Relationship Between Morphology and Itaconic Acid Production by *Aspergillus terreus*

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The morphology of filamentous fungi closely correlates with the productivity in submerged culture. Using itaconic acid (IA) production by *Aspergillus terreus* as a research model, the quantitative relationship between the growth form of *A. terreus* and IA production was investigated. IA fermentation was scaled up from shake flasks to a 7 L stirred tank bioreactor based on the quantitative relationship. Our results demonstrated the following: (1) Three morphologies of *A. terreus* were formed by changing the inoculum level and shape of the flask. (2) Investigation of the effects of the three morphologies on broth rheology and IA production revealed the higher yield of IA on dry cell weight (DCW, IA/DCW) and yield of glucose on DCW (consumed glucose/DCW) were achieved during clump growth of *A. terreus*. (3) By varying the KH\(_2\)PO\(_4\) concentration and culture temperature, the relationships between clump diameter and IA production were established, demonstrating that the yield of IA on DCW (\(R^2 = 0.9809\)) and yield of glucose on DCW (\(R^2 = 0.9421\)) were closely correlated with clump diameter. The optimum clump diameter range for higher IA production was 0.40–0.50 mm. (4) When the clump diameter was controlled at 0.45 mm by manipulating the mechanical stress in a 7 L fermentor, the yield of IA on DCW and yield of glucose on DCW were increased by 25.1% and 16.3%, respectively. The results presented in this study provide a potential approach for further enhancement of metabolite production by filamentous fungi.

**Keywords:** *Aspergillus terreus*, itaconic acid, morphology, quantitative relationship

**Introduction**

Filamentous fungal fermentation is widely used to produce valuable primary and secondary metabolites such as organic acids, enzymes, and antibiotics [3, 13]. For example, *Aspergillus niger* is an important industrial strain for the production of citric acid, which is in heavy demand as a bulk chemical for food and other industries [1]. *Trichoderma reesei* is the principal source of cellulase, which is widely used in the food, feed, and textile industries [2]. One of the most intriguing and often-problematic characteristics of filamentous fungi is their complex morphology in submerged culture. Fungal morphology not only has a significant impact on mixing and mass transfer, but also determines the overall process productivities and subsequent economics [22]. Thus, fungal morphology is considered as a key bioprocess parameter for submerged culture.

In general, the morphology of filamentous fungi varies between dispersed mycelia, clump, and pellet [21]. Under this condition, broth rheology is significantly affected by the complex morphological changes. The morphology of dispersed mycelia is favorable to ensure high production of some enzymes, because it allows almost all the individual hyphal elements to be in contact with the medium [7]. However, a major drawback of this morphology is that the high viscosity of the broth leads to insufficient mixing, which increases energy consumption for mechanical stress [18]. In comparison, clump formation ensures a lower broth viscosity, but disadvantages related to a decline of nutrient availability in the inner part of the compact pellet can occur.
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as the mean ± SD, were obtained from a population size of approximately 100 events per sample.

Rheological Measurements

Samples were taken from the culture broth based on the growth kinetics of dispersed mycelia, clump, and pellet. Then the wet cell weight was adjusted to the same value in order to avoid the influence of biomass concentration. Rheological parameters were examined using 25 ml of fermentation broth in a 50 ml glass vessel by a Brookfield viscometer (Brookfield, USA), fitted with a disc-spindle impeller #1. The consistency index (K) and flow behavior index (n) were determined as previously described [12, 19].

Results

Effect of Culture Conditions on A. terreus Morphology in Shake Flasks

Three different fungal morphologies were achieved at three different culture conditions in shake flasks: 500 ml Erlenmeyer flask, inoculum level 10^9 spores/ml (dispersed mycelia, Figs. 1A–1B); 500 ml Erlenmeyer flask, inoculum level 10^8 spores/ml (clump, Figs. 1C–1D); and 750 ml baffled flask, inoculum level 10^8 spores/ml (pellet, Figs. 1E–1F).

Effect of Morphologies on Broth Rheology and IA Production of A. terreus

The effects of morphologies on the fermentation broth rheological parameters were investigated at three points: the point of the mid-exponential growth phase, late-exponential growth phase, and end of fermentation, as summarized in Fig. 2. From the figure, the following can be observed: (i) With regard to the clump, K exhibited a 290.4% increase, while n presented 18.8% decrease during the period from the mid-exponential growth phase to late-exponential growth phase. Furthermore, the value of K increased by 29.9%, while that of n maintained constancy during the period from the late-exponential growth phase to the end of fermentation. A similar result was observed for dispersed mycelia. (ii) With regard to the pellet, the value of K increased by 29.9%, while that of n maintained constancy during the period from the late-exponential growth phase to the end of fermentation. A similar result was observed for dispersed mycelia. (iii) At the end of fermentation, the value of K of the dispersed mycelia was 83.8% and 844.3% higher than that of the clump (0.31 Pa·s^n) and pellet (0.06 Pa·s^n), respectively. The value of n of the pellet was 95.6% and
28.1% higher than that of the dispersed mycelia (0.36 s\(^{-1}\)) and clump (0.56 s\(^{-1}\)), respectively.

The effect of morphologies on IA production is presented in Table 1. The yield of IA on DCW and yield of glucose on DCW of the clump presented the highest values, which were 318.2% and 98.8% higher than those of the dispersed mycelia, and 45.7% and 10.6% higher than those of the pellet, respectively. Thus, it can be concluded that *A. terreus* growth in clump contributed to IA fermentation.

**Effects of Adding KH\(_2\)PO\(_4\) and the Fermentation Temperature on Clump Size and IA Production**

The effect of adding KH\(_2\)PO\(_4\) on the clump diameter and IA production is presented in Table 2. When the concentration of adding KH\(_2\)PO\(_4\) was increased from 0 to 0.2 g/l, the clump diameter increase was from 0.53 to 0.75 mm. However, the yield of IA on DCW and yield of glucose on DCW were decreased by 44.7% and 36.2%, respectively. In order to provide a clear description of the effect of clump diameter on IA production and glucose consumption, the time course of IA fermentation at two nutrition conditions (0 and 0.05 g/l of KH\(_2\)PO\(_4\)) is illustrated in Fig. 3. It can be observed that the diameter increased during the period of rapid growth and then remained at a relatively constant value from about 24 h to the end of fermentation. Approximately 60% of IA was produced during the stationary phase. Consequently, the average diameter value during the stationary phase was calculated as being representative of a given fermentation. When 0 g/l KH\(_2\)PO\(_4\) was added to the acid production medium, the clump diameter (0.53 mm) was 15.9% smaller than that of the control (0.05 g/l KH\(_2\)PO\(_4\)). Addition of 0.05 g/l of KH\(_2\)PO\(_4\) increased the DCW but decreased the IA production and glucose consumption, resulting in the highest yield of IA on DCW of 3.27 g/g and yield of glucose on DCW of 6.57 g/g, which were 24.0% and 21.7% lower than those obtained with the addition of 0 g/l of KH\(_2\)PO\(_4\), respectively.

The effect of culture temperature on the clump diameter and IA production is presented in Table 3. When the culture temperature was decreased from 37°C to 30°C, the

**Table 1. Effect of the three morphologies on IA production.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Morphology</th>
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<tbody>
<tr>
<td></td>
<td>Dispersed mycelia</td>
</tr>
<tr>
<td>Titer of IA (g/l)</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>Glucose consumption (g/l)</td>
<td>29.4 ± 1.1</td>
</tr>
<tr>
<td>Maximum DCW (g/l)</td>
<td>9.1 ± 0.2</td>
</tr>
<tr>
<td>Yield of IA on DCW (g/g)</td>
<td>0.77 ± 0.03</td>
</tr>
<tr>
<td>Yield of glucose on DCW (g/g)</td>
<td>3.21 ± 0.04</td>
</tr>
</tbody>
</table>

Culture conditions: dispersed mycelia (500 ml Erlenmeyer flask, inoculum level 10\(^8\) spores/ml); clump (500 ml Erlenmeyer flask, inoculum level 10\(^8\) spores/ml); pellet (750 ml baffled flask, inoculum level 10\(^8\) spores/ml). All the media contained 0.05 g/l of K\(_2\)PO\(_4\) and were cultured at 37°C.
clump diameter decreased from 0.53 to 0.28 mm. Similarly, the yield of IA on DCW and yield of glucose on DCW decreased along with the decrease in temperature from 35°C to 30°C.

By using the above-mentioned experimental data, the correlation among the yield of IA on DCW, yield of glucose on DCW, and clump diameter is summarized in Fig. 4. It can be noted from the figure that the yield of IA on DCW ($R^2 = 0.9809$) and yield of glucose on DCW ($R^2 = 0.9421$) were closely correlated with clump diameter. The optimum clump diameter range for higher yield of IA on DCW and yield of glucose on DCW was 0.40–0.50 mm. The yield of IA on DCW and yield of glucose were 4.42 g/g and 8.38 g/g, which were 35.2% and 27.5% higher than the corresponding value of the control (0.05 g/l KH$_2$PO$_4$, 37°C), respectively.

**Enhanced IA Production by Controlling Clump Diameter in a 7 L Stirred Tank Bioreactor**

Based on the above-mentioned results, we could obtain the highest IA production in a stirred tank bioreactor with

![Fig. 3. Time course of IA production by A. terreus at different KH$_2$PO$_4$ concentrations.](image)

(A) Clump diameter; (B) IA production; (C) glucose consumption; and (D) DCW. Symbol (○) represents the control with 0.05 g/l of KH$_2$PO$_4$, whereas (■) represents no KH$_2$PO$_4$. Fermentation conditions: 37°C, 500 ml Erlenmeyer flask, inoculum level $10^8$ spores/ml.
A. terreus fermented within the optimum diameter range. The changes in morphology and IA production of A. terreus over a range of mechanical stress (agitation and aeration) were investigated. As shown in Table 4, the conditions of optimum mechanical stress were 300 rpm of agitation speed and 0.8 vvm of aeration rate. The time course of IA fermentation under the optimum condition is illustrated in Fig. 5. The clump diameter (0.45 mm) was within the optimum diameter range (0.40–0.50 mm) under the optimum condition, which led to 25.1% and 16.3% increases in the

Table 3. Effect of temperature on clump diameter and IA production by A. terreus in 500 ml Erlenmeyer flasks.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37</td>
</tr>
<tr>
<td>Clump diameter (mm)</td>
<td>0.53 ± 0.06</td>
</tr>
<tr>
<td>Titer of IA (g/l)</td>
<td>32.7 ± 0.2</td>
</tr>
<tr>
<td>Glucose consumption (g/l)</td>
<td>63.1 ± 0.6</td>
</tr>
<tr>
<td>Maximum DCW (g/l)</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>Yield of IA on DCW (g/g)</td>
<td>4.18 ± 0.04</td>
</tr>
<tr>
<td>Yield of glucose on DCW (g/g)</td>
<td>8.06 ± 0.07</td>
</tr>
</tbody>
</table>

Conditions: 500 ml Erlenmeyer flask, inoculum level 10⁸ spores/ml, without KH₂PO₄.

Table 4. Effect of mechanical stress (agitation and aeration) on clump diameter and IA production by A. terreus in a stirred tank bioreactor.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mechanical stress (agitation and aeration)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 rpm 0.4 vvm</td>
</tr>
<tr>
<td>Clump diameter (mm)</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>Titer of IA (g/l)</td>
<td>26.1 ± 1.1</td>
</tr>
<tr>
<td>Glucose consumption (g/l)</td>
<td>54.3 ± 1.0</td>
</tr>
<tr>
<td>Maximum DCW (g/l)</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>Yield of IA on DCW (g/g)</td>
<td>3.66 ± 0.27</td>
</tr>
<tr>
<td>Yield of glucose on DCW (g/g)</td>
<td>7.65 ± 0.31</td>
</tr>
</tbody>
</table>

Fermentation conditions: 500 ml Erlenmeyer flask, inoculum level 10⁸ spores/ml, 35°C, without KH₂PO₄, and initial pH of 3.1 (not adjusted).

Fig. 4. The relationships between clump diameter and (A) yield of IA on DCW and (B) yield of glucose on DCW, respectively. (A) \( y = 118.2395x^3 - 217.6035x^2 + 123.3799x - 17.6514, R^2 = 0.9809; \) (B) \( y = 158.7866x^3 - 219.8184x^2 + 163.5855x - 20.3128, R^2 = 0.9421. \) Fermentation conditions: 30–37°C, 500 ml Erlenmeyer flask, inoculum level 10⁸ spores/ml, and with 0–0.2 g/l KH₂PO₄.
yield of IA on DCW and yield of glucose on DCW, respectively, when compared with those of the control (200 rpm, 0.4 vvm).

Discussion

The present study demonstrated that the inoculum level and shape of the flask had a significant influence on the formation of the three fungal morphologies. Different morphological growth forms affected the rheology of the fermentation broth and IA production. By altering the KH$_2$PO$_4$ concentration and culture temperature, the quantitative relationships between the yield of IA on DCW ($R^2 = 0.9809$), yield of glucose on DCW ($R^2 = 0.9421$), and clump diameter were developed. Furthermore, higher yield of IA on DCW and yield of glucose on DCW were achieved by controlling A. terreus morphology based on the quantitative relationship in a 7 L fermentor.

Fungal morphology is strongly influenced by medium composition and culture condition [8, 22]. The inoculum level may affect conidial aggregation, and then influence fungal morphology. Experiments with A. terreus FMME033 indicated a clear transition from clump to dispersed mycelia when the inoculum level was increased from $10^5$ to $10^6$ spores/ml in 500 ml Erlenmeyer flasks. The results seem to be comparable with those presented in the literature with respect to A. niger, which demonstrated that when the inoculum level was varied from $10^5$ to $10^6$ spores/ml, the morphology changed from pellet, to clump, to dispersed mycelia [14]. Compact pellets were formed in baffled flasks because they can provide higher shear force and higher dissolved oxygen when compared with Erlenmeyer flasks at the same rotation speed. A similar result was also reported by Teng et al. [19]. Different inoculum levels and shapes of flasks were used to develop distinctive morphologies, and growth of A. terreus in clumps was suggested to achieve high IA production. The reason for this may be the higher value of K and lower value of n induced by the dispersed mycelia (Fig. 2), which may have led to a low nutrient and oxygen supply [9]. Although the compact pellet showed the lowest K and highest n (Fig. 2), nutrient and oxygen transfer might be limited in the central part of the pellet [9]. The results based on the sensitivity of the IA-producing mechanism to the lack of oxygen have been reported by Kuenz et al. [10] and Gyamera [5], who showed that insufficient oxygen diffusion reduced the production of IA significantly. Moreover, the maximum DCW of dispersed mycelia was 23.0% higher than that of the pellet (Table 1), which may limit mass transfer more severely. This may be the reason why the yield of IA on DCW and yield of glucose on DCW of the dispersed mycelia were lower than that of the pellet.

The clump diameter and DCW of A. terreus increased with the increase of phosphate concentration, but the yield of IA on DCW and yield of glucose on DCW decreased (Table 2). Similar changes were also observed in A. niger [15]. Therefore, it is necessary to restrict the phosphate concentration to obtain a small clump diameter and high IA yield. The results seem to be comparable to literature results with A. terreus NRRL 1960, where limiting phosphorus was very important for IA formation, and most of the IA was produced when phosphorus was depleted from the medium [16]. When 0 g/l KH$_2$PO$_4$ was added to the acid production medium, about 30 mg/l of phosphorus was noted in corn steep liquor powder at the start of the fermentation [25], as well as the residual phosphorus from the inoculum medium, which supported reasonable cell growth. Temperature, which is easy to control, may influence the nutritional and pH requirements for growth [13]. The present study indicated that the clump diameter decreased along with the decrease in temperature from 37°C to 30°C, similar to that observed in Rhizopus delemar [26]. However, this finding is not in agreement with that of the study by Spohr et al. [17] on the production of α-amylase by A. oryzae, where the pellet size distribution was found to be independent of temperature.

Many studies have only focused on the qualitative relationships between morphology and production of metabolites in submerged cultures of filamentous fungi,
including the growth form of *A. terreus* and IA production [10, 24]. In the present study, the quantitative relationships between detailed morphological forms and the production of IA in flasks were investigated. Based on the quantitative relationship, scale-up of *A. terreus* fermentation from shake flasks to a 7 L stirred tank bioreactor fermentor was successfully performed by manipulating the mechanical stress, which has been proved to be an effective approach to influence fungal morphology [11]. However, in order to further improve the IA production in the fermentor, a deeper understanding of the clump formation mechanism and a systematic study on the effect of different fermentation conditions on the changes in morphology are necessary.

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**References**

