the late phase of logarithmic growth, and it reached maximum flocculation rate at the end of the stationary phase (the day 6). Strain HHE-A8 demonstrated flocculation rate after two days cultivation at 30°C. The flocculation was rather good in the day 4 at the late phase of logarithmic growth, with the maximum flocculation rate in the day 8 at the end of the stationary phase. HHE-A8 grew in acid conditions. In the first five days, the pH value descended, then slowly ascended. From the day 6, the pH value descended again and eventually tended to be stable. During the cultivation of HHE-P21, the flocculant-producing bacteria had the highest flocculation rate at the end of the stationary phase (the day 6). For HHE-P24, the flocculation rate was the highest, nearly 97.1%. At the end of the stationary phase, the HHE-A26 attained the highest flocculation rate, 98.7%.

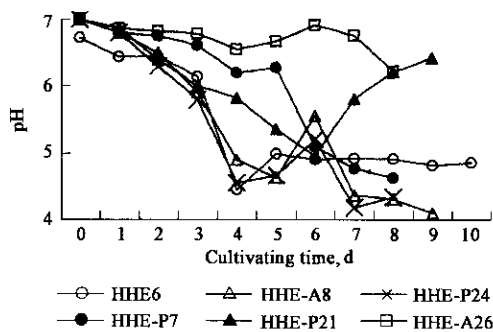


Fig. 3 Curve of pH change of flocculant-producing strains

3 Discussion

Generally, soil and activated sludge are considered as the best sources of screening and isolation of flocculant-producing strains. A variety of bioflocculant producing microorganism was isolated from soil and activated sludge by conventional method. However, bioflocculant-producing strains obtained by mutation breeding technology were few reported, especially with chemical mutagenesis NTG unreported. In this study, bioflocculant-producing strains were isolated from activated sludge by combining conventional method with NTG mutagenesis. The results show that this method is reliable and could greatly shorten the screening period. Six flocculant-producing strains with high flocculating rate, i. e. HHE6, HHE-P7, HHE-A8, HHE-P21, HHE-P24, HHE-A26, were obtained. Five strains (HHE-P7, HHE-A8, HHE-P21, HHE-P24, HHE-A26) of them with high flocculation efficiency was obtained by treatment with NTG. Although the curves were different in accordance with strains, there was one thing in common: all strains grew well under acidic condition. During the cultivating process, pH

declined at the beginning, then decreased slowly and eventually tended to be stable. The flocculation rate curves were almost synchronous with the growth curves. The flocculation rate was directly proportional to the number of microorganism. These results were consistent with the literature (Yokoi, 1996; Salehizadeh, 1998).

Although many microorganisms producing flocculating substance including bacteria, fungi, yeast, and algae have been isolated from soil and wastewater, few bioflocculant-producing strains of *Penicillium* have been reported. The results show that HHE6, HHE-P7 and HHE-P24 belong to *Penicillium purpurogenum*. HHE-P21 belongs to *Penicillium cyclopium*. HHE-A26 belongs to *Aspergillus versicolor* and HHE-A8 belongs to *Aspergillus fumigatus*. Culture liquids of all strains have flocculation rate of over 90% for kaolin suspension. The further research results on culture conditions of flocculant-producing strains will be reported in another paper.

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