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Correlation of oxygen consumption with growth and red pigment production of *Monascus purpureus* in solid state fermentation of rice

[1]*Chysirichote, T., [1]Sakoolkaew, P. and [2]Klinthong, W.

[1]*Department of Food Engineering, School of Engineering, King Mongkut's Institute of Technology Ladkrabang, Chalongkrung 1, Ladkrabang, Bangkok 10520, Thailand*
[2]*Greenday Global Co., Ltd., 90/38, Soi Boonmeesup, Bangplee-Tumru Rd., Bangpleeyai, Samut, Prakan 10540, Thailand*

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Abstract

Since aeration has a crucial role in mass and heat transfer during solid-state fermentation (SSF), the aim of the present work was to find out how it related to biomass and red pigment production of *Monascus purpureus* TISTR3615 during SSF. The fungal consumption of oxygen, one of the air's constituents important for fungal metabolism, was used to explain the relationship between the aeration rate, the fungal growth, and the red pigment production during the SSF of rice. Findings showed that at increased aeration rates, the fungus absorbed more oxygen, which boosted fungal growth but decreased red pigment production. Conversely, red pigment production increased when the fungus' oxygen consumption decreased. Regression equations with an acceptable R^2 were used to explain the relationship between *M. purpureus*' red pigment production and its oxygen consumption and aeration rate. The ideal oxygen concentration for increasing *M. purpureus*' capacity to produce red pigment was 0.0685 mmol/day/g of dry biomass, which produced red pigment at around 2,897 AU_{500nm}/g of dry biomass. However, the optimal oxygen consumption of the fungus for overall red pigment production (including growth impact) was 1.390 mmol/day/g of dry biomass, which produced red pigment at around 2,787 AU_{500nm}/g of dry fermented rice. The results of the present work could potentially be utilised in developing *M. purpureus*' red pigment production approach.

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Introduction

Natural red pigment extracted from red mould rice has been used as a food additive (Dufosse, 2018; Techaoei *et al.*, 2021) and colorant in cosmetic products (Manan *et al*., 2017; Koli *et al*., 2019; Silbir and Goksungur, 2019). Red mould rice has been traditionally produced by the solid-state fermentation (SSF) of rice by *Monascus purpureus* in a tray chamber (Farawahida *et al.*, 2022). Based on a previous work (Chysirichote *et al.*, 2011), the mycelium of *Monascus* spp. was composed of aerial mycelium growing on the surface of a solid substrate and substrate mycelium penetrating through the solid substrate, in which the ratio of aerial and substrate mycelium content was affected by the oxygen concentration in the fermentation environment. Also, the ratio affected the red pigment production because red pigment was produced from the substrate mycelium. Many studies (Said *et al.*, 2010; Chysirichote, 2016; Razali and Said, 2017; Duad *et al.*, 2021) found that aeration rate affected the red pigment production of *Monascus* spp. Yang *et al*. (2015) and Liu *et al*. (2018) studying submerged fermentation (SmF) found that increasing the rotating rate increased the red pigment production of *Monascus* spp., which was similar to increasing aeration in the solid-state fermentation (SSF) of Razali and Said (2017), while the work of Duad *et al*. (2021) indicated that too much aeration reduced red pigment production. Even if the aeration is important for oxygen supply, and heat and moisture transfers, especially in large-scale processes (Figueroa-Montero *et al*., 2011), few studies have examined oxygen consumption (Han and Mudgen, 1992; Kim *et al*., 2016). The aim of the present work was therefore to explain the relationship between oxygen consumption, red pigment production, and the growth

*Corresponding author.
Email: teerin.ch@kmitl.ac.th

of *M. purpureus* during the SSF of rice. The findings of the present work could be useful to understand the oxygen consumption of *M. purpureus* to produce the red pigment, and to be able to supply an appropriate concentration of oxygen for its pigment production and growth.

Materials and methods

Spore suspension preparation

A spore suspension of *M. purpureus* TISTR3615 was prepared for an inoculum by incubating the fungal strain for 7 d at 30°C on potato dextrose agar (PDA). The spores were collected by adding 3 mL of sterilised water to the sporulated culture, and scraping under aseptic conditions. The density of spores was measured using a haemocytometer. The spore density in the suspension was adjusted to 1×10^6 spores/mL by adding sterilised water.

Substrate preparation

The rice [*Oryza sativa*, moisture content = 10% (wet basis)] was purchased from a local market. Its moisture content was determined before analyses. The rice was then mixed with distilled water to obtain a final moisture content of 50% (w/w), including the initial moisture contained in the rice, and the moisture in the added spore suspension. Then, it was sterilised by autoclaving at 121°C for 20 min. After cooling to ambient temperature, it was inoculated with the spore suspension (1×10^6 spores/g_{DS}).

Experimental setup

Ten gram of the prepared substrate mixed with *M. purpureus* spores was placed in a 9 cm-diameter Petri dish (without a lid), and then placed in the UV-sterilised opaque plastic black box ($20 \times 20 \times 50$ cm^3). The rice bed thickness in each dish was 0.8 cm. Ten dishes were placed in the box. During fermentation, the humid air entered the box from the bottom, and exited at the top at 0.02, 0.05, 0.10, and 0.15 vvm (volume of air/volume of bed/minute). One dish was collected for analysis at 1-day intervals. After removing one dish, the aeration rate was decreased by 10% to obtain the constant aeration rate. The gas inlet and outlet were collected with a syringe at 1-day intervals, and transferred to a vacuumed glass vial to determine the oxygen concentration in the gas. Each study condition was conducted in triplicate.

Growth evaluation

Due to the strong connection between the fungal mycelium and solid substrate, the fungal biomass was determined by a measurement of glucosamine, which was used as an indirect biomass content (Chysirichote *et al*., 2013). To evaluate the biomass content, the factor for converting the content of glucosamine in biomass was identified. Briefly, *M. purpureus* was cultured in the rice synthetic medium containing rice flour at different concentrations (2, 4, and 6%, w/v) and solidified with 1.5% of agar. They were cultivated at 30°C for 7 d. The mycelium was collected by melting the cultured solid media in boiling water and filtering through No. 1 Whatman filter paper. They were washed with hot water three times to remove the growth medium, and obtain only the fungal mycelium (biomass). They were then dried for 24 h at 60°C to obtain the dry biomass.

Approximately, 10 mg of dry biomass (known actual weight) was determined for its glucosamine content using a colorimetric method (Chysirichote *et al*., 2021). In short, the sample was hydrolysed by soaking it for 24 h at room temperature in 1 mL of 60% (v/v) sulphuric acid. After that, the solution was heated for 1 h at 121°C, and diluted with deionised water to reach the final concentration of sulphuric acid at 0.5 mol/L. After neutralising the hydrolysate with 10 and 1 mol/L NaOH, the volume was adjusted with water to 100 mL. Next, 0.5 mL of the corrected solution was combined with 0.5 mL of 5% (w/v) $KHSO_4$ and 0.5 mL of 5% (w/v) $NaNO_2$. After 2 min of centrifugation at 1,500 *g*, 0.6 mL of supernatant was mixed with 0.2 mL of 12.5% (w/v) $NH_4SO_3NH_2$. For the colorimetric assay, 0.2 mL of 0.5% (w/v) methyl-2-benzothiazolinone hydrazone hydrochloride was added to the mixture, which was then heated for 3 min, and immediately cooled to room temperature before adding 0.2 mL of 0.5% (w/v) $FeCl_3 \cdot 7H_2O$. After 30 min in the dark, the absorbance of each mixture was measured at 650 nm using a spectrophotometer. The standard curve was constructed using a standard HCl-glucosamine solution (10 - 100 mg/mL). The value of the glucosamine content and biomass of *M. purpureus* (mg of glucosamine per g of dry biomass, mg_{Glu}/g_{DB}) was further used to calculate the biomass dry weight in the dry fermented rice.

The result of glucosamine in the dry biomass was used to convert the value of glucosamine content in substrate to the dry biomass weight in substrate

since glucosamine was found in the fungal mycelium only (not found in substrate). Therefore, the biomass dry weight of *M. purpureus* was calculated using Eq. 1.

$$m_{DB} = m_{Glu} / \text{conversion factor} \quad \text{(Eq. 1)}$$

where, m_{DB} = biomass dry weight (g), m_{Glu} = glucosamine content in the sample (mg/g), and the conversion factor ($0.0074 g_{DB} / mg_{Glu}$) obtained previously.

The biomass dry weight values were plotted against the fermentation duration, and their values during the growth phase were utilised to calculate the specific growth rate using an exponential equation, as shown in Eq. 2.

$$m_{DB,t} = m_{DB,0} \cdot e^{\mu t} \quad \text{(Eq. 2)}$$

where, $m_{DB,t}$ = biomass dry weight in the fermented rice (mg/g) at any time, $m_{DB,0}$ = biomass dry weight in the dry fermented rice (mg/g_{DS}) at the start (t = 0), and μ = growth phase specific growth rate (-/d).

Moisture content determination

The moisture content of the rice substrate and the fermented rice were determined to evaluate the dry weight of the substrate. About 1 g of a sample with a known accurate weight was placed in a hot air oven at 65°C for 24 h. The weight was measured after cooling the samples to room temperature in a desiccator. The moisture content was calculated from the ratio of the reduced weight to the initial weight of the sample.

Red pigment determination

The red pigment, produced on the surface and inside the fermented rice, was extracted by soaking 0.5 g of the dry ground sample in 50 mL of 95% ethanol at 30°C for 1 d in a flask covered with parafilm to avoid the evaporation of solvent. After centrifugation, the supernatant was measured for absorbance at 500 nm wavelength (Chysirichote *et al*., 2013) using a spectrophotometer (Model Genesys 10S; Thermo Fisher Scientific).

Oxygen consumption determination

The concentration of O_2 in the gas sample was analysed using gas chromatography and a thermal conductivity detector (GC-320, GL Science Inc.; molecular sieve 13X column; nitrogen gas carrier; 315 K of column temperature; 333 K of injector temperature; 353 K of detector temperature). The oxygen consumption of the fungus was calculated using Eq. 3, according to Smits *et al*. (1998):

$$O_{2consumed} = V_{air} (C_{O2,in} - C_{O2,out}) \quad \text{(Eq. 3)}$$

where, $O_{2consumed}$ = amount of consumed oxygen (mmol/d), V_{air} = volume of supplied air (mL/d), $C_{O2,in}$ = concentration of oxygen in the inlet air, and $C_{O2,out}$ = concentration of oxygen in the outlet air.

Statistical analysis

The results were presented as mean ± standard deviation. An analysis of variance (ANOVA) and Tukey's comparison tests were used to present the significance of the data at 95% confidence level. All statistical analyses was conducted using Minitab 19 software.

Results and discussion

Conversion factor for glucosamine content of biomass dry weight of M. purpureus

The glucosamine content in the biomass of *M. purpureus* obtained from the cultivation on the synthetic rice media containing 2, 4, and 6% ground rice was 134.0 ± 1.6, 135.1 ± 2.8, and 135.6 ± 0.5 mg/g, which were not significantly different. The conversion factor to convert the glucosamine content in the biomass dry weight of the fungus, 1 mg of glucosamine = 0.0074 g of dry biomass weight, led to its use as an indicator for biomass dry weight calculation using Eq. 4:

$$m_{DB} = 0.0074 m_{Glu} \quad \text{(Eq. 4)}$$

where, m_{DB} = biomass dry weight (g) and m_{Glu} = weight of glucosamine analysed from the sample with known weight (mg/g_{DB}).

Fungal growth

The SSF of *M. purpureus* on the rice was performed at constant aeration rates of 0.02, 0.05, 0.10, and 0.15 vvm throughout the fermentation. The fungal biomass production, as shown in Figure 1, revealed that exponential growth occurred from the first to the third day of fermentation. The lag phase was not observed. The biomass content becoming stable or starting the stationary growth phase was detected in the SSF with 0.05, 0.10, and 0.15 vvm of

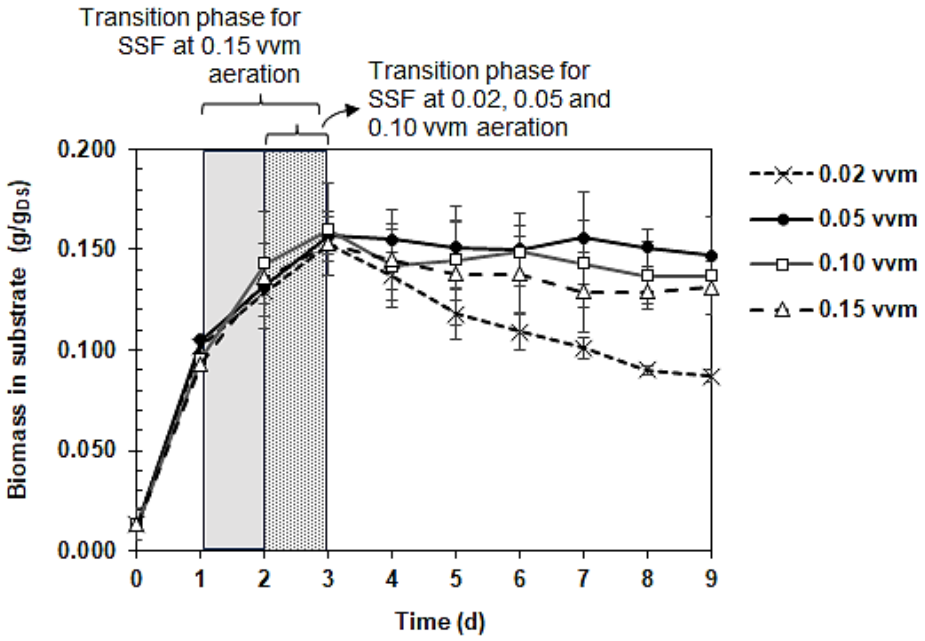

Figure 1. Biomass production of *M. purpureus* per weight of rice fermented at different aeration rates.

aeration, while the fungus cultured with 0.02 vvm of aeration immediately entered the death phase after reaching the maximum biomass content. It was most likely due to a lack of oxygen which caused autolysis (Xu *et al*., 2021), in which the organism produces enzymes and degrades itself (Giovannoni *et al*., 2020). The maximum biomass content and their specific growth rate obtained from the SSF with any aeration rates were quite similar, about 0.150 g/g_{DS} and 2.0/d, respectively. Nevertheless, it showed the death phase in the SSF with 0.02 vvm of aeration after day 3. This could have been due to the depletion of oxygen and nutrients (Vrabl *et al*., 2019). Moreover, the longer transition phase (day 0 - 3) was detected in the SSF with 0.15 vvm of aeration, while the other aeration rates were found from day 2 - 3. This indicated that a limitation of carbon sources and energy for the fungal metabolism occurred (Vrabl *et al*., 2019). The statistical analysis shown in Table 1A showed that both the fermentation time and the aeration rate affected the fungal growth.

Red pigment production

The red pigment was observed on the surface of the rice on day 2 of fermentation, and increased rapidly until day 7 before decreasing until day 9, due to oxidation caused by the higher concentration of left oxygen in the air, since the lower fungal growth, the less oxygen consumption (Chaudhary *et al*., 2021). The highest total red pigment content of about 2500 AU was found in the SSF under aeration conditions of 0.05 and 0.10 vvm at day 7, as shown in Figure 2A. The fungal ability to produce red pigment was expressed as the red amount per fungal dry weight, as shown in Figure 2B. The potential of *M. purpureus* to produce red pigment (red pigment production per biomass) was shown to be greater in the SSF with a lower aeration rate, although biomass production and fungal development were interrupted. Overall red pigment production was reduced in the SSF with low aeration rates (0.02 vvm) due to lower fungal growth. Although the high aeration rate (0.15 vvm) promoted fungal biomass (growth), it inhibited the ability to produce red pigment, as demonstrated in Figure 2B. High aeration was thought to be advantageous for fungal growth; however, it inhibited red pigment production.

Based on the statistical analysis shown in Table 1B, both aeration rate and fermentation time had substantial impact on red pigment production. Furthermore, their interaction factor had considerable effect on red pigment production. The regression equation, as indicated in Eq. 5, was able to predict red pigment production, yielding an adequate R^2 of 92.2%. The negative coefficient of aeration suggested that the rate of aeration was inversely proportional to red pigment production. The statistical analysis also revealed that the aeration rate and fermentation duration were important determinants in the ability of the fungus to produce red pigment. The interaction factors of aeration rate and fermentation duration, on the other hand, had no effect on the fungal ability to

Table 1. Analysis of variance.

A) Biomass production *versus* aeration rate and fermentation time

Source	DF	Adj SS	Adj MS	F-value	*p*-value
Regression	3	33.975	11.3250	93.86	0.000
Aeration rate (vvm)	1	7.331	7.3308	60.76	0.000*
Time (d)	1	4.618	4.6177	38.27	0.000*
Aeration rate × Time	1	2.198	2.1983	18.22	0.000*
Error	33	3.982	0.1207		
Total	36	37.957			

B) Ability of *M. purpureus* on red pigment production *versus* aeration rate and fermentation time

Source	DF	Adj SS	Adj MS	F-value	*p*-value
Regression	3	91193136	30397712	160.17	0.000
Aeration rate (vvm)	1	2295074	2295074	12.09	0.001*
Time (d)	1	38375086	38375086	202.21	0.000*
Aeration rate × Time	1	117	117	0.00	0.980
Error	33	6262800	189782		
Total	36	97455936			

C) Biomass production of the *M. purpureus versus* amount of consumed oxygen (mmol/d) and fermentation time

Source	DF	Adj SS	Adj MS	F-value	*p*-value
Regression	3	34.174	11.3912	99.37	0.000
Oxygen consumption	1	6.932	6.9325	60.47	0.000*
Time (d)	1	7.832	7.8325	68.32	0.000*
Oxygen consumption × Time	1	3.243	3.2432	28.29	0.000*
Error	33	3.783	0.1146		
Total	36	37.957			

D) Ability of *M. purpureus* on red pigment production *versus* amount of consumed oxygen (mmol/d) and fermentation time

Source	DF	Adj SS	Adj MS	F-value	*p*-value
Regression	3	91321767	30440589	163.89	0.000
Oxygen consumption	1	2409357	2409357	12.97	0.001*
Time (d)	1	44811297	44811297	241.07	0.000*
Oxygen consumption × Time	1	360011	360011	1.94	0.173
Error	33	6134169	185884		
Total	36	97455936			

*significant factor on the total content of biomass at 95% confident level.

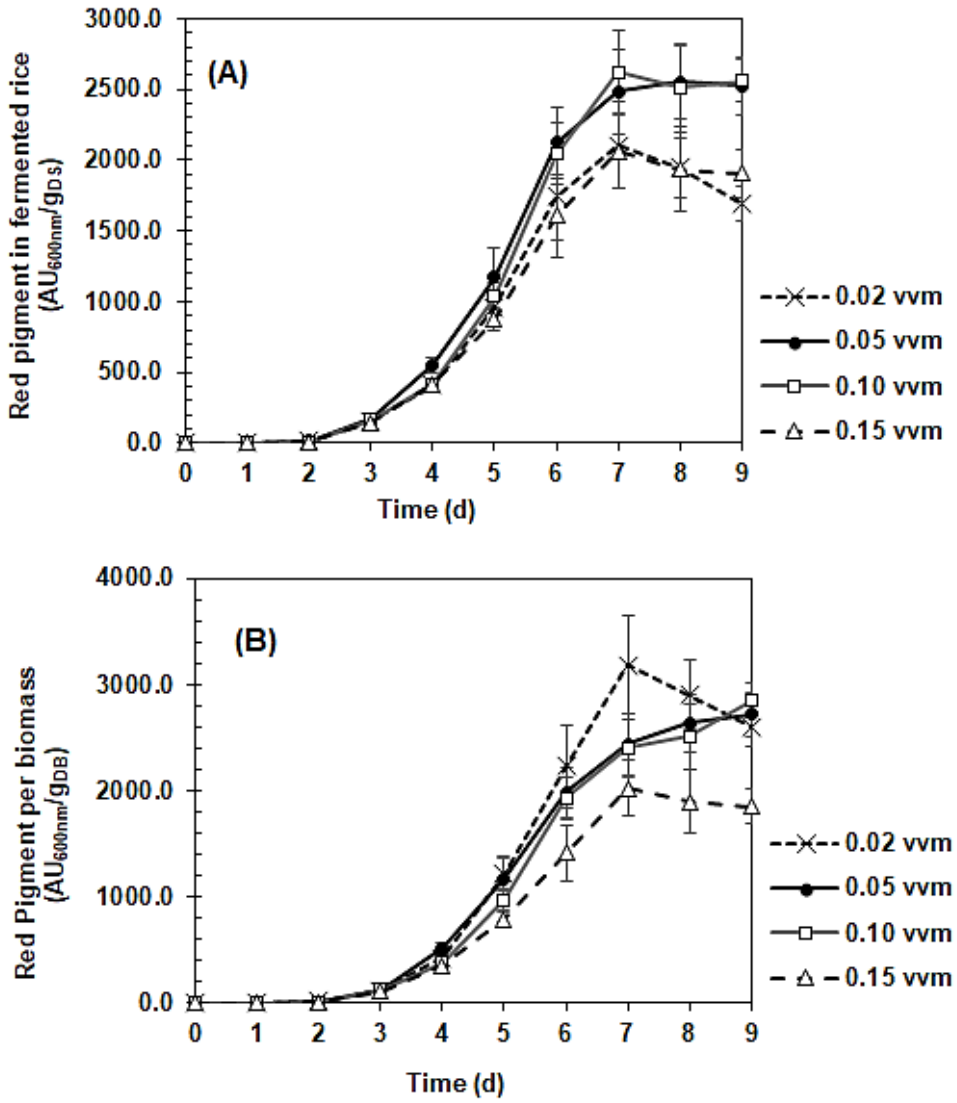

Figure 2. Red pigment production of *M. purpureus* during SSF on rice at different aeration rates: **(A)** red pigment production per one tray, and **(B)** red pigment production per dry biomass.

produce red pigment. In Eq. 6, which was obtained by the regression analysis, the inverse variation between the fungal ability to produce red pigment and aeration rate was also observed. The regression equation performed well, with an R^2 of 93.6%.

$$RP_{total} = 240.9\ t - 5014\ A + 800\ At \quad \text{(Eq. 5)}$$

where, RP_{total} = total red pigment production (AU), A = aeration rate (vvm), and t = time (d).

$$RP_{cell\ ability} = 348.7\ t - 5850\ A + 9\ At \quad \text{(Eq. 6)}$$

where, $RP_{cell\ ability}$ = fungal ability of red pigment production (AU/g_{DB}), A = aeration rate (vvm), and t = time (d).

Oxygen consumption of M. purpureus

Figure 3 displays the amount of oxygen consumed based on the concentration of oxygen in the fermentation chamber's input and output gases. It showed that increasing aeration rates enhanced *M. purpureus*' consumption of oxygen. The maximum amount of oxygen utilised in the SSF was recorded with aeration at 0.15 vvm, followed by 0.10, 0.05, and 0.02 vvm. The visual investigation revealed an abundance of aerial mycelium, interpreting the oxygen limit (Rahardjo *et al*., 2002) in the chamber to be 0.02 vvm of aeration.

The regression analysis of the fungal biomass production and the content of oxygen consumption shown in Table 1C revealed that the amount of oxygen consumed, fermentation time, and their

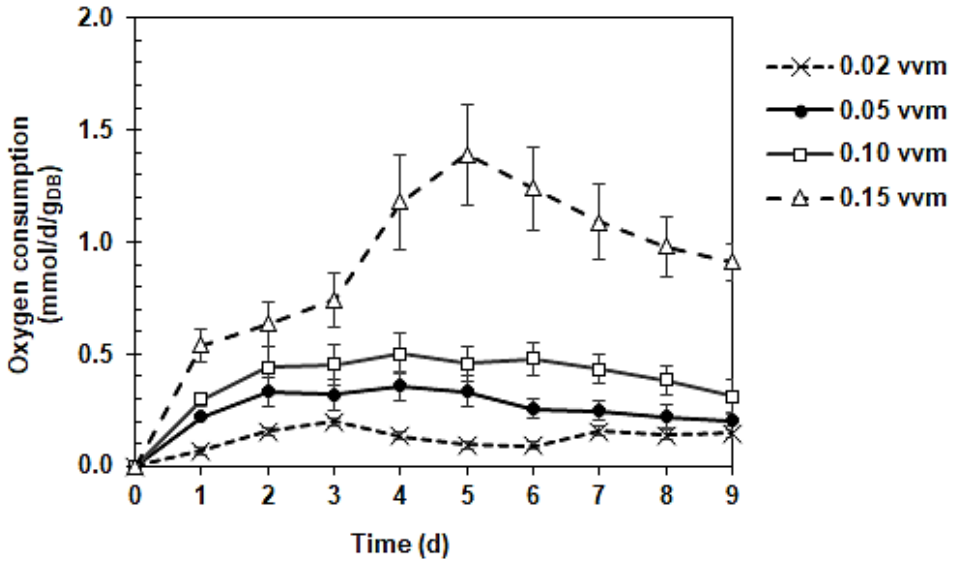

Figure 3. Amount of consumed oxygen of *M. purpureus* during SSF at different aeration rates.

interaction all had significant impact on fungal growth. Contrary to the findings of Arora *et al*. (2018) who found that the oxygen consumption rate was directly related to *Rhizopus oryzae* cell biomass production, the present work showed the impact of the growth phase and the amount of oxygen supply on the growth since they affected the content of oxygen consumption. This was supported by the work of Bastos *et al*. (2016) in which increased biomass and protein production using polyurethane foam as an inert support to increase oxygen transfer, since oxygen uptake affected enzyme production, which was required for fungal growth (Zhou *et al*., 2018; Abdella *et al*., 2020).

Table 1D shows the statistical analysis of red pigment production data and oxygen consumption content to determine the significant factors for red pigment production. It indicated that *M. purpureus* red pigment production was impacted by both single and multiple interactions of oxygen consumption and fermentation duration. The correlation was expressed using Eq. 6, which produced an R^2 of 93.4%. It was found that the influence of consumed oxygen content on red pigment production was completely different from the effect of aeration rate since negative coefficient of oxygen consumption was obtained in Eq. 3, while positive coefficient was obtained in Eq. 7. It was interpreted that the high oxygen consumption of the fungus decreased red pigment production, but the high aeration rate enhanced biomass production. To improve *M. purpureus'* ability to produce red pigment, conditions affecting fungal oxygen consumption should be considered, since low oxygen consumption increased red pigment production, as expressed by the negative coefficient in Eq. 8 (R^2 of 93.7%).

$$RP_{total} = 235.7t - 1213O_{2consumed} + 213.3O_{2consumed}\,t \quad \text{(Eq. 7)}$$

where, RP_{total} = total red pigment production (AU), $O_{2consumed}$ = amount of consumed oxygen (mmol/d), and t = time (d).

$$RP_{cell\ ability} = 320.7\,t - 1190\,O_{2consumed} + 98.4\,O_{2consumed}\,t \quad \text{(Eq. 8)}$$

where $RP_{cell\ ability}$ = fungal ability of red pigment production (AU/g_{DS}), $O_{2consumed}$ = amount of consumed oxygen (mmol/d), and t = time (d).

The results of oxygen consumption and aeration rate during SSF were used to optimise the conditions appropriate for red pigment production of *M. purpureus* as shown in Table 2. SSF of rice with aeration to regulate fungal oxygen consumption at 0.0685 mmol/d/g_{DB} was suitable for red pigment production.

Conclusion

The rate of aeration during fermentation had a significant impact on *M. purpureus* growth and red pigment production. The high aeration rate increased the ability of *M. purpureus* to consume oxygen during fermentation. The present work demonstrated that the high oxygen consumption of the fungus promoted its

Table 2. Multiple response prediction for maximum red pigment production.

Variable	Setting			
Oxygen consumption (mmol/d/g_{DB})	1.390			
Time	9			
Response	**Fit**	**SE Fit**	**95% CI**	**95% PI**
Total red pigment (AU_{500nm}/g_{DS})	2,787.17	347	(2082, 3492)	(1727, 3847)
Variable	**Setting**			
Oxygen consumption (mmol/d/g_{DB})	0.0685			
Time	9			
Response	**Fit**	**SE Fit**	**95% CI**	**95% PI**
Red pigment / biomass (AU_{500nm}/g_{DB})	2,897	156	(2580, 3215)	(2023, 3772)

growth, but reduced red pigment production. Therefore, the important key to red pigment production was the reduction of the fungal capacity to consume oxygen during the fermentation. The optimum oxygen consumption of *M. purpureus* for red pigment production on rice in a tray-type bioreactor was 0.0685 mmol/d/g_{DB}, which could be achieved by the appropriate aeration rate.

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