e-ISSN:2320-3528
p-ISSN:2347-2286

Research & Reviews: Journal of Microbiology and Biotechnology

Mycelial pellet formation of marine-derived fungus: new formation pathway directly from hyphae

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Research Article

Received date: 05/03/2015
Accepted date: 26/05/2015
Published date: 02/05/2015

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Keywords: Mycelial pellet, Pellet-forming mechanism, Mycelium inoculation, Marine-derived fungi.

ABSTRACT

Mycelial pellets are microbial particles formed during fungi culturing and their formation mechanisms are still under investigation. Spore aggregation is often considered as a crucial step for fungi to form mycelial pellets. In this work, the pellet formation processes of three fungi were investigated for proving the mycelial pellet formation without spores, and a marine-derived *Aspergillus niger* was selected as an example for the mechanism studies of mycelial pellet formation, and detailed processes of mycelium agglomeration and pellet formation were recorded. Experimental results showed that mycelial pellets can be formed directly from hyphae and the spore aggregation may not be an essential step. Moreover, the culture media pH had substantial effects on the pellet formation processes. Scanning electron microscope and infrared spectroscopy results indicated that the decrease of pelletization capacity was related to the change of extracellular polymeric substances. However, mycelium could not grow into mycelial pellets after adding 100 g/L talc into the culture media, while the spore inoculation method could still result in the formation of mycelial pellets under the same condition. This result indicated that although spore aggregation may not be essential, it is likely an enhancing factor for the mycelial pellet formation.

INTRODUCTION

Mycelial pellets are microbial particles of fungi and actinomycetes formed by culturing processes and have found applications in certain bioengineering processes. As self-immobilized and bioactive particles, these pellets show advantages over mycelium for some industrial applications, such as strong surviving ability, fast settlement rates, easy solid-liquid separation and good reusability. Currently, mycelial pellets are mostly used in the fermentation industry, such as production of citric acid, enzymes and antibiotics [1]. They have also been used as biosorbents in dye decolorization and heavy metal adsorption for wastewater treatments [2-7].

Studying on pellets formation processes are important in understanding pellet formation mechanisms, which can facilitate the design of mycelial pellets for various applications. Research shows that the formation of mycelial pellets can be affected by many external factors, such as spore inoculum concentration, pH value, surfactants, osmotic pressure and stirring rates [8-11]. Moreover, the addition of talc or other particles can also impact pellet formation processes [12]. Currently, several pellets formation mechanisms have been reported [13]. In the non-coagulative type, one pellet is generated from one spore. While in the coagulative type, the spores of fungi, such as *A. niger*, will aggregate and germinate, and eventually form mycelial pellets. A kinetic model of conidial aggregation of *A. niger* [14] was proposed to elucidate the coagulative mechanism, which described the“inoculum - two conidial aggregation steps - mycelial pellet formation” process. This model has been widely accepted and many researchers consider spore aggregation as a necessary condition for the mycelial pellet formation.

However, hyphal inocula are also widely used [1] and mycelial pellets can be formed without spore inoculation. Furthermore,

Priegnitz et al. [15] found that spores of certain mutants of *A. niger* which did not aggregate with each other can also form mycelial pellets. These results indicate that the pellet-forming mechanism should concern mycelium in addition to spores.

Two marine-derived strains of molds, *Penicillium janthinellum* P1 and *Aspergillus niger*, were collected from the East China Sea and studied in our research group previously [3,16], and results showed that both can form mycelial pellets. Moreover, the hyphae of these molds can combine with other microorganisms and form mixed mycelial pellets to achieve multiple functions [3,4]. Research showed that mycelial pellets can be produced when single hyphae of the marine *A. niger* were inoculated in a new medium and cultured under shaking conditions, . This phenomenon indicates that the marine-derived *A. niger* mycelial pellets can be prepared with their mycelium and does not necessarily need the participation of spores.

Since the pellet-forming mechanism is important in preparing proper pellets for different bioengineering applications, such as enzyme immobilization and bio adsorption, the key mechanism of the pellet-forming process need to be explored. In this study, a type fungus *A.niger* ATCC16888*, a* marine *A. niger* and a marine *P. janthinellum* P1 were chosen as the model fungi to investigate the possibility of mycelial pellet formation directly via hyphae. Scanning Electron Microscope (SEM) and Attenuated Total Reflection-Flourier Transformed Infrared Spectroscopy (ATR-FTIR) were used to study the relationship between mycelial pelletization capacity and Extracellular Polymeric Substances (EPS) and the pellet formation mechanism was explained.

MATERIALS AND METHODS

Strains

Three strains of fungi were used. A marine-derived *A. niger* collected from the East China Sea was identified as a moderately halophilic *A. niger* [16]. It is currently stored in the Center for Type Culture Collection of China with conservation number CCTCCM2010132. Another fungus is a marine-derived *P. janthinellum* P1 which has a conservation number CCTCCM2012006 [3]. A type fungus *A.niger* ATCC16888 was bought from China Center of Industrial Culture Collection (CICC).

Culture Media

The culture medium for the marine-derived *P.janthinellum* P1 had a formulation of: 10 g glucose, 2 g ammonium tartrate, 2 g KH_2PO_4, 0.5 g $MgSO_4$ and 2 g yeast extract in one liter water (pH 5.5) The medium for the marine-derived *A. niger* and the type *A.niger* had a formulation of: 10 g glucose, 2 g NH_4Cl, 2g KH_2PO_4, 0.5 g $MgSO_4$ and 2 g yeast extract in one liter water (pH 5.5). All these chemicals were bought from Sangon Biotech (Shanghai, China).

Inoculation and cultivation

Inoculation with conidia: Spore suspension with concentration of approximately 10^7 spores per milliliter was prepared and 1 ml spore suspension was inoculated into 100 ml culture media in 250 ml Erlenmeyer flasks, which were then stored in rotary shakers under 160 rpm, 28°C for 48 h.

Inoculation with mycelia: Mycelial pellets produced by conidia were homogenized for 2 min to prepare hyphae suspension under sterile conditions. Hyphae suspension with cell dry mass of approximately 6 g/L was investigated with a stereo microscope (Phenix-XTL, 165-VT, China) to insure single hyphae dispersion, and then 1 ml hyphae suspension was inoculated into 100 ml culture medium in 250 ml Erlenmeyer flasks. Same incubation conditions were used as the preparation with conidia.

Alkali treatment

The hyphae suspension was centrifuged and the precipitate (hyphae) was re-suspended with distilled water and the pH of hyphae suspension was shifted to 12.5 for 10 minutes. The suspension was centrifuged and the precipitate was washed twice with distilled water, which was finally re-suspended with fresh medium as the alkali-treated hyphae sample.

Characteristics of mycelia pellets

Mycelium morphology at different cultivation stages was observed with the stereo microscope and recorded with a digital camera (Cannon S5 IS). Moreover, mycelia pellet samples were lyophilized for 24 h and the freeze-dried samples were coated with gold and examined by SEM (Hitachi SU-70, Japan). The hyphae lyophilized were also scanned from 4000 to 400 cm^{-1} by ATR-FTIR (Nicolet 5700) to analysis their chemical structures.

Influence of microparticles on pellet formation

Hydrous magnesium silicate microparticles (2500 mesh) were added to the culture medium to study the influence of talc on pellet formation. Talc was added at varying concentrations from 1 ~100 g/L and the culturing process was the same as mentioned above.

Reproducibility

The experiment was repeated at least three times in each group and similar results were obtained. These experimental results can be reproducible.

e-ISSN:2320-3528
p-ISSN:2347-2286

RESULTS

Mycelial pellets of marine-derived A. niger prepared with mycelial fragments

Mycelial pellets shown in Figure 1A were prepared by inoculation of spores of the marine-derived *A. niger*, which indicates that this fungus could easily form mycelial pellets from spores. In order to investigate the formation of mycelial pellets direct from fungus hyphae, these pellets were crushed into single mycelial fragments (Figure 1B) and then added into fresh culture media. Figure 1C indicates that mycelial pellets were formed after 48 h of incubation and the appearance of the mycelial pellets does not have an obvious discrepancy from those prepared with spores.

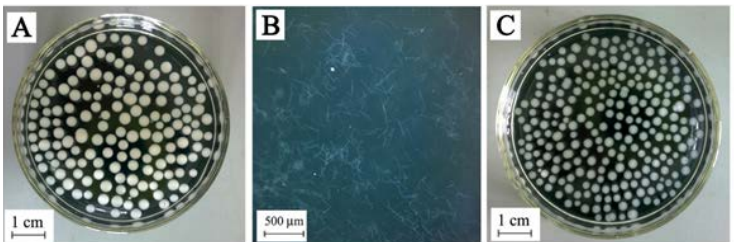

Figure 1: Mycelial morphology of marine-derived *A. niger*. (A) Pellet formation of molds by spore inoculation after 48 h shaking culture (B) Hyphae suspension (C) Pellet formation of mycelium pellets in Figure A being smashed and added into fresh medium after 48 h culture.

Microscopic study on mycelial pellets formation

Figure 2 shows microscopic pictures of the mycelial pellet formation, which recorded in detail a whole pellets formation process within 36 h. Most of the mycelia smashed were single initially with few branched ones that were relatively short (Figure 2A). Small aggregation groups of the mycelia were formed after 2 h of inoculation (Figure 2B), and further aggregation could be clearly observed between 4-8h (Figures 2C and 2D). The growing rate was fast from 12-16 h with the diameter increased sharply and these pellets became nearly spherical at 16 h, and their surface showed newly formed mycelia (Figures 2E-2G). At 20 - 28 h, mycelia of the outer layer were denser and interconnected together under the shear force of running water in shack flasks (Figures 2H-2J). The mycelial pellets became smoother and denser at 32 h with little diameter change (Figure 2K). At 36 h, the surface of the mycelial pellets was fluffy (Figure 2L). This process clearly shows an easy aggregation of the mycelia.

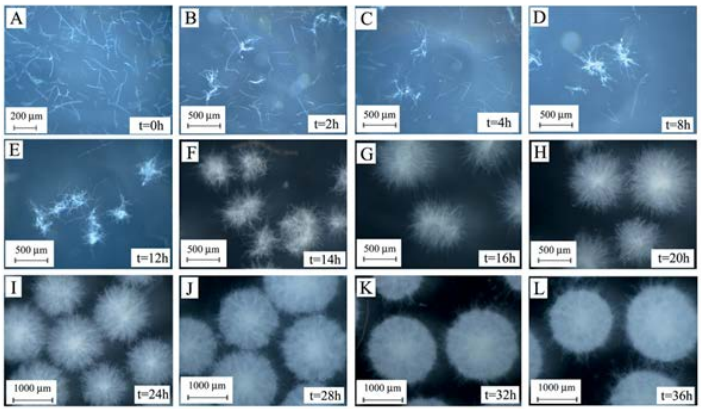

Figure 2: Pellet-forming processes of free mycelia of marine *Aspergillus niger* in fresh medium during 36 h. A-L showed detailed processes of free breaking mycelia slowly aggregating and growing up into pellets within 36 h.

Effects of initial pH on pellet formation

The agglomeration of mycelia is strongly influenced by pH values [17]. Experimental results showed that the most suitable initial pH for the marine-derived *A.niger* pellet-forming was 5.5 and the mold could not grow in alkaline conditions (pH>11). While initial pH was 2.0, the mycelia could still grow, but not able to form pellets (Figure 3A). A detailed view with higher magnification shows that the mycelia can partly aggregate, but could not form pellets (Figure 3B). Pellets cannot be formed with spore inoculation under pH 2.0 either.

Direct impact of pH conversion on pellet formation of mycelia

A further study was conducted to understand the impact of environmental pH on mycelium pellet formation. Mycelia in the medium of pH 2.0 were pulverized and inoculated into fresh medium at pH 5.5. It was found that the mycelia could easily form pellets, and their appearance was similar as pellets studied (Figures 4A-4D). Meanwhile, the mycelial pellets grown in pH 5.5 media were directly added into pH 2 media after being smashed (Figure 4E-4H). The results show that the aggregation rate of the mycelia was slow, and no pellet was formed with only a small amount of mycelia aggregated at 12 h. These results indicate that mycelia were difficult to aggregate in an acidic environment. However, they can rapidly re-agglomerate after moving to a suitable culturing environment. Their aggregation rate was similar as that of mycelia cultured at pH 5.5. This result indicates that the

e-ISSN:2320-3528
p-ISSN:2347-2286

influence of pH on pellet-forming from mycelia was obvious and reversible.

Effects of alkali treatment on pellet-formation

On the fungal hyphae surface there are many Extracellular Polymeric Substances (EPS), which act as natural flocculants and lead to microbial aggregation [18]. The main components of EPS are polysaccharide. Alkali treatments can increase polysaccharide solubility thus result to the change of mycelium surface structure. A suitable alkali treatment was ensured to damage mycelial surface structure while keeps the hyphae alive. Figure 5A shows that untreated mycelia could easily aggregate, and there was almost no free hypha in the culture medium at 12 h. These aggregates then gradually formed mycelial pellets (Figure 5B-5C). However, the alkali-treated mycelia did not form agglomeration at 12 h (Figure 5D)and a small amount of mycelial agglomeration was appeared (24 h) which then formed mycelial pellets after 48 h cultivation (Figures 5E-5F). Therefore, the alkali treatment disturbed the aggregating ability of dispersed mycelia, but this kind of ability can be slowly recovered during the culture process because the pellets could eventually formed. Surprisingly, this recovery rate would be significantly enhanced when adding fermentation broth (Figure 5G-5I), in which case the mycelium aggregation rate appeared only a slight delay compared to the control group. Accordingly, it is suspected that the molds secrete the EPS - or analogues of them - to the environment, and the hypha could utilize these secretions to restore the surface structure when it is damaged.

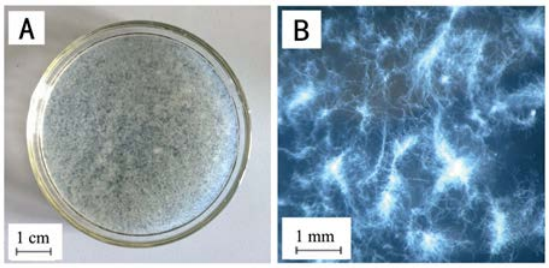

Figure 3: The mycelia could still grow but not able to form pellets under pH 2.0 (A) and detailed views with higher magnification was shown in B.

Furthermore, Figure 5C shows an interesting phenomenon that two pellets can adhere to each other, which indicates that the particles formed initially may not be the core of the final pellets. This phenomenon could be caused both by the hyphal viscous characteristic and particle population balances [19] in the culture media.

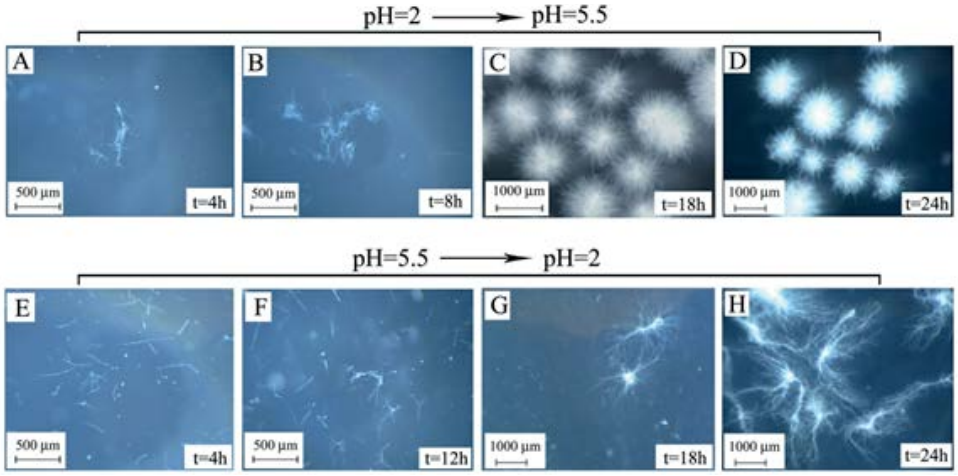

Figure 4: Direct impact of pH conversion on pellet formation of mycelia. Mycelia in the medium at a pH of 2 were pulverized and inoculated into a fresh medium at a pH of 5.5 for a shaking culture, mycelia could form into pellets again (A-D). The phenomenon of reverse conversion was displayed as E-H.

The change of mycelial surface structure under different treatments

In order to investigate the mechanism of inhibition on aggregation of dispersed mycelium in acidic environments or after alkali treatment, the surface structures of the mycelia were compared with SEM and ATR-TIR. The SEM micrographs show that the hyphae were rugged-looking under pH 2 (Figure 6A), which were much smoother under pH 5.5 (Figure 6C). The mycelia treated by alkali had a strange surface, just like being scattered with wood chips (Figure 6E). The infrared spectra of three samples (Figures 6B, 6D and 6F) all showed stretch vibration of O–H and C=O at 3196–3263 cm^{-1} and 1633-1635 cm^{-1}, saturated C–H at 2922–2923 cm^{-1}, and asymmetric vibration of the glucosidic bond C–O–C at 1012-1030 cm^{-1}, which are also the characteristic peaks of several polysaccharides [20]. However, compared to the control, the overall absorption characteristics were quite disparate when pH was 2.0 (Figure 6B). The difference was particularly evident at 1100-1500 cm^{-1}, which was the area of stretch vibration of C–N and bending vibration of N–H. Meanwhile, the peak at 1416 cm^{-1} was apparently lacking, which is likely to be the characteristic peak of deformation vibration C-H in the fragments of acetates and ketones, or the C=O symmetric stretching of COO^- group in amino acids and fat acids [21]. It is demonstrated that hyphae grown under acid environment had quite a different surface

e-ISSN:2320-3528
p-ISSN:2347-2286

structure compared to the control group. The infrared spectra of alkali-treated mycelium were similar to which grown under pH 2, but with even lower absorptivity. The results above indicated that alkali treatment could elute some kinds of substances attaching to the mycelial surface, which might play an important role in mycelial pellet formation. And these important substances are not able to generate or their structure are changed in a large degree in the acidic environment.

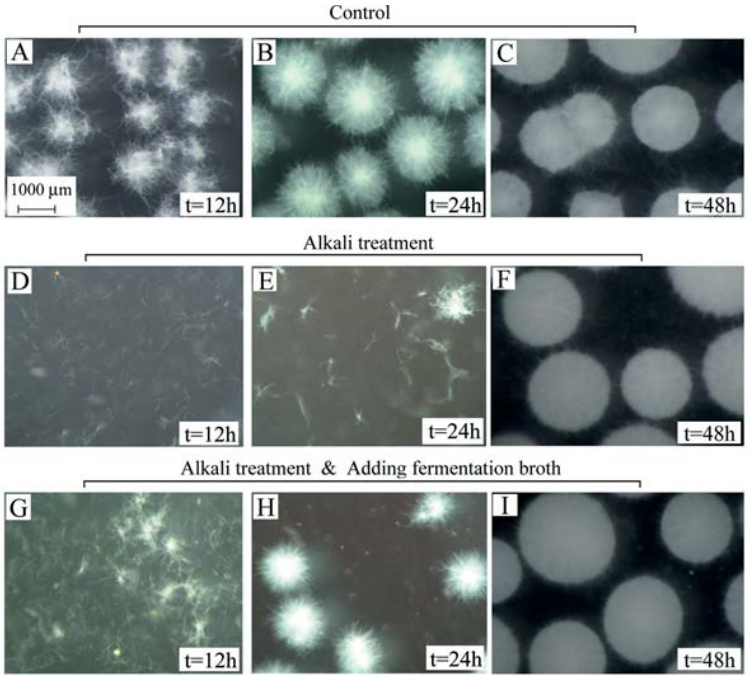

Figure 5: Effects of alkali treatment on pellets formation process. In control conditions (A-C) the mycelium aggregated and formed pellets smoothly. The alkali treatment disturbed the aggregating ability of dispersed mycelia (D-F), but this ability can be enhanced after adding the fermentation broth (G-I).

Effects of adding microparticulates on pellet formation

The addition of particulates into medium could affect mycelia pellet formation [22]. When 100 g/L talc was added, marine *A. niger* inoculated with mycelia had no sign of pellet-forming, however, when the mold was inoculated with spores, it could still form small pellets. In our previous experiments, we are committed to prove that the formation of mycelial pellets does not depend on the agglomeration of spores, but under such an adverse environment at a high concentration of talc, a stronger pellet-forming capacity can be obtained by spore inoculation.

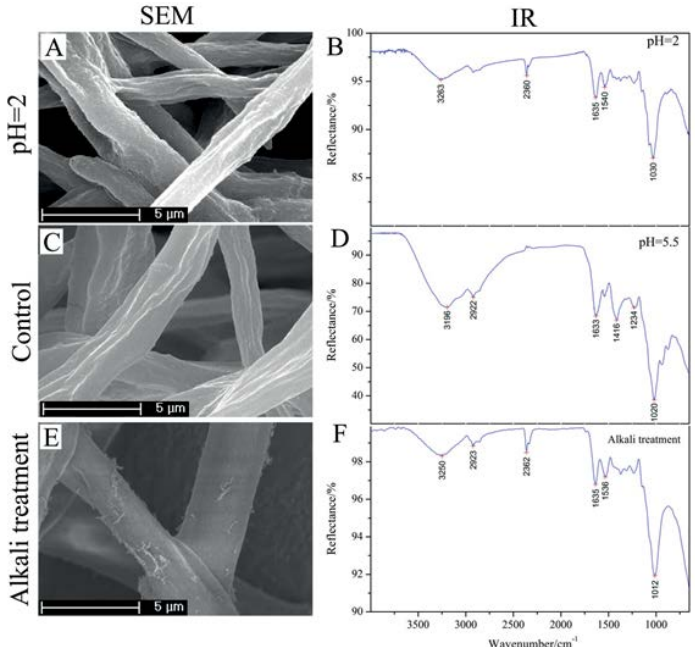

Figure 6: SEM and IR resluts of mycelium grown in different conditions. A&B: culture medium's initial pH=2, C&D: control condition, i.e. initial pH=5.5, E&F: mycelium grown in control condition were treated by alkali.

The universality of pelletization directly from hyphae

The works mentioned above were all focused on marine-derived *A. niger*, and two other fungi were also used in similar

e-ISSN:2320-3528
p-ISSN:2347-2286

experiments to prove the universality of this phenomenon. Mycelial pellets shown in Figures 7A and 7B were prepared by inoculation of spores of the *P. janthinellum* P1 and the type strain of *A. niger* ATCC16888, respectively. These pellets were fully crushed into single mycelial fragments and then added into fresh culture media, and they could form the mycelial pellets after 48 h of incubation (Figures 7C and 7D). A type strain of *A. niger* and a *P. janthinellum* can both form mycelial pellets directly from hyphae, indicating that the pellet-forming ability of hyphae is not just belong to marine-derived fungi or *A. niger*.

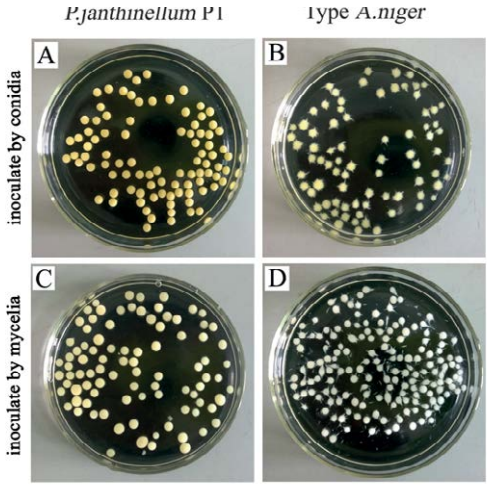

Figure 7: Mycelial morphology of marine-derived *P. janthinellum* P1 and type *A. niger* after 48 h shaking culture. A&B: Pellets formed by spore inoculation. C&D: Pellets formed by inoculating hyphae suspension.

DISCUSSION

This study shows that the strains investigated can all form mycelial pellets with mold hyphae. Similar experiments that reseeding of pellet populations through mycelial fragments were reported much earlier [23,24], but the difference was that the mycelia they used were directly derived from cultivation process which may already have formed mycelial agglomerations, rather than single hyphae. However, people did not explain the pellet-forming mechanism according to this phenomenon, instead to connect the mechanism with conidial aggregation, for the conidial inocula were used more extensively. The results above illustrates that the pellet formation of the marine-derived *A. niger* mycelia was dominated by the agglomeration of mycelia and did not necessary need the aggregation of spores.

The adhesion force between *A. niger* spores at pH 2.5 is even higher than that at pH 5 [25], but the case of mycelial pellets formation is the opposite, indicating that the adhesion between spores may not dominate the formation of mycelial pellets. Moreover, deletion of gene alb1 in *A. niger* will lead to changes in the structure of the spore surface [15], thus spores could hardly form agglomeration, but it has no significant effect on the ultimate formation of mycelial pellets. Actually, the coagulating and non-coagulating types can be partly unified, because the point should be focused on the hypha itself rather than the spore. We consider in this work that spore aggregation is a general process rather than an essential step in mycelial pellets formation. In the model of conidial aggregation of *A. niger* [14], the most important step should be the growth stage after secondary aggregation, in which hyphae growth rate comes to the maximum and the growing hyphae interact with each other and finally formed mycelial pellets.

Mycelial pellets have important applications in wastewater treatment. For instance, it has good adsorption ability on dyes [26] and good flocculating effect on particles in sewage water. The EPS at the surface of fungal hyphae plays an important role in the biological flocculation processes [18,27]. Structural damage of hyphae surface like alkali treatment will seriously affect the flocculation ability of hyphae. Therefore, these macromolecules adhering to the hyphae surface are exactly the structural basis of pellet formation. The fundamental mechanism of fungus pelletization should be counted on the molecular mechanism. In *Penicillium chrysogenum*, deletion of the Pcku70 gene will result in a dramatic reduction of mycelial pellet formation [28]. It is probably because Pcku70 in *P. chrysogenum* can influence PcchiB1 gene expression, which is closely related to the integrity of the cell wall. Therefore the integrity of mycelial surface structure plays a decisive role in pellet formation.

All kinds of external factors, e.g., high salinity, temperature, surface active agent and pH value, can influence the chemical properties of EPS or their interactions, thus change the agglomerate ability of hyphae and finally affect the formation of mycelial pellets. It needs the mutual contact of mycelium to make EPS adhesive together, thus the opportunity of hyphae's collision is also a decisive factor for the formation of mycelial pellets, which is concerned with biological reactors' shape, stirring rate, aeration intensity and so on. In the culture media with high concentration of talc, a stronger pellet-forming capacity can be obtained by spore inoculation. It could be due to the conidia of molds have a strong reunion ability and mycelium germination in a same spores group would obtain more opportunities by contacting with each other. To sum up, it can be said that the influence factors to mycelial pellet formation are almost achieved by EPS, i.e. the surface structure of hyphae, and the action modes of these factors can be divided into nothing more than two types: change the structure of mycelial surface and impact the interaction between hyphae.

In conclusion, the research on hyphal inocula demonstrates that the formation of mycelial pellets of the marine-derived *A. niger* was possible by mycelia agglomeration. The EPS structure of the mycelia and their interaction might influence the pellet-forming process. This article is a first step for the detailed analysis of interaction between hyphae and these results will provide a theoretical foundation for further exploration of pellet-forming mechanism and application of the marine *A. niger*.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China, Scientific Research Fund of Zhejiang Provincial Education Department and Scientific Technology Program of Zhoushan City (2013C51021).

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