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preliminary communication

Factors Affecting the Growth and Production of Milk-Clotting Enzyme by *Amylomyces rouxii* in Rice Liquid Medium**

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Summary

Amylomyces rouxii is one of the main fungi usually coexisting with yeasts in Chinese yeast ball, the starter of chiu-niang, a traditional Chinese fermented product from rice. In the present study, growth and production of milk-clotting enzyme (MCE) in gelatinous rice liquid culture of *A. rouxii* as influenced by waxy (gelatinous) rice content in the medium (5–20 %), temperature (25–40 °C), cultivation time (1–6 days), shaking speeds (0–150 rpm) and metal ions (Na^+, K^+, Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Fe^{3+} and Al^{3+}) were investigated. Results revealed that rice content in the medium, shaking speed, temperature and cultivation time all affected the mycelial propagation and the production of milk-clotting enzyme by *A. rouxii* in the rice liquid culture. The maximum milk-clotting enzyme activity of ca. 1.22 unit/mL of medium was observed in the 3-day static culture of test organism grown at 30 °C in the medium containing 20 % of gelatinous rice, while mycelial propagation increased with the increase of cultivation time and shaking speed. Furthermore, a significant increase ($p<0.05$) in the milk-clotting enzyme activity of ca. 1.90 unit/mL of medium, which was about 1.55-fold of the control, was observed when Al^{3+} was added to the rice liquid medium.

Key words: *Amylomyces rouxii*, milk-clotting enzyme, rice liquid culture

Introduction

Calf rennet, one of the important enzymes, is traditionally used for cheese making all over the world, but due to the shortage of animal rennet supply, tremendous effort has been made to seek the alternative substitute for calf rennet.

Owing to the rapid growth and the relative inexpensive growth substrate, the microbial rennet like milk--clotting enzyme (MCE) has become a popular rennet substitute. Although there are still some shortcomings when compared with the traditional calf rennet, microbial rennet has been accepted in the markets of several countries (*1*). So far, various organisms including *Mucor miehei* (*2*), *Rhizopus* spp. (*3*), *Aspergillus niger* (*4*), *Amylomyces* spp. (*5*), *Bacillus subtilis* (*6*) have been reported to produce rennet-like MCE.

Amylomyces rouxii, one of the major fungi existing in Chinese yeast ball, is the starter of chiu-niang, which is a Chinese trational fermented product of rice (*7*). It produces an MCE, which causes the curding of milk, and contributes to the gelatinous structures of gua-nai, an oriental style fermented milk product (*5*,*8*). *A. rouxii* was also the main organism involved in the fermentation of

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Indonesian tape ketan, a partially liquefied, sweet-sour, mildly alcoholic rice paste (*9*). With solid-state fermentation, we had previously investigated the MCE production by *A. rouxii* and found that waxy rice was the best fermentation substrate (*10*). Considering the advantages of submerged liquid state fermentation, such as simple preparation, easy scale-up, and operation control, low cost and reduced chance of infection over solid state fermentation (*11*), production of MCE by *A. rouxii* in the liquid rice culture as influenced by various culture conditions was further examined in the present study.

Materials and Methods

Test organism and preparation of spore suspension

Amylomyces rouxii Calmette was obtained from Professor C. Y. Chein, Dept. of Biology, National Taiwan Normal University (Taipei, Taiwan).

Before experiment, organism was activated by two successive transfers to potato dextrose agar (Difco, Detroit, MI, USA) slants and incubated at 30 °C for 5 days. Spores of test organism were harvested by flooding the surface of the agar with sterile distilled water containing 0.1 % Tween 80. The spore suspension was adjusted with sterile distilled water to a concentration of ca. 10^8/mL and used as the inoculum.

Culture conditions

Fermentation was carried out by transferring an aliquot (1 mL) of the spore suspension to a 250-mL Erlenmeyer flask containing 100 mL of rice culture medium. To prepare the rice culture medium, waxy rice (Taichung wax 70) obtained from local market was first mixed with 0.5 part of distilled water and held at temperature of ca. 22 °C for 8 h. The mixture was then boiled at 121 °C for 15 min. The rice liquid medium was then prepared by mixing the appropriate amount of cooked rice with distilled water, homogenized with a blender and then sterilized by autoclaving at 120 °C for 15 min.

To examine the effect of rice concentration, various amounts of cooked rice substrate were mixed with distilled water to give the rice liquid media containing 5–20 % of cooked rice substrate. To determine the effect of culture temperature, the culture was maintained at 20–40 °C. To investigate the effect of pH, the initial pH of the medium was adjusted to various pH values (4–8) with sterile 1 M NaOH or 1 M HCl solution. To determine the effect of shaking speed, the culture was subjected to a shaking speed of 0–150 rpm. All fermentation experiments were carried out for 3 days unless otherwise specified in the section Results and Discussion.

Determination of MCE activity and other analyses

To determine the milk-clotting enzyme activity, the liquid culture was first filtered through triple-layer cheesecloth. The filtrate was further centrifuged (16000 × *g* for 5 min) and the supernatants served as the enzyme source. Method of Arima *et al.* (*12*) was followed to examine the milk-clotting enzyme activity. A volume of 0.5 mL of enzyme solution was added to 5 mL of skim milk solution (10 %), supplemented with 0.01 M $CaCl_2$, and kept at 35 °C. The time of clotting was set with stopwatch. One unit was defined as the amount of enzyme that clotted 1 mL of substrate within 40 min.

To measure the spore concentration, a Petroff-Hausser Counting Chamber was used. To assess the mycelial propagation, the dry mass of the mycelia, collected by filtrations, was then determined by drying overnight at 105 °C.

Statistical analysis

The mean values and the standard deviation were calculated from the data obtained with triplicate trials. These data were then compared by the Duncan's multiple range method (*13*).

Results and Discussion

Effect of rice content

In our previous work (*10*) we compared the MCE production on various rice solid-state cultures of *A. rouxii* using five different varieties of rice as substrate and we found that waxy rice, among the various rice varieties tested, was the best substrate for MCE production by *A. rouxii*. In the present study, the rice liquid medium containing 5–20 % of waxy rice was examined for the growth and MCE production by *A. rouxii* because the tests on rice liquid media containing more than 20 % of waxy rice had been found to be too viscous to be manipulated.

As shown in Fig. 1, rice content in the liquid medium significantly affects the growth and the production of MCE by *A. rouxii*. Both mycelial propagation and MCE production were enhanced as the content of rice in the medium increased. In the liquid medium containing 20 % of rice substrate, *A. rouxii* showed the highest mycelial propagation and MCE production.

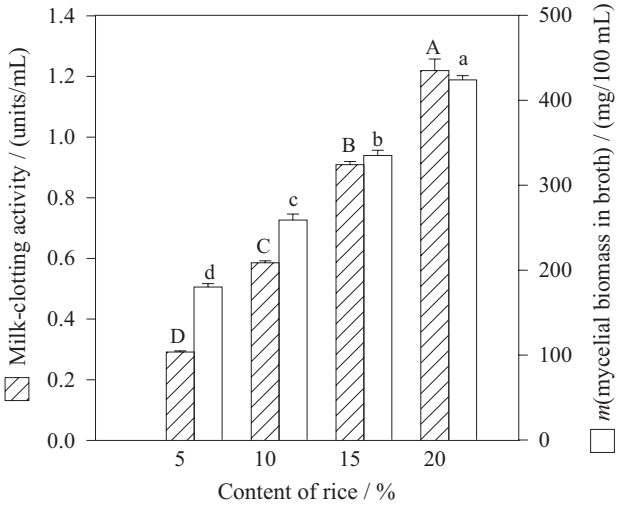

Fig. 1. Effect of waxy rice content on the growth and milk-clotting enzyme production by *A. rouxii* in liquid medium. Determinations were made after 3 days of static cultivation at 30 °C. Means (bar values) with the same letter do not differ at 5 % level by Duncan's multiple range test

Effect of cultivation temperature

Tubesha and Al-Delaimy (*14*), Thakur *et al.* (*2*) and Su and Chen (*15*) all observed that the production of MCE by microorganisms was affected by cultivation temperature. Similar phenomenon was also observed with *A. rouxii* when grown in the rice liquid medium at various temperatures. As shown in Fig. 2, *A. rouxii* exhibited the highest mycelial propagation and MCE production at 30 °C. Increasing or lowering the cultivation temperature resulted in the reduction of both mycelial propagation and the production of MCE by test organisms. The optimal MCE production temperature found in the present study is consistent with that reported by Hung and Chou (*16*) and in our previous work (*10*). The optimal cultivation temperature of 30 °C found with *A. rouxii* for MCE production is also similar to that reported by Tubesha and Al-Delaimy (*14*) for *Mucor* J20 and comes close to that (28–30 °C) observed with *Mucor pusillus* and *M. mucedo* (*15*), while it is different from that (42 °C) reported for *M. miehei* (*2*).

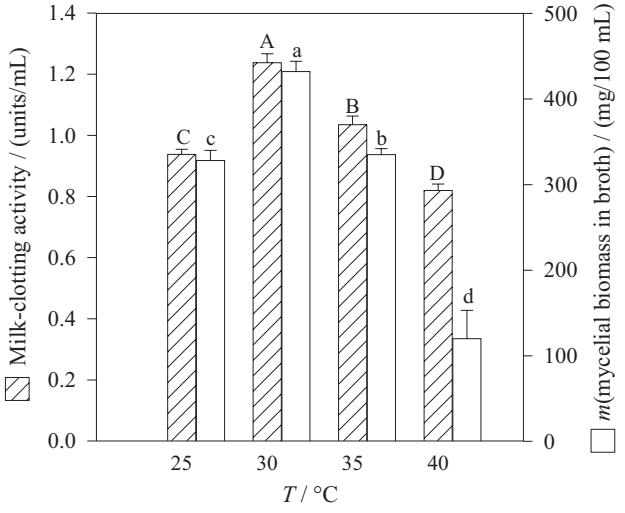

Fig. 2. Effect of cultivation temperature on the growth and milk--clotting enzyme production by *A. rouxii* in liquid medium containing 20 % of rice. Determinations were made after 3 days of static cultivation at 30 °C. Means (bar values) with the same letter do not differ at 5 % level by Duncan's multiple range test

Effect of cultivation time

Fig. 3 shows the mycelial propagation and MCE production by *A. rouxii* in the liquid medium containing 20 % of waxy rice after various periods of cultivation at 30 °C. Although in the solid-state fermentation the highest MCE activity was detected after 5 days of cultivation (*10*), the highest MCE activity in the liquid medium of rice culture was found after 3 days of cultivation. A reduced MCE activity was observed in the culture with extended cultivation time. On the other hand, mycelial propagation of *A. rouxii* increased as the cultivation time increased. Among the various cultivation periods tested, *A. rouxii* exhibited the highest mycelial propagation in the rice liquid medium after 6 days of incubation.

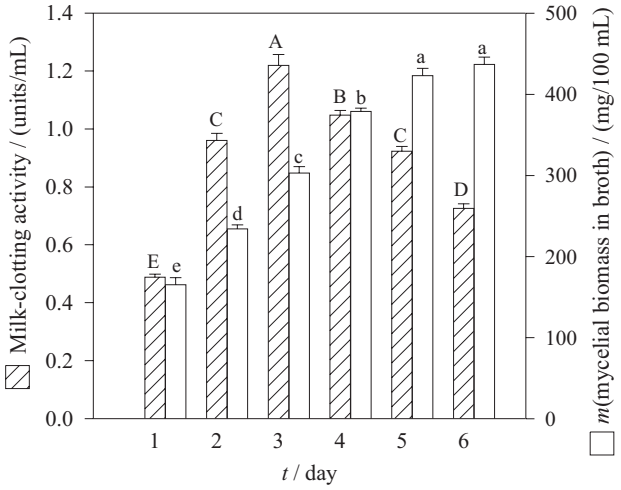

Fig. 3. Effect of incubation time on the growth and milk-clotting enzyme production by *A. rouxii* in liquid medium containing 20 % of rice. Determinations were made after 1–6 days of static cultivation at 30 °C. Means (bar values) with the same letter do not differ at 5 % level by Duncan's multiple range test

Effect of initial pH

Effect of initial pH of the medium on the growth and MCE production has been illustrated. D'Souza and Pereira (*17*) indicated that MCE production by *Bacillus licheniformis* is the highest in the medium with an initial pH=7, while maximum MCE production by *M. pusillus* (*18*), *M. mucedo* (*19*), and *Fusarium subglutinans* (*20*) was reported to be in the culture with an initial pH of 6.0, 3.7, 9.0 and 6.0, respectively.

Fig. 4 shows the mycelial propagation and MCE production by *A. rouxii* in the rice liquid medium adjusted to different initial pH values. It was observed that when the initial pH was increased, both the propagation

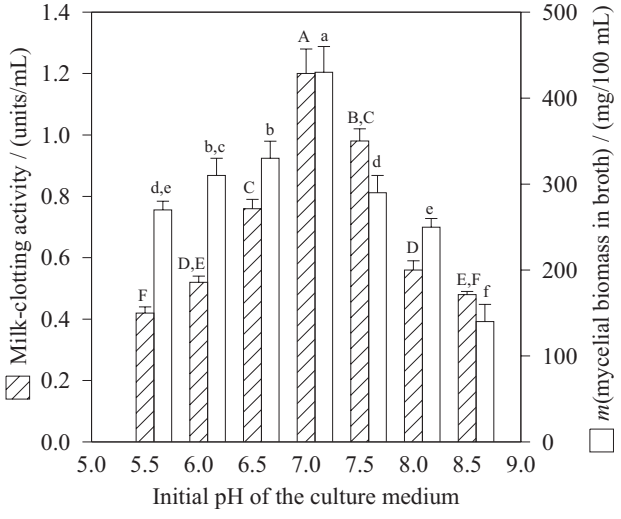

Fig. 4. Effect of initial pH on the growth and milk-clotting enzyme production by *A. rouxii* in liquid medium containing 20 % of rice. Determinations were made after 3 days of static cultivation at 30 °C. Means (bar values) with the same letter do not differ at 5 % level by Duncan's multiple range test

of mycelia and MCE production also increased, and they reached their maximum when the initial pH of the medium was adjusted to 7. Further increase of the initial pH resulted in the reduced mycelial propagation and MCE production by the test organism. The optimal initial pH for MCE production observed is similar to that reported by Hung and Chou (*16*) who cultivated *A. rouxii* in medium E containing yeast extract, soluble starch and various mineral salts. However, they indicated that the mycelial propagation in medium with an initial pH ranging from 5 to 8 was similar, while we observed a significantly lower ($p<0.05$) growth of *A. rouxii* in the culture with an initial pH=5 or 8 compared with that in the culture with an initial pH of 7. These observations along with the reports of previous investigators (*17–20*) suggest that the optimal initial pH of the medium for microbial growth and MCE production may vary depending on the culture medium and the test organism.

Effect of shaking speed

Mycelial propagation and MCE production by *A. rouxii* in the culture with various shaking speeds is shown in Fig. 5. It was found that the mycelial propagation increased with the increase of the shaking speed. Among the various shaking speeds examined, mycelial propagation is the highest in the rice liquid culture with a shaking speed of 150 rpm. On the contrary, MCE production decreased as the shaking speed increased. The highest MCE production of 1.21 unit/mL was found in the *A. rouxii* culture without shaking. The lowest MCE production of only 0.87 unit/mL, ca. 71 % of that produced without shaking, was observed in the culture with a shaking speed of 150 rpm. In contrast with the report of Ghareib *et al.* (*20*), who observed the highest MCE activity in the shaking flask culture of *Fusarium subglutinans*, the highest MCE production by test organism in rice liquid culture without shaking observed in the present study is similar to the findings of Hashem (*21*), who observed a higher MCE activity in still culture than in shaking flask culture of *Penicillium oxalicum*.

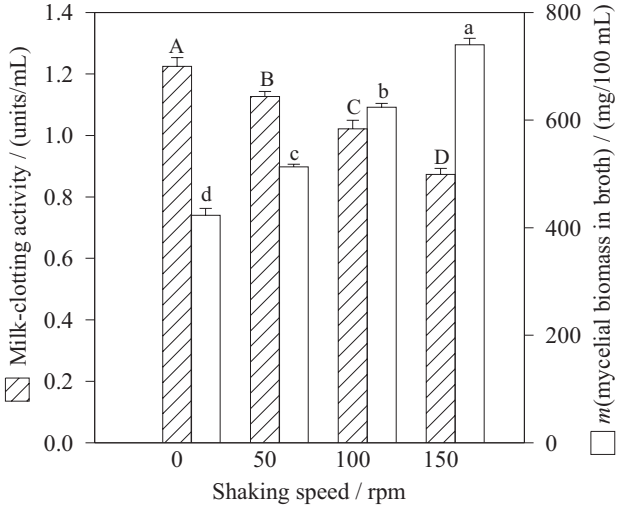

Fig. 5. Effect of shaking speed on growth and milk-clotting enzyme production by *A. rouxii* in liquid medium containing 20 % of rice. Determinations were made after 3 days of shaking cultivation at 30 °C. Means (bar values) with the same letter do not differ at 5 % level by Duncan's multiple range test

Effect of metal ions

Metal ions have been reported to affect the MCE production by microorganisms. Nigam *et al.* (*22*) indicated that the addition of Na^+ to the medium reduced the MCE production by *Bacillus subtilis*. Kobayashi *et al.* (*23*) reported that the addition of Mn^{2+}, Ca^{2+} or Mg^{2+} in SPY medium increased MCE production by *Trametes ostreiformis*, while the supplementation of Cu^{2+}, Zn^{2+}, Co^{3+} or Fe^{3+} reduced MCE production. To examine the effect of metal ions on the growth and MCE production by *A. rouxii*, various metal ions were added to the rice liquid medium. Table 1 shows the mycelial propagation and MCE production by test organism after 3 days of fermentation. It was found that the addition of Mg^{2+} to the rice liquid medium increased the mycelial production of *A. rouxii*, while the reduction in the mycelial propagation was observed when Cu^{2+} or Fe^{3+} were added. On the other hand, the addition of Cu^{2+} or Al^{3+} significantly enhanced ($p<0.05$) the MCE production by *A. rouxii* in the rice liquid medium. The effect of the enhanced production of MCE was most marked in the culture with added Al^{3+}. In the rice liquid medium containing Al^{3+}, the highest MCE production of 1.90 unit/mL of medium, about 1.55-fold of the control, was noted. Furthermore, although Cu^{2+} was found to enhance the MCE

Table 1. Effect of metal ions on the growth and milk-clotting enzyme production by *A. rouxii* in rice liquid culture

Metal salts[a]	Liquid culture[b]		
	m(mycelium in broth)	Milk-clotting activity	Relative activity
	mg/100 mL	units/mL	%
None	426.2±7.8 B,C,D[c]	1.3±0.0 C,D	100
NaCl	423.4±13.0 B,C,D,E	1.2±0.0 D	100
KCl	429.7±14.8 B,C,D	1.3±0.1 C,D	106
$ZnCl_2$	444.1±14.3 A,B	1.4±0.0 B,C	114
$MgCl_2$	449.7±4.1 A	1.4±0.0 B,C	111
$MnCl_2$	430.1±8.8 A,B,C	1.3±0.0 C,D	102
$CuCl_2$	407.9±9.4 E,F	1.5±0.0 B	116
$CaCl_2$	422.5±9.4 C,D,E,F	1.3±0.1 C,D	101
$FeCl_3$	405.9±7.2 F	1.4±0.0 B,C	114
$AlCl_3$	418.3±7.7 D,E,F	1.9±0.1 A	153

[a]Substrate (100 mL) contained 0.1 mmol of ion

[b]Liquid medium contained 20 % of rice substrate. Determinations were made after 3 days of cultivation at 30 °C

[c]Means within the same column with the same letter do not differ at 5 % level by Duncan's multiple range test

production by *A. rouxii* in the present study, a reduced MCE production was noted with *M. racemosus* (*24*) and *Trametes ostreiformis* (*23*). Therefore, the effect of metal ions on microbial MCE production may vary with microorganisms.

Conclusion

Based on the data obtained, it was concluded that rice content, cultivation time, temperature, shaking speed and metal ions all affect the growth and MCE production by *A. rouxii* in the rice liquid medium. Changing the medium composition and culture growth conditions, a 1.55-fold increase of MCE production can be achieved. These results provided valuable information for the production of MCE by *A. rouxii* in the relatively inexpensive rice liquid medium.

Acknowledgements

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Čimbenici koji utječu na rast *Amylomyces rouxii* i proizvodnju enzima za grušanje mlijeka u tekućoj podlozi s rižom

Sažetak

Amylomyces rouxii jedna je od glavnih plijesni što se pojavljuju zajedno s kvascima u »kineskom kvascu« koji je starter kultura za »chiu-niang«, tradicionalni kineski fermentirani pripravak od riže. U radu su ispitani rast plijesni *A. rouxii* i proizvodnja enzima za grušanje mlijeka u tekućoj podlozi želatinaste riže. Istražen je utjecaj udjela želatinaste riže u podlozi (5–20 %), temperature (25–40 °C), vremena uzgoja (1–6 dana), brzine mućkanja (0–150 rpm) i prisutnosti metalnih iona (Na^+, K^+, Zn^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Fe^{3+} i Al^{3+}). Utvrđeno je da količina riže u podlozi, brzina mućkanja, temperatura i vrijeme uzgoja utječu na rast micelija i proizvodnju enzima za grušanje mlijeka. Maksimalna aktivnost enzima za grušanje mlijeka od približno 1,22 jedinice/mL podloge opažena je trećeg dana u statičkoj kulturi test-organizma pri 30 °C, u podlozi koja je sadržavala 20 % želatinaste riže. Produženjem vremena uzgoja i brzine mućkanja povećava se razmnožavanje micelija. Značajno je bilo povećanje ($p<0,05$) aktivnosti enzima za grušanje mlijeka od 1,9 jedinica/mL podloge, što je približno 1,55 puta više od kontrolnog uzorka, opaženog kada je Al^{3+} dodan u podlogu.