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Abstract

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Keywords (separated by '-') *Rhizopus oryzae* - Morphology - Exogenous calcium - L-lactic acid - Pellet

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Chapter 25
Effects of Calcium on the Morphology of *Rhizopus oryzae* and L-lactic Acid Production

Yong-Qian Fu, Long-Fei Yin, Ru Jiang, Hua-Yue Zhu and Qing-Cheng Ruan

Abstract The effects of exogenous calcium on fungal pellet morphology during preculture and L-lactic acid production were studied. The results showed that addition of exogenous calcium could induce pellet formation. The diameter of the pellet increased with increasing concentration of exogenous calcium, including $CaCl_2$ and $CaCO_3$. The smaller pellet precultured with low concentration of soluble calcium ($CaCl_2$) was beneficial for L-lactic acid production because the pellet was dense and the large inner part of the pellet was inactive. By contrast, the larger pellet precultured with high concentration of insoluble calcium ($CaCO_3$), except 8.0 g/L $CaCO_3$, was beneficial for L-lactic acid production. Supported by the $CaCO_3$ powder, the entire biomass layer was fully active, and the highest L-lactic acid productivities of 1.22 g/L h and 58.6 g/L L-lactic acid were reached using the 1.5 mm pellet.

Keywords *Rhizopus oryzae* · Morphology · Exogenous calcium · L-lactic acid · Pellet

25.1 Introduction

Submerged cultures of filamentous fungi are widely used to provide important biotechnological products, such as enzymes, organic acid, and antibiotics, which have a lot of applications in food, medical, pharmaceutical, chemical, and textile industries [1]. However, the filamentous fungi growth characteristic brings a number of process engineering problems attributed to the morphological change accounted during the fermentation process in large scale [2]. Three extreme morphologies of

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filamentous fungi have been reported, namely suspended mycelial, pellet, and clump morphology [3]. The morphology of filamentous fungi in submerged cultivation has been a subject of considerable interest for many years. The fungal growth in pellet form is a favorable alternative [4, 5] because it makes repeated-batch fungal fermentation possible. This form is also favored because it significantly improves culture rheology, which results in better mass and oxygen transfer into the biomass and lower energy consumption for aeration and agitation. Numerous studies have been carried out to control fungal morphology in pellet form [6–8]. However, most early studies mainly focused on environmental factors, such as medium composition, inoculum, pH, medium shear, additives (polymers, surfactants, and chelators), culture temperature, and medium viscosity. For individual strains, each factor has a different importance to the growth morphologies; some strains (e.g., *Rhizopus* sp.) need strong agitation to form pellets, whereas other strains (e.g., *Penicillium chrysogenum*) require high pH [4, 9]. Thus, most studies on fungal pellet formation are limited to the level of the individual strain because of the shortage of mechanisms of morphogenesis.

Environmental conditions maybe markedly influence the growth pattern of filamentous fungi, which can range from a dispersed filamentous form to pellet. However, Braun and Vecht-Lifshitz [10] reported in their study that the pellet morphology of a filamentous microorganism developing in any fermentation system may be represented as a final result of the competing influences, which is the equilibrium between the forces of cohesion and disintegration. Shear forces may be unambiguously assigned the function of disintegrating factors. The hyphal extension and branching rate thereby affected the mycelium cohesion. The morphology of a mycelium and final fungal morphology are mainly determined by the mechanisms that regulate the polarity and direction of hyphal growth, as well as the frequency with which they branch [11, 12]. A typical fungal hypha grows out of a single cell-spore as a multinucleate tube containing cytoplasm, which moves within a hypha toward the hyphal tip, where it grows. During normal tip growth, a delicate balance must exist among the deposition of new material, synthetic activity, lytic activity, and turgor pressure, which provides the force for elongation [2, 3, 11]. Phosphoinositides, calcium, calmodulin, and cyclic nucleotides, especially Ca^{2+}, are involved in the mechanisms that regulate hyphal extension and branching, and ultimately affect fungal morphology [13, 14]. Several studies have investigated the effects of calcium on the hyphal extension and branching. For example, Robson et al. [15] studied the effects of Ca^{2+} on the regulation of hyphal extension and branching in *Fusarium graminearum* A 3/5, and illustrated that low Ca^{2+} concentrations increase the mean hyphal extension rate and hyphal growth unit length. Their results showed that the treated mycelia become more sparsely branched in *F. graminearum*, which was similar to the results of Robson et al. [16]. Jackson and Heath [17] also found in their research that increasing the external Ca^{2+} concentration generally resulted in an increased rate of hyphal extension and in a decreased frequency of branching. Very high concentration of external Ca^{2+} (>50 mM) will inhibit tip extension. A few studies also researched the effects of Ca^{2+} on the morphology of fungi in the macro-morphology. For example, Žnidaršič et al. [13] found that the

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addition of 1×10^{-3} M Ca^{2+} to basal medium resulted in the formation of smooth large pellets and clump. The average diameter of pellets was 3.84 ± 0.84 mm. When the medium was supplemented with Ca^{2+} in concentration above 1×10^{-2} M, the whole mycelium of *Rhizopus nigricans* was aggregated in clumps.

In conclusion, most studies mainly focused the effects of Ca^{2+} on the morphology of filamentous fungi in micro-morphology, including hyphal tip growth, morphology, extension, and branching. No systematic reports have been published to discuss the effect of exogenous calcium on the macro-morphology of filamentous fungi, especially *Rhizopus oryzae*. The present study addresses the challenging task of investigating the influence of exogenous calcium on the macro-morphology of *R. oryzae*, and controlling the morphology of *R. oryzae* in pellet form to produce L-lactic acid efficiently. In this work, the effects of exogenous calcium, including soluble and insoluble calcium, on the pellet form, growth characteristics, and L-lactic acid production in a mutant strain of *R. oryzae* is discussed.

25.2 Materials and Methods

25.2.1 Microorganism and Growth

Rhizopus oryzae TZ-45, the mutant of *R. oryzae* NRRL 395, was used in this study. The fungus was grown on a potato dextrose agar (PDA) plate at 30 °C for 7 d. For the experiments, fungal spores were collected by shaving the PDA surface with a sterile loop and extracting spores with sterile water. Fungal spores were then stored at 4 °C.

25.2.2 Preculture Conditions

The preculture medium contained the following components (per liter): 20.0 g of glucose; 2.0 g of peptone; 0.2 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$; 0, 2.0, 4.0, and 6.0 g of $CaCl_2$ or 0, 2.0, 4.0, 6.0, and 8.0 g of $CaCO_3$ (the initial concentration in the preculture medium); and natural pH. Approximately 50 mL of medium without $CaCl_2/CaCO_3$ was loaded into a 250 mL Erlenmeyer flask and heat sterilized (121 °C for 20 min).

Before preculture, 15.0 mL of 100.0 g/L $CaCl_2$ solution and 0.1–0.4 g of $CaCO_3$ powders were separately placed in a 20 mL vial. All vials were then tightly closed with screw caps to avoid moisture adsorption from the outside, and autoclaved at 115 °C for 30 min. The sterilized $CaCl_2$ solution with definite volume and $CaCO_3$ powders were then added to each sterilized 250 mL Erlenmeyer flask to ensure that $CaCl_2/CaCO_3$ in the medium reached the initial concentration. The spore solution was inoculated in the Erlenmeyer flask with a spore concentration of 1×10^6 spores/mL, and cultured in a rotary shaker (150 rpm) at 30 °C for 18 h. All values presented in this study are averages of at least three independent trials.

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25.2.3 Fermentation

The production medium contained the following components (per liter): 80.0 g of glucose, 3.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.04 g of $ZnSO_4 \cdot 7H_2O$, 0.2 g of KH_2PO_4, and 40 g of $CaCO_3$. The media without $CaCO_3$ were autoclaved at 121 °C for 20 min. The calcium carbonate powder was sterilized separately (115 °C at 30 min). L-lactic acid production was performed in a 3.0 L stirred tank (New Brunswick Scientific, USA) with a 2.0 L working volume, which was inoculated with 300 mL of preculture. The cultivation temperature in the stirred tank was maintained at 30 °C throughout the experiments. The aeration rate and agitation speed were set at 0.5 vvm and 300 rpm, respectively. Sterile $CaCO_3$ was used as a neutralizer, which was added to the tank before fermentation to maintain a pH of approximately 6.0 during culture. The cultivation time in the experiments ranged from 48 to 68 h. Samples were periodically obtained for high-performance liquid chromatography (HPLC) analysis.

25.2.4 Analytical Methods

To determine glucose and ethanol concentrations, samples were centrifuged, and the resulting supernatants were used. To determine lactic acid concentration, samples were diluted by the addition of distilled water and hydrochloric acid, heated at 80 °C until the broth was clear, and centrifuged. The resulting supernatants were then used for analysis.

Glucose, ethanol, and lactic acid concentrations were measured by HPLC (Summit P 680 HPLC, Dionex, USA; Shodex RI-101 Refractive Index Detector, Showa Denko, Japan; Aminex HPX-87 H Ion Exclusion Column 300 mm × 7.8 mm, Bio-Rad, USA) under the following conditions: sample volume, 20 μL; mobile phase, 0.005 M H_2SO_4; flow rate, 0.8 mL/min; and column temperature, 60 °C [18]. Biomass was determined by weighing the mycelial mass after drying at 60 °C overnight. Seed morphology was determined using an Olympus microphotograph (Tokyo, Japan).

25.3 Results and Discussion

25.3.1 Effects of Exogenous Calcium on the Growth of R. oryzae

All morphologies observed during the cultivation of *R. oryzae* with different exogenous calcium and different initial concentrations are summarized in Table 25.1. The diameter and other characteristics of the obtained pellets are also included in Table 25.1.

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Table 25.1 Overview of the morphology of *R. oryzae* after 18 h of growth with different calcium compounds and concentrations

Additive	Concentration (g/L)	Morphology	pH		Pellet diameter (mm)		Pellet characteristics	Dry weight (g/L)
			Initial	Final	Average	Size range		
$CaCl_2$	6.0	Clumps	5.3	4.8	–	–	–	6.01 ± 0.30
	4.0	Pellets	5.2	3.7	1.2	1.0–1.5	Radial, slightly hairy	6.42 ± 0.32
	2.0	Pellets	5.2	3.6	0.8	0.5–1.0	Smooth	6.21 ± 0.31
	0	Filaments	5.0	4.5	–	–	–	5.90 ± 0.30
$CaCO_3$	2.0	Pellets and filaments	5.5	4.6	1.0	0.5–1.5	Small, hollow, sticking together	6.30 ± 0.32
	4.0	Pellets	5.8	5.1	1.2	1.0–1.8	Smooth	6.86 ± 0.34
	6.0	Pellets	6.0	5.8	1.5	1.0–2.0	Radial, fluffy	7.02 ± 0.35
	8.0	Pellets	5.9	6.1	2.3	1.5–3.0	Large, smooth, mixed with $CaCO_3$	8.14 ± 0.41

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The morphology and final cell dry weight clearly varied with different exogenous calcium. Table 25.1 showed that the final cell dry weight initially increased, and then decreased when the $CaCl_2$ concentration changed from 0 to 6.0 g/L. The maximum cell dry weight of 6.42 g/L was obtained at 4.0 g/L $CaCl_2$, which differed by 8.8 % from the minimum weight (5.9 g/L). The final cell dry weight increased with the $CaCO_3$ increased from 0 to 8.0 g/L. The maximum cell dry weight reached 8.14 g/L, which differed by 38 % from the minimum weight (6.3 g/L), possibly because the pellet was mixed with excess $CaCO_3$ powder. The changes in pH also proved this phenomenon.

25.3.2 *Effects of Exogenous Calcium on the Morphology of* R. oryzae

Figure 25.1 illustrates the representative morphological forms generated in the precultures with different calcium types. It was found that exogenous calcium significantly influenced the morphology and pellet size of *R. oryzae*. The experiments performed in shake flasks using the precultures with exogenous calcium mostly resulted in pellets, except 6.0 g/L $CaCl_2$. Long and entangled filaments (Fig. 25.1d) were formed in the preculture without exogenous calcium, whereas large mycelial clumps were observed in the preculture with 6.0 g/L $CaCl_2$ (Fig. 25.1a). Fluffy pellets mixed with filaments were observed in the preculture with 2.0 g/L $CaCO_3$. Small and smooth pellets with an average pellet diameter of 1.0 and 1.2 mm (Fig. 25.1b, f) were found in the precultures with 2.0 g/L $CaCl_2$ and 4.0 g/L $CaCO_3$, respectively. Larger radial and hairy pellets (Fig. 25.1c, g) were formed in the precultures with 4.0 g/L $CaCl_2$ and 6.0 g/L $CaCO_3$ as the

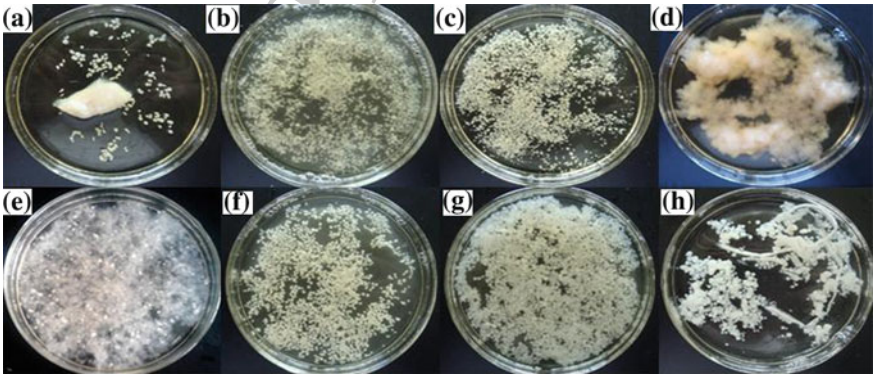

Fig. 25.1 Influence of exogenous calcium on the morphology of *R. oryzae* NRRL 395 in submerged cultures in shaken flasks. $CaCl_2$ and $CaCO_3$ were added at various concentrations: **a** 6.0 g/L $CaCl_2$; **b** 4.0 g/L $CaCl_2$; **c** 2.0 g/L $CaCl_2$; **d** 0 g/L $CaCl_2$; **e** 2.0 g/L $CaCO_3$; **f** 4.0 g/L $CaCO_3$; **g** 6.0 g/L $CaCO_3$; and **h** 8.0 g/L $CaCO_3$

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concentrations of $CaCl_2$ and $CaCO_3$ increased. The average pellet diameters were 1.2 and 1.5 mm. Large and smooth pellets mixed with $CaCO_3$ powder were formed in a higher $CaCO_3$ concentration (8.0 g/L). The average pellet diameters were 2.3 mm. Notably, higher soluble calcium ($CaCl_2$) was not beneficial for pellet formation, but the opposite was observed in insoluble calcium ($CaCO_3$).

This study showed that an appropriate concentration of calcium (different solubilities with different concentrations) could promote pellet formation. Very low or very high concentrations were not beneficial for pellet formation. Similar results were reported by Pera and Callieri [14], Jackson and Heath [12], and Robson et al. [15]. These results indicated that in low or deficient exogenous calcium, fungi lack a Ca^{2+}-CTC membrane-associated gradient, grow slowly, display hyper-branching, and have abnormal swollen hyphae. The increase in the external Ca^{2+} concentration resulted in an increased rate of hyphal extension [16, 17, 19] and a decrease rate in the branching frequency [17]. Very high concentrations of external Ca^{2+} can inhibit tip extension [16]. However, whether this inhibition is due to the direct Ca^{2+} interactions with the cell wall (i.e., Ca^{2+} induces rigidity of the apical cell wall [16]) or a general toxic response to high cytosolic Ca^{2+} concentration remains unclear. Žnidaršič and Pavko [11] reported that the pellet morphology of a filamentous microorganism developing in any fermentation system may be represented as a final result of the competing influences, which is the equilibrium between the forces of cohesion and disintegration. Shear forces may be unambiguously assigned the function of disintegrating factors. Ca^{2+} may affect the hyphal extension and branching rate, thereby affecting mycelium cohesion. This result possibly explains why the pellet cannot form without exogenous calcium (Fig. 25.1d) or high exogenous calcium (Fig. 25.1a). Meanwhile, pellets obtained from a low concentration of exogenous calcium showed a smooth surface (Fig. 25.1c, f), whereas pellets obtained from a high calcium concentration showed a rough surface (Fig. 25.1b, g).

25.3.3 Effect of Exogenous Calcium on L-lactic Acid Production

Table 25.2 summarizes the L-lactic acid production, residual glucose concentration, lactic acid yield, lactic acid productivity, and by-product concentrations as measurable indicators of different *R. oryzae* morphologies, which resulting from different precultures. It was found that the residual glucose concentrations reached 21.4 and 32.2 g/L when the precultures with 0 g/L $CaCl_2$ and 8.0 g/L $CaCO_3$, respectively. The final L-lactic acid production increased from 30.3 to 57.2 g/L in the precultures with $CaCl_2$. The highest L-lactic acid production of 57.2 g/L was obtained in the fermentation with 2.0 g/L $CaCl_2$. Meanwhile, the final L-lactic acid production increased from 39.2 to 58.6 g/L in the precultures with $CaCO_3$. The highest L-lactic acid production of 58.6 g/L was obtained when using 6.0 g/L $CaCO_3$. Although the highest L-lactic acid production using precultures with $CaCl_2$

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Table 25.2 Experimental data from *R. oryzae* fermentation carried out using precultures with different exogenous calcium

Calcium factor	$CaCl_2$			$CaCO_3$ powder			
	0	2.0	4.0	2.0	4.0	6.0	8.0
Initial glucose concentration (g/L)	82	81	83	82	84	83	82
Residual glucose concentration (g/L)	21.4	2	3	9	2	3	32.2
Final L-lactic acid production (g/L)	30.3	57.2	54.1	39.2	53.2	58.6	25.1
Average L-lactic acid yield on glucose (g/g)	0.50	0.72	0.68	0.54	0.65	0.73	0.504
Average L-lactic acid productivity (g/L^{-1} h^{-1})	0.446	1.02	0.902	0.576	1.02	1.22	0.369
Final ethanol (g/L)	7.8	5.4	5.8	6.1	4.2	2.7	9.4
Fermentation time (h)	68	56	60	68	52	48	68

and $CaCO_3$ had little difference, the average L-lactic acid productivity had a difference of 20 % (1.02 g/L^{-1} h^{-1} for 2.0 g/L $CaCl_2$ and 1.22 g/L^{-1} h^{-1} for 6.0 g/L $CaCO_3$). Ethanol production was also higher for 2.0 g/L $CaCl_2$ than that for 6.0 g/L $CaCO_3$.

Pellets have been reported with desired morphology for the production of lactic acid [20], itaconic acid [21], citric acid [22], or penicillin [23]. Mass transfer within a typically dense pellet is regarded as a severe disadvantage, and the smaller pellet is much more beneficial for fermentation [11, 24]. However, a large pellet was more beneficial for fermentation in our study.

25.3.4 Microscopic Analysis for the Morphology of Mycelial Pellet

Microscopic analysis (Fig. 25.2) revealed that the application of pellets was limited by the low mass transfer inside the fungal aggregate [25, 26].

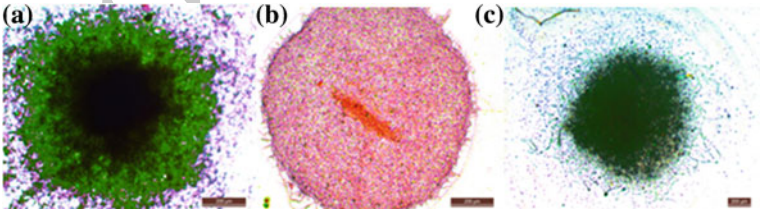

Fig. 25.2 Morphology of mycelial pellet generated by growing *R. oryzae* with different exogenous calcium (**a** pellet with 2.0 g/L $CaCl_2$; **b** pellet with 6.0 g/L $CaCO_3$; and **c** pellet with 8.0 g/L $CaCO_3$). The images were captured using a Leica microscope (dm2500p)

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Pellets are fully supplied with nutrients or oxygen only up to a critical diameter. For *Aspergillus niger* pellets, this diameter is approximately 0.4 mm. Recent fluorescence analysis of GFP reporter strains of *A. niger* confirmed that only a thin layer at the pellet surface contributes to protein production, whereas the large inner part of the pellet is inactive [27]. The pellets of approximately 0.8 mm in size and precultured with 2.0 g/L $CaCl_2$ consisted of a dense outer layer of biomass, and exhibited an unfilled center (Fig. 25.2a). The thickness of the outer layer was approximately 0.2 mm. The supply of oxygen and other nutrients to the cell in the interior layer was limited. The addition of $CaCO_3$ in the preculture, especially a certain amount of $CaCO_3$, significantly changed the morphology (Fig. 25.2b). Most strikingly, the insoluble $CaCO_3$ powder was associated with the biomass and occurred inside the pellets, which created a loose interior structure with a better biomass filling in the pellet core. Thus, the $CaCO_3$ powder was randomly distributed within the aggregate [28, 29]. At higher levels, core shell aggregates were formed (Fig. 25.2b). Thus, the larger pellets were mainly composed of $CaCO_3$ powder. Supported by the $CaCO_3$ powder, the entire biomass layer was fully active. The created inner pellet structure was rather loose, which enabled higher mass transfer than the pellets precultured with $CaCl_2$. When the $CaCO_3$ concentration increased, the excess $CaCO_3$ enveloped the pellet (Fig. 25.2c) and decreased L-lactic acid productivity.

25.4 Conclusion

Exogenous calcium regulates the polarity and direction of hyphal growth, as well as the frequency with which they branch, ultimately determining the mycelial morphology. The results show that the addition of exogenous calcium could induce pellet formation. The diameter of the pellet increased with the concentration of exogenous calcium increased, including $CaCl_2$ and $CaCO_3$. The pellet precultured with soluble $CaCl_2$ (Fig. 25.2a) formed a dense interior structure, and the large inner part of the pellet was inactive. Thus, the smaller pellet precultured with low concentration of soluble calcium ($CaCl_2$), the better for the benefit of L-lactic acid production. By contrast, the larger pellet precultured with high concentration of insoluble calcium ($CaCO_3$), except 8.0 g/L $CaCO_3$, was beneficial for L-lactic acid production. Microscopic analysis revealed that the addition of $CaCO_3$ in the preculture, especially a certain amount of $CaCO_3$ (6 g/L $CaCO_3$ in this study), resulted in a remarkable change in the morphology, and a loose interior structure with a better biomass filling in the pellet core was created (Fig. 25.2b). Supported by the $CaCO_3$ powder, the entire biomass layer was fully active and the highest L-lactic acid production rates of 1.22 g/L^{-1} h^{-1} and 58.6 g/L L-lactic acid were obtained using the 1.5 mm pellet. The excess $CaCO_3$ enveloped the pellet and decreased L-lactic acid productivity when precultured with 8.0 g/L $CaCO_3$. AQ1

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