Stimulatory Effects of Sugarcane Molasses on Fumigaclavine C Biosynthesis by *Aspergillus fumigatus* CY018 via Biofilm Enhancement^S

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Biofilms are of vital significance in bioconversion and biotechnological processes. In this work, sugarcane molasses was used to enhance biofilms for the improvement of the production of fumigaclavine C (FC), a conidiation-associated ergot alkaloid with strong anti-inflammatory activities. Biofilm formation was more greatly induced by the addition of molasses than the addition of other reported biofilm inducers. With the optimal molasses concentration (400 g/l), the biofilm biomass was 6-fold higher than that with sucrose, and FC and conidia production was increased by 5.8- and 3.1-fold, respectively. Moreover, the global secondary metabolism regulatory gene *laeA*, FC biosynthetic gene *fgaOx3*, and asexual central regulatory genes *brlA* and *wetA* were upregulated in molasses-based biofilms, suggesting the upregulation of both asexual development and FC biosynthesis. This study provides novel insight into the stimulatory effects of molasses on biofilm formation and supports the widespread application of molasses as an inexpensive raw material and effective inducer for biofilm production.

Keywords: Fumigaclavine C, sugarcane molasses, biofilm, *Aspergillus fumigatus* CY018.

Introduction

Fumigaclavine C (FC) is a conidiation-associated ergot alkaloid isolated from the filamentous endophytic fungus *Aspergillus fumigatus* CY018 [1]. It displays strong anti-inflammatory activity [2, 3], as well as antitumor and anti-atherogenic effects [4, 5], indicating the potential of this clavine alkaloid for widespread application.

FC (shown in Fig. S1), composed of a tetracyclic ergoline ring, is the end product of the ergot alkaloid pathway in *A. fumigatus* [6]. The biosynthesis of FC is initiated by the prenylation of L-tryptophan in the presence of dimethylallyl diphosphate [7]. Through several biosynthetic steps, including methylation, acetylation, cyclization, and prenylation, the major intermediates are ultimately converted to FC [8]. FgaOx1 and FgaOx3 are involved in the cyclization of 4-dimethylallyl-L-tryptophan and chanoclavine-1 aldehyde, respectively, to form tricyclic and tetracyclic ergoline scaffolds; these enzymes are coordinated by the global secondary metabolic regulator LaeA [9]. Additionally, the expression of BrlA, the central activator of *A. fumigatus* conidiation, is indispensable for FC biosynthesis [10],

indicating an interesting correlation between asexual development and FC biosynthesis. Moreover, several conidiation-inducing methods, such as ultrasonic stimulation and two-stage procedures, can effectively result in FC production [1, 11]. However, further investigation is required to examine the stimulatory effects of conidiation on genes involved in FC biosynthesis.

A. fumigatus conidiation is observed during biofilm maturation, which, along with the planktonic state, is a natural stage of fungal growth [12]. Biofilm growth is considered a developmental shift towards preservation, wherein structured polymicrobial biofilms form on natural or inanimate surfaces [13]. Fungal biofilms are multicellular communities that possess vastly distinct phenotypes compared with their planktonic counterparts [14]. Moreover, biofilms are of vital significance in bioconversion and biotechnological processes. In one of the earliest publications on the topic, *Aspergillus niger* biofilm was adhered to a vertical disc rotating fermenter, and a 4-fold increase in the production of citric acid was observed [15–17]. It was also reported that *A. niger* biofilm attached to the surface of polyurethane foam in a rotating biological contactor attained

3-fold higher citric acid production than in a stirred-tank fermenter [18]. Biofilms perform better at producing a wide range of bioproducts, such as kojic acid by *Aspergillus oryzae* [19], fructooligosaccharides by *Aspergillus phoenicis* [20], red pigment by *Penicillium novae-zelandiae* [21], and cellulase by *A. niger* [22]. Gliotoxin, one of the major secondary metabolites produced by *A. fumigatus*, increases with biofilm formation, and several genes involved in secondary metabolic biosynthesis are significantly upregulated in the mature phase [23]. Nevertheless, whether the biosynthesis of FC, another secondary metabolite produced by *A. fumigatus*, is related to biofilm formation remains unknown.

Previous studies have evaluated sugarcane molasses, a low-cost waste feedstock, as an efficient supplemental ingredient in two-stage culture medium [24]. However, the mechanism underlying the promotion of conidiation and FC biosynthesis in *A. fumigatus* by molasses remains unknown. In this work, the distributions of conidia and FC were examined, and found to be concentrated in the biofilm instead of the planktonic state. Several additives reported to enhance biofilm formation in other studies were tested with *A. fumigatus*, and molasses was observed to be an excellent additive that contains various carbon sources. The concentration of molasses as a sole carbon source was optimized and compared with sucrose. Biofilm morphologies were observed via scanning electron microscopy (SEM). Furthermore, the expression profiles of genes involved in FC biosynthesis (*laeA*, *fgaOx1*, and *fgaOx3*) and asexual development (*brlA*, *wetA*, and *abaA*) were analyzed to elucidate transcriptional changes in biofilm cells in molasses-based medium. This study suggests the widespread application of molasses as an inexpensive raw material and effective inducer for biofilm production, and provides useful information for further investigation on FC biosynthetic regulation.

Materials and Methods

Strain and Culture Conditions

A. fumigatus CY018, provided by Nanjing University, was maintained on potato-dextrose-agar (PDA) slants and cultured at 28°C for 7 days. Afterwards, 2 agar blocks (10 mm × 10 mm) were transferred to a 500-ml shake flask containing 200 ml of potato-dextrose broth. After shaking at 150 rpm and 28°C for 72 h, 7 ml of the seed broth was inoculated in a 250-ml Erlenmeyer flask containing 43 ml of fermentation medium. Each liter of the cultivation medium contained 1 g of NaNO₃, 1.5 g of KH₂PO₄, 0.7 g of Mg₂SO₄, 0.02 g of FeSO₄·7H₂O, 3 g of sodium glutamate, 1.5 g of sodium tartrate, and either 60 g of sucrose or different concentrations of molasses as a carbon source. The final pH of the

medium was adjusted to 5.2 with 1 M HCl. FC was produced in a two-stage process. First, the culture was incubated at 28°C and 130 rpm for 72 h, and then the culture was moved to a 9-cm-diameter plate for static biofilm formation.

Sampling and Determination of Biomass, Conidia, and Fumigaclavine C Production

Three flasks or plates were sampled in each experimental replicate. Biomass production was examined by determining the dry cell weight (DCW) during different fungal phases. For planktonic samples, the broth was filtered through a preweighed filter paper under suction and dried in an oven at 60°C to a constant weight; for biofilm samples, the biofilm was washed three times with distilled water and dried as described above [22]. The number of conidia was determined with a hemocytometer.

The broth and the biofilm were separated, extracted 3 times with 50 ml of ethyl acetate, and evaporated using a vacuum rotary evaporator at 45°C. The crude extract was dissolved with methanol and filtered by a 0.22-μm polyvinylidene difluoride syringe filter for high-performance liquid chromatography analysis. An Agilent ZORBAX Eclipse SB-C18 column (4.6 mm × 250 mm, 5 μm) was used for quantitative analysis of FC. Analytes (20 μl) were injected and eluted in the mobile phase in solvent A (35% acetonitrile) and solvent B (65% H₂O, 1% trifluoroacetic acid) at the flow rate of 1 ml/min for 25 min in an Agilent 1290 Infinity LC system. The UV absorption was measured at 227 nm and the operating temperature was set at 25°C. FC concentrations were determined using a standard curve prepared with FC powder provided by Prof. RX Tan, Nanjing University.

Biofilm Quantification

The biofilm biomass was estimated according to a modified Crystal Violet staining method [25]. In brief, *A. fumigatus* biofilms were formed on commercially available 96-well microtiter plates after 3 days of shaking in flasks. A standardized cell suspension (200 μl) was statically incubated for 8 days. After incubation, the culture medium was removed, and the biofilm was washed at least 3 times with phosphate-buffered saline (PBS), stained with 200 μl of 0.1% Crystal Violet for 5 min, and washed with deionized water to remove excess dye. The biofilms were destained with 200 μl of 95% ethanol, 150 μl of the solution was transferred into a clean 96-well microtiter plate, and the absorbance was measured at 570 nm. The absorbance values were proportional to the biofilm biomass, which comprises the hyphae and extracellular polymeric substances.

Scanning Electron Microscopy

SEM was used to observe the morphology of *A. fumigatus* using sucrose or molasses as a carbon source. Cells in different growth phases were fixed in 2.5% (v/v) glutaraldehyde at 4°C for 12 h. The cells were washed twice in PBS and dehydrated with an ethanol concentration gradient. Finally, imaging was performed on a scanning electron microscope (S-3400; Hitachi, Japan).

Measurement of Gene Expression by Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from 100 mg of fresh cells via the RNAiso Plus kit (Takara Bio Inc., Japan), and cDNA was prepared with the PrimeScript RT reagent kit according to the manufacturer’s protocols.

The transcriptional levels of *brlA*, *abaA*, *wetA*, *fgaOx1*, *fgaOx3*, and *laeA* were analyzed using a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Each 25 µl real-time quantitative polymerase chain reaction (qPCR) contained 2 µl of cDNA, and was performed using the SYBR Premix Ex Taq II kit (Takara Bio Inc., Japan). After 30 sec of denaturation at 95°C, the cDNA was amplified with 40 cycles of denaturation at 95°C for 5 sec, annealing at 55°C for 30 sec, and extension at 72°C for 60 sec. Identical thermal cycling conditions were utilized for all targets, and the transcriptional levels were normalized to *A. fumigatus* β-actin. Each reference sample was set as 1.0, and the results of others were expressed as fold-increase in mRNA level over reference samples. Primers for target genes are presented in Table 1.

Statistical Analysis

All data represent the mean ± standard deviation of three independent experiments. One-way analysis of variance was used to determine the significance of differences between cultures under different conditions. A value of $p < 0.05$ was considered significant.

Results and Discussion

Distribution of Conidia and Fumigaclavine C in Molasses-Containing Culture

In our previous work, aerial mycelia (*i.e.*, biofilm) were

Fig. 1. Distribution of dry cell weight (DCW), number of conidia, and fumigaclavine C (FC) production in the broth versus biofilm from 60 g/l sucrose on day 12 of fermentation.

observed at the gas-liquid surface, which stimulated conidiation and facilitated oxygen transport during the static culture stage [1, 24]. Thus, the two phases of growing cells, the planktonic cells in the culture broth and the biofilm on the static surface, coexist during the two-stage process. However, the precise contributions of *A. fumigatus* biofilms and planktonic cells to conidiation and FC production in the two-stage process remain unclear.

The biofilm and culture broth were separated and analyzed with sucrose-based medium. As shown in Fig. 1, the majority of conidia and FC were retained in the biofilm, with 10.5-fold more conidia and 4.0-fold greater FC production in the biofilm, which had a 2.2-fold higher DCW compared with the cells in the broth. Metabolites produced by *A. fumigatus* in the broth and biofilm were compared (Fig. S2), to further demonstrate the differences between them. The results demonstrate that by-product titers were higher in the broth, whereas the FC concentration was obviously greater in the biofilm. This indicates that FC is the main metabolite produced by the biofilm, and suggests a potential role for biofilm in conidia production and FC biosynthesis. Biofilm had a similar stimulatory impact on the production of gliotoxin, another conidiation-associated metabolite produced by *A. fumigatus* [23].

Effects of Different Inducers on Biofilm Formation and Fumigaclavine C Production in *A. fumigatus*

To attain higher FC production, different additives were tested for the ability to enhance biofilm formation. Different concentrations of additives previously reported to stimulate biofilm formation were evaluated in sucrose-based medium,

Table 1. Primers used for the qPCR of *Aspergillus fumigatus* gene expression.

Gene	Primer sequence
β-actin	F: 5'-AGACCTTCAACGCTCCCGCCT-3' R: 5'-GGCGTGGGGGAGAGCGAAAC-3'
<i>brlA</i>	F: 5'-ACACCAAGACCCACAGCAA-3' R: 5'-AGTAAGTGGCCCCGATAGT-3'
<i>wetA</i>	F: 5'-AAGCCCGTCAAAGGGCCAAA-3' R: 5'-AACTCCCACTCGGAGCGACT-3'
<i>abaA</i>	F: 5'-CAGGGCGACAAAGCCTTCCA-3' R: 5'-ACATTCTCCAGCGGCATGGG-3'
<i>laeA</i>	F: 5'-GTTGGCAGCCAATGGTTCC-3' R: 5'-CGGCAGCTCGAAAGAAGGGA-3'
<i>fgaOx1</i>	F: 5'-TTTCCTTGACACCCATCCCC-3' R: 5'-TCTTCAGTGCGACCAACCTC-3'
<i>fgaOx3</i>	F: 5'-TGAGGTGTTGTGCGTGCT-3' R: 5'-GCCACATTGGTGTAAAGTCGC-3'

Fig. 2. Effect of different additives on the formation of biofilm (Crystal Violet A570), fumigaclavine C (FC) production, and number of conidia.

Ctrl: 0.94 ± 0.17 for Crystal violet A570, $1.23 \pm 0.02 \times 10^7$ conidia/ml for Number of conidia, and 34.03 ± 0.32 mg/l for FC concentration.

including molasses [26], Fe^{2+} [27], casein [28], and sheep blood [29]. As shown in Fig. 2, the addition of molasses (100 and 200 g/l) and sheep blood (30 g/l) significantly enhanced biofilm formation after 12 days of fermentation, with 1.83-, 1.97-, and 1.91-fold increases compared with the control, whereas Fe^{2+} and casein addition slightly increased biofilm growth. At the same time, the production of FC was also markedly improved with the addition of molasses and sheep blood, reaching 89.98 mg/l and 110.80 mg/l, respectively, compared with 34 mg/l in the control. Regarding asexual sporulation, molasses markedly increased the conidia number, whereas sheep blood exerted no significant effect. This might be due to the enrichment of nitrogen sources in sheep blood, which inhibit sporulation [30]. The results suggest that both molasses and sheep blood are excellent inducers for biofilm formation and FC production. However, molasses is less expensive and easier to sterilize, and can also serve as a carbon source during fermentation [31]. Therefore, molasses was selected for further study, and biofilms were subjected to further analyses to illuminate the stimulatory effects of molasses on the conidiation and FC production of *A. fumigatus*.

Enhancement of Biofilm Growth and Fumigaclavine C Production through the Optimization of Molasses Concentration

The biosynthesis of ergot alkaloids requires significant amounts of carbon, primarily sourced from sucrose or glucose [32], which makes it economically unfavorable for

Fig. 3. Effect of molasses concentration on dry cell weight (DCW), number of conidia, and fumigaclavine C (FC) production in the biofilm.

Ctrl: 0.94 ± 0.17 for Crystal violet A570, $1.23 \pm 0.02 \times 10^7$ conidia/ml for Number of conidia, and 34.03 ± 0.32 mg/l for FC concentration.

industrial FC production. On the contrary, molasses is a waste product of the sugar industry, and shows promise as a supplementary ingredient for *A. fumigatus* fermentation. Moreover, molasses, with a similar amount of carbon (*i.e.*, residue sugar) as sucrose, performs better in FC production (Table S1). Furthermore, 60 g/l of sucrose was dispensable in medium containing molasses (data not shown); therefore, molasses was utilized as a sole carbon source in the subsequent experiments, which might further decrease fermentation costs. The molasses concentration was optimized to promote biofilm growth. As shown in Fig. 3, *A. fumigatus* biofilm growth was increased in molasses-based medium harvested on day 12. The DCW of the biofilm increased in a dose-dependent manner, and peaked at 52.9 g/l with 400 g/l molasses. Conidiation and FC production also increased with increasing molasses concentration. Clearly, 400 g/l molasses was optimal for cell growth, conidiation, and FC production, with 37.1×10^6 conidia/ml and 155.1 mg/l of FC produced. However, this effect was not observed at 500 g/l molasses, when the growth of *A. fumigatus* was severely diminished, possibly because of unfavorable osmotic pressure. *A. fumigatus* macro- and microstructures grown in sucrose- and molasses-based media were observed by SEM. Compared with the control in Fig. 4A, the morphology of *A. fumigatus* grown in 400 g/l molasses-based medium (Fig. 4B) formed a much curlier biofilm, whereas 500 g/l molasses failed to form a complete biofilm (Fig. 4C). In addition, the biofilm cultivated in molasses-based medium produced markedly more conidia

Fig. 4. Macro- (A–C) and micro-structures (D and E) of *A. fumigatus* grown under 60 g/l sucrose-based (A and D) and 400 g/l molasses-based (B and E) media after 144 h of fermentation.

Note that, the macro-structure of *A. fumigatus* grown in 500 (g/l) molasses (C) was also observed.

with more extracellular matrix (Figs. 4D, 4E), implying the promotion of asexual development with enhanced biofilm formation.

Effects of Molasses as a Sole Carbon Source on Biofilm Growth, Conidiation, and Fumigaclavine C Production

To study the kinetic effects of molasses on the fermentation process of *A. fumigatus*, biofilm growth, the number of conidia, and FC production were compared in 60 g/l sucrose- and 400 g/l molasses-based media. As shown in Fig. 5, compared with sucrose, molasses had a positive impact on biofilm DCW throughout the fermentation process. The biofilm DCW of sucrose- and molasses-based media both reached their maximal levels (7.38 and 52.90 g/l, respectively) after 288 h of cultivation. Meanwhile, the amount of conidia peaked at 240 h (12.1×10^6 and 37.1×10^6 conidia/ml, respectively), followed by slight decreases afterwards, which might be due to the germination of a small portion of the conidia. FC biosynthesis began after 96 h of cultivation, and the highest FC production values of sucrose- and molasses-based media (26.49 and 155.10 mg/l, respectively) were obtained after 288 h. The kinetic data demonstrated a strong facilitating role for molasses in the biofilm growth stage (static stage) of *A. fumigatus*.

A. fumigatus Asexual Development Gene Expression in Molasses-Based Medium

To better understand the mechanism underlying the

stimulatory role of molasses, the transcriptional profiles of *A. fumigatus* biofilms grown in sucrose- and molasses-based media were examined. Conidiation is the major reproductive mode of aspergilli, and is mainly controlled by three central regulators, BrlA, AbaA, and WetA [33]. The expression of *brlA*, *wetA*, and *abaA* was assayed at the transcriptional level in *A. fumigatus* biofilms (Fig. 6). In molasses-based medium, *brlA* expression was significantly upregulated on the first day of static culture, and was 11.24-fold higher than in sucrose-based medium. The expression of *wetA* and *abaA* was downregulated, possibly due to unfavorable components present in the raw molasses [1]. However, these inhibitory effects were reduced by the third day of static culture, possibly as a result of earlier BrlA expression. Molasses promoted the expression of *brlA* and *wetA* for 8 and 6 days, respectively, but for less than 2 days for *abaA*, suggesting that *brlA* and *wetA* are two major genes affected by molasses. This may be due to *wetA* feedback inhibition during asexual development signaling [33], which meanwhile verified the upregulation of *brlA* and *wetA* in the asexual development of *A. fumigatus*. The results suggest that molasses significantly increases the expression of asexual development genes, especially *brlA* and *wetA*, during *A. fumigatus* biofilm growth.

Fumigaclavine C Biosynthesis Gene Expression in Molasses-Based Medium

To further investigate the mechanism underlying the

Fig. 5. Time course of dry cell weight (DCW) of biofilm (A), number of conidia (B), and fumigaclavine C (FC) production (C) in sucrose-based and molasses-based media.

Fig. 6. Transcriptional levels of *brlA* (A), *wetA* (B), and *abaA* (C). The levels of gene transcripts (B) from cultures with sucrose on day 4 were set to 1 (black column) to normalize those from cultures with molasses. The mean fold of gene transcriptional level over the control is presented for the molasses-based medium. The error bars in the figure indicate the standard deviations from three independent samples, * $p < 0.05$.

stimulatory role of molasses, the expression of two FC biosynthetic genes, *fgaOx1* and *fgaOx3*, as well as the global

Fig. 7. Transcriptional levels of *fgaOx1* (A), *fgaOx3* (B), and *laeA* (C).

The levels of gene transcripts (B) from cultures with sucrose on day 4 were set to 1 (black column) to normalize those from cultures with molasses. The mean fold of gene transcriptional level over the control is presented for the molasses-based medium. The error bars in the figure indicate the standard deviations from three independent samples; * $p < 0.05$.

secondary metabolism regulatory gene *laeA*, were quantified by qPCR (Fig. 7). The two biosynthetic genes were downregulated initially, whereas *laeA* expression was similar to that in the control, indicating the preliminary activation of secondary metabolism in both conditions. Afterwards, the transcriptional levels of *laeA*, *fgaOx1*, and *fgaOx3* were upregulated 2.92-, 6.59-, and 3.60-fold, respectively on day 3 of static culture, in accordance with the increased FC production; however, *fgaOx1* upregulation lasted no more than 48 h, after which decreased expression was observed. This may suggest that *fgaOx1* is not limited in the FC biosynthetic cluster. However, *fgaOx3* expression remained higher than in the control during the fermentation process, indicating a crucial role for *FgaOx3* in molasses-induced FC biosynthesis. The expression of *laeA* remained upregulated until the end of static culture, indicating increased secondary metabolism activity in the molasses-based medium, and partially explaining the increased FC production. A similar relationship between *laeA* expression and productivity was observed during the fermentation of penicillin [34] and monacolin K [35]. Moreover, *LaeA* is also required for conidia differentiation [36]. The *laeA* transcriptional profile seemed to be consistent with that of *brlA* in the initial short period stage of static culture, not synchronously. Hence, the inner correlation of *laeA* and *brlA* still requires further study. Together, the results demonstrate that molasses upregulates the expression of the secondary metabolism regulatory gene *laeA* and the biosynthetic gene *fgaOx3* during static culture, and transiently upregulates *fgaOx1*.

Finally, based on our study, a genetic model for regulation of FC biosynthesis and asexual development is shown (Fig. 8). The stimulatory effects of molasses on FC biosynthesis in the biofilm at the transcriptional level was first presented. The key role of the biofilm in FC biosynthesis was verified by the transcriptional analyses of asexual development and FC biosynthetic genes. Key asexual development genes (*brlA* and *wetA*), biosynthetic genes (*fgaOx3*), and the global secondary metabolism regulatory gene *laeA* were upregulated in biofilm grown with molasses, indicating the induction of both asexual development and FC biosynthesis. This study provides novel insight into the stimulatory effects of molasses on biofilm growth, and may aid further research on the regulation of *A. fumigatus* FC production.

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Fig. 8. Stimulatory effects of molasses on fumigaclavine C biosynthesis at the transcriptional level.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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