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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*

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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

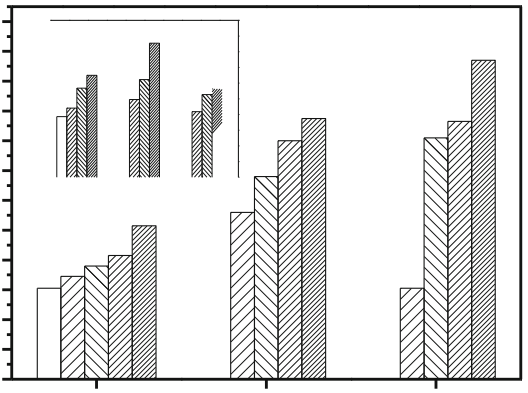
To determine glucose concentration, samples were centrifuged, and the supernatants were tested. To determine lactic acid concentration, samples were diluted with distilled water and hydrochloric acid, heated at 80 °C until the broth was clear, and centrifuged. The resulting supernatants were then used 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



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, whiltono1ntic acid0002(av)20.5(e)-216.8999996138.5(p)0(r)19.296999961(t)12.39961(e)11.
a55fa1.hasa7.39999961(s)0(s)-338.1000061(pm(3)Tj9.4645996(t)12.39999961(i)0(os99998(.79999923
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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).

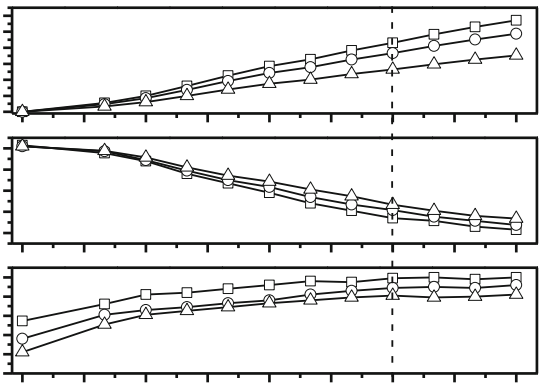
Subsequently, in the logarithmic phase (phase B, 12–36 h), the concentrations of glucose at 36 h decreased to 14, 21.7, and 26.7 g/L for densities of 57.3, 32.6, and 9.18 kg/m^3, respectively. When significant differences in L-lactic acid production began to become

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.



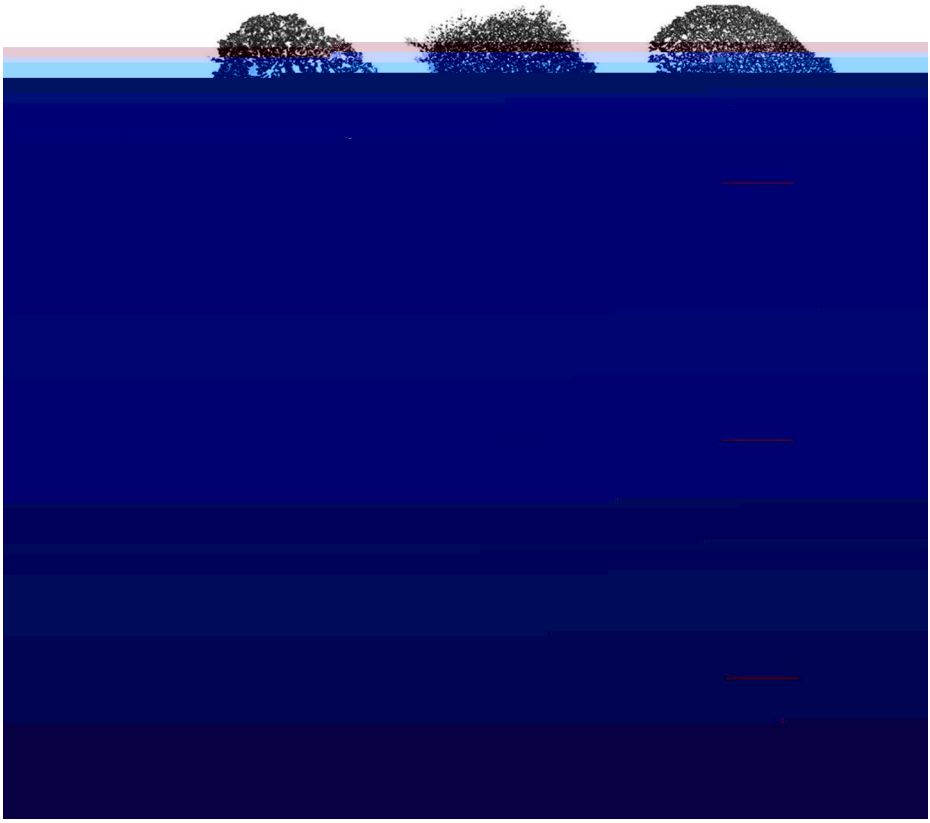
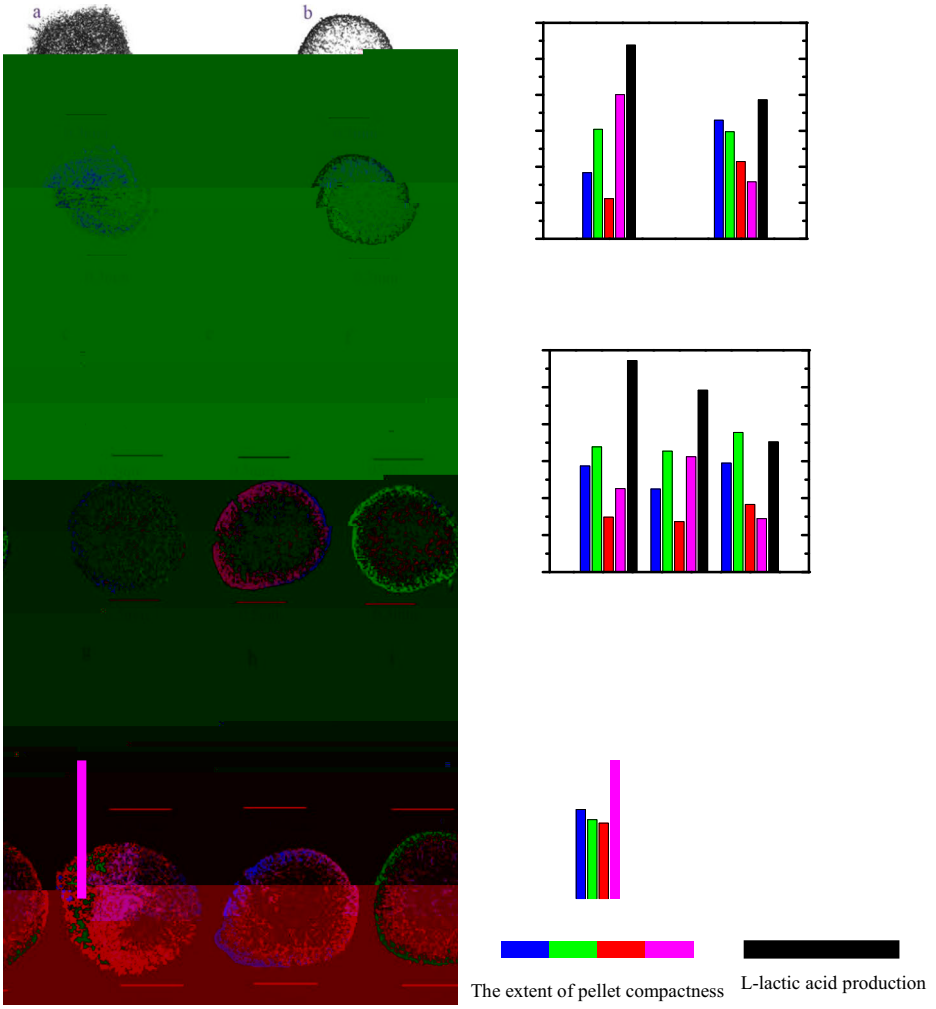


Fig. 3 Changes in pellet inner structure of *R. oryzae* for different pellet densities in different fermentation phases

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



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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