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Research article

Simultaneous isoquercitin and gallic acid production of *Aspergillus niger* on Triphala byproduct under solid state fermentation in packed-bed bioreactor

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Abstract: Triphala byproduct from hot-water extraction (TPB), which was a traditional process, was valorized by solid state fermentation in this research. Since the leftovers from the extraction contain high rutin and tannin contents, they were hydrolysable to isoquercitin and gallic acid, which were their monomers, respectively. *Aspergillus niger*, a producer of α-L-rhamnosidase and β-glucosidase, was cultured on the TPB to produce both isoquercitin and gallic acid, which were powerful antioxidants used in medical applications. The solid-state fermentation (SSF) was conducted in the three-layered packed-bed bioreactor aerated with humid air at different rates (0.1, 0.2 and 0.3 L/L/min or vvm). The highest isoquercitin and gallic acid production rates were found in the SSF, with 0.1 vvm at 1.14/h and 0.3 vvm at 3.12/h, respectively. The interaction of aeration rate and fermentation time significantly affected the fungal growth and the production of gallic acid, while the isoquercitin production was affected only by the fermentation time. Moreover, the differences of their production yields in different positions of bed along the height of bioreactor found to be useful to design the harvesting period of the

fermentation products including isoquercitin or gallic acid or simultaneous isoquercitin and gallic acid. The results clearly showed that aeration, harvesting time, and position of the bioreactor were crucial in designing the process for isoquercitin, gallic acid, or both.

Keywords: isoquercitin; gallic acid; *Aspergillus niger*; solid state; fermentation; triphala

1. Introduction

Triphala, the herbal formula of Thai traditional medicine, is a combination of three dried fruits, including amala, bibhitaki, and haritaki. The main active component of Triphala is polyphenol, which has been reported for its uses in medical applications such as anti-gastric ulcer and anti-peptic agents [1]. Many active ingredients have been found in Triphala, such as tannins, gallic acid, corilagin, chebulagic acid, chebulinic acid, and rutin [2,3].

Generally, Tri-Phala is extracted by boiling water, with the ratio of Triphala to water being 3:10 [4]. The leftover is ignored even if it still contains a high amount of tannin that can be hydrolyzed into gallic acid. Presently, microbial production of the tannase enzyme is mostly used for the bioconversion of tannins into gallic acid because of its cost-effectiveness [5]. Also, some rutin was left in the residue due to low water solubility [6]. Incidentally, the methods to transform rutin to isoquercetin have also been studied, including acid hydrolysis [7], heating [8], and microbial transformation [9], since it is useful in biological activities such as anti-mutagenesis, anti-virus, and anti-hypertension [10]. Because of *Aspergillus niger*'s ability to produce α-L-rhamnosidase [11,12], which is specific for releasing isoquercitin from rutin by derhamnosylation. Both gallic acid and isoquercitin and their derivatives are used in various industries, especially in the food and cosmetic industries [13]. SSF has mostly been tested and studied in laboratories using Erlenmeyer flasks or petri dishes [14–16]. Packed-bed bioreactors are an appealing and well-considered choice for scaling up SSF due to their simple structure and ease of operation; however, the critical factors such as heat accumulation from microbial metabolism, oxygen supply, and substrate moisture content must be taken into account [17,18]. Multi-layer packed-bed bioreactors, according to Chysirichote [19], improve heat transfer during operation. Because of its low water activity requirement, *A. niger* is better suited to SSF than SMF [20,21]. However, because it is a filamentous fungus, it is unsuitable for mixing fermentation because shear force will damage the mycelium and inhibit its growth [22].

According to the research of Chysirichote and Pakaweerachat [23], which indicated that the Triphala byproduct from extraction (TPB) supplemented with 0.75% sodium nitrate was appropriate to produce gallic acid and isoquercitin, the purposes of this research were to scale up the production of gallic acid and isoquercitin by a solid-state fermentation of *A. niger* in a 125-mL Erlenmeyer flask to 127-L packed bed bioreactor (PBB). The optimal aeration rate in the PBB for production and the optimal harvest time were also investigated.

2. Materials and methods

2.1. Microorganism

The inoculum was prepared using the method described by Pakaweerachat and Chysirichote [24]. *A. niger* ATCC 16888 used in this study was obtained from MicroBiologics Inc., USA, and cultured on potato-dextrose agar (PDA) for 3–5 days at 30 ℃. Fresh spores were collected by scraping with sterilized water. The number of spores in the suspension was counted using a neubuacer haemocytometer. The concentration of the spore suspension was then diluted to 1×10^5 spores/mL by adding sterilized water. The spore suspension was freshly prepared before use.

2.2. Solid substrates

Triphala, a byproduct of its extraction (TPB) was obtained from the Institute of Thai Traditional Medicine. It contained rutin (72.1 mg/g) and tannin (21.7 mg/g), which were determined using an HPLC system with the Water 717 Plus autosampler and an acetonitrile/1% acetic acid gradient (85:15) as a mobile phase. The flow rate was 1 mL/min and the detection was at 280 nm. The column was a Hypersil gold C18 column (250 mm × 4.6 mm, 5 μm), the injection volume of the TPB sample was 25 μL and the gravimetric method was described by Makkar et al. [25]. It was dried at 60 ℃ for 24 h and ground to less than 600 microns by a hammer mill. It was kept in a cool (10 ℃) and dark place. Before conducting a fermentation, the moisture content of TPB was adjusted the moisture content to 55% (w/w) using 0.75% sodium nitrate solution, which was used as a nitrogen source.

2.3. Solid state fermentation (SSF)

A 127-L bioreactor with a 45-cm diameter and 80-cm height for fermenting 32 L substrate was constructed, which was modified from the work of Chysirichote [19]. The substrate bed was separated into three layers using the screen tray, as shown in Figure 1. The height of each layer of f substrate was 12 cm. The bioreactor was constructed using three trays for separating the bed layer. Each tray contained 2 kg of TPB powder mixed with the spores of *A. niger* 5×10^5 spores/g. The air was forced from the bottom of the PBB to flow through the bottom to the top of the substrate bed in the PBB. The SSF was performed at 0.1, 0.3, and 0.5 vvm aeration for 120 h. The samples were collected at 12-h intervals from both trays, dried at 60 ℃ for 24 h, and milled into a powder for analysis. The fermented samples were sampled from three positions in each tray to calculate the average values. Each treatment was carried out in triplicate.

2.4. Fungal growth determination

Glucosamine content (*Glu*), an indirect indicator for biomass content, was analyzed using the method colorimetric of Chysirichote et al. [26]. The obtained values of glucosamine content in the fermented samples were converted to the biomass content by Eq 1, which was retrieved from the research of Pakaweerachat and Chysirichote [15]. Biomass content was plotted with time, and then the specific growth rate was calculated using an exponential equation during the growth phase, as in Eq 2.

$$X = 5.03 \times Glu, \tag{1}$$

where X and Glu are the biomass dry weight and the glucosamine content in the sample (g/g dry substrate).

$$X_t = X_0 e^{\mu t}, \tag{2}$$

where X is biomass dry weight (g/g dry substrate) in the exponential growth phase, μ is maximum specific growth rate (/h) and t is time (h).

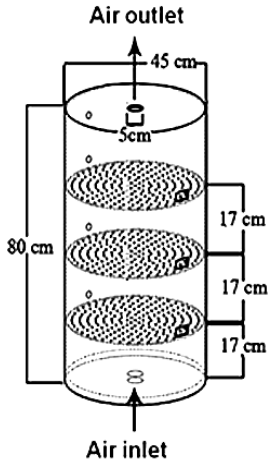

Figure 1. Schematic diagram of the three-layer packed-bed bioreactor used in this research.

2.5. Isoquercitin and gallic acid determination

The fermented sample was extracted and analyzed according to the methods of Chysirichote and Pakaweerachat [23]. Briefly, the sample was sonicated with methanol at 30 ºC for 30 min (at 40 kHz) to extract gallic acid. A HPLC system consisting of the Water 717 Plus autosampler, Waters 600E pump controller and Waters 2487 dual absorbance UV/Vis detector was used to carry out the analysis. The mobile phase was the gradient of acetonitrile and acetic acid (0.1%) in water (85:15). The flow rate was 1 mL/min. The detection was at 280 nm [27]. The column was a Phenomenex® Luna Omega C18 column (150 mm × 4.6 mm, 5 µm) and the injection volume of the aqueous sample was 10 µL. Prior to determining the isoquercitin and gallic acid, calibration plots were obtained using standard solutions of isoquercitin (Sigma-aldrich, Germany) and gallic acid (Sigma-aldrich, Germany) with concentrations ranging from 0 to 100 ppm, as shown in Figure 2(A),(B), respectively. Gallic acid and isoquercitin peaks appeared at a retention time of 2.40 and 3.00 min, respectively, as presented in Figure 2(C). The calibration curve was determined by linear regression in the range of 0–100 ppm. Therefore, the concentrations of isoquercitin and gallic acid in the sample were calculated by Eqs 3 and 4. The isoquercitin and gallic acid yields were calculated using Eqs 5 and 6, respectively.

$$\text{Isoquercitin concentration (ppm)} = [(\text{Peak area, AU}) + 5800.2]/2271.7, \tag{3}$$

$$\text{Gallic acid concentration (ppm)} = [(\text{Peak area, AU}) + 5102.5]/3583.5, \quad (4)$$

$$\text{Isoquercitin yield (\%)} = (\text{mg of isoquercitin/g of dry substrate}) \times 100, \quad (5)$$

$$\text{Gallic acid yield (\%)} = (\text{mg of gallic acid/g of dry substrate}) \times 100, \quad (6)$$

where the molecular weights of isoquercitin and rutin were 464.1 and 170.1 g/mol, respectively.

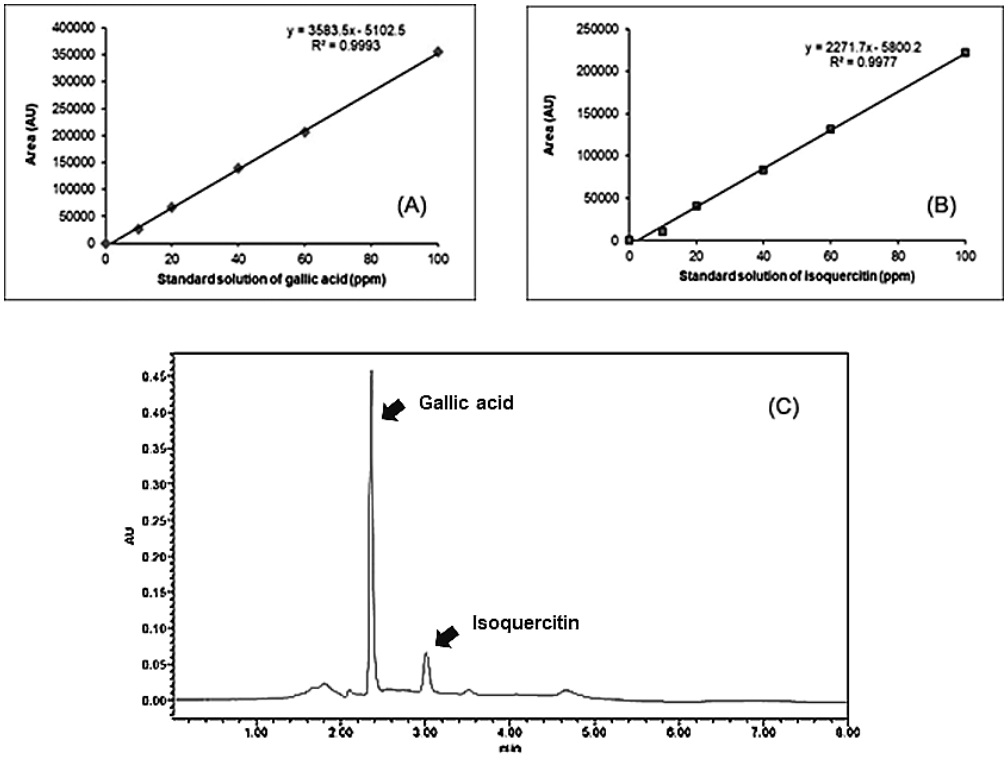

Figure 2. Calibration curves of (A) gallic acid and (B) isoquercetin. (C) the chromatogram of the fermented TPB, showing the peak at the retention time 2.40 and 3.00 min for the gallic acid and isoquercetin, respectively.

2.6. Data analysis

The Tukey test with analysis of variance (ANOVA) at the 95% significance level was used for analyzing the significance of the data. The regression optimization was conducted using Minitab 19.

3. Results and discussion

3.1. Fungal growth

The dry weight of the biomass analyzed from the fermented TPB shown in Figure 3 represented the fungal growth in the SSF with 0.1, 0.3, and 0.5 vvm aeration. At 0.1 vvm aeration, the fungal growth phase was extended until 48 h during SSF in all zones. The highest specific growth rate was

1.36 ± 0.02/h found in the bottom zone, as shown in Table 1. Not only were the growth rates in the SSF with 0.3 vvm (1.92 ± 0.03/h) and 0.5 vvm (1.96 ± 0.02/h) higher than in the SSF with 0.1 vvm, but the growth phase lasted longer. Therefore, the maximum biomass obtained from the SSF with 0.3 vvm (113.89 ± 11.39 mg/g in the middle zone at 60 h) and 0.5 vvm (117.59 ± 7.45 mg/g in the middle zone at 60 h) was higher than that obtained with 0.1 vvm (86.22 ± 7.83 mg/g in the bottom zone at 48 h). The fungal growth in the upper zones decreased with the height of the bioreactor because an increased amount of oxygen in the air was consumed in the lower ones, resulting in an insufficiency for its growth. The high fungal growth seemed to move to the upper zone when the aeration rate increased since the moisture from the lower zone moved to the upper one in accordance with the flow of air, resulting in a dehydration of the substrate [19,28].

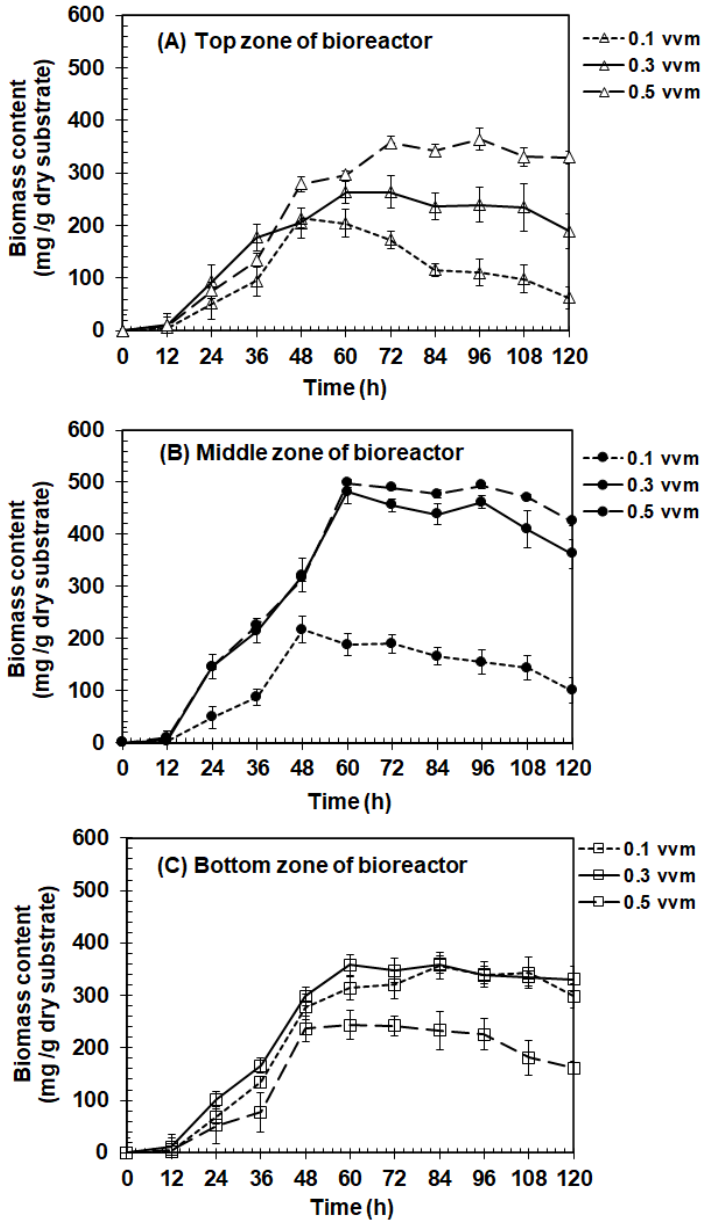

Figure 3. Biomass dry weights of *A. niger* during the SSF of TPB with different aerations.

Figure 4 clearly shows that the moisture transferred from the bottom zones to the upper ones. This explained that the lower fungal growth in the bottom zone occurred because the moisture content of the substrate in this zone was not suitable for the growth [24]. Also, moisture in the substrate affected both oxygen and nutrient transfers during the SSF, which are responsible for the growth of *A. niger* [29] because water mainly affected the swelling of the substrate and the effective nutrient absorption of fungi [30]. On the other hand, higher moisture contents resulted in a reduction of substrate porosity and a limitation of oxygen transfer within the substrate, resulting in poor growth [31,32].

Table 1. Specific growth rate, production rates of isoquercitrin and gallic acid on the SSF of *A. niger* with different aeration.

Aeration rate	Position of PBB	Specific growth rate (-/h)	Isoquercitrin production rate (-/h)	Gallic acid production rate (-/h)
0.1 vvm	Top	1.08 ± 0.01^{h}	1.41 ± 0.06^{a}	1.77 ± 0.09^{f}
	Middle	1.09 ± 0.02^{h}	1.14 ± 0.08^{c}	1.82 ± 0.07^{f}
	Bottom	1.36 ± 0.02^{e}	0.80 ± 0.09^{d}	2.12 ± 0.09^{e}
0.3 vvm	Top	1.22 ± 0.02^{f}	1.27 ± 0.09^{b}	2.27 ± 0.09^{d}
	Middle	1.92 ± 0.03^{b}	0.78 ± 0.06^{d}	3.12 ± 0.09^{a}
	Bottom	1.56 ± 0.03^{c}	0.53 ± 0.08^{e}	2.53 ± 0.08^{c}
0.5 vvm	Top	1.43 ± 0.02^{d}	0.93 ± 0.08^{d}	2.35 ± 0.09^{cd}
	Middle	1.96 ± 0.02^{a}	0.78 ± 0.07^{d}	2.75 ± 0.08^{b}
	Bottom	1.14 ± 0.04^{g}	0.45 ± 0.09^{e}	2.12 ± 0.07^{e}

Note: * Different superscript letters in the column represent the statistic difference.

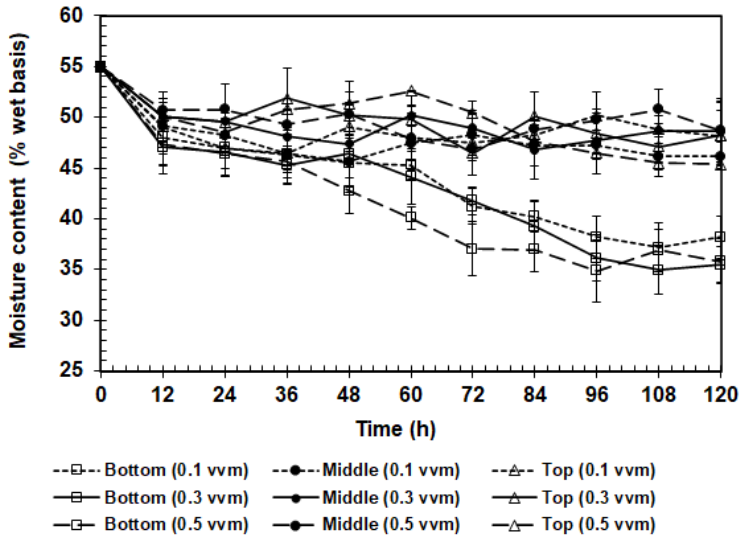

Figure 4. Moisture content of substrate during the SSF of TPB with different aeration rates.

However, the highest growth of *A. niger* in the SSF with 0.1 vvm was found in the bottom zone, even if the moisture content was still optimal. The fungi detected in the SSF with 0.1, 0.3, and 0.5 vvm

aerations had different morphologies, with the 0.1 vvm condition having abundant aerial mycelium (white mycelium on the surface of TPB particles). It was suggested that the oxygen content in the bioreactor was too low for some metabolisms of fungi, so that the fungi preferred a proration to other metabolisms [33]. Due to the moisture uniformity of the substrate and a sufficient oxygen supply along the packed bed bioreactor, the results indicated that 0.3 vvm was appropriate for biomass production. Furthermore, the small amount of aerial mycelium and high fungal growth found in the SSF with 0.3 vvm suggested that a significant portion of the biomass was substrate mycelium, resulting in hydrolase secretion and substrate utilization [19,34,35].

3.2. Isoquercitin production

Rutin is another active compound found in TPB, and it can be converted into isoquercetin and quercetin by the enzymes α-L-rhamnosidase and β-glucosidase that are produced by *A. niger* besides tannase [11,12]. The findings revealed a link between fungal growth and isoquercitin production. It showed that faster growth produced more isoquercetin, which was converted from rutin. According to Figure 5, isoquercitin increased until 48 h and then decreased to near zero.

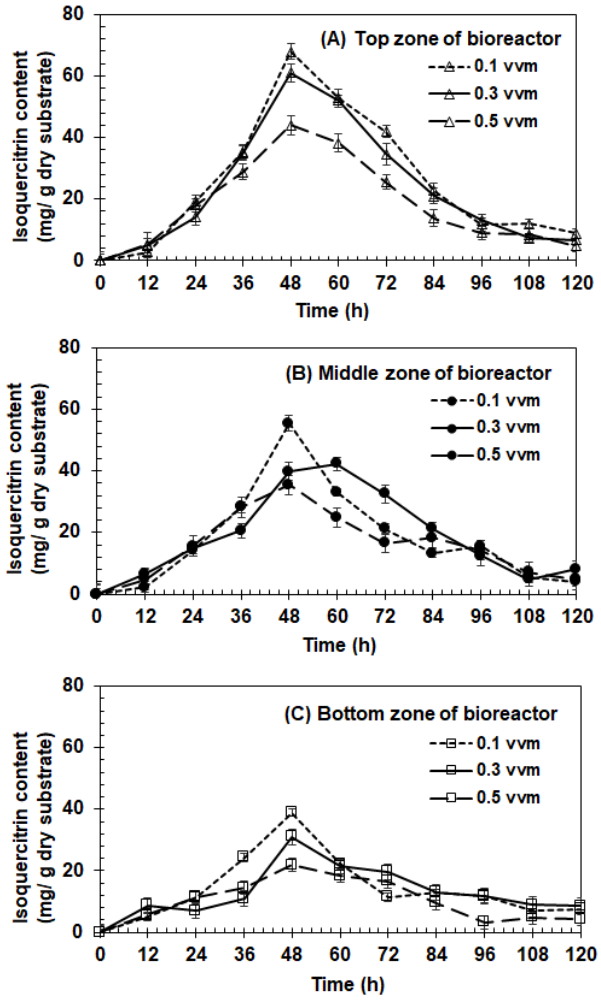

Figure 5. Isoquercitin production of *A. niger* during the SSF of TPB with different aeration rates.

Puri and Sukirti [36] and Puri [37] used naringinase, a multienzyme that consisted of α-L-rhamnosidase and β-glucosidase, to convert rutin to isoquercitin. α-L-rhamnosidase hydrolyzed rutin into isoquercitin and rhamnose, while β-glucosidase, that hydrolyzed isoquercitin to quercetin and glucose. When rutin was present in the substrate, the activity of α-L-rhamnosidase was higher than that of β-glucosidase; however, once rutin was depleted, the activity of β-glucosidase increased over that of α-L-rhamnosidase, resulting in the degradation of isoquercitin to glucose for metabolism [38]. This is why, despite some of it being converted into quercetin by β-glucosidase, isoquercitin continued to rise, potentially depleting rutin after 48 or 60 h. The results also showed that the upper zone was the zone with the highest isoquercitin content in all 0.1, 0.3, and 0.5 vvm aerations, with values of 67.97 ± 2.55, 60.95 ± 2.92 and 44.07 ± 3.17 mg/g, respectively.

The isoquercitin content result was consistent with the isoquercitin production rate (Table 1), with the highest values obtained from the top zone from 0.1 and 0.3 vvm aeration as 1.41 ± 0.06 and 1.27 ± 0.09/h, respectively, but on 0.5 vvm aeration as 0.78 ± 0.07/h, which was not significantly different from the upper zone. Beside the presence of rutin, the temperature change during SSF affected the activities of α-L-rhamnosidase and β-glucosidase because the optimum temperatures for them were 50 ℃ and 70 ℃, respectively. According to the work of Chysirichote [19] which used the same configuration of bioreactor, the temperature differences along the three layers were quite similar, and they reached the peaks of temperature when the growths were at their maximum. It was possible that the temperature rising for 48 or 60 hours due to heat generated by *A. niger* during a growth phase was suitable for activating α-L-rhamnosidase. When the temperature was higher and suitable for β-glucosidase, isoquercitin was converted to quercitin. Based on the capacity of *A. niger* for isoquercitin production in Table 2, the highest isoquercitin production related to α-L-rhamnosidase activity was detected at the bottom zone of the bioreactor because the temperature was suitable for α-L-rhamnosidase production at around 28 ℃. The statistical analysis shown in Table 3 indicated that only fermentation time affected the production of isoquercitin.

Table 2. Yields of isoquercitin and gallic acid per biomass on the SSF of *A. niger* with different aeration in the packed-bed bioreactor.

Aeration rate	Position of PBB	Isoquercitin/biomass (mg/g)	Gallic acid/biomass (mg/g)
0.1 vvm	Top	275.90 ± 8.62^{f}	$1{,}500.00 \pm 58.39^{e}$
	Middle	234.90 ± 5.03^{g}	$1{,}566.67 \pm 43.01^{e}$
	Bottom	525.50 ± 6.65^{d}	$1{,}863.34 \pm 24.90^{d}$
0.3 vvm	Top	492.20 ± 26.45^{e}	$2{,}986.67 \pm 47.85^{a}$
	Middle	662.00 ± 18.77^{b}	$2{,}573.13 \pm 60.68^{c}$
	Bottom	867.50 ± 14.57^{a}	$1{,}493.53 \pm 39.80^{e}$
0.5 vvm	Top	538.80 ± 15.14^{d}	$2{,}863.35 \pm 40.20^{b}$
	Middle	486.50 ± 34.94^{e}	$2{,}943.93 \pm 94.04^{ab}$
	Bottom	577.10 ± 10.96^{c}	$1{,}300.00 \pm 97.61^{f}$

Note: Different superscript letters in the column represent the statistic difference.

Table 3. Analysis of variance for the production condition of isoquercitin and gallic acid of *A. niger* on TPB.

Isoquercitin content					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Regression	3	11783.0	3927.68	33.27	0.000
Aeration (vvm)	1	98.3	98.29	0.83	0.374
Time (h)	1	3691.2	3691.18	31.27	0.000*
Aeration (vvm) × time (h)	1	207.5	207.52	1.76	0.201
Error	18	2125.1	118.06		
Total	21	13908.1			
Gallic acid content					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Regression	3	227832	75944.1	194.99	0.000
Aeration (vvm)	1	9	8.6	0.02	0.884
Time (h)	1	17011	17010.8	43.68	0.000*
Aeration (vvm) × time (h)	1	7569	7568.5	19.43	0.000*
Error	18	7010	389.5		
Total	21	234843			

Note: * represents significant factor on the production of isoquercitin and gallic acid of *A. niger* at 95% confidence level (n = 3).

3.3. Gallic acid production

Gallic acid contents analyzed from the fermented TW with different aeration rates (0.1, 0.3, and 0.5 vvm) are shown in Figure 6. The gallic acid production in the SSF with 0.1 vvm aeration was distinctly lower than the others. The SSF produced the most gallic acid, with 0.3 and 0.5 vvm as 3.12 ± 0.09 and 2.75 ± 0.08/h in the middle zone after 60 hours. The capacity of gallic acid production by biomass in the bottom zone, which was supplied with enough oxygen for growth resulting in low aerial mycelium (white mycelium), was clearly higher than those below (Table 2). Moreover, the enhancement of gallic acid was affected by the moisture content of the bed as detected in the middle and top zones of the SSF with 0.3 and 0.5 vvm since gallic acid was produced from both tannin hydrolysis and the shikimate pathway, which required oxygen.

However, forcing air at an aeration rate of 0.3 vvm results in higher gallic acid production. At 60 h, the middle zone had the highest gallic acid content of 187.23 ± 11.05 mg/g. It was implied that the proper aeration rate greatly increased fungal growth and led to increased production of tannase [39], hydrolase, and N-acetylglucosamine [40] affecting gallic acid production. From the results of all conditions, the fermentation time and the interaction of fermentation time and aeration statistically affected the gallic acid production, as shown in Table 3.

Forced aeration increased the oxygen partial pressure in the bioreactor, which was used for *A. niger* growth, and it also affected the gallic acid production. However, insufficient, or excessive aeration had a negative effect on the production, as low aeration caused heat accumulation in the bioreactor while too high aeration caused damage to *A. niger* mycelium by shear force. Low aeration in the upper zone, on the other hand, was ideal for isoquercitin production because it made it difficult for *A. niger* to grow, resulting in reduced β-glucosidase activity, which converted isoquercitin to

quercetin; as a result, this was the condition with the most remaining isoquercitin compared to the other zones.

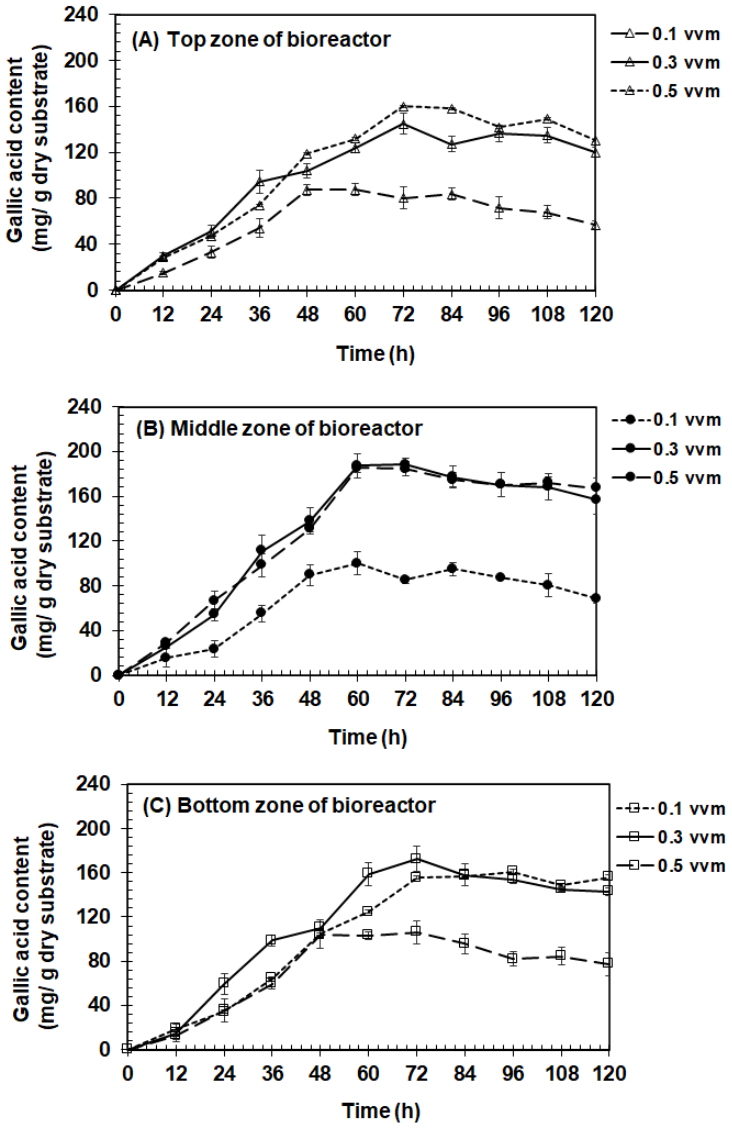

Figure 6. Gallic acid production of *A. niger* during the SSF of TPB with different aeration rate.

3.4. Harvesting design of isoquercitin and gallic acid

Due to the different production yields of isoquercitin and gallic acid in different zones of bioreactor (top, middle and bottom trays) and aerated at different rates, the method to harvest could be designed in different ways as shown in Table 4. It suggested that the fermented materials harvested from all trays after 48 h fermentation (0.1 vvm) provided the highest isoquercitin yield and the lowest gallic acid, whereas the highest gallic acid was obtained from the SSF with 0.3 vvm harvested at 60 h. The pattern of harvesting from the top, middle and bottom trays at 48 h, 60 h and 60 h from the SSF with 0.3 vvm aeration was recommended to get high contents of both isoquercitin and gallic acid.

Table 4. Amounts of isoquercitin and gallic acid obtained from the SSF with different aeration rates and different positions of trays placed in the packed-bed bioreactor (top/middle/bottom).

Aeration (vvm)	Harvesting design in different tray (top/middle/bottom)	Isoquercitin ($mg/g_{dry\ substrate}$)	Gallic acid ($mg/g_{dry\ substrate}$)
0.1	48 h/48 h/48 h	54.1 ± 1.92^{a}	93.8 ± 5.43^{c}
	48 h/60 h/60 h	41.1 ± 1.8^{bc}	104.0 ± 6.0^{bc}
	60 h/60 h/60 h	36.0 ± 1.9^{c}	104.0 ± 6.1^{bc}
0.3	48 h/48 h/48 h	43.9 ± 2.6^{b}	117.3 ± 8.3^{b}
	48 h/60 h/60 h	41.6 ± 2.2^{bc}	150.2 ± 9.2^{a}
	60 h/60 h/60 h	38.6 ± 1.8^{bc}	156.8 ± 8.7^{a}

Note: Different superscript letters in the column represent the statistic difference.

4. Conclusions

A triphala byproduct from extraction was studied as a main substrate for the production of gallic acid and gallic acid using *A. niger* in the packed-bed bioreactor. The rate of supplied aeration for fermentation was discovered to be important for fungal growth, but the interaction of fermentation time and aeration rate was found to be significantly important for gallic acid production. In addition, it was found that only fermentation time affected the isoquercitin content obtained from the SSF. The findings in this research could be used to design the harvesting time of the fermented material from each tray along the height of the multi-layered packed bed bioreactor.

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Conflict of interest

The authors declare that they have no competing interests.

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