Effects of Pellet Characteristics on L-Lactic Acid Fermentation by R. oryzae: *Pellet Morphology, Diameter, Density, and Interior Structure*

Yong-Qian Fu, Long-Fei Yin, Hua-Yue Zhu, Ru Jiang, Shuang Li & Qing Xu

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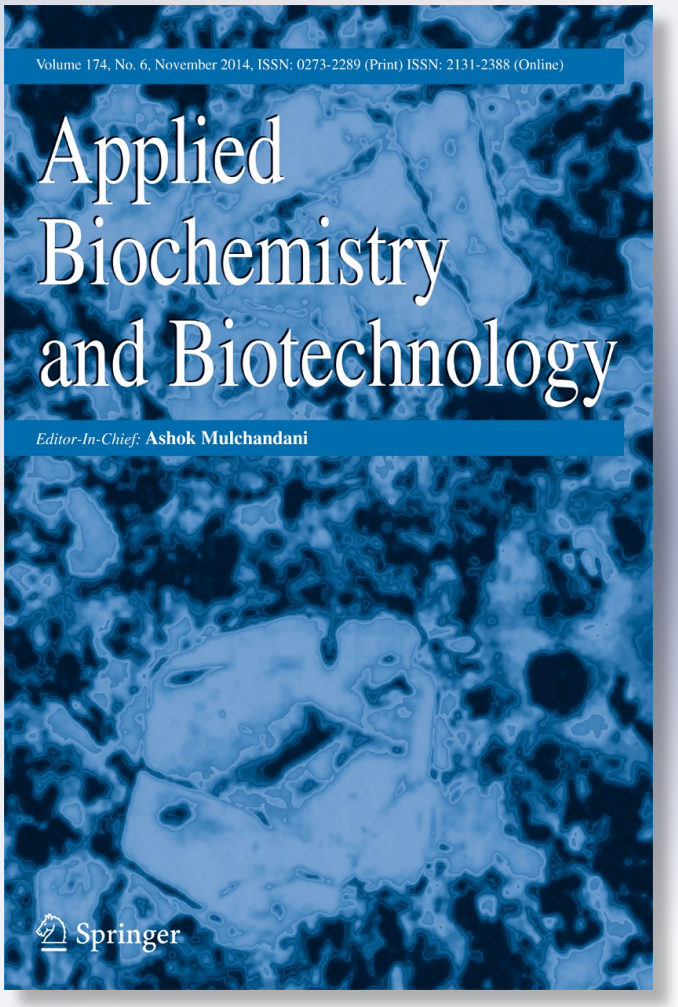

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Effects of Pellet Characteristics on L-Lactic Acid Fermentation by *R. oryzae*: Pellet Morphology, Diameter, Density, and Interior Structure

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Abstract The effects of pellet morphology, diameter, density, and interior structure on L-lactic acid fermentation by *Rhizopus oryzae* were characterized for different inoculum sizes and concentrations of peptone and $CaCO_3$. Inoculum size was the most important factor determining pellet formation and diameter. The diameter decreased with increasing inoculum size, and larger pellets were observed for lower inoculum sizes. Peptone concentration had the greatest effect on pellet density, which increased with increasing peptone concentration. L-lactic acid production depended heavily on pellet density but not on pellet diameter. Low-density pellets formed easily under conditions of low peptone concentration and often had a relatively hollow structure, with a thin condensed layer surrounding the pellet and an extraordinarily loose biomass or hollow center. As expected, this structure greatly decreased production. The production of L-lactic acid increased until the density reached a certain level (50–60 kg/m^3), in which the compact part was distributed homogeneously in the thick outer layer of the pellet and loose in the central layer. Homogeneously structured, denser pellets had limited mass transfer, causing a lower overall turnover rate. However, the interior structure remained nearly constant throughout all fermentation phases for pellets with the same density. $CaCO_3$ concentration only had a slight influence on pellet diameter and density, probably because it increases spore germination and filamentous hypha extension. This work also provides a new analysis method to quantify the interior structure of pellets, thus giving insight into pellet structure and its relationship with productivity.

Keywords Density · Interior structure · L-lactic acid · Pellet characteristics · *Rhizopus oryzae*

Y.-Q. Fu (✉) · L.-F. Yin · H.-Y. Zhu · R. Jiang
Institute of Biomass Resources, Key Laboratory of Plant Evolutionary Ecology and Conservation, Taizhou University, Jiaojiang, 318000 Zhejiang, People's Republic of China
e-mail: fuyq@tzc.edu.cn

Y.-Q. Fu · S. Li · Q. Xu
State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 210009, People's Republic of China

Introduction

Interest in L-lactic acid production has increased recently, as L-lactic acid may serve as a raw material for the manufacture of green solvents, such as ethyl lactate and poly-L-lactic acid, which can be used as biodegradable plastics [1]. The increasing use of L-lactic acid in novel applications and the potential for use in biodegradable plastics have made the production of L-lactic acid an attractive investment. Pure L-lactic acid can be produced by *Rhizopus oryzae*. In the past 2 decades, L-lactic acid fermentation with *R. oryzae* has attracted great interest because of its amylolytic ability and low nutritional requirements compared to lactic acid bacteria [2, 3]. As filamentous mycelia, *R. oryzae* can grow in different morphologies, such as filaments, clumps, and pellets [4]. The morphology significantly affects the oxygen transfer and the productivity of *R. oryzae*. For example, during large-scale production, the growth of cells as filaments or clumps may be problematic. Many studies have concluded that pellets are often the preferable morphological form for industrial fermentation processes [5, 6].

Moreover, production performance is largely determined by the underlying pellet shape. For many decades, this consideration has stimulated efforts to control *R. oryzae* in pellet form [7–9]. Many researchers [10, 11] believe that pellet size is the most important factor affecting production. They believe that although a well-stirred fermentor containing mycelial pellets appears fairly homogeneous on a macroscopic scale, the heterogeneity caused by mass transport limitations within the pellet would be apparent on a microscopic scale. Pellets are subjected to both external and internal mass transfer and heat transfer limitations, which cause growth to occur only in peripheral zones where ample oxygen is available [9]. Thus, once a pellet reaches a certain size, the substrate concentration may be depleted below a critical value. For example, previous research has shown that the critical pellet size of *R. oryzae* for fumaric acid production was 1.2 mm [8]. Attempts have also been done to control the pellet size and investigate the affect of pellet size on organic acid production [12, 13]. However, pellet size is not the only morphological determinant of organic acid yield.

In other work, researchers found that the structure of the pellet periphery and the proportion of inactive mycelium inside the pellet strongly influence production [14]. Wittier et al. [15] used histological staining to discern four different layers in the pellets of *Penicillium chrysogenum*: an outer layer (L1, 60 % of the volume of a 2-mm pellet), two intermediate layers (L2 and L3, 38 %), and a central layer (L4, 2 %). The outer layer was rich in cytoplasm and was best suited for metabolite production. Further studies also indicated that the structure throughout the biopellet is inhomogeneous. The effective mass transfer and heat transfer are closely related to the interior structures of pellets and ultimately affect the generation of metabolites [16, 17]. However, none of these studies explain the relationship between pellet parameters, such as pellet morphology, diameter, density, interior structure, and metabolite production.

This article focuses on the effect of pellet parameters, including pellet form, diameter, density, and interior structure, on the production of L-lactic acid. Furthermore, this work also provides a novel method for quantitatively analyzing the interior structures of pellets, thus providing insight into the relationship between pellet structure and productivity.

Material and Methods

Microorganism

R. oryzae TZ-45 from our laboratory was used in this study. The fungus was grown on a potato dextrose agar (PDA) plate at 30 °C for 7 days. For the experiments, fungal spores were

collected by shaving the PDA surface with a sterile loop and extracting spores with sterile water and then were stored at 4 °C.

Medium and Culture Method

Culture Medium

Preculture medium contained glucose (20.0 g/L), KH_2PO_4 (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), peptone (1.0–3.0 g/L), and $CaCO_3$ (2.0–4.0 g/L), depending on the experimental design. The fermentation medium consisted of glucose (80.0 g/L), $(NH_4)_2SO_4$ (2.0 g/L), KH_2PO_4 (0.2 g/L), $MgSO_4 \cdot 7H_2O$ (0.25 g/L), $ZnSO_4 \cdot 7H_2O$ (0.04 g/L), and $CaCO_3$ (50 g/L).

Culture Method

The precultures inoculated with spores were grown in a 250-mL flask containing 50 mL preculture medium and shaken at 150 rpm for 24 h at 30 °C. The initial inoculation concentration ranged from 0.009 to 107.6×10^7 spores/L. The precultures were then inoculated at 10 % (*v*/*v*) into the fermentation medium. The batch cultivations were incubated in another 250-mL flask containing 50 mL fermentation medium and shaken at 150 rpm for 48 h at 30 °C. Excess sterile $CaCO_3$ was added to the shaking flask to maintain a pH of 5.5.

Analytical Methods

L-lactic Acid, Sugar, and Biomass Assay

To determine glucose concentration, samples were centrifuged, and the supernatants were tested. To determine lactic acid concentration, samples were diluted with 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 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). HPLC measurements used a sample volume of 20 μL, a mobile phase of 0.005 M H_2SO_4, a flow rate of 0.8 mL/min, and a column temperature of 60 °C [18]. Biomass was determined by weighing the mycelial mass after drying at 60 °C overnight.

Pellet Size and Pellet Density Assay

Suspended pellets were imaged with a CCD camera using an exposure time of 40 ms. Using digital image analysis (Image J 3.0), pellet size was determined by measuring the cross-sectional area of the pellets. Pellet diameter and volume were calculated, assuming that the pellets were perfectly spherical. Pellet size was calculated by averaging 100–300 pellets for each sample. The pellet density was defined as cell dry mass per wet volume, determined using the method reported by Hille [19].

Pellet Interior Structure Analysis

Representative pellets (i.e., average size) were chosen for interior structure analysis. First, pellets were embedded in a frozen embedding medium for 20 min and frozen on a rapid freezing station of a microtome at −20 °C. Wrinkle-free slices were cut with a knife mounted in

the cryochamber microtome, which had reached the sectioning temperature of −20 °C. The microtome was programmed to section the sample at a predetermined thickness of 70 μm. Slices from the equatorial region of the pellets were selected with precooled tweezers, quickly transferred onto a room temperature slide, and immediately defrosted. Images of the pellet slices were collected using a light microscope. The slice with the largest diameter was chosen to represent the interior structure of the pellet.

The monochrome images of the slices were stored for structure analysis. Using image analysis software, an adaptive binary mask was computed, and the binary image was automatically segmented into four zones based on gray-level segmentation. At this time, the ratio of zone area to total pellet area was calculated. The gray level of the zones corresponded to the extent of pellet compactness. Finally, the zones were enhanced by different false colors. Thus, the proportion of different compactness extent and their precise location in the pellet was obtained.

Results and Discussion

Influence of Environmental Factors on Pellet Form and Characterization

The effects of environmental factors such as inoculum size and peptone and $CaCO_3$ concentration on pellet formation, diameter, density, and biomass are shown in Fig. 1. Figure 1a mainly illustrates the influence of these factors on pellet formation and diameter. These results show that pellet formation and diameter depended much more on inoculum size than on peptone and $CaCO_3$ concentration. The diameter of the pellet decreased with increasing inoculum size, regardless of the peptone and $CaCO_3$ concentration in the culture. As expected, larger pellets were observed for smaller inoculum sizes; however, a clear transition from pelleted to dispersed form is apparent with increasing inoculum size [6, 20]. A large increase in the main and total hyphal lengths and in the branching frequency was observed in mycelial as the inoculum level increased from 0.009 to 107.4×10^7 spores/L, but in aggregated forms, the number of particles and their compactness decreased [21]. At constant inoculum size and $CaCO_3$ concentration, the diameter increased with increasing peptone concentration. At the lowest peptone concentration, the pellets were small, very light, and fluffy, and they tended to form filaments. However, at high peptone concentrations, *R. oryzae* tended to form clumps, which are not suitable for fermentation [3, 20, 22]. The effect of $CaCO_3$ concentration on diameter was different from the effect of peptone concentration, probably because Ca^{2+} is involved in the mechanisms that regulate hyphal extension and branching [23–25]. The rate of hyphal extending and branching, which was different in differ peptone concentration, leading to the diversity change [5].

Peptone concentration had the greatest impact on pellet density (Fig. 1b). Bai et al. [2] showed that increasing concentrations of nitrogen (NH_4NO_3) caused fungal pellets to increase in size but decrease in density. However, our results showed that pellet density increased with increasing peptone concentration at constant inoculum size and $CaCO_3$ concentration, in agreement with Pavko et al. [6]. Pavko et al. found that an increase in nitrogen source concentration resulted in higher biomass concentrations and a substantial increase in average pellet dry weight. However, mean pellet size and number of pellets only varied slightly, suggesting that higher nitrogen concentrations result in a denser pellet structure. The difference of these researches may be caused by the different nitrogen sources. Liao et al. [5] reported that the type of nitrogen compound used had a considerable influence on fungal pellet formation [26]. A study of the influence of different nitrogen sources on *R. oryzae* ATCC 20344 showed

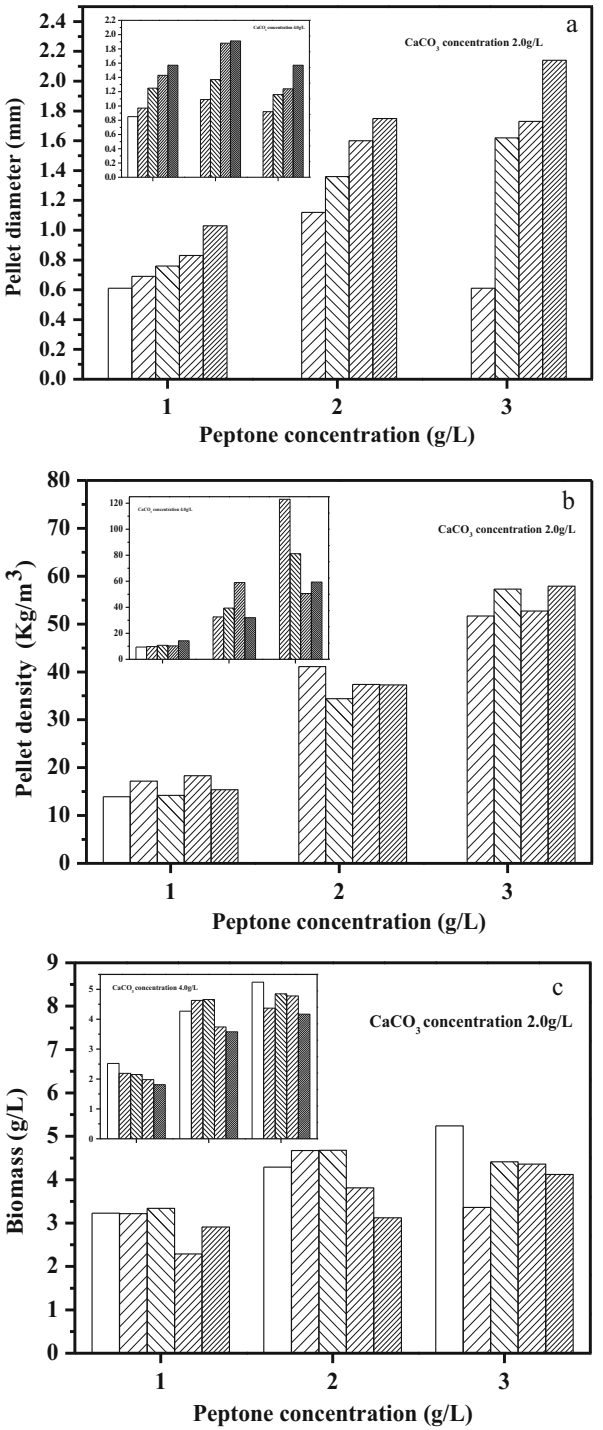

Fig. 1 The effects of peptone, inoculum size (from left to right: 107.4, 11.05, 1.212, 0.104, and 0.009× 10^7 spores/L), and $CaCO_3$ concentration on pellet diameter (**a**), pellet density (**b**), and biomass (**c**)

that peptone produced much smaller, more unique, and heavier pellets than other nitrogen sources. Interestingly, increasing inoculum size had almost no effect on pellet density when other conditions remained constant.

Peptone concentration also had the greatest impact on biomass (Fig. 1c). The biomass only increased when peptone concentration increased. However, biomass remained constant when the peptone concentration exceeded 2.0 g/L. Inoculum size and $CaCO_3$ concentration almost had no effect on the biomass when other parameters were held constant.

The effects of inoculum size, peptone concentration, and $CaCO_3$ concentration on *R. oryzae* morphology, including pellet diameter, density, and biomass, are summarized in Table 1. These process parameters have been reported to influence fungal morphology. Table 1 shows that inoculum size and peptone concentration both influence the morphology of *R. oryzae*. When inoculum size is held constant, pellet diameter varies only slightly. However, pellet biomass and density are highly dependent on peptone concentration. Inoculum size has a much greater effect on pellet size than on biomass because biomass is mainly controlled by the nutrients rather than inoculum size in a certain range of inoculum sizes [3]. $CaCO_3$ concentration slightly influences pellet diameter and density, probably because it increases spore germination and filamentous hypha extension [5, 20].

Effects of Pellet Density on L-Lactic Acid Production

No previous work has shown whether pellet size or pellet density has a significant influence on organic acid production. In order to investigate the relationship between pellet density and lactic acid production, pellets with the same diameter but different densities were assayed to study their L-lactic acid production (Table 2). Some studies have reported that pellet size was an important factor affecting organic acid production: Smaller pellets produce more organic acid. However, as shown in Table 2, pellet density was a much more important factor in the production of L-lactic acid. For pellets with the same diameter, lactic acid production first increased and then decreased with increasing pellet density. The L-lactic acid production reached a maximum when the density was between 50–60 kg/m^3. This suggests that pellet size was not an important factor in organic acid production. Lactic acid production could be higher for larger pellets than for smaller ones. For example, a 1.5-mm pellet produced 55.9 g/L lactic acid, while a 0.6-mm pellet produced 38.6 g/L lactic acid.

Analysts believe that pellet density largely determines the interior structure and maturity of pellets. In this study, at low peptone concentration, low-density pellets with low biomass formed easily (Table 1). In this case, pellet activity was low and led to low lactic acid production. The optimal density for lactic acid production was 50–60 kg/m^3, when the activity was high and the interior structure was most conducive to oxygen transfer and mass transfer. At higher densities, the pellet becomes too dense and is not conducive to the transfer of oxygen and mass, causing a decline in lactic acid production.

Comparison of Fermentation Performance of Different Densities

Dynamic detections of the correlative fermentation parameters, such as glucose consumption and L-lactic acid production, aimed to provide sufficient information to analyze the effects of density on L-lactic acid production. A batch culture was carried out in a 3.0-L fermentor in order to investigate L-lactic acid production at the densities of 57.3, 32.6, and 9.18 kg/m^3. As shown in Fig. 2, *R. oryzae* LA-UN-1 cultured at different densities displayed identical fermentation cycles, which could be divided into a lag phase (phase A), a logarithmic phase (phase B), and a stationary phase (phase C). In the lag phase (phase A), all of the nutrients

Table 1 The effects of $CaCO_3$ concentration, peptone concentration, and inoculum size on pellet formation

$CaCO_3$ concentration (g/L)	Peptone concentration (g/L)	Inoculum sizes ($\times 10^7$ spores/L)	Pellet diameter (mm)	Pellet density (kg/m^3)	Biomass (g/L)
2.0	1.0	107.4	0.61±0.05	13.9±0.5	3.23±0.45
2.0	1.0	11.05	0.69±0.08	17.2±0.3	3.22±0.32
2.0	1.0	1.212	0.76±0.11	14.2±0.4	3.34±0.36
2.0	1.0	0.104	0.83±0.13	18.3±0.3	2.29±0.28
2.0	1.0	0.009	1.03±0.12	15.4±0.4	2.91±0.43
2.0	2.0	107.4	Clump	–	4.29±0.55
2.0	2.0	11.05	1.12±0.16	41.1±0.6	4.67±0.65
2.0	2.0	1.212	1.36±0.07	34.4±0.5	4.68±0.49
2.0	2.0	0.104	1.60±0.04	37.4±0.2	3.81±0.18
2.0	2.0	0.009	1.75±0.08	37.3±0.1	3.12±0.08
2.0	3.0	107.4	Clump	–	5.24±0.24
2.0	3.0	11.05	0.92±0.11	51.7±0.4	3.36±0.35
2.0	3.0	1.212	1.16±0.07	57.3±0.3	4.41±0.27
2.0	3.0	0.104	1.24±0.05	52.7±0.6	4.36±0.54
2.0	3.0	0.009	1.57±0.08	57.9±0.4	4.12±0.34
4.0	1.0	107.4	0.85±0.05	9.42±0.3	2.52±0.27
4.0	1.0	11.05	0.97±0.12	9.18±0.4	2.19±0.38
4.0	1.0	1.212	1.25±0.11	10.8±0.5	2.15±0.29
4.0	1.0	0.104	1.43±0.08	10.3±0.2	1.98±0.16
4.0	1.0	0.009	1.57±0.18	14.2±0.3	1.81±0.11
4.0	2.0	107.4	Clump	–	4.27±0.45
4.0	2.0	11.05	1.09±0.09	32.6±0.4	4.63±0.36
4.0	2.0	1.212	1.37±0.07	39.4±0.3	4.66±0.18
4.0	2.0	0.104	1.88±0.12	59.0±0.4	3.74±0.28
4.0	2.0	0.009	1.91±0.14	32.1±0.5	3.58±0.29
4.0	3.0	107.4	Clump	–	5.24±0.54
4.0	3.0	11.05	0.61±0.13	123±0.6	4.37±0.39
4.0	3.0	1.212	1.62±0.18	81.2±0.6	4.85±0.34
4.0	3.0	0.104	1.73±0.09	50.6±0.5	4.78±0.42
4.0	3.0	0.009	2.14±0.14	59.4±0.4	4.17±0.47

were consumed for cell growth, and a small amount of L-lactic acid was produced in this phase (9.9, 8.4, and 6.1 g/L at densities of 57.3, 32.6, and 9.18 kg/m^3, respectively).

Subsequently, in the logarithmic phase (phase B, 12–36 h), the concentrations of glucose at 36 h decreased to 14, 21.7, and 26.7 g/L for densities of 57.3, 32.6, and 9.18 kg/m^3, respectively. When significant differences in L-lactic acid production began to become apparent, at 36 h, the accumulation of L-lactic acid reached 43, 36.6, and 26.5 g/L for densities of 57.3, 32.6, and 9.18 kg/m^3, respectively.

During the stationary phase (phase C, 36–48 h), cell growth stopped accumulating due to the exhaustion of the nitrogen source. The average glucose consumption rates in this stage were 0.92, 1.12, and 0.71 g/L/h for densities of 57.3, 32.6, and 9.18 kg/m^3, respectively, which were all lower compared with the glucose consumption rates in the logarithmic phase. The

Table 2 The effect of pellet compactness on L-lactic acid fermentation by *R. oryzae* with same pellet diameter

Pellet diameter (mm)	Pellet density (kg/m^3)	Biomass (g/L)	Lactic acid (g/L)	Productivity (g/L/h)
0.6±0.1	123±0.6	4.37±0.39	53.8	0.93
	17.2±0.3	3.22±0.32	41.2	0.858
	13.9±0.5	3.23±0.45	38.6	0.804
1.0±0.2	57.3±0.3	4.41±0.27	57.2	1.192
	51.7±0.4	3.36±0.35	56.2	1.171
	41.1±0.6	4.67±0.65	49.2	1.025
	32.6±0.4	4.63±0.36	45.7	0.952
	15.4±0.4	2.91±0.43	40.3	0.840
	9.18±0.4	2.19±0.4	35.2	0.733
1.5±0.2	81.2±0.6	4.85±0.34	52.3	0.968
	57.9±0.4	4.12±0.34	55.9	1.164
	37.4±0.2	2.81±0.18	46.6	0.978
	14.2±0.3	1.91±0.26	39.5	0.823
	10.3±0.2	1.986±01	34.6	0.721

production rates of L-lactic acid were only 42, 43, and 42 % of the values in phase B for densities of 57.3, 32.6, and 9.18 kg/m^3, respectively.

Next, we sampled and sliced the pellets from the three phases of fermentation to observe the difference in pellet interior structure for different densities (57.3, 32.6, and 9.18 kg/m^3) with the same diameter (1.0±0.2 mm), as shown in Fig. 3. In the same phase, large differences in the interior structure of pellets with different densities were apparent. The interior structure was much more compact for a density of 57.3 kg/m^3 and much looser for a density of 9.18 kg/m^3. In all fermentation phases, the change in pellet size was relatively small for pellets with the same density. In addition, the interior structure remained almost constant throughout the entire fermentation cycle for pellets of the same density, except that the pellet became a little denser at later stages.

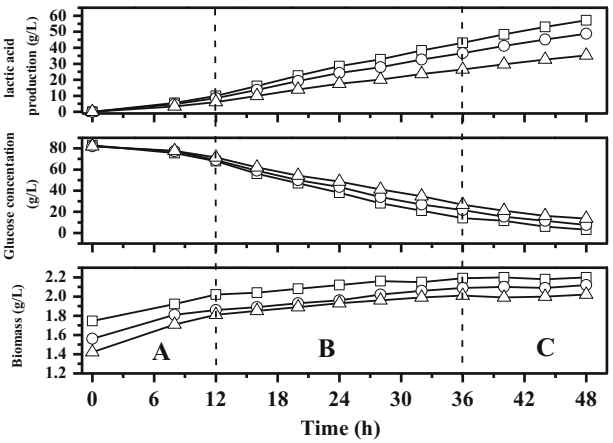

Fig. 2 The time course of lactic acid fermentation by *R. oryzae* (pellet diameter, 1.0±0.2 mm, with different pellet densities, *square* 57.3 kg/m^3, *circle* 32.6 kg/m^3, and *triangle* 9.18 kg/m^3)

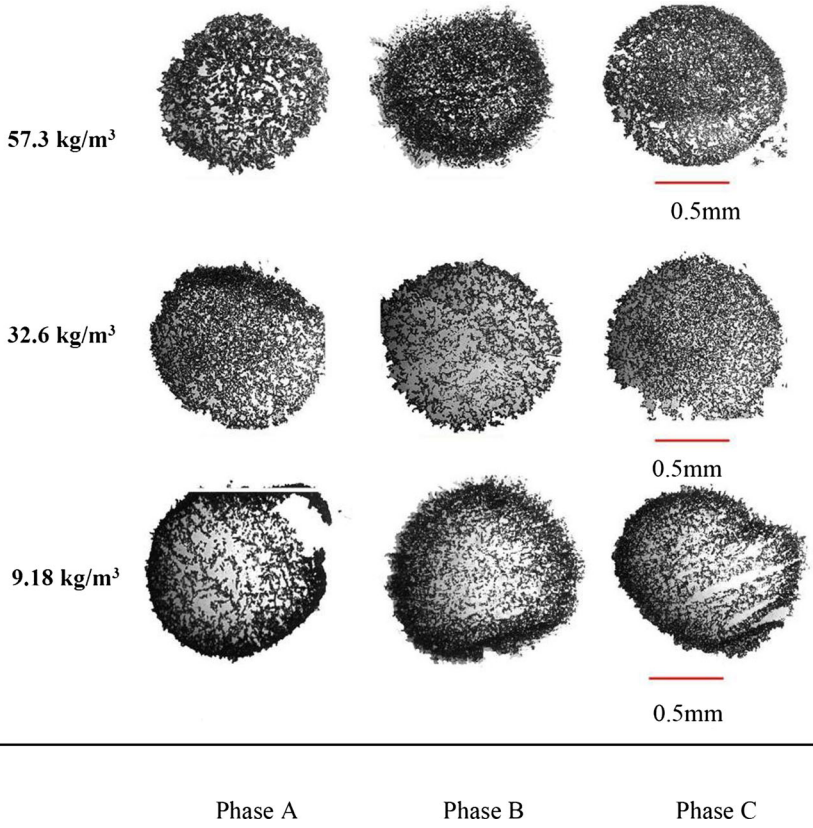

Fig. 3 Changes in pellet inner structure of *R. oryzae* for different pellet densities in different fermentation phases

Correlation Between Pellet Interior Structure and Lactic Acid Fermentation

Quantitative analysis of the typical microscopic structure of the biopellets was performed using K-means algorithms, as shown in Fig. 4. In general, mass transfer is limited in homogeneously structured, dense pellets causing a lower overall turnover rate. However, for lactic acid producing systems, the small denser (123 kg/m^3) pellet (a) is capable of outstanding secretion (53.8 g/L), probably because the pores are highly connected in the outer part of the structure and mass transfer is not limited inside the pellet where the transport distance is short (0.3 mm). Recently, Driouch [27] designed a type of core shell structure that could improve the overall performance of the interior structure. Our results show that this structure can also be obtained by adjusting the pellet density. As shown in image c, the compact part (blue and green) was distributed almost homogeneously in the thick outer layer of the pellet and loose in the central layer. A key factor affecting productivity is the active biomass layer inside the pellet [19, 28, 29]. The proper pellet diameter with incompact and homogeneously distributed interior structure benefitted for the nutrition supply and L-lactic acid production. The highest production of L-lactic acid is observed in this structure. Unfortunately, direct detection of the active layer has proven difficult when using our system, so we could only conclude that the core shell structure facilitates lactic acid production. Another typical interior structure for low-density pellets is a hollow structure (images b, f, h, and i), which has a thin condensed layer (red and

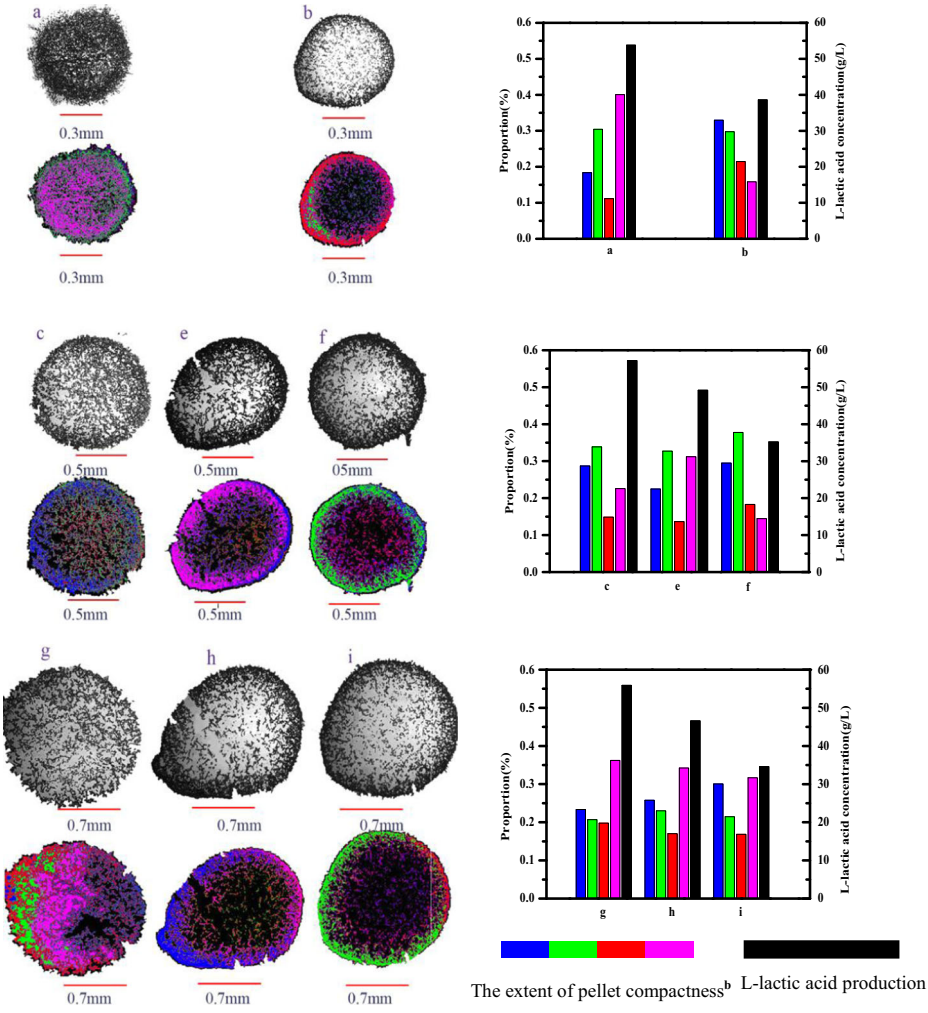

Fig. 4 The correlation between pellet interior structure and lactic acid fermentation by *R. oryzae*[a] (**a** 0.6±0.1 mm with 123±0.6 kg/m^3, **b** 0.6±0.1 mm with 17.2±0.3 kg/m^3, **c** 1.0±0.2 mm with 57.3±0.3 kg/m^3, **e** 1.0±0.2 mm with 41.1±0.6 kg/m^3, **f** 1.0±0.2 mm with 9.18±0.4 kg/m^3, **g** 1.5±0.2 mm with 57.9±0.4 kg/m^3, **h** 1.5±0.2 mm with 37.4±0.2 kg/m^3, **i** 1.5±0.2 mm with 10.3±0.2 kg/m^3).
[a]Since the software could not calculate the ratio of hollow pellets, the ratio of hollow pellets is not given in the figure, and the *black part* of the internal structure of the pellets represent hollow portions. [b]Compactness degree: *blue* was the most compact and *pink* was the loosest (Color figure online)

green) around the outside of the pellet, while the center is composed of extraordinarily loose biomass or may even be hollow (b and i). As expected, the hollow structure resulted in a large decrease in production, mainly due to the inhomogeneous distribution of biomass and the overabundance of inactive biomass inside the pellet. Moreover, the hollow structure was found in pellets of many sizes, i.e., not only in pellets exceeding the critical size but also in small pellets. Overall, the relationship between the interior structure of the pellet and lactic acid fermentation exhibited by a valuable combination of approaches suggests that targeted morphology control is crucial.

Conclusion

Our findings confirm that environmental factors greatly affect pellet characteristics, including pellet morphology, diameter, density, and interior structure, which ultimately affect L-lactic acid production. Pellet formation and diameter depended more heavily on inoculum size than on peptone and $CaCO_3$ concentrations. The diameter decreased with increasing of inoculum size, and larger pellets were observed for lower inoculum sizes, but a clear transition from the pelleted form to the dispersed form occurred with increasing the inoculum size.

Peptone concentration had the greatest effect on pellet density. Pellet density increased with increasing peptone concentration and was a more important factor for L-lactic acid production than pellet diameter. Low-density pellets formed easily at low peptone concentrations and often had a hollow structure with a thin condensed layer around the outside of the pellet and a center composed of extraordinarily loose biomass. In some cases, these pellets were completely hollow, which, as expected, greatly reduced production. L-lactic acid production was optimal when the density reached a certain level (50–60 kg/m^3), and the compact part was distributed almost homogeneously in the thick outer layer of the pellet, while the inner was composed of loose biomass. A key factor affecting productivity is the active biomass layer inside the pellet. The proper pellet diameter with incompact and homogeneously distributed interior structure benefitted for the nutrition supply and L-lactic acid production. The highest L-lactic acid production was observed in pellets with this structure (57.2 g/L L-lactic acid for 1.0 mm and 57.3 kg/m^3). Mass transfer was limited in homogeneously structured, dense pellets, causing a lower overall turnover rate. The interior structure also had almost no effect on the fermentation in pellets with same density.

$CaCO_3$ concentration has only a small influence on pellet diameter and density, probably because it increases spore germination and filamentous hypha extension.

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