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Mycelial Pellet Formation by *Rhizopus oryzae* ATCC 20344

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Abstract

Factors in a cultivation medium affecting fungal growth morphology and fumaric acid production by *Rhizopus oryzae* ATCC 20344 were investigated. These factors included the initial pH value and trace metals such as zinc, magnesium, iron, and manganese in the cultivation medium. It was found that a significant change in the growth morphology of *R. oryzae* ATCC 20344 occurs when the initial pH value is varied. A lower initial pH value in the cultivation medium was inhibitory to fungal growth, and fast growth in the cultivation medium at a higher initial pH value promoted the formation of large pellets or filamentous forms. Trace metals in the cultivation media also had significant effects on pellet formation and fumaric acid fermentation.

Index Entries: Pellet formation; fumaric acid; *Rhizopus oryzae*.

Introduction

Many fungi can grow in submerged culture in different forms ranging continuously from discrete compact pellets of hyphae to filamentous forms depending on a number of factors such as the components of the medium, the size of the inoculum, and the physical environment *(1)*. Filamentous growth increases the viscosity of the medium, thereby requiring a higher power input to maintain adequate mixing and aeration. Also, filamentous forms may wrap around impellers, foul agitation blades, and block spargers. Growing fungi in the form of pellets is very attractive in industrial fermentation processes because of the following advantages:

1. The slime-forming tendencies of the fungus are eliminated.
2. The ease of aeration is increased.

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3. The viscosity of the suspension is considerably lower and the mass transfer condition is considerably better.
4. The subsequent separation of the mycelium from the medium is simpler *(2)*.

However, the limitation of nutrients and oxygen may cause the death of pellet interiors or cause the pellet interiors to become anaerobic and have lower yield. Therefore, control of the formation of small uniform pellets is a prerequisite for industrial applications to ensure adequate mass and heat transfer or metabolite production.

A significant change in morphology is often observed when the composition of the growth medium is varied. Generally, growth morphology can be influenced by carbon and nitrogen sources; pH value; and heavy metals such as magnesium, zinc, iron, copper, and manganese in the medium *(3,4)*. In this article, we deal with factors such as pH, heavy metal ions in a cultivation medium affecting growth morphology, and fumaric acid production by *Rhizopus oryzae* ATCC 20344.

Materials and Methods

Microorganism and Inoculum

R. oryzae ATCC 20344, purchased from the American Type Culture Collection (Rockville, MD), was used. This fungal strain is a good producer of fumaric acid and has been shown to have the best specific productivity of fumaric acid from glucose among five different *Rhizopus* strains *(5)*. The fungus grows and forms spores on yeast extract, malt extract, and peptone (YMP) agar plates that consist of yeast extract (0.3%), malt extract (0.3%), peptone (0.3%), glycerol (2%), and agar (2%). After 8 to 9 d, agar plates containing sporulated fungi were washed with sterilized deionized water to obtain a spore suspension. The spore suspension was maintained at 4°C for later inoculation.

Culture Methods

The bioprocess for fumaric acid production by *R. oryzae* undergoes two phases: the cell growth phase and the fermentation phase. Because separation of the cell growth phase and the nongrowth acid fermentation phase can provide better understanding of the characteristics of *R. oryzae*, two kinds of media were employed: cultivation and fermentation media. The cultivation medium is used to grow cells and it consisted of 50 g of glucose, 2 g of urea, 0.6 g of KH_2PO_4, 0.50 g of $MgSO_4 \cdot 7H_2O$ (50 ppm Mg^{++}), 0.0176 g of $ZnSO_4 \cdot 7H_2O$ (4 ppm Zn^{++}), and 0.498 mg of $FeSO_4 \cdot 7H_2O$ (100 ppb Fe^{++}) in 1 L of deionized water. The fermentation medium is a nitrogen-free medium to use for fumaric acid production. In this study, glucose was the carbon source of the fermentation.

Effect of pH

To investigate the influence of pH in the cultivation medium on fungal growth morphology and fumaric acid production, the pH of the cultivation medium was adjusted with the addition of HCl.

Effect of Metal Ions

The effects of four metal ions (Mg^{++}, Zn^{++}, Mn^{++}, and Fe^{++}) present in the cultivation medium on fungal growth and fumaric acid fermentation were evaluated. The sources of these ions were $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot H_2O$. For each experiment, glucose, nitrogen, and salt solutions were sterilized separately and added together prior to the incubation of spores. Incubation was carried out in 250-mL Erlenmeyer flasks containing 100 mL of media in an incubator-shaker at 35°C and 200 rpm. After incubation for 48 h, the mycelia were harvested and then transferred into fermentation media to carry out fermentation in the same incubator-shaker at the same conditions (35°C and 200 rpm).

Analytical Methods

High-Performance Liquid Chromatograph

A high-performance liquid chromatograph (Model L-6200A) with a refractive index detector (Model L-3350 RI), an automatic injector (Model AS-4000), and an integrator (Model D-2500) (Hitachi Instruments, Inc., San Jose, CA) was used to analyze glucose, fumaric acid, and byproduct concentrations. The mobile phase was 0.005 *M* H_2SO_4 at a flow rate of 0.8 mL/min through a Bio-Rad Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA) at 60°C.

Final Fumaric Acid Concentration and Dry Weight

Owing to the low solubility of calcium fumarate, fumarate may precipitate during fermentation. To obtain an accurate total amount of the fumarate produced, the final culture broth was diluted by the addition of distilled water to dissolve the fumarate, and HCl to neutralize the excess $CaCO_3$. To help the fumarate dissolve, the final culture broth was heated at 80°C until the broth was clear. Samples were collected for high-performance liquid chromatography analysis. Once the broth was cooled, it was filtered on preweighed Whatman filter paper to recover the biomass. The solid fraction was washed completely with deionized water, dried at 85°C for 24 h, and weighed to yield the final dry weight.

Microphotography

A microphotograph (Olympus, Tokyo, Japan) was introduced to observe cell morphology and take pictures during the cell growth phase. When pellets were formed, samples of pellets were taken from the culture. The diameter of pellets is the largest size of the pellets. Because the diameter is not in the magnitude of micrometers, it can be determined by observation under a magnifier with a ruler.

Table 1
Effect of pH in Cultivation Medium on Growth Morphology of *R. oryzae*

pH	Cell morphology
5.59	Pellet form, elliptical, ~1.5 mm
3.93	Filamentous form
3.36	Pellet form, uniformly distributed, smooth, spherical, ~1 mm
3.05	Pellet form, uniformly distributed, smooth, spherical, <1 mm
2.88	Pellet form, uniformly distributed, smooth, spherical, <1 mm
2.78	Pellet form, uniformly distributed, smooth, spherical, <1 mm
2.68	Pellet form, uniformly distributed, smooth, spherical, <1 mm
2.60	Pellet form, uniformly distributed, smooth, spherical, <1 mm
2.50	Spores unable to germinate

Table 2
Effect of pH in Cultivation Medium on Fumaric Acid Fermentation by *R. oryzae*

pH	Weight yield of fumaric acid produced/ glucose consumed (%)	Residual glucose (g/L)	Fumaric acid produced (g/L)	Ethanol produced (g/L)
5.59	42.12	0	31.76	15.79
3.36	46.29	0	34.89	12.42
3.05	42.98	0	32.40	18.39
2.88	43.13	2.32	31.51	16.35
2.78	42.70	2.27	31.22	8.95
2.68	38.89	0	29.32	12.49
2.60	38.39	2.37	28.03	15.78

Results and Discussion

Effect of pH

The influence of pH in the cultivation medium on fungal cell morphology and fumaric acid production was investigated; Tables 1 and 2 give the results. In the fermentation stage, the initial glucose was 75.38 g/L, and the fermentation time was 67 h. According to Tables 1 and 2, when the initial pH of the cultivation medium was in the range of 3.36–2.60, small (<1 mm in diameter), uniformly distributed, spherical pellets were formed. The size of the formed spherical pellets decreased slightly with a decrease in pH; the fumaric acid weight yield decreased slightly as well. When the pH was <2.50, spores could not germinate. When the pH was about 5.59, which is the natural pH of the cultivation medium, larger, not uniformly distributed, elliptical pellets were formed. However, when a small amount of HCl was added to the medium, the pellet formation was not consistent. At a pH of about 3.93, the filamentous form occurred. It is evident that formation of smaller pellets takes place at a lower pH in the range from 3.36 to 2.60.

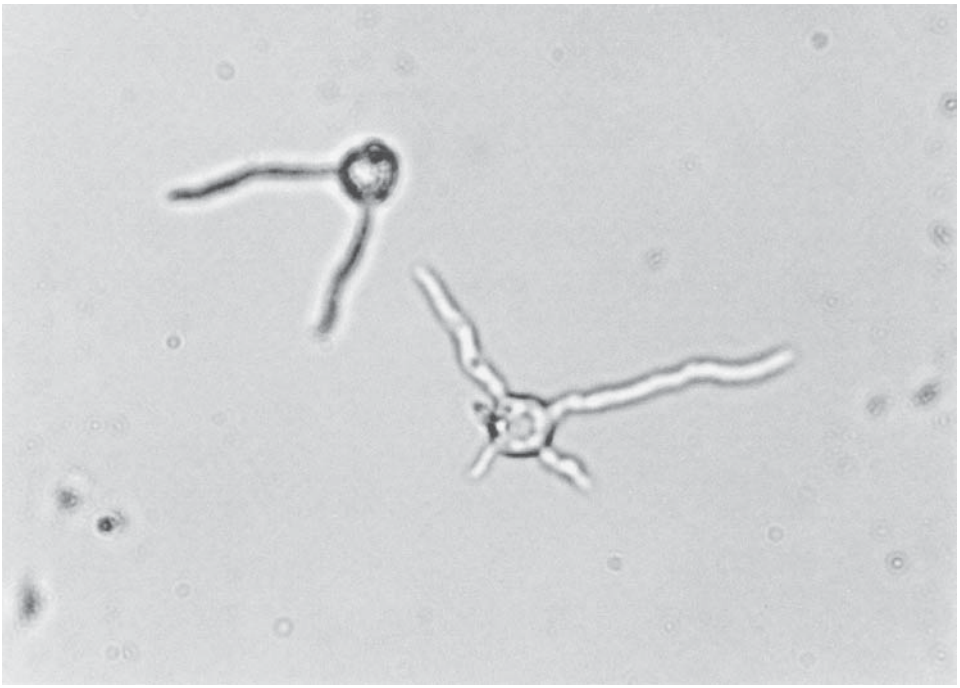

Fig. 1. Image of fungal growth at h 5 in the cultivation medium at an initial pH of 5.59. Magnification: ×150.

During the fungal growth phase, pictures were taken at h 5 and 22 with the Olympus Microphotograph. Figures 1 and 2 represent the images of fungal morphology at h 5 in the cultivation media with pH values of 5.59 and 2.88, respectively. Germinated spores in the medium at pH 5.59 had much longer tubes than spores in other media at lower pH values. Spore germination of this strain was retarded in the presence of acid. Figures 3 and 4 represent the images of pellets formed at h 22 in media at pH 5.59 and 2.88, respectively. As the pH decreased, the pellets were less smooth, more hairy and fluffy, and much smaller. Figure 4 represents a typical pellet required in the fermentation.

Therefore, a significant change in morphology occurred when the initial pH in the cultivation medium was varied. A lower initial pH in the cultivation medium was inhibitory to fungal growth, and fast growth in the cultivation medium at a higher pH was seen to promote the formation of large pellets or filamentous forms. However, mycelium formed at a lower pH may slightly lower acid weight yield in the submerged fermentation phase.

Effect of Metal Ions

Magnesium and Zinc

Various amounts of $MgSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ were added to the cultivation media to study the effects of magnesium and zinc on fungal

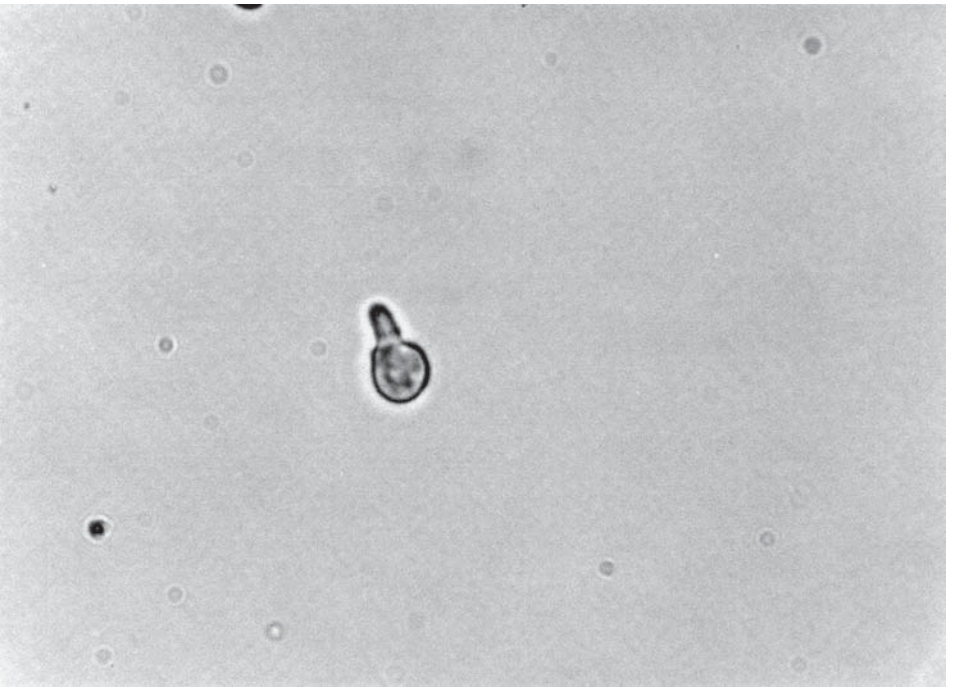

Fig. 2. Image of fungal growth at h 5 in the cultivation medium at an initial pH of 2.88. Magnification: ×150.

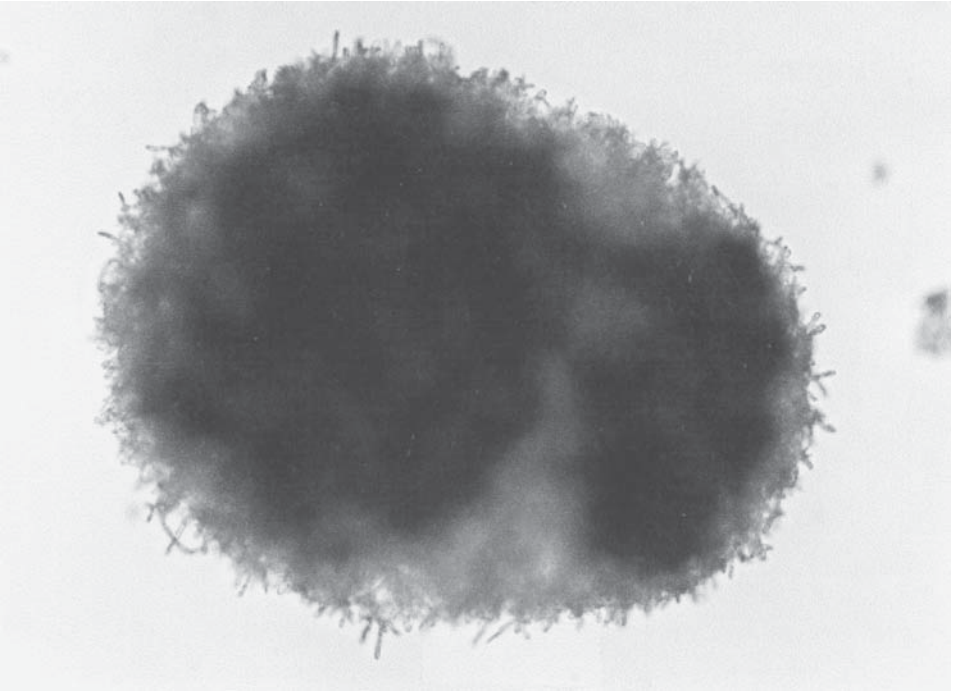

Fig. 3. Image of fungal growth at h 22 in the cultivation medium at an initial pH of 5.59. Magnification: ×25.

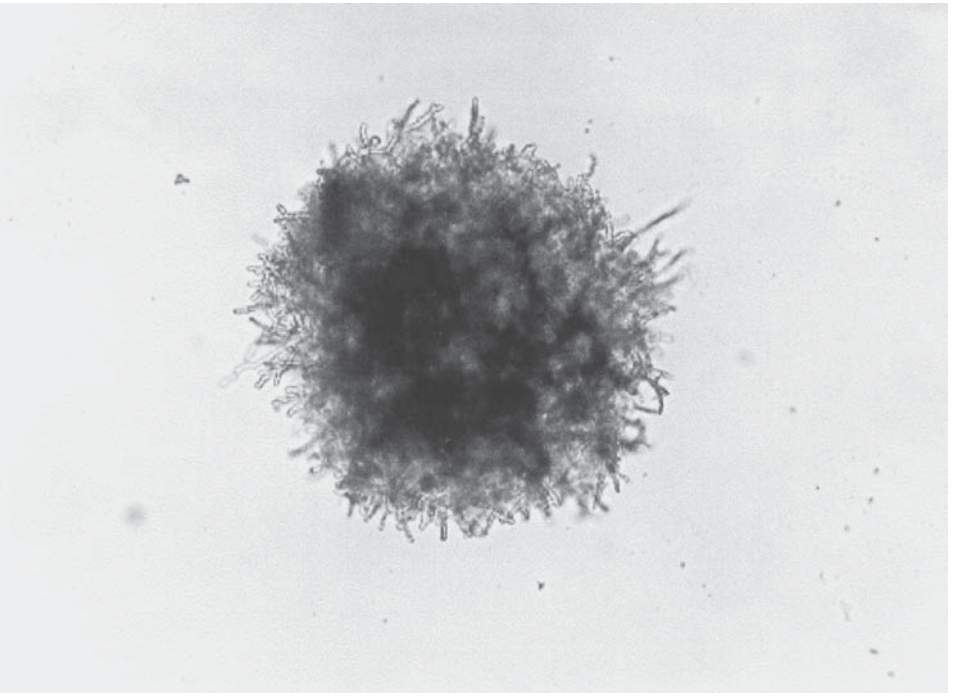

Fig. 4. Image of fungal growth at h 22 in the cultivation medium at an initial pH of 2.88. Magnification: ×25.

growth and fumaric acid production. In the cultivation media, four levels of Zn^{++} concentrations were used—0, 4, 20, and 40 ppm—and four levels of Mg^{++} concentrations—0, 5, 25, and 50 ppm. After the mycelia were harvested, they were transferred to the fermentation media containing 90.12 g/L of glucose and an excess of $CaCO_3$ to carry out fermentation for 72 h. Tables 3 and 4 present the results.

As can be seen from Table 3, when magnesium was not present in the cultivation media, spores of *R. oryzae* could not germinate. It is obvious that magnesium is indispensable for fungal growth of the strain of *R. oryzae*. It is also evident that in the absence of zinc, pellet formation does not take place, whereas it does in the media with certain amounts of magnesium and zinc. Therefore, it is concluded that both magnesium and zinc are essential for pellet formation of this fungal strain. However, it seems that the medium with a higher concentration of magnesium and a lower concentration of zinc can form smaller and uniformly distributed pellets. The optimal medium to form better pellets was the medium with 4 ppm of Zn^{++} and 50 ppm of Mg^{++}. The pellets formed were about 1–1.5 mm and elliptical.

From Table 4, it is found that a lower zinc concentration and a higher magnesium concentration can increase the weight yield and volumetric productivity of fumaric acid. Regarding fumaric acid weight yield and small pellets, the optimal combination of Mg^{++} and Zn^{++} in the cultivation medium was 50 ppm of Mg^{++} and 4 ppm of Zn^{++}.

Table 3
Effect of Magnesium and Zinc in Cultivation Medium on Growth Morphology of *R. oryzae*

Mg^{++} (ppm)	Zn^{++} (ppm)	Cell morphology
0	0	Spores unable to germinate
	4	Spores unable to germinate
	20	Spores unable to germinate
	40	Spores unable to germinate
5	0	Small pellet-matrix
	4	Pellet form, 2–3 mm, elliptical
	20	Filamentous form, mats
	40	Filamentous form, mats
25	0	Filamentous form, mats
	4	Pellet form, 2–3 mm, elliptical
	20	Pellet form, 2–3 mm, elliptical
	40	Filamentous form, mats
50	0	Filamentous form, mats
	4	Pellet form, 1–1.5 mm, elliptical
	20	Pellet form, 1–4 mm, not uniformly distributed
	40	Pellet form, 2–3 mm, elliptical

Table 4
Effect of Magnesium and Zinc in Cultivation Medium on Fumaric Acid Fermentation by *R. oryzae*

Mg^{++} (ppm)	Zn^{++} (ppm)	Weight yield of fumaric acid produced/glucose consumed (%)	Fumaric acid produced (g/L)	Residual glucose (g/L)	Ethanol produced (g/L)	Dry weight of mycelium (g/100 mL)
5	0	41.38	13.66	57.20	4.60	0.18
	4	37.74	26.05	21.10	6.60	0.16
25	0	42.01	30.60	17.28	11.30	0.16
	4	39.38	30.60	12.52	9.23	0.11
	20	30.01	17.70	31.12	11.60	0.12
50	0	42.27	20.70	19.86	6.59	0.14
	4	40.10	34.80	3.32	7.19	0.16
	20	27.14	17.90	24.30	16.40	0.23

Iron

The influence of iron in the cultivation on fungal cell growth was studied. The iron concentration in the cultivation medium varied from 0 to 3 ppm. Mycelia grown in the cultivation media with different concentrations of iron were transferred to the fermentation medium containing 86.75 g/L of glucose. The fermentation time was 72 h, and glucose was

Table 5
Effect of Iron in Cultivation Medium on Growth Morphology of *R. oryzae*

Fe^{++} (ppb)	Cell morphology
0	Pellet form, 1–1.5 mm, elliptical
50	Pellet form, 1–2 mm, spherical
100	Pellet form, 1–1.5 mm, elliptical
250	Pellet form, ~3 mm, spherical
500	Pellet form, 1–4 mm, not uniformly distributed, elliptical
1500	Pellet form, 1–4 mm, not uniformly distributed, elliptical
3000	Pellet form, 3–4 mm, elliptical

Table 6
Effect of Iron in Cultivation Medium on Fumaric Acid Fermentation by *R. oryzae*

Fe^{++} (ppb)	Weight yield (%)[a]	Fumaric acid produced (g/L)	Residual glucose (g/L)	Ethanol produced (g/L)	Dry weight of mycelium (g/100 mL)
0	39.97	28.8	0	9.8	0.21
50	41.54	36.1	0	14.2	0.20
100	45.32	39.3	0	15.8	0.24
250	44.38	38.5	0	15.4	0.28
500	44.76	38.8	0	12.8	0.24
1500	38.04	32.6	0	11.7	0.34
3000	37.90	32.9	0	17.1	0.31

[a]Weight yield = grams of fumaric acid produced/gram of glucose consumed (%).

consumed completely. Tables 5 and 6 give the results. It can be observed that the presence of iron does not make a great contribution to the formation of small pellets. Generally, formed pellets were still larger than 1 mm, and a small amount of iron in the cultivation medium could increase the fumaric acid weight yield.

Manganese

To study the effect of manganese on fungal growth, various amounts of $MnSO_4 \cdot H_2O$ were added to the cultivation media with two combinations of concentration levels of Mg^{++} and Zn^{++}: 25 ppm of Mg^{++} and 20 ppm of Zn^{++}; and 50 ppm of Mg^{++} and 4 ppm of Zn^{++}. Concentrations of Mn^{++} in the cultivation media were 2, 10, 20, 50, 100, and 200 ppm. The initial concentrations of glucose for fermentation were 89.6 and 86.75 g/L for the cultivation medium with 25 ppm of Mg^{++} and 20 ppm of Zn^{++}, and for the cultivation medium with 50 ppm of Mg^{++} and 4 ppm of Zn^{++}, respectively. The fermentation time was 72 h. Tables 7 and 8 summarize the results.

Table 7
Effect of Manganese in Cultivation Medium
on Growth Morphology of *R. oryzae*

Mg^{++} (ppm)	Zn^{++} (ppm)	Mn^{++} (ppm)	Cell morphology
25	20	0	Pellet form, ~2 mm, spherical
		2	Pellet form, ~2 mm, spherical
		10	Pellet form, 1–2 mm, spherical
		20	Pellet form, 1–2 mm, spherical
		50	Pellet form, ~1.5 mm, uniform, spherical
		100	Spores unable to germinate
		200	Spores unable to germinate
50	4	0	Pellet form, ~1 mm, uniform, elliptical
		2	Filamentous form
		10	Filamentous form, cloudier
		20	Filamentous form, cloudier and denser
		50	Filamentous form, cloudier and denser
		100	Filamentous form
		200	Spores unable to germinate

Table 8
Effect of Manganese in Cultivation Medium
on Fumaric Acid Fermentation by *R. oryzae*

Mg^{++} (ppm)	Zn^{++} (ppm)	Mn^{++} (ppm)	Weight yield (%)[a]	Fumaric acid produced (g/L)	Residual glucose (g/L)	Ethanol produced (g/L)	Dry weight of mycelium (g/100 mL)
25	20	0	30.00	11.80	56.26	5.65	0.11
		2	32.50	12.55	51.05	7.83	0.09
		10	36.40	19.76	35.32	10.74	0.12
		20	37.10	20.12	35.51	17.21	0.12
		50	25.60	13.20	38.04	10.86	0.14
50	4	0	39.97	28.80	10.70	9.80	0.13
		2	40.50	27.00	20.12	9.40	0.12
		10	42.76	37.10	0	13.70	0.38
		20	45.32	38.20	2.38	14.20	0.34
		50	35.66	22.10	24.78	10.80	0.22
		100	25.71	22.30	0	23.30	0.26

[a]Weight yield = grams of fumaric acid produced/gram of glucose consumed.

In the cultivation medium with 25 ppm of Mg^{++} and 20 ppm of Zn^{++}, when the concentration of manganese was lower than 50 ppm, pellet formation occurred. When the concentration of manganese in the cultivation medium was higher than 100 ppm, no spores could germinate. When the concentration of manganese increased from 0 to 50 ppm, the weight yield of fumaric acid increased and reached the maximum value, then decreased.

In the cultivation medium with 50 ppm of Mg^{++} and 4 ppm of Zn^{++}, no pellet formation occurred in the presence of a manganese concentration from 2 to 100 ppm. When the manganese concentration in the cultivation medium was 200 ppm, no spores would germinate. It was also found that manganese had a similar effect on the weight yield of fumaric acid as observed before.

From the results of the investigation on initial pH and metal ions in the cultivation medium on fungal cell morphology and fumaric acid production, it was found that optimal concentration levels for Mg^{++}, Zn^{++}, and Fe^{++} were 50 ppm, 4 ppm, and 100 ppb, respectively. In the cultivation medium with optimal concentration levels of these three metal ions (Mg^{++}, Zn^{++}, and Fe^{++}), no additional Mn^{++} and an initial pH of about 3.05 were optimal conditions for the formation of small (diameter <1 mm), spherical, and uniformly distributed pellets.

Acknowledgments

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