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Studying Pellet Formation of a Filamentous Fungus *Rhizopus oryzae* to Enhance Organic Acid Production

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Abstract

Using pelletized fungal biomass can effectively improve the fermentation performance for most of fugal strains. This article studied the effects of inoculum and medium compositions such as potato dextrose broth (PDB) as carbon source, soybean peptone, calcium carbonate, and metal ions on pellet formation of *Rhizopus oryzae*. It has been found that metal ions had significantly negative effects on pellet formation whereas soybean peptone had positive effects. In addition PDB and calcium carbonate were beneficial to *R. oryzae* for growing small smooth pellets during the culture. The study also demonstrated that an inoculum size of less than 1.5×10^9 spores/L had no significant influence on pellet formation. Thus, a new approach to form pellets has been developed using only PDB, soybean peptone, and calcium carbonate. Meanwhile, palletized fungal fermentation significantly enhanced organic acid production. Lactic acid concentration reached 65.0 g/L in 30 h using pelletized *R. oryzae* NRRL 395, and fumeric acid concentration reached 31.0 g/L in 96 h using pelletized *R. oryzae* ATCC 20344.

Index Entries: Calcium carbonate; fumaric acid; fungal pellet; inoculum size; lactic acid; metal ions; potato dextrose broth; *Rhizopus oryzae*.

Introduction

Filamentous fungal fermentation is widely used to commercially produce useful products such as organic acids, enzymes, antibiotics and the cholesterol lowering drugs (Statins), and so on *(1–7)*. Fungi can be grown in submerged cultures by several different morphological forms: suspended mycelia, clumps, or pellets *(8)*. Many studies have discussed the advantages and disadvantages of growth morphologies in terms of different product *(9–11)*. It has been concluded that the fungal growth in pellet form is a favorable alternative to benefit the most of fungal fermentations because it not only makes fungal biomass reuse possible but also significantly improve the culture rheology, which results in better mass and oxygen transfer into the biomass, and lower energy consumption for aeration and agitation *(12)*.

The change of fungal morphology is mainly influenced by medium compositions, inoculum, pH, medium shear, additives (polymers, surfactants, and chelators), culture temperature, and medium viscosity *(5,8,13,14)*. For individual strains, each factor has different importance to the growth morphologies; some strains such as *Rhizopus* sp. need strong agitation to form pellets, whereas some strains such as *Penicillium chrysogenum* require high pH to form pellets *(8)*. Thus, the study on fungal pellet formation is limited at the level of the individual strain.

Strains of *R. oryzae* have the capability to produce fumaric acid, lactic acid, pectinase, amylogucosidase, α-amlyase, and so on *(1,4,5,15)*. Zhou et al. *(16)* investigated the effects of different metal ions (Mg^{2+}, Zn^{2+}, and Fe^{2+}) and pH on the pellet formation of *R. oryzae* ATCC 20344 under glucose as a carbon source and urea as a nitrogen source. Byrne studied the effects of glucose concentration, peptone concentration, pH, and some additives on the pellet formation of *R. oryzae* ATCC 10260 *(17,18)*. However, the comprehensive investigation of the effects of medium compositions and inoculum on the pellet formation and the effects of pellets on the improvement of organic acid production has not been fulfilled. This article is focused on studying the factors such as carbon source, nitrogen source, metal ions, neutralizer, and inoculum in order to develop a new approach to form *R. oryzae* pellets, and further enhance organic acid production.

Materials and Methods

Microorganism

R. oryzae ATCC 20344 and *R. oryzae* NRRL 395 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The strains were first cultured on potato dextrose agar (Difco, Franklin Lakes, NJ) slants, and further propagated on potato dextrose agar in 500-mL Erlenmeyer flasks to form spores. The culture temperature was 25°C. The spores were washed from the agar with sterile distilled water, and collected as a spore suspension for the study. The spore concentrations of the suspension were 7.5×10^7 spores/mL for *R. oryzae* ATCC 20344, 9.0×10^7 spores/mL for *R. oryzae* NRRL 395, respectively. *R. oryzae* ATCC 20344 was used for studying pellet formation.

Pellet Formation

The effects of factors such as different carbon source, nitrogen source, mineral ions, and neutralizer on pellet formation were carried out by a 2^4 full-factorial design with replicates (Table 1). Two different carbon sources Potato dextrose broth (PDB) and glucose under two levels of nitrogen (with and without), mineral ions (with and without), and neutralizer (with and without) were studied. The glucose concentration in the carbon sources was 20.0 g/L. The nitrogen source was soybean peptone (Sigma,

Table 1
2^4 Full-factorial Design With Replicates for Pellet Formation[a,b]

Run	Factors			
	Carbon source	Nitrogen source	Mineral ions	Neutralizer
1	PDB ((+1)	Yes (+1)	Yes (+1)	Yes (+1)
2	PDB (+1)	Yes (+1)	Yes (+1)	No (–1)
3	PDB (+1)	Yes (+1)	No (–1)	Yes (+1)
4	PDB (+1)	Yes (+1)	No (–1)	No (–1)
5	PDB (+1)	No (–1)	Yes (+1)	Yes (+1)
6	PDB (+1)	No (–1)	Yes (+1)	No (–1)
7	PDB (+1)	No (–1)	No (–1)	Yes (+1)
8	PDB (+1)	No (–1)	No (–1)	No (–1)
9	Glucose (–1)	Yes (+1)	Yes (+1)	Yes (+1)
10	Glucose (–1)	Yes (+1)	Yes (+1)	No (–1)
11	Glucose (–1)	Yes (+1)	No (–1)	Yes (+1)
12	Glucose (–1)	Yes (+1)	No (–1)	No (–1)
13	Glucose (–1)	No (–1)	Yes (+1)	Yes (+1)
14	Glucose (–1)	No (–1)	Yes (+1)	No (–1)
15	Glucose (–1)	No (–1)	No (–1)	Yes (+1)
16	Glucose (–1)	No (–1)	No (–1)	No (–1)

[a]Code values are in the parentheses.
[b]The strain is *R. oryzae* ATCC 20344.

St. Louis, MO) with a concentration of 6.0 g/L. The mineral ions included: 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 0.088 g/L $ZnSO_4 \cdot 7H_2O$. The neutralizer was $CaCO_3$ (6.0 g/L). The cultures were performed at 27°C for 48 h on a rotary shaker at 190 rpm.

Effects of Inoculum Size on Pellet Formation and Growth

Four spore concentrations (1.5×10^8, 3.75×10^8, 7.5×10^8, and 1.5×10^9 spores/L) were run on the selected media, which were identified in the previous section as favorable media to form pellets. The cultures were carried out under the same conditions described under "Pellet Formation."

Comparison of Pelletized Fungal Fermentation and Clump-Like Fungal Fermentation on Organic Acid Production

R. oryzae NRRL 395 was used to produce lactic acid. The fermentation conditions for lactic acid production were: 120.0 g/L of glucose, 60.0 g/L of $CaCO_3$, and 6.5 g/L (dry basis) of biomass for both pellets and clump. *R. oryzae* ATCC 20344 was the strain used to produce fumaric acid. The fermentation conditions for fumaric acid production were: 100.0 g/L of glucose, 60.0 g/L of $CaCO_3$, and 11.5 g/L (dry basis) of biomass for both pellets and clumps. The cultures were carried out at 27°C in 250-mL flasks

containing 100 mL of culture medium on a rotary shaker at 190 rpm. All media were autoclaved at 121°C for 15 min before inoculation.

Statistical Analysis

The effects of carbon source, nitrogen, mineral ions, and neutralizer on pellet formation were compared using the special property of factorial designs whose effects can be simply estimated by the differences in average response values between the high and low codes of each factor. A ranked list that presented the relative importance among factors was formed by the comparison. The list is given in the Pareto chart *(19)*.

Analytical Methods

The morphology of the cultures was determined by examining submerged cultures dispersed on Petri dishes. An Olympus microphotograph (Tokyo, Japan) was used to observe the pellet morphology and measure the size of the pellets. The pH value was measured with a Fisher portable pH meter (Fisher Scientific, Pittsburgh, PA). Dry biomass was determined by washing the pellet mycelia with 6 *N* HCL to neutralize excess $CaCO_3$ attached in the pellets, and then washing to pH 6.0 with deionized water. The washed biomass was dried at 100°C overnight before weight analysis. The fumaric acid and lactic acid in the broth were analyzed using a Dionex DX-500 system (Sunnyvale, CA) including an AS11-HC (4 mm 10-32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 autosampler *(20)*.

Results

Pellet Formation

The effects of carbon source, nitrogen source, metal ions, and neutralizer on fungal morphology were investigated (Table 2). Fungal morphologies varied from different combination of factors (Fig. 1). There were only four runs (Nos. 3, 4, 11, and 12), which were able to form pellets. In order to run statistical analysis on a qualitative data of morphologies, the qualitative value has to be assigned to describe it. The uniform pellet form of fungal biomass was represented by the value of 1, and other nonpellet forms such as clump, less/nongrowth, and nonuniform pellet/clump were represented by the value of 0. The analysis demonstrated that peptone, metal ions, and their combined interaction together had 100% of total effect, which means that peptone and metal ions were the two main factors on pellet formation (Fig. 2). The data also showed that peptone had a positive effect (33% of total effect) on pellet formation, whereas metal ions and the interaction of metal ions and peptone (33% each of total effect) had negative effects.

Table 2
Experimental Results From 2^4 Full-Factorial design[a,b]

Run	Fungal morphology[c]	Pellet size (mm)[d]	Biomass (g dry matter/L)	Initial pH	Final pH
1	Clump (0)	–	6.344	6.06	7.10
2	Clump (0)	–	3.62	5.76	7.13
3	Uniform pellet (1)	1.98 ± 0.41	3.992	6.84	6.81
4	Uniform pellet (1)	1.03 ± 0.15	3.158	5.99	3.77
5	Clump (0)	–	2.818	5.58	5.59
6	Nonuniform pellet and clump (0)	–	2.318	4.65	3.11
7	Nonuniform pellet and clump (0)	–	2.366	6.58	6.21
8	Nonuniform pellet and clump (0)	–	0.912	4.93	3.31
9	Clump (0)	–	4.574	6.44	7.18
10	Clump (0)	–	3.03	6.20	6.11
11	Uniform pellet (1)	2.57 ± 0.22	2.408	7.42	5.88
12	Uniform pellet (1)	1.47 ± 0.35	1.842	7.13	3.43
13	Nongrowth (0)	–	0.008	5.43	5.84
14	Nongrowth (0)	–	0.008	4.64	4.52
15	Less-growth (0)	–	0.016	6.81	6.74
16	Less-growth (0)	–	0.036	6.05	6.09

[a]All data except pellet size are the mean of two replicates, pellet size is the mean of 200 replicates with standard deviation at $\alpha = 0.05$.
[b]The strain is *R. oryzae* ATCC 20344.
[c]Code values are in the parentheses. 1 is the value to represent the pellet, 0 is the value to represent the nonpellet forms such as clump, nongrowth, and so on.
[d]"–" means nonpellet.

Statistical analysis concluded that the other two factors of carbon source and neutralizer were not the main factor on pellet formation. However, both of them had significant influences on fungal pellet growth. Pellets from cultures with calcium carbonate and PDB had an average diameter of 1.98 mm and 3.99 g biomass, whereas pellets from corresponding cultures without calcium carbonate only had 1.03 mm and 3.16 g, respectively (Fig. 3). Pellets cultured on glucose had the same trend as those on PDB (Fig. 3). In addition, pellets cultured on both PDB and glucose with calcium carbonate were much smoother than those

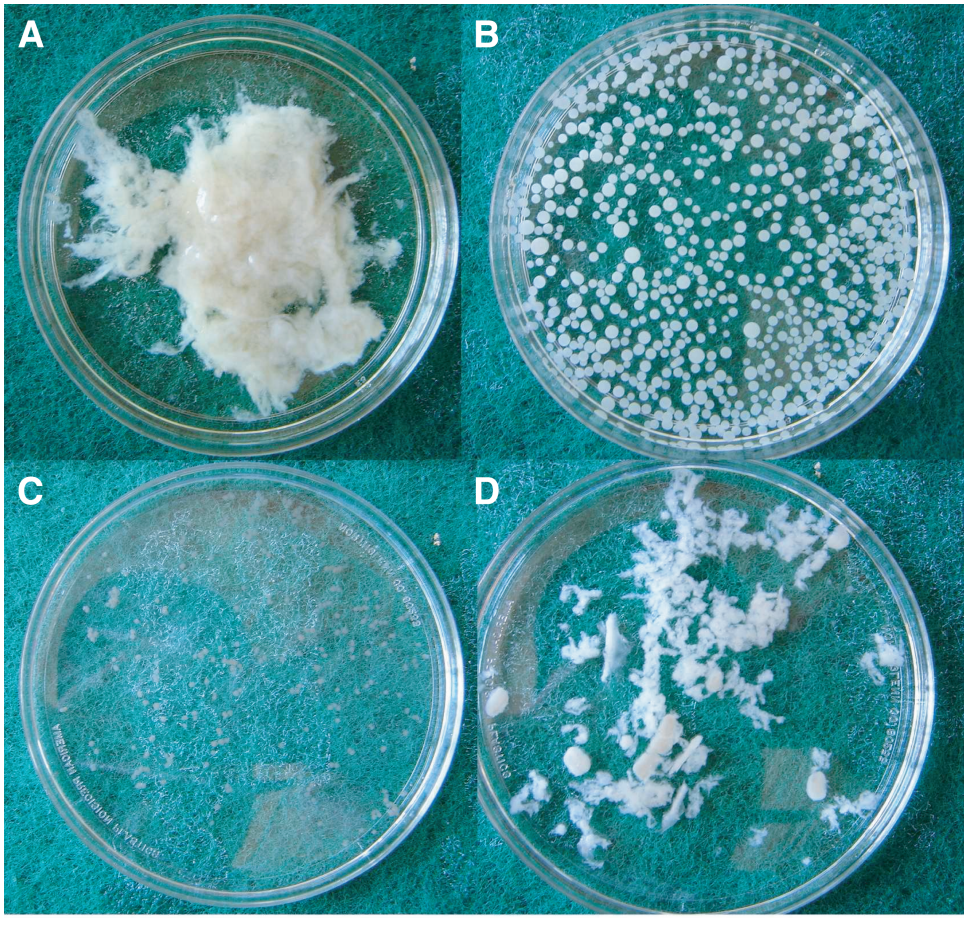

Fig. 1. Morphology of fungal biomass (the strain is *R. oryzae* ATCC 20344). **(A)** Clump from run 2, **(B)** pellet from run 3, **(C)** less-growth from run 15, and **(D)** nonuniform pellet and clump.

without calcium carbonate (Fig. 4). As for carbon sources, on the media with calcium carbonate the size of pellets cultured on PDB was 1.98 mm, which was smaller than the 2.57 mm from cultures on glucose, and PDB medium produced 1.58 g/L more biomass than the glucose medium (Fig. 3). The same trend was on the media without calcium carbonate (Fig. 3). In terms of effects of carbon sources on fungal pellets, PDB was a benefit for producing more biomass and pellets with a smaller size compared with glucose. The data also demonstrated that pH differences caused by different components in the culture medium among experimental runs had no significant influence on pellet formation compared with other factors (Table 2). Run Nos. 3 and 4 formed pellets at final pH 6.81 and 3.77, respectively. Run No. 11 and 12 formed pellets at the pH values of 5.88 and 3.43.

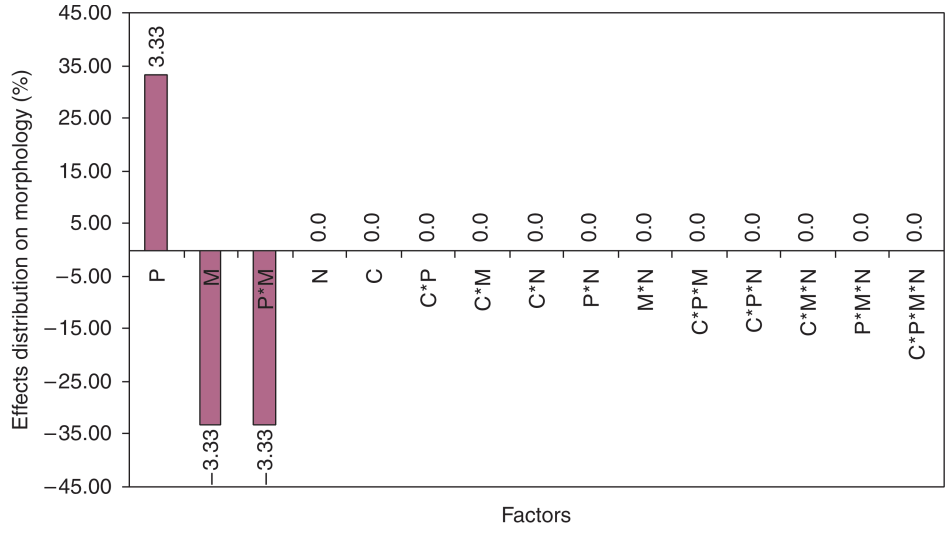

Fig. 2. Pareto charts of effects of medium composition on pellet formation. Where P is peptone, M is mineral ions, N is neutralizer, and C is carbon source. The strain is *R. oryzae* ATCC 20344.

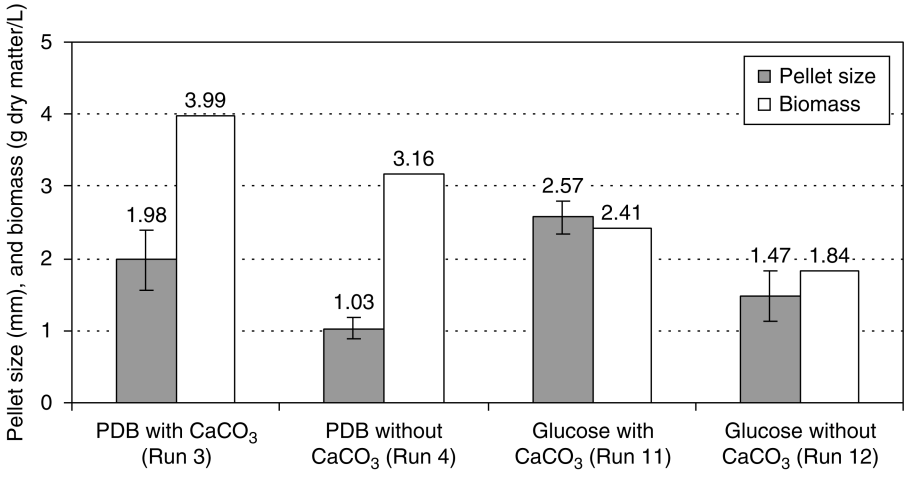

Fig. 3. Effects of carbon source and neutralizer on pellet morphology during pellet formation (the strain is *R. oryzae* ATCC 20344).

Effects of Inoculum Size on Pellet Formation and Growth

Spore concentration did not influence the pellet formation. All four different spore concentrations (1.5×10^8, 3.75×10^8, 7.5×10^8, and 1.5×10^9 spores/L) formed smooth pellets on both PDB pellet-formed culture medium (24.0 g/L PDB, 6.0 g/L soybean peptone, and 6.0 g/L $CaCO_3$) and glucose pellet-formed culture medium (20.0 g/L glucose, 6.0 g/L soybean peptone, 6.0 g/L $CaCO_3$). Pellet numbers and total amount of biomass

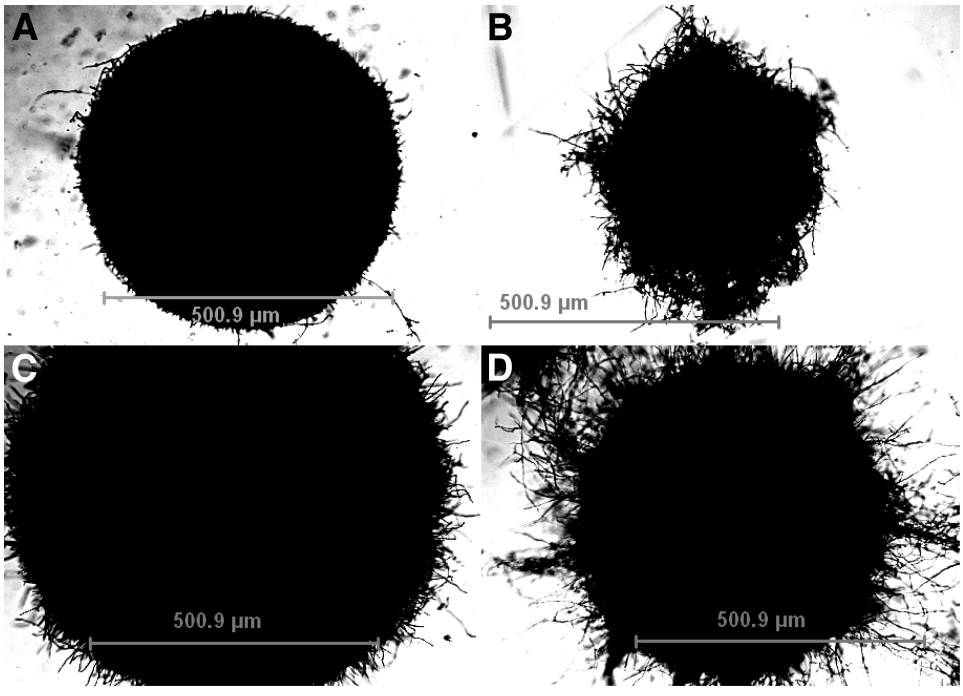

Fig. 4. Surface structure of fungal pellets from different cultural conditions (the strain is *R. oryzae* ATCC 20344). **(A)** From run 3 (PDB with $CaCO_3$), **(B)** from run 4 (PDB without $CaCO_3$), **(C)** from run 11 (glucose with $CaCO_3$), and **(D)** from run 12 (glucose without $CaCO_3$).

increased, and pellet size decreased following the increase of spore concentration no matter what type of carbon source the cultures were on (Fig. 5). The changes of total pellet numbers and pellet size with the increase of inoculum concentration was much more significant than the change of biomass because the biomass increase in the certain range of inoculum is mainly controlled by the nutrients rather than spore concentration.

Comparison of Pelletized Fungal Fermentation and Clump-Like Fungal Fermentation on Organic Acid Production

The pelletized fungal biomass for both lactic acid and fumaric acid production were obtained from culturing *R. oryzae* NRRL 395 and *R. oryzae* ATCC 20344 on the medium (24.0 g/L PDB, 6.0 g/L soybean peptone, 6.0 g/L $CaCO_3$) with inoculum size of 1.5×10^9 spores/L at 27°C and 190 rpm for 48 h. The average pellet diameters of biomass for *R. oryzae* NRRL 395 and *R. oryzae* ATCC 20344 are 1.7 mm and 1.5 mm, respectively. Organic acid fermentations comparing clump and pellet morphologies demonstrated that there were significant ($p < 0.05$) differences on lactic acid and fumaric acid production between clump and pellet morphologies. The lactic acid concentration of clump fermentation reached 32.0 g/L in 60 h of culture duration,

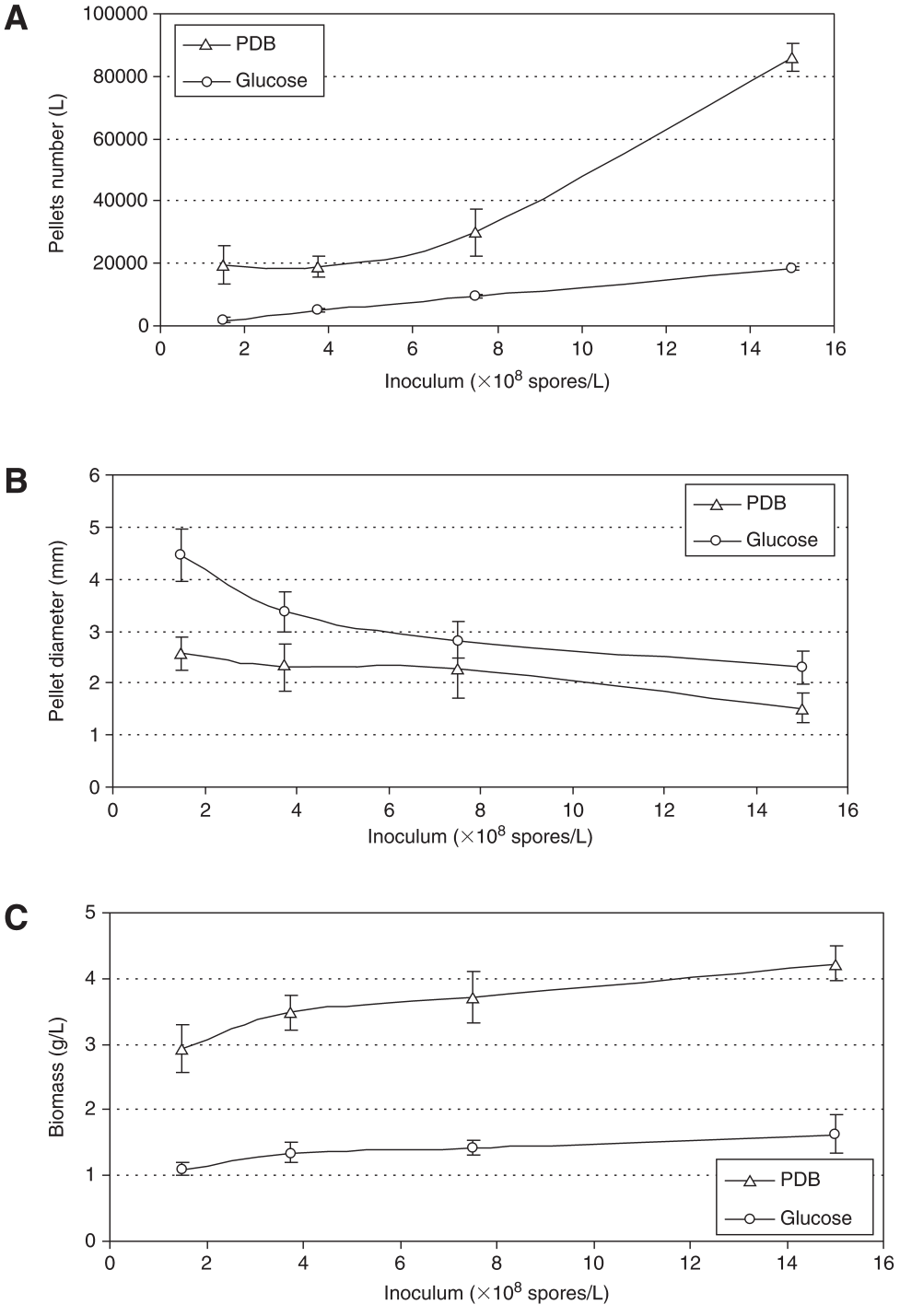

Fig. 5. Effects of inoculum on pellet formation (the strain is *R. oryzae* ATCC 20344). **(A)** Pellet number, **(B)** pellet size, and **(C)** biomass.

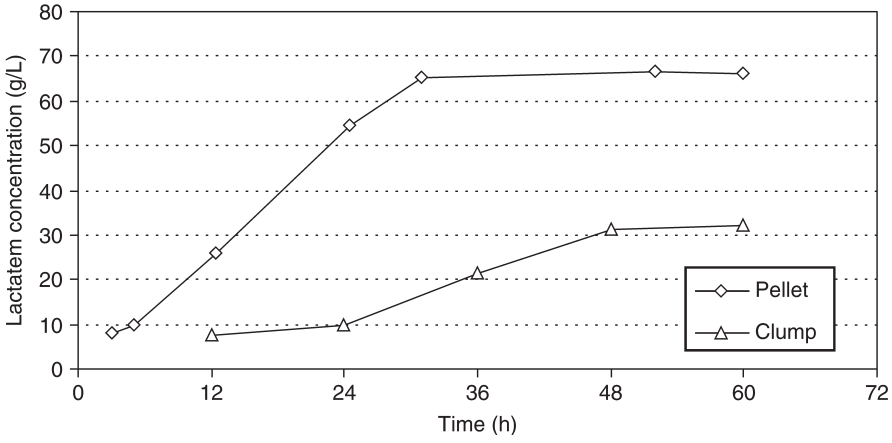

Fig. 6. Comparison of lactic acid production using pellet and clump morphology (the strain is *R. oryzae* ATCC NRRL 395). Data are presented as the mean of two replicates.

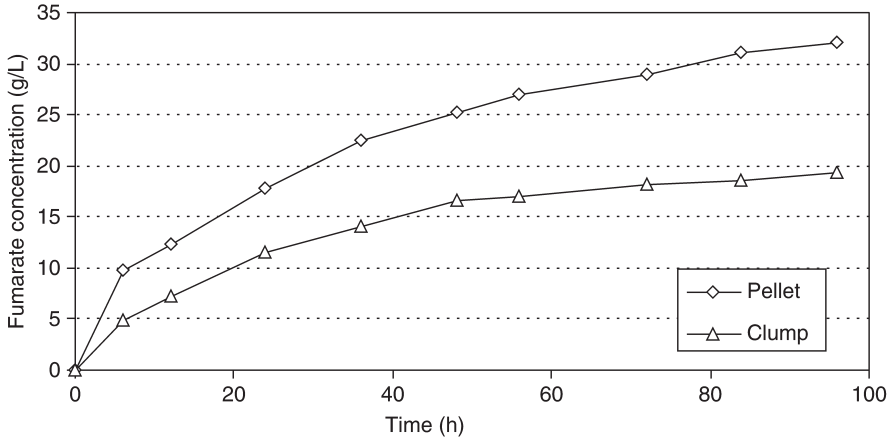

Fig. 7. Comparison of fumaric acid production using pellet and clump morphology (the strain is *R. oryzae* ATCC 20344). Data are presented as the mean of two replicates.

whereas the pellet fermentation produced 65.0 g/L in 30 h of culture duration (Fig. 6). Meanwhile, the clump and pellet fermentations of fumaric acid reached 21.5 and 31.0 g/L, respectively, during 96 h of culture duration (Fig. 7). The data indicated that both of lactic acid and fumaric acid production were significantly increased using pelletized fungal fermentation.

Discussion

Metal ions are very important factor in the metabolism of *R. oryzae*. The organism utilized the energy three times more efficiently when metal ions were added to the medium, which made for a relatively fast and abundant fungal growth *(21)*. It has been proved by the study that the cultures with metal ions produced more biomass than those without metal

ions (Table 2). Meanwhile, in terms of fungal morphology, metal ions in the media made *R. oryzae* difficult to form the pellet as well, because the fungus grew so fast on the media with metal ions, the filaments tangled with each other and leaned to clumpy morphology (Fig. 1A). Thus, in order to form *R. oryzae* pellet, metal ions had to be eliminated in the culture media.

The pH of medium has also been reported as a very important factor for various fungi to form pellets. Generally, pH could change the surface properties of fungi, further influencing the pellet formation, and different strains have different sensibility to pH value *(8)*. However, for this particular strain of *R. oryzae*, the results showed that there were no significant differences on pellet formation with a pH range of 3.0–7.0, which means that this strain is not as sensitive to pH as some other strains such as *Aspergillus niger* and *P. chrysogenum (7,22,23)*.

Inoculum size is generally recognized as of great importance in the process of fungal pellet formation. Generally, the interaction of hyphae is considered as the main force to form clump. In the early stage of growth, the higher the inoculum size, the more interaction with the hyphae, and the more possibility the clump would be formed. Thus, it has been concluded by other researchers that low inoculum concentrations are beneficial for pellet production *(24)*. However, the maximum inoculum size varied from strain to strain *(8)*. Most of studies on pellet formation of strains of *Rhizopus* were conducted at relatively low concentrations ($<10^7$ spores/L) *(14,17,18,25)*. The study on the effects of inoculum on *R. oryzae* pellet formation demonstrated that there were no significant ($p > 0.05$) influences on pellet formation once the inoculum concentration was increased up to 10^9 spores/L. The result elucidated that the particular strain of *R. oryzae* is able to prevent hyphae growth from forming clumps at relatively higher inoculum concentrations compared with other strain in the genus *Rhizopus*.

Peptone as nitrogen source was one of the two main factors on fungal pellet formation based on the statistical analysis. It had positive effect on pellet formation mainly because nitrogen is the limiting factor on the growth of *R. oryzae (26)*. Meanwhile, the type of nitrogen compound also has a considerable influence on fungal pellet formation *(23)*. A study of different nitrogen sources on *R. oryzae* ATCC 20344 showed that peptone produced much smaller, more unique, and heavier pellets than other nitrogen sources such as urea (W. Liao and S. Chen, unpublished data).

Although the carbon source was not a main factor on pellet formation, the result showed that PDB is a better carbon source on the pellet formation of *R. oryzae* compared with glucose. PDB as a good nutrient source has been widely used for fungal and yeast cultures. It contains mainly glucose, some vitamins, and a little nitrogen. The effects of PDB on fungal morphology have not been reported to date. It has been found by this study that PDB had a large impact on pellet such as pellet size and total biomass. This means that

the vitamins in PDB might be the main substances causing the difference in fungal pellet growth. Furthermore, studies on the effects of PDB components on pellet growth need to be carried out.

Calcium carbonate, as a neutralizer, prevents pH from dropping into the low pH range of 2.0–3.0, which is not favorable for the biomass accumulation *(16)*. Total amount of biomass from a medium with calcium carbonate were significantly higher than those without it (Fig. 3). In addition, during fungal pellet formation, calcium carbonate is not only a neutralizer to keep pH stable, but it also supplies Ca^{2+} ions. It has been reported that calcium ions were usually recognized to induce mycelial aggregation during fungal growth *(27)*, which has been proved by this study that media with calcium carbonate produced smoother and larger pellets than those without calcium carbonate (Fig. 4).

Conclusion

This study developed a new, simple culture medium to grow pellets for *R. oryzae*. The fungal pellets can be formed from the culture on a medium with only three components of PDB, soybean peptone, and calcium carbonate without any additives such as metal ions, polymers, and so on. Pelletized fungal fermentation significantly enhanced organic acid production. Lactic acid concentration reached 65.0 g/L in 30 h using pelletized *R. oryzae* NRRL 395, and fumeric acid concentration reached 31.0 g/L in 96 h using pelletized *R. oryzae* ATCC 20344.

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