Full Length Research Paper

Enhanced production of mycelial biomass and ganoderic acid in submerged culture of *Ganoderma applanatum* ACCC-52297 elicited by feeding rutin

Zhicai Zhang[1,2,3]*, Wangli Shen[1], Dan Liu[1] and Jiashao Li[1]

[1]School of Food and Biotechnology, Jiangsu University, Zhenjiang, 212013, P. R. China.
[2]Beijing Green Technology and Natural Biotechnology Co. Ltd., Beijing 102300, P. R. China.
[3]Parchn Sodium Isovitamin C Co. Ltd., Dexing, 334221, China.

In this study, it was found that supplementation of 0.6 g/L rutin could improve the fermentation performance of *Ganoderma applanatum* ACCC-52297 in terms of mycelial growth and ganoderic acid (GA) production. Albeit retarded mycelial growth was detected at the first 36 h in the submerged culture of *G. applanatum* ACCC-52297 with the addition of rutin, kinetic model analysis revealed that rutin could improve mycelial growth and GA synthesis at the later phase of the fermentation (after 36 h). As such, rutin post-feeding strategy was proposed to elevate the final GA titer and at the same time minimize the inhibitory effect of rutin on mycelia growth. As a result, higher GA production (293 mg/L) and dry mycelia weight (DCW) (30.5 g/L) were achieved, GA were increased by 102.1 and 7.32%, and DCW were increased by 200 and 130% compared with those culture without addition of rutin and addition of 0.6 g/L rutin at the beginning of fermentation, respectively. Rheology analysis showed that addition of rutin was adversely related with the broth consistency coefficient and apparent viscosity, possibly due to the inhibitory effect of rutin on the biosynthesis of some macromolecules such as proteins and polysaccharides, which could partially account for the improved production of mycelia and GA during *G. applanatum* ACCC-52297 fermentation. Quericean, a metabolite of rutin, was also found to accumulate within the mycelium. It was concluded that rutin does not participate in the synthesis of GA as a precursor but rather facilitates the synthesis of GA and mycelia biomass by increasing the dissolved oxygen concentration during fermentation.

Key words: *Ganoderma applanatum*, rutin, ganoderic acid, dry cell weight, rheology.

INTRODUCTION

Ganoderma species have been used in traditional Chinese and Japanese medicine for control of blood pressure, for treatment of chronic bronchitis, as immune enhancers and for stress alleviators. Moreover, some *Ganoderma* species also display cytotoxicity against hepatoma cells *in vitro* (Toth et al., 1983), inhibit the proliferation of human and mouse carcinoma cell lines (Liu et al., 2002; Sliva, 2003, 2004). So far, more than 100 polyoxygenated triterpenes, which are one of the major bioactive ingredients of *Ganoderma* species (Bao et al., 2002; Hu et al., 2002; Min et al., 2001; Miyamoto et al.,

2009; Chen et al., 2008), have been isolated from their fruiting bodies and cultured mycelia. Most of them belong to the lanostane triterpenes and contain a terminal carboxylic groups (Gao et al., 2005), which are termed as ganoderic acids (GA).

GA and their derivatives are obtained not only from *Ganoderma lucidum*, but also from other kinds of *Ganoderma* genus. For example, GA is also mainly extracted from the solid cultivated fruiting bodies of *Ganoderma applanatum*. However, solid cultivation of higher fungus has suffered from several limitations, such as long period of production cycle, uncontrolled production conditions and intensive labor input. As a result, submerged fermentation has emerged as an alternative approach for efficient production of higher fungus metabolites (Fang and Zhong, 2002). Indeed,

*Corresponding author. E-mail: zhangzhicai2001@yahoo.com.cn. Tel: +86-511-8780226. Fax: 86-511-8780201.

Figure 1. The structure of isoflavonid and rutin.

there are several advantages of submerged fermentation over solid cultivation for GA production such as high productivity, low costs and availability of convenient control as well as easy downstream processing steps (Tang and Zhong, 2002).

Strategies for improvement of GA production have been primarily focused on nutritional (medium) and environmental (pH, dissolved oxygen and agitation speed) optimization. According to our previous study, peptone was found to be a suboptimal nitrogen source for mycelial biomass formation and GA biosynthesis compared to soybean powder in the submerged culture of *Ganoderma* species, although peptone has higher protein content and was more easily utilized than soybean powder (Xu et al., 2008). Soybean powder, a naturally occurring complex media, consisting of large quantities of proteins and isoflavonoids, is a more favorable nitrogen source for secondary metabolites biosynthesis. In addition to be a nutritional factor, isoflavonoids, may function as an elicitor which facilitates triterpene biosynthesis in the *G. lucidum*. Nevertheless, little information has been available with respect to facilitating GA production elicited by isoflavonoids in submerged culture of *Ganoderma* species. To examine whether isoflavonoids in soybean powder could elevate the GA production and mycelia formation, different concentration of rutin, a structural analogue of isoflavonoids (Figure 1), was supplemented to the *Ganoderma* culture with peptone instead of soybean powder as nitrogen source. Effect of rutin on GA production was investigated and the rheological property of fermentation broth was also analyzed in the present study. These studies would provide the information that isoflavonoids, which were neither components of medium nor precursor of final products, could increase the biosynthesis of some secondary metabolites by changing the rheological property of fermentation broth, that is, consistency coefficient, apparent viscosity and shear stress.

MATERIALS AND METHODS

All chemicals used were of analytical or reagent grade. Soybean powder and wheat bran were purchased from a local market and then filtered with a 60 screen mesh.

Microorganism and media

G. applanatum ACCC 52297 used in the study was deposited in the Agricultural Culture Collection of China. It was maintained on potato glucose agar (PGA) slants. The slants were inoculated and incubated at 30°C for 7 days, then stored at 4°C for about 2 months. The seed medium consisted of (g/L) glucose 20, soybean powder 5, KH_2PO_4 3, $MgSO_4 \cdot 7H_2O$ 2. The basal fermentation medium in shake flask was as follows: (g/L) glucose 40, peptone 2, KH_2PO_4 4, $MgSO_4 \cdot 7H_2O$ 2.

Inoculum and shake flask culture

For the seed culture, three pieces of agar culture in pea size from a slant culture were inoculated into a 250 mL flask with 80 mL seed medium, and then cultivated for 6 days on a reciprocal shaker at 150 rpm. The seed culture was then inoculated either into a 250 mL flask containing 80 mL fermentation medium or into a 5 L stirred fermentor containing 3 L fermentation medium with an inoculum size of 8% (v/v).

Shake flask culture was carried out on a reciprocal shaker at 150 rpm, 30°C for 6 days. Submerged fermentation was carried out in a 5 L stirred fermentor (JSF-5, made in Jiangsu University, China). The agitation speed and aeration rate were controlled at 200 rpm and 4 vvm, respectively. The pH was fixed in the range of 3.0 to 7.0 with addition of either 0.5 mol/L HCl or 0.5 mol/L NaOH at 30°C. All experiments were done in triplicate.

Assay of mycelial biomass, ganoderic acids, residual glucose, polysaccharide and protein

The mycelial biomass of *G. applanatum* was determined by weighing the dry cell amount. Samples collected at different times were centrifuged for 20 min at 3000 rpm, and then the resulting pellet was washed repeatedly with distilled water and dried at 105°C until a constant weight was achieved. At the same time, the supernatant was concentrated under vacuum. The residues were suspended in purified water and then extracted with chloroform. The GA in the chloroform phase was extracted with saturated $NaHCO_3$ aqueous solution. Then upper phase ($NaHCO_3$ phase) was cooled on ice and acidified to pH 3.0 with 2 mol/L HCl and re-extracted with chloroform. After removal of chloroform by evaporation at 40°C, GA was dissolved in chromatographic grade methanol. Quantitative analysis of GA was determined by measuring the absorbance at 243 nm in a Unico 2100 spectrophotometer (Chang, 2006). Residual glucose, gross sugar and protein in the supernatant were measured
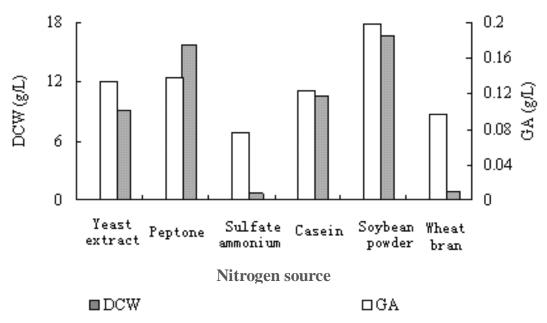
Figure 2. Effect of nitrogen sources on GA production by *G. applanatum* ACCC-52297; ■: DCW, □: GA.

according to the 3, 5-dinitrosalicylic acid method (Miller, 1959), the anthrone sulphate method (Texier et al., 1984) and the biuret reaction (Joyce and Sanford, 1970), respectively. The level of polysaccharides was equal to gross sugar content minus residual glucose content.

Assay of rheological properties

After filtering with filter paper (Xinhua1[#]), the clarified liquid was obtained and used for rheological properties measurement. Rheological properties were determined on a Brookfield LVDV-II rotating Viscometer. In the course of measuring, the temperature was kept in the range of 29 to 30°C and the speed of the rotor in the range of 5 to 100 rpm.

Rheological model for *G. applanatum* fermentation broth was fitted according to the power-law model, namely, $\tau = K\ r^n$, where τ is the shear stress, r is the shear rate, K is the consistency coefficient and n is the flow behavior index. Apparent viscosity was calculated as: $\eta_a = K\ r^{n-1}$. For the case of flask culture, r_{max} represented the maximum shear rate in 250 mL Erlenmeyer flasks, $r_{max} = 2\pi R/60$, where R is the rotational speed. In the trial, R was 150 rpm, so $\eta_a = K\ 15.7^{n-1}$.

Assay of rutin's metabolite

The fermentation broth of *G. applanatum* in the presence and absence of rutin were both centrifuged for 10 min at 8000 × g. The supernatant was concentrated under reduced pressure and dried to constant weight. The resulting residue (HBr) was weighted. The pellet (mycelium) was dried to constant weight and then extracted with ethanol (ethanol volume: precipitation weight 1:30). The resulting liquor was filtered and analyzed by HPLC-MS.

Chromatographic separation was performed on a Waters Alliance 2690 series LC/UV/ESI-MSD SL (single quadrupole) spectrometer equipped with ESI-mode sources, Masslynx 3.0 Chem Station, and Waters 996 diode-array detector (DAD). Liquid chromatography was on a Spherisorb C18 reverse-phase column (0.5 μm, 3.9 × 250 mm). The active ingredient was eluted with a linear gradient from 25 to

95% acetonitrile in 0.1% formic acid in 30 min (with holding at 30% for 5 min). The sample was eluted at a constant flow rate of 1.0 mL/min. The injection volume was 5 μL each of the sample solutions. The column temperature was maintained at 25°C. The UV spectra were monitored over the range of 210 to 400 nm. The scan measurements, using negative ESI mode in the mass spectrometer, were performed with the following settings: heater temperature of nitrogen gas, 300°C; flow of heated dry nitrogen gas, 5.0 L/min; nebulizer gas pressure, 40 psi; capillary voltage, 2500 V for negative and 4000 V for positive; and scan range, 100 to 1000 m/z. These parameters were optimized in preliminary experiments to get the highest abundance of the targeted molecular-related ions. The content of responding metabolites was calculated according to the formula of external standard method (Deng, 2008):

$$C_{\text{sample}} = \frac{A_{sample} \times C_{\text{standard}}}{A_{sample}}$$

RESULTS AND DISCUSSION

Synergistic effect of peptone and rutin on GA production

In our experiment, six different nitrogen sources (yeast extract, peptone, ammonium sulfate, casein, soybean powder and wheat bran) were added to the basal medium (Figure 2). Both higher mycelial biomass and GA production were achieved when soybean powder was used as nitrogen source, which was consistent with the previous literature (Xu et al., 2008). To investigate whether rutin would promote the biosynthesis of GA, different concentration of rutin, were synchronously added to the medium. As shown in Figure 3, when peptone was used as the sole nitrogen source and rutin was added   at
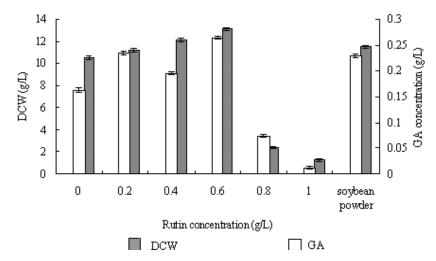
Figure 3. Effect of rutin concentration on GA production by *G. applanatum* ACCC-52297; ■: DCW; □: GA.

the range of 0.2 to 0.6 g/L, the mycelial biomass and GA production were significantly improved in comparison to the culture without addition of rutin; however, high level of rutin (0.8 g/L) was found to inhibit the mycelial biomass and GA production, presumably due to the cytotoxic effect of flavonoids on cell growth. When using peptone as the nitrogen source supplemented with rutin of 0.4 to 0.6 g/L, the mycelial biomass and GA production in the broth were even higher than that of the culture with soybean powder as nitrogen source (Figure 3). This significant increase in the mycelia biomass and GA production could possibly be ascribed to the synergistic effect of peptone and rutin, which on another hand could substantiate the fact that soybean powder was a better nitrogen source for triterpene biosynthesis than the defined nitrogen source like casein and yeast extract. These results also demonstrated that rutin or other isoflavonids would function as an elicitor which could facilitate the mycelial biomass and GA production in the submerged culture of *Ganoderma* speices.

Time course of GA fermentation in optimal medium supplemented with different concentration of rutin

To further investigate how rutin could promote the production of mycelia biomass and GA, time course of GA and biomass production supplemented with different concentration (from 0 to 1.0 g/L) of rutin in the submerged culture of *G. applanatum* ACCC-52297 were recorded. As shown in Figure 4, before 48 h, the dry cell weight (DCW) and glucose consumption were lower in the culture supplemented with rutin than that of the culture without supplementation of rutin (Figure 4). Obviously, these results showed rutin could inhibit the mycelial growth of *G. applanatum* ACCC-52297. Almost no mycelial growth was observed when the supplemented rutin concentration reached 1.0 g/L. However, when supplementing less than

0.6 g/L of rutin, the mycelia biomass and GA production were significantly enhanced after 36 h of fermentation. As shown in Figure 4, the maximum GA concentration (0.273 g/L) and the maximum DCW (22.54 g/L) were achieved with the addition of 0.6 g/L rutin at 48 h, and the residual glucose concentration in the broth decreased to the lowest level (8.2 g/L). The maximum values of biomass and GA concentration were achieved at 96 h and 120 h, respectively (Figure 4), with a short lag time, which was congruent with the previous literature that GA was growth-unassociated metabolites. It is concluded that supplementation of 0.6 g/L of rutin at 36 h of fermentation is favorable for achievement of higher GA yield. To prove this conclusion, the rutin-feeding experiment at 36 h of fermentation was carried out in a 5 L bioreactor. The results showed that the maximum DCW (30.5 g/L) was achieved at 96 h and higher than that of culture with addition of 0.6 g/L rutin at the beginning of fermentation (Figure 5); moreover, the concentration of GA achieved 0.293 g/L at 108 h and increased by 102.1 and 7.32%, respectively, compared with that of medium without addition of rutin and addition of 0.6 g/L rutin at the starting point. More interestingly, with rutin post-feeding strategy, the residual glucose concentration of the culture broth at the maximum GA production was 5.6 g/L, which was about 65.8 and 11.1% lower than that of medium without addition of rutin and addition of 0.6 g/L rutin at the starting point.

Difference in rheological property of *G. applanatum* ACCC-52297 in the presence and absence of rutin

There are a large numbers of industrial fermentations that utilize filamentous microorganisms (e.g. antibiotic production, enzymes, organic acids etc.) and it is well established that the heterogeneous broths generated in such processes have highly viscous and   non-Newtonian
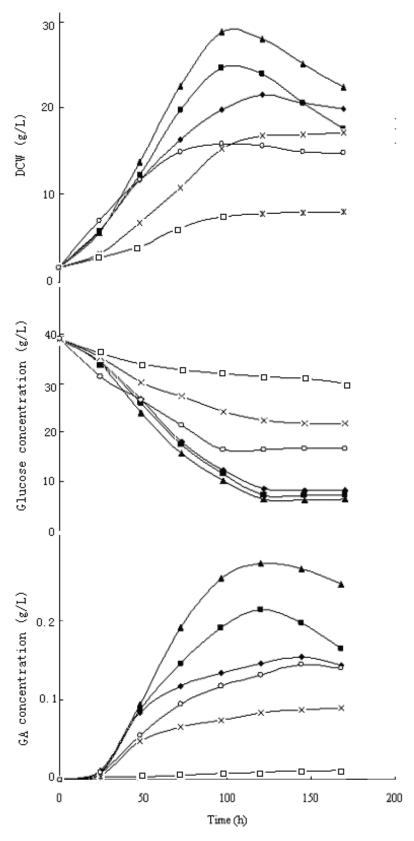
Figure 4. Time courses of GA production by *G. applanatum* ACCC-52297 in the medium added the different concentration of rutin. (A): DCW; (B):glucose concentration; (C): GA; rutin concentration added was presented as ♦:0.2; ■: 0.4; ▲: 0.6; ×:0.8; □:1; ◊:0 (g/L)
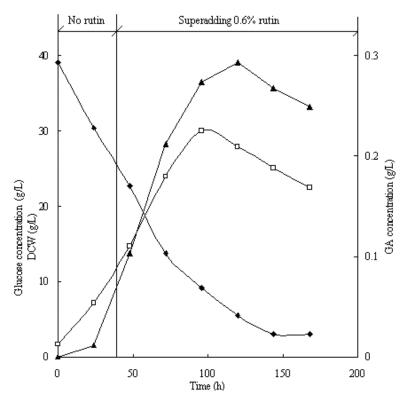
Figure 5. Time courses of GA production by *G. applanatum* ACCC-52297 with post-feeding strategy . ♦: glucose concentration; ♦: glucose concentration; □: DCW; ▲: GA.
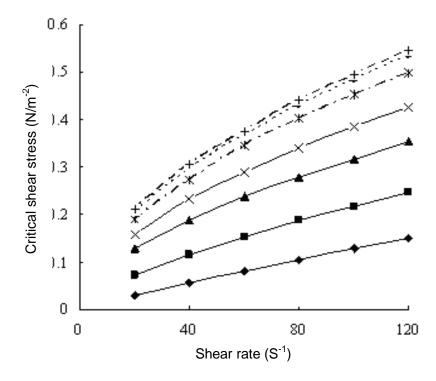
rheological properties. High apparent viscosity and consistency coefficient is directly related to the dissolved oxygen level which could further impair the metabolite accumulation and cell growth. From the previous report, strategies for elevation of desired end-product formation have been mainly carried out by optimizing the nutritional (medium composition) and environmental (pH, temperature) factors and few were reported by modulating the non-Newtonian rheological behavior of fermentation broth. Especially, the high consistency coefficient and apparent viscosity are characteristic of filamentous fungi culture, which were a major bottleneck for large-scale application of fungi fermentation. The increase in consistency coefficient and apparent viscosity is often related with the synthesis of some hydrophilic macromolecules, such as proteins and polysaccharides. Once the synthesis of these hydrophilic metabolites was inhibited, the rheological properties of broth would change, and mycelial growth and the synthesis of end products would therefore change due to the increased dissolved oxygen level. In our previous report (Xu et al., 2008), it was accidently found that soybean powder could decrease the culture viscosity. These findings led to our assumption that there must be some substances that could change the rheological behavior of the fermentation broth within the soybean powder, which might be associated with the improved production of mycelial

biomass and GA.

To verify this assumption, the rheological behavior of *G. applanatum* ACCC-52297 culture at different time point was analyzed (Figure 6). The fluid consistency coefficient and flow behavior index were deduced from the fitting curve according to the power function (Table 1). As shown in Figure 6 and Table 1, the deviation between the *G. applanatum* ACCC-52297 culture fluid and standard Newtonian fluid became greater with increase of consistency coefficient and the apparent viscosity; whereas this difference became smaller with decrease of flow behavior index when the fermentation time prolonged from 24 to 168 h. The varying tendency of rheological curve, consistency coefficient, apparent viscosity and flow behavior index of *G. applanatum* ACCC-52297 broth were very similar to each other. However in the fermentation process with post-supplementation of rutin, the consistency coefficient and apparent viscosity became bigger, and shear stress and flow behavior index became smaller compared with the culture without addition of rutin.

Metabolism of rutin in *G. applanatum* ACCC-52297 culture

To examine whether rutin was involved as a precursor for
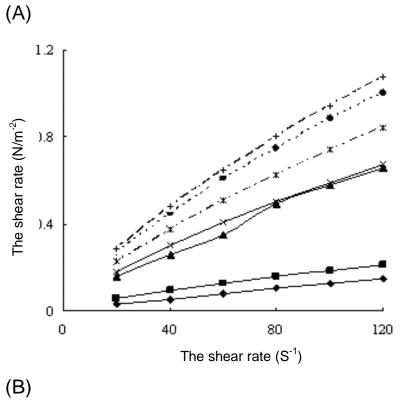
(A)

(B)

Figure 6. Influence of superadded rutin on the *G. applanatum* ACCC-52297 broth's rheological curve. (A): No added rutin; (B): superadded rutin in 36 h; sampling times were presented as: ♦: 24; ■: 48; ▲: 72; ×: 96; *: 120;* 144; +: 168 (h).

the biosynthesis of GA, ethanol extracts of *Ganoderma* mycelium were analyzed by waters ZMD4000 LC-ESI/MS

(Figure 7). For the culture with addition of rutin, a new peak at retention time 7.21 min (Figure 7a) was found   in
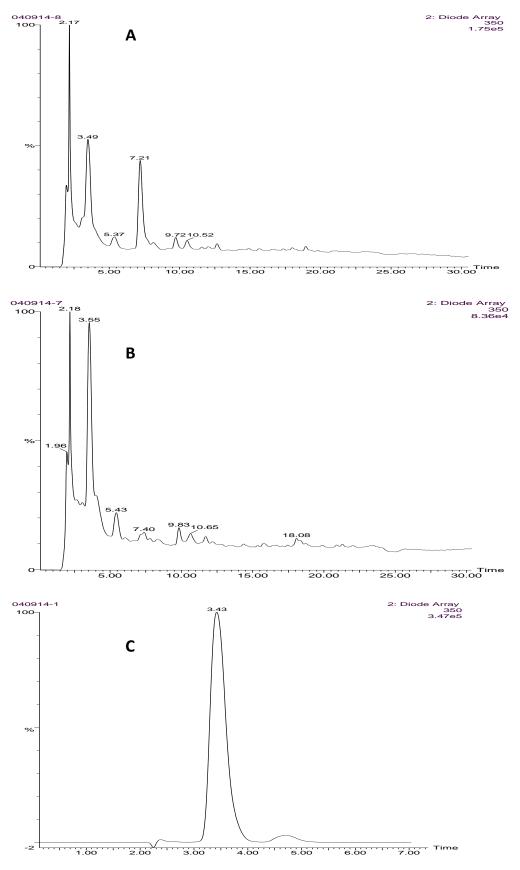
Figure 7. Compare with the chromatogram map of ethanol extraction of mycelia obtained from cultures medium fed rutin (A), not added rutin (B) and rutin (C).

Table 1. The comparison of consistency coefficient, flow behavior index and apparent viscosity of *G. applanatum* ACCC-52297' broth superadded rutin with not added rutin.

Time (h)		Consistency coefficient (Nm^{-2})	Flow behavior index	Apparent viscosity (PaS^{-1})
Not added rutin	24	0.0019	0.9142	0.0015
	48	0.0090	0.6963	0.0039
	72	0.0235	0.5653	0.0071
	96	0.0294	0.5578	0.0087
	120	0.0368	0.54109	0.0104
	144	0.0407	0.5282	0.0111
	168	0.0422	0.5279	0.0115
Superadded rutin*	24	0.0019	0.9142	0.0015
	48	0.0073	0.7532	0.0027
	72	0.0180	0.6070	0.0051
	96	0.0200	0.6028	0.0067
	120	0.0250	0.5628	0.075
	144	0.0300	0.5668	0.08
	168	0.0320	0.5628	0.082

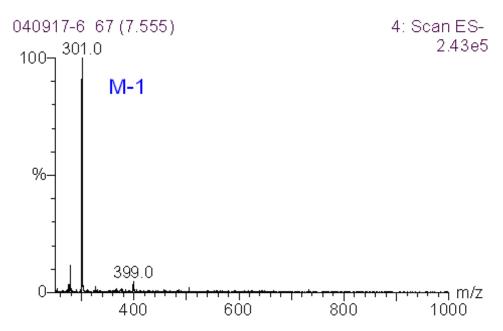∗ Rutin was added at 36 h in the course of fermentation.

Figure 8. The mass map of the peak at the retention time 7.21 min in the chromatogram map of the extraction of mycelia from cultures medium fed rutin.

the chromatographic map; whereas this peak did not present the culture without addition of rutin (Figure 7b) and rutin control sample (Figure 7c). Also the standard rutin peak at 3.43 min (Figure 7c) was almost disappeared for the rutin-feeding culture sample (Figure 7a), indicating that rutin has been transformed to another compound. To validate this assumption, mass spectrometry was applied to identify this new compound that appeared in the rutin feeding culture sample. In Figure 8, these ion fragments showed complete match with the standard quercetin, a glucoside derative of rutin. These results demonstrated that rutin could be transformed to quercetin at a conversion rate of 97% in the submerged culture of *G. applanatum* ACCC-52297. It was concluded that rutin does not participate in the synthesis of GA as a precursor but rather facilitates the synthesis of GA and mycelia biomass by increasing the dissolved oxygen concentration during fermentation.

Conclusion

Supplementation of 0.2 to 0.6 g/L rutin, could significantly increase the mycelial biomass and GA productions in the submerged culture of *G. applanatum* ACCC-52297. The growth profile showed that rutin could inhibit mycelial growth before 36 h but facilitate mycelia growth and GA synthesis after 36 h. As such, rutin post-feeding strategy was proposed to further boost GA production. Rutin as an elicitor could improve the mycelial growth and GA synthesis by decreasing the consistency coefficient and apparent viscosity of the *G. applanatum* ACCC-52297 culture instead of participating in the synthesis of GA as a precursor. The rutin or flavonoids post-supplementation strategy could also be applied to other bioprocess for efficient production of valuable secondary metabolites.

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