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Aeration and agitation strategies for the improvement of red pigment production by *Monascus purpureus* FTC 5391

(Strategi pengudaraan dan pemutaran untuk membaiki penghasilan pigmen merah oleh *Monascus purpureus* FTC 5391)

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Key words: *Monascus purpureus,* agitation speed, aeration, non-growth associated

Abstract

The influence of agitation speed and aeration rate on mycelial morphology and red pigment production of *Monascus purpureus* FTC 5391 was investigated in 2-litre stirred tank bioreactor. At agitation speed of 400 rpm or less, serious mycelia aggregation and fluffy pellets were present with pigment yields of approximately below 0.27 UA_{500}/g.litre. At this speed, *M. purpureus* FTC 5391 formed long hairy mycelia, resulting in the formation of viscous cultures. As the agitation speed increased from 200 to 1,000 rpm, the K_La value also increased gradually. The specific oxygen uptake rates (QO_2) at 800 and 1,000 rpm were lower than the rate at 600 rpm.

Mycelia had short branches and appeared to be damaged by mechanical shear forces due to high agitation speed. The dissolved oxygen tension (DOT) levels were varied within the ranges of 30–100% saturation in fermentation with agitation speed fixed at 600 rpm. The maximum cell concentration of *M. purpureus* FTC 5391 was about 13.2 g/litre and yield of red pigment production of about 0.411 UA_{500}/g.litre was achieved with DOT of 100% saturation. The highly branched cell morphology and vacuolated cell morphology were observed in a high DOT level (100% saturation). Fermentation was successfully developed in 2-litre stirred tank bioreactor based on a constant agitation speed of 600 rpm and DOT levels higher than 90% saturation, which was the best condition for pigment production and cell morphology.

Introduction

Microorganisms are normally varied in their oxygen requirement. The largest utilization of oxygen during growth of aerobic microorganisms is for respiration. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities (Forage et al. 1985). Productivity of several fermentations is limited by oxygen availability and therefore, it is important to consider the factors, which affect the efficiency in supplying microbial cells with oxygen (Standbury and Whitaker 1984).

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Agitation provided in the fermentation process affects the mycelium formation. Pigment production is proportional to mycelial growth. The pigment production in stirred tank fermenter is high at high agitation rates (Yoshimura et al. 1975). In fermentation with agitation speed of 300 rpm, the mycelial growth is in the form of pulp and the pigment production is low. At agitation speed of 600 rpm and 800 rpm, the mycelial growth is in the form of intermediate pulp and pellet, where pigment production is substantially high. However, when the agitation speed is increased to 1,200 rpm, growth of the fungus is mainly in pellet form and the pigment production dropped to about half compared to fermentation at 600–800 rpm. The high agitation speed provides high shear rates that will cause the mycelium of the fungus to split, which in turn, reduce pigment production (Yoshimura et al. 1975).

Krairak et al. (1999) reported that at low agitation speed, sedimentation of cell was observed. Therefore, the agitation speed must be sufficiently high to obtain cell culture in the form of cell suspension. Moreover, increase in cell concentration during fermentation causes high broth viscosity. Higher agitation speed is necessary during fermentation to avoid cell sedimentation especially for growth in pellet form and high cell concentrations. Krairak et al. (1999) also suggested that during *Monascus* fermentation, agitation speed at 100 rpm should be used at early stage of fermentation and increased to 250 rpm (at maximum cell growth) for improvement of red pigment fermentation.

Pigment production is greatly influenced by medium composition and oxygen supply (Suh and Shin 2000). The *Monascus* species requires sufficient aeration and therefore submerged cultivation can proceed only in shaken, preferably baffled flasks (Juzlova et al. 1994) or in a well-stirred and aerated fermenter such as fermenter equipped with an airlift system (Malfait et al. 1981). The mass transfer of oxygen to the aqueous phase of the fermentation volume is improved significantly through the use of airlift fermenter (Malfait et al. 1981). Shear forces that may destroy the mycelium can be overcome by using roller bottles (Mak et al. 1990).

To understand how pigment production in *Monascus* cultures was regulated, the effects of agitation speeds and dissolved oxygen on growth of *M. purpureus* FTC 5391 and red pigment production were studied. The main objective of the study was to find the optimal agitation and aeration strategies for the improvement of red pigment production.

Materials and methods

Microorganism and medium

The monospore isolate MP 3 of *M. purpureus* FTC 5391 (Musaalbakri 2004), was used throughout this study. The fungus was maintained on potato dextrose agar (PDA) plate for 7 days at 37 °C. The inoculum medium (YMP broth) consisted of yeast extract (3 g/litre), malt extract (3 g/litre), peptone (5 g/litre) and glucose (20 g/litre). Four pieces of 4 mm mycelial blocks of *M. purpureus* FTC 5391 were used to inoculate the inoculum cultures in 250 ml flask containing 100 ml YMP broth. The flasks were incubated in orbital shaker at 37 °C, agitated at 250 rpm for 4 days. The fermentation medium consisted of glucose as a carbon source (50 g/litre), monosodium glutamate – MSG (12 g/litre), K_2HPO_4 (2.5 g/litre), KH_2PO_4 (2.5 g/litre), $MgSO_4.7H_2O$ (1.0 g/litre), KCl (0.5 g/litre), $ZnSO_4.7H_2O$ (0.01 g/litre), $FeSO_4.7H_2O$ (0.01 g/litre) and $MnSO_4.H_2O$ (0.03 g/litre).

Fermentation in 2-litre stirred tank bioreactor

The 2-litre stirred tank fermenter (Biostat B. Braun, Germany) with a working volume of 1.5 litres was used in this study. The agitation of the culture was achieved using two 0.052 m diameter six bladed Rushton turbine impellers. The agitation speed was

set at 200 rpm (impeller tip speed = $2\pi ND_i$ = 1.09 m/s), 400 rpm (impeller tip speed = $2\pi ND_i$ = 2.19 m/s), 600 rpm (impeller tip speed = $2\pi ND_i$ = 3.27 m/s), 800 rpm (impeller tip speed = $2\pi ND_i$ = 4.36 m/s) and 1,000 rpm (impeller tip speed = $2\pi ND_i$ = 5.45 m/s). In all experiments, the DOT control level was set at 90% saturation by varying air flow rate ranged from 0.1 litre/min (0.067 vvm) to 1.5 litres/min (1.0 vvm) using mass flow controller. During the fermentation, pH of the culture was measured using Ingold pH probe, which was calibrated using pH buffer at 4.0 and 7.0 prior to the fermenter sterilization (121 °C, 20 min). The initial pH of all media was adjusted to 6.5 and recorded throughout the experiment.

A polarographic dissolved oxygen probe (Ingold) was used to measure the DOT levels. The calibration was conducted after sterilization by sparging the medium with oxygen free nitrogen for 0% reading followed by air until saturation was achieved at 100% saturation. To study the effect of DOT level, the agitation speed was fixed at 600 rpm (impeller tip speed = $2\pi ND_i$ = 3.27 m/s) and DOT was controlled at 30, 60, 80 and 100% saturation throughout the fermentation, by varying the air flow rate at a range of 0.1 litre/min (0.067 vvm) to 1.5 litres/min (1.0 vvm) using mass flow meter. The DOT levels were recorded continuously during the fermentation.

The relationship between cell growth, red pigment production and cell morphology of mycelium as a function of different agitation speeds (200, 400, 600, 800 and 1,000 rpm) was investigated. To prevent mycelium floatation and adhesion on the inner wall of bioreactor, the agitation speed was set at 200 rpm during the first 12 h of cultivation and then adjusted and maintained at the required speed for the rest of the fermentation time. All experiments were performed at least in duplicate using a 2-litre B. Braun stirred-tank fermenter.

Determination of cell concentration

Cell concentration was determined using filtration and oven dry method. A known volume of culture sample (3–5 ml) withdrawn from the shake flask and fermenter was filtered through a pre-weighed filter paper (Whatman No. 1) by using vacuum pump. After drying period of more than 24 h in an oven at 80 °C i.e., until a constant weight was achieved, the filter paper and cells were re-weighed and the cell dry weight was calculated by difference.

Glucose analysis

Glucose concentration in the culture broth was measured by using Glucose Analyzer (YSI 2700 Select Biochemistry Analyzer). Samples were prepared by filtering the supernatant through sep – pack to remove the particles and pigments that may interfere with the determination.

Determination of red pigment

Samples collected during the fermentation were centrifuged at 3,000 rpm for 10 min using laboratory centrifuge (Centrifuge 5810R, Germany). The red pigment was present in both fractions, filtrate and cell pellet. In order to measure red pigment in cell pellet, extraction of the pigment was carried out using 95% ethanol. The method of extraction used was as follows: 10 ml of ethanol was added to 1 g wet cell in 20 µl test tube, shaken for a while and then kept at room temperature overnight. The mixture was then filtered through a filter paper (Whatman No. 1) and the filtrate was used for pigment determination.

For measurement of absorbance for filtrate from culture broth, uninoculated medium was used as blank while for filtrate from the extract, ethanol was used as blank. The wavelength at 500 nm represents absorption maxima for the red pigment. Whenever necessary, the samples were diluted with distilled water (filtrate) or ethanol (extract) prior to absorbance measurement. The pigment production was

calculated by multiplying the absorbance units by the dilution factor. The spectra of the red pigment were measured using a Cecil CE 2502 2000 series scanning spectrophotometer.

Determination of ethanol

Ethanol was measured using high performance liquid chromatography (HPLC) (Waters 2690, USA) with refractive index detector (Waters 2410). The separation of ethanol was obtained by using Biorad aminex HPX-87H cation-exchange resin column (300 x 7.8 I.D.) as stationary phase. The mobile phase was 7 mM sulfuric acid (H_2SO_4). Flow rate of the mobile phase and column temperature was controlled at 1.0 ml/min and 50 °C respectively. Samples were prepared by filtering fermentation broth with nylon filter paper (Whatman 0.2 µm pore size, 47 mm diameter). The concentration of ethanol was quantified using a pre-determined calibration curve.

Determination of QO_2 and K_La values

The measurement of specific oxygen uptake rate *(QO_2)* and oxygen transfer rate (K_La) during the fermentation was carried out using the dynamic gassing out technique (Taguchi and Humphrey 1966). It is based on the respiratory activity of organisms which are actively growing in the fermenter.

Image analysis of cells

To analyse morphologic images of *M. purpureus* cells, culture broths were randomly sampled six times. For light microscope completed with image analysis (LEICADMLAB) mycelial observations, approximately 50 µl of culture broths containing mycelia was placed on a glass slide with a cover glass.

Mathematical model

The following simplified batch fermentation kinetic models for cell growth, substrate consumption and product formation based on Logistic and Luedeking-Piret equations, which have been described, elsewhere (Weiss and Ollis 1980) were used to evaluate the kinetics of red pigment production by *M. purpureus* FTC 5391. The fungus, *M. purpureus* FTC 5391, employed in pigment fermentation was very stable as shown by the high viability, even at extreme growth conditions. From preliminary kinetics study, it was found that growth of *M. purpureus* FTC 5391 followed logistic equation. Thus, the simplified batch fermentation kinetic models for cell growth, substrate consumption and product formation based on logistic and Leudeking-Piret equation, are proposed for red pigment fermentation by *M. purpureus* FTC 5391 and are expressed as follows:

Cell growth	$dX/dt = [\mu_{max}(1 - X/X_{max})]X$	(1)
Substrate consumption	$-dS/dt = \alpha(dX/dt) + \beta X$	(2)
Product formation	$dP/dt = m(dX/dt) + nX$	(3)

The batch fermenter models (equations 1–3) were fitted to the experimental data by non-linear regression with MS EXCEL computer software. The model parameter values were evaluated by solving equations (1–3). The predicted values were then used to simulate the profiles of cell, substrate and product concentrations during the fermentation. To determine whether the deviations between the experimental and calculated data are significant or not-significant, statistical analysis (unpaired T-test) was also carried out by using SAS program (SAS Inst. 1990).

Results and discussion

Effect of agitation speed

The time courses of red pigment production by *M. purpureus* FTC 5391 at different agitation speeds are shown in *Figure 1.* In this set of experiment, the fermentation was carried out at different agitation speeds (200–1,000 rpm) and the DOT control level was set at 90% saturation by varying air flow rate ranged from 0.1 litre/min (0.067 vvm) to 1.5 litres/min (1.0 vvm).

Different growth profiles were obtained at different agitation speeds *(Figure 1A).* In all cases, growth was increased

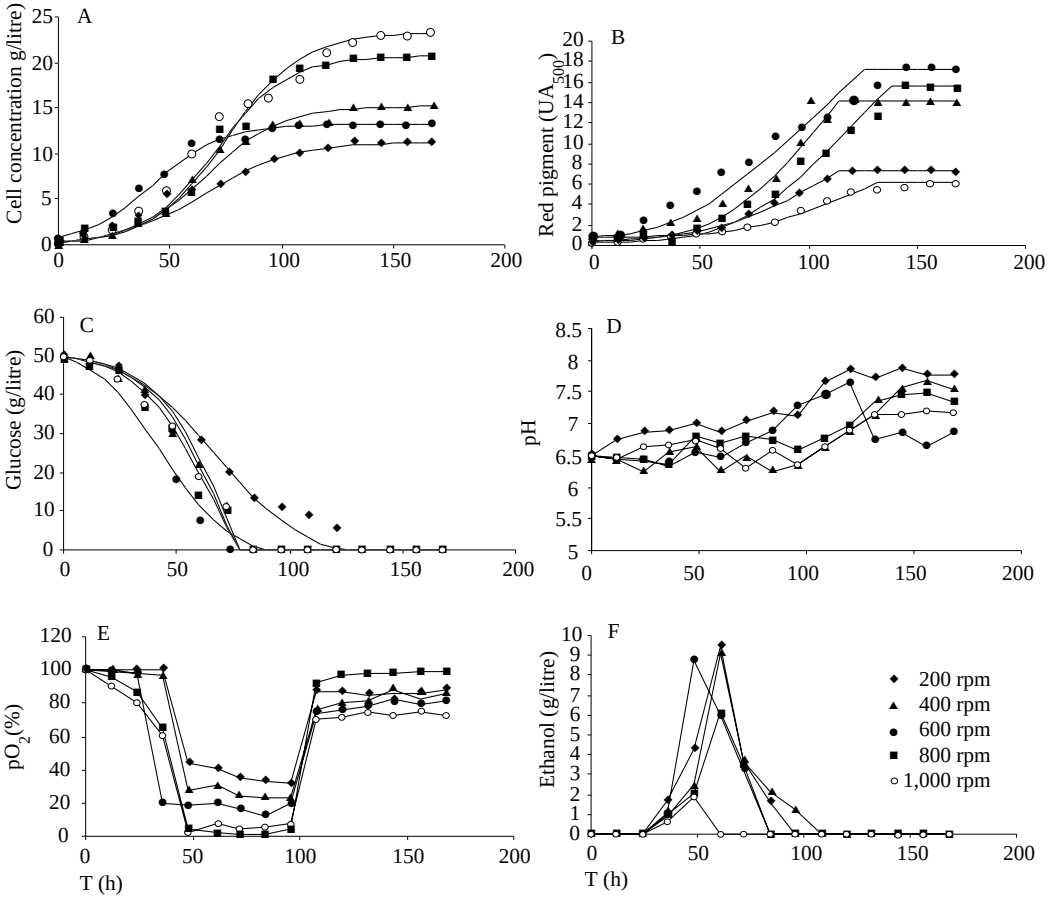

*Figure 1. Batch red pigment fermentation by **Monascus purpureus** FTC 5391 in 2-litre stirred tank fermenter using different agitation speeds which also includes the comparison between the calculated experimental data*

exponentially with time in all cultures studied with more or less the same value of maximum specific growth rate (μ_{max}), ranged from 0.05 to 0.065/h during the 84 h of fermentation. In cultures with an agitation speed of 1,000 and 800 rpm, the cell growth increased with time until it reached 23.3 g/litre and 20.73 g/litre respectively after 132 h and remained constant towards the end of the fermentation. On the other hand, in cultures with an agitation speed of 600 rpm, the cell growth increased with time until it reached 13.2 g/litre after 96 h and remained constant towards the end of the fermentation. In fermentation carried out at 400 rpm, no further increased in cell growth was observed after 132 h and the cell concentration of about 14.99 g/litre was constant for the rest of the fermentation. On the other hand, at 200 rpm, cell growth continued up to 84 h reaching the maximum concentration of 11.7 g/litre and remained constant towards the end of fermentation.

The agitation speed was inversely proportional to cell growth. Although the agitation speed did not show a strong influence on growth rate during fermentation, the agitation speed played a significant role in red pigment production. Maintenance of high DOT levels is important for high yields of red pigment production by *M. purpureus* FTC 5391 fermentation. Thus, the culture broth viscosity should be kept low throughout the

fermentation. This may be achieved by controlling the cell morphology by varying the agitation speed.

After a lag phase of about 10–15 h, red pigment was produced with different rates depending on the agitation speed *(Figure 1B).* Red pigment production was increased from 200 to 600 rpm and decreased when the agitation speed was increased up to 1,000 rpm. When the agitation speed was controlled at below 400 rpm, yield for red pigment production was low and growth in the form of fluffy pellets and aggregated mycelia were observed. As a result, culture broth viscosity was very high and DOT was reduced to very low levels. The pigment yield increased with increasing agitation speed up to 600 rpm, at which the maximum red pigment (P_{max}) and ($Y_{p/s}$) yield of 20.63 UA_{500} and 0.411 UA_{500}/g.litre respectively was obtained at DOT levels of 90% saturation with the highest cell efficiency to produce red pigment (P_{max}/X_{max}) 1.563 UA_{500}/g.litre. It is important to note that the pigment yields at 800 and 1,000 rpm were reduced significantly.

The profile of volumetric oxygen transfer rate (K_La) and specific oxygen uptake rate (QO_2) during fermentation carried out at different agitation speeds are shown in *Table 1*. As the agitation speed increased from 200 to 1,000 rpm, the (K_La) value decreased gradually. At agitation speeds of 800 and 1,000 rpm, the DOT levels were maintained at a value lower than 10% saturation. Despite the fact that DOT levels and K_La values at 800 and 1,000 rpm were slightly higher than those values measured at 600 rpm, the pigment yields at 800 and 1,000 rpm were lower than the yield at 600 rpm, suggesting that there are other key factors involved that influenced red pigment production.

The highest specific oxygen uptake rate was obtained at 600 rpm *(Table 1)*. As the agitation speed increased to 800 rpm or higher, the QO_2 value decreased by approximately relative to the value at 600 rpm. At rates less than 400 rpm, the specific oxygen uptake rate was reduced by 10% of the value measured at 600 rpm, resulting in reduced cell growth and pigment yield.

Rate of oxygen transfer is directly proportional to the driving force for transfer and the area available for the transport process to take place (Charles and Wilson 1994). For each fluid on either side of the phase boundary, the driving force for oxygen transfer can be expressed in terms of concentration difference. Since oxygen is one of the limiting reactant in the present process, a high level of dissolved oxygen will result in a higher oxygen uptake rate and thus a higher production rate of red pigment as in the case of high agitation speed culture. A higher oxygen uptake rate should lead to a more effective oxygen transport since oxygen is supplied continuously at a constant rate (Charles and Wilson 1994).

Table 1. The profile of volumetric oxygen transfer rate *(K_La)* and specific oxygen uptake rate (QO_2) during red pigment fermentation by *Monascus purpureus* FTC 5391 in stirred tank fermenter operated at different agitation speeds. [DOT was controlled at 90% saturation and air flow rate was set at 0.1 litre/min (0.067 vvm) to 1.5 litres/min (1.0 vvm)]

Agitation speed (rpm)	Impeller tip speed, $2\pi ND_i$ (m/s)	Volumetric oxygen transfer rate, K_La (h^{-1})			Specific oxygen uptake rate, QO_2 (mg O_2/g.cell/h)		
		12 h	24 h	48 h	12 h	24 h	48 h
200	1.09	3.12	0.25	0.09	0.0055	0.0042	0.0025
400	2.19	3.21	0.53	0.27	0.0085	0.0063	0.0048
600	3.27	3.59	2.99	1.97	0.029	0.0285	0.025
800	4.36	3.32	2.6	1.48	0.0264	0.024	0.021
1,000	5.45	3.25	2.26	0.9	0.0175	0.0165	0.0128

The specific oxygen uptake rate (QO_2) at 600 rpm was higher than the rates at 800 and 1,000 rpm, despite the fact that the results for oxygen transfer rate were opposite. This result suggested that the presence of mechanical cell damage was caused by the strong shear stress at high agitation speeds. This was supported by cells grown at different agitation speeds when observed microscopically. As shown in *Plate 1*, cells grown at 200 and 400 rpm consisted fluffy pellets and aggregated mycelia. As the agitation speed increased to 600 rpm, the mycelia were shortened and less branched. However, at 800 and 1,000 rpm, a large number of short, seriously damaged mycelia fragments were observed. This was confirmed by determining the amount of red pigment in cultures with different agitation speeds. At the optimum agitation speed of 600 rpm in a 2-litre stirred tank fermenter, high cell activities and low viscosities can be maintained, leading to high pigment yields.

The kinetic parameter values calculated by the models for fermentation in a 2-litre stirred tank fermenter can be used to describe the kinetics of the production of red pigment by *M. purpureus* FTC 5391 *(Table 2)* by using different agitation speeds. According to the proposed models (Equations 1–3), the calculated data fitted to the experimental data of fermentation at different agitation speeds *(Figure 1A–C)*. In most cases the calculated data fitted well to the experimental data with more than 95% confidence. In all cases, the values of *m* were zero while the value of *n* was not zero. The values of α were not significantly different at different agitation speeds. These

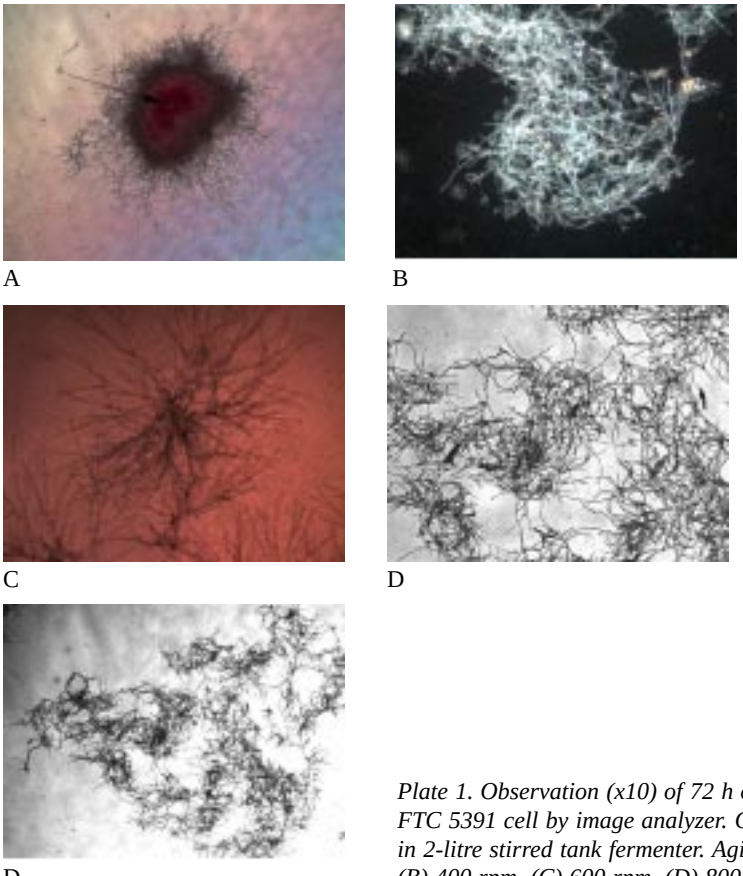

Plate 1. Observation (x10) of 72 h old ***Monascus purpureus*** *FTC 5391 cell by image analyzer. Cultivations were carried out in 2-litre stirred tank fermenter. Agitation speed: (A) 200 rpm, (B) 400 rpm, (C) 600 rpm, (D) 800 rpm and (E) 1,000 rpm*

Table 2. Comparison of the performance and kinetic parameter values of red pigment fermentation in 2-litre stirred tank fermenter using different agitation speeds. [DOT was controlled at 90% saturation and air flow rate was set at 0.1 litre/min (0.067 vvm) to 1.5 litres/min (1.0 vvm)]

Kinetic parameter values	Agitation speed (rpm)				
	200	400	600	800	1,000
Impeller tip speed $2\pi ND_i$ (m/s)	1.09	2.19	3.27	4.36	5.45
X_{max} (g/litre)	11.7	14.99	13.2	20.73	23.3
X_0 (g/litre)	0.37	0.28	0.25	0.26	0.25
μ_{max} (h^{-1})	0.0512	0.06	0.065	0.062	0.060
P_0 (UA_{500})	0.482	0.394	0.385	0.375	0.40
P_{max} (UA_{500})	7.16	13.90	20.63	15.47	5.90
$Y_{x/s}$ (g cell/g glucose)	0.227	0.294	0.248	0.410	0.461
P (g cell/litre.h)	0.080	0.111	0.077	0.155	0.137
$Y_{p/s}$ (UA_{500}/g.litre)	0.150	0.270	0.411	0.302	0.110
P (UA_{500}/h)	0.056	0.113	0.122	0.105	0.033
P_{max}/X_{max} (UA_{500}/g.litre)	0.612	0.927	1.563	0.746	0.253
α (g glucose/g cell)	4.54	5.0	4.0	4.0	4.0
β (g glucose/g cell.h)	0.005	0.005	0.005	0.005	0.005
m(UA_{500} red pigment/g cell)	0	0	0	0	0
n(UA_{500} red pigment/g cell.h)	0.012	0.019	0.02	0.011	0.004

results confirmed the previous results using a batch fermenter, which showed that the red pigment production was non-growth associated process.

Effect of aeration rate

In this set of experiment, the fermentation was carried out at a fixed agitation speed of 600 rpm and the DOT was controlled at various values 30–90% by varying air flow rate ranged from 0.1 litre/min (0.067 vvm) to 1.5 litres/min (1.0 vvm). The concentration of glucose sharply decreased from the beginning of the fermentation with corresponding increase in cell concentration and red pigment production *(Figure 2)*. Complete glucose utilization was observed for all fermentations with the different degrees of aeration, whereas 3.5 g/litre glucose remained in the fermentation culture at a low DOT of 30% during the same fermentation period.

Increased in cell concentration corresponded well to glucose consumption and ethanol formation, then the ethanol consumption with subsequent red pigment accumulation. Red pigment formation started when glucose was completely assimilated and rapid ethanol consumption was observed. Maintaining a low DOT resulted in an increase in the levels of ethanol accumulated, which did not favour the fermentative but the oxidative metabolism. The pH profiles for all fermentations were more or less the same.

In general, increased in air flow rate simply increases the oxygen mass transfer coefficient and thus allows the cells to uptake more oxygen. The maximum cell concentration (X_{max}) attained during the fermentation was varied with different DOT levels in the culture during fermentation. Maximum cell concentration of 13.2 g/litre was achieved at the DOT of 90% saturation. At low DOT (30% saturation) red pigment production was greatly reduced.

The growth kinetic parameter of *M. purpureus* FTC 5391 on glucose medium with different aeration rates is illustrated in *Table 3*. Growth of *M. purpureus* FTC 5391 and red pigment production were greatly affected by the DOT levels during the fermentation. Growth was excellent at DOT levels of 90% saturation. Inhibition of

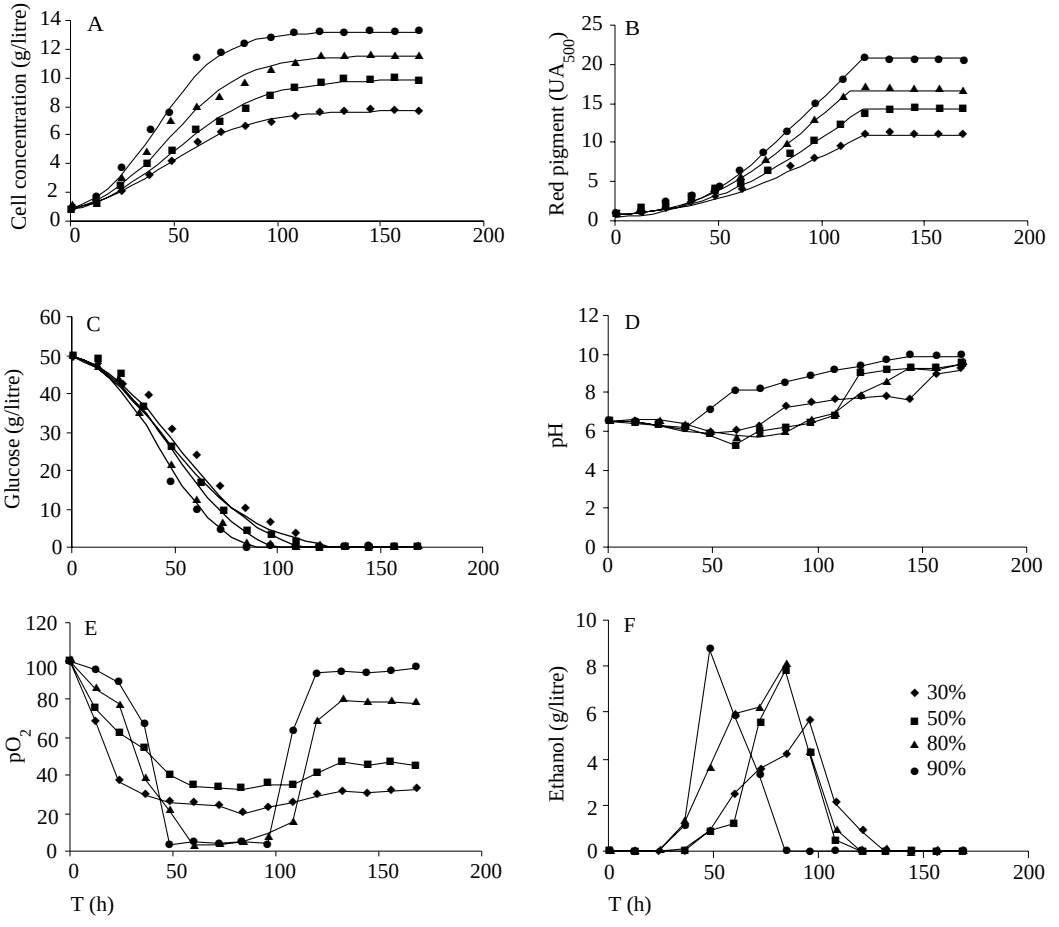

Figure 2. Batch red pigment fermentation by ***Monascus purpureus*** *FTC 5391 in 2-litre stirred tank fermenter at different DOT levels, which also includes the comparison between the calculated and experimental data. A) cell concentration, B) red pigment production C) glucose consumption, D) pH profile, E) profile of dissolved oxygen tension and F) ethanol production*

growth was observed in oxygen-limited condition (less than 50% saturation). The effect of DOT on red pigment production was similar to the effect on growth, suggesting that red pigment production was closely related to the rate of growth and cell concentration in the culture.

The highest P_{max} values were obtained at DOT levels of 90% saturation. In addition, the μ_{max} was also higher at DOT levels of 90% saturation. The maximum amount of the main components of red pigment should be determined per cell weight *(P_{max}/X_{max})* to indicate whether the fungus produced the red pigment under certain culture conditions or the production was simply proportional to cell mass. The values of P_{max}/X_{max} was not significantly different at 30, 50 and 80% saturation and the value of P_{max}/X_{max} was highest at 90% saturation, suggesting that production of red pigment was enhanced at very high DOT *(Table 3)*. The yield ($Y_{p/s}$) and productivity *(P)* of red pigment production was 0.411 UA_{500}/g.litre and 0.122UA_{500}/h respectively, while the cells efficiency to produce red pigment *(P_{max}/X_{max})* was 1.563 UA_{500}/g.litre.

According to the proposed models (Equations 1–3), the calculated data fitted to the experimental data of fermentation at

Table 3. Comparison of the performance and kinetic parameter values of red pigment fermentation in 2-litre stirred tank fermenter using different DOT levels. Agitation speed was fixed at 600 rpm and DOT was controlled via flow rate by varying the air flow rate at a range of 0.1 litre/min (0.067 vvm) to 1.5 litres/min (1.0 vvm)

Kinetic parameter values	Dissolved oxygen tension (DOT) (% saturation)			
	30	50	80	90
X_{max} (g/litre)	7.72	9.99	11.55	13.2
X_0 (g/litre)	0.78	0.81	0.78	0.85
μ_{max} (h^{-1})	0.0478	0.0475	0.055	0.065
P_0 (UA_{500})	0.798	0.812	0.807	0.805
P_{max} (UA_{500})	10.79	13.93	16.35	20.63
$Y_{x/s}$ (g cell/g glucose)	0.139	0.182	0.215	0.248
P (g cell/litre.h)	0.053	0.069	0.09	0.077
$Y_{p/s}$ (UA_{500}/g. litre)	0.20	0.262	0.311	0.411
P (UA_{500}/h)	0.07	0.091	0.13	0.122
P_{max}/X_{max}	1.40	1.41	1.42	1.563
α (g glucose/g cell)	6.9	5.5	4.65	4.0
β (g glucose/g cell.h)	0.005	0.005	0.005	0.005
m (UA_{500} red pigment/g cell)	0	0	0	0
n (UA_{500} red pigment/g cell.h)	0.018	0.02	0.021	0.02

different DOT levels *(Figure 2A–C).* In most cases the calculated data fitted well to the experimental data with more than 95% confidence. In all cases, the values of *m* were zero while the value of *n* was not zero confirming the previous results which showed that the red pigment production by *M. purpureus* FTC 5391 was non-growth associated process. The values of α were not significantly different at different DOT levels. In a defined liquid medium with oxygen limiting conditions, red pigment production is growth associated, whereas in oxygen excess, pigment production may be inhibited by the effect of an unknown product (Hajjaj et al. 2000).

The dissolved oxygen level at the DOT levels studied was reduced from set point saturation at the beginning of fermentation to almost 0% saturation at 50–100 h of fermentation, as the oxygen uptake by the mycelium was higher. The dissolved oxygen level was increased slowly as the growth shifted to the stationary phase; thereafter low dissolved oxygen level was maintained throughout the fermentation *(Figure 2E).* This result implies that DOT 80% of above saturate, although it might enhance the production of other metabolites (red pigments in this study). The enhancement of red pigment production at high aeration condition may possibly be related to the result from oxidation reaction of the pigment produced thereby increasing the optical density (measured red pigment concentration in this study). However, there was no possible explanation why increased pigment production was achieved at high DOT.

The profile of volumetric oxygen transfer rate *(K_La)* and specific oxygen uptake rate *(QO_2)* during fermentation at which the DOT was controlled at different DOT levels is shown in *Table 4.* The QO_2 was higher during the active growth. A decrease in QO_2 was observed in the fermentation with a decreasing DOT level. The profile of QO_2 during the fermentation confirms the preceding results, which showed that very high oxygen demand was required during the active growth phase with concomitant production of red pigment.

Among many parameters which affect the fungal morphology and product yield, the effect of aeration has been studied by a number of researchers (Wecker and Onken 1991; Stasinopoulos and Seviour 1992). The

Table 4. The volumetric oxygen transfer rate *(K_La)* and specific oxygen uptake rate *(QO_2)* taken at different times during red pigment fermentation by *Monascus purpureus* FTC 5391 in stirred tank fermenter carried out at different DOT levels

DOT levels (%)	Volumetric oxygen transfer rate, K_La (h^{-1})			Specific oxygen uptake rate, QO_2 (mg O_2/g.cell/h)		
	12 h	24 h	48 h	12 h	24 h	48 h
30	1.115	0.715	ND	0.0036	0.00085	ND
50	1.245	1.187	ND	0.011	0.0027	ND
80	2.951	1.820	0.585	0.027	0.0023	0.0014
90	3.59	2.99	1.97	0.029	0.0285	0.025

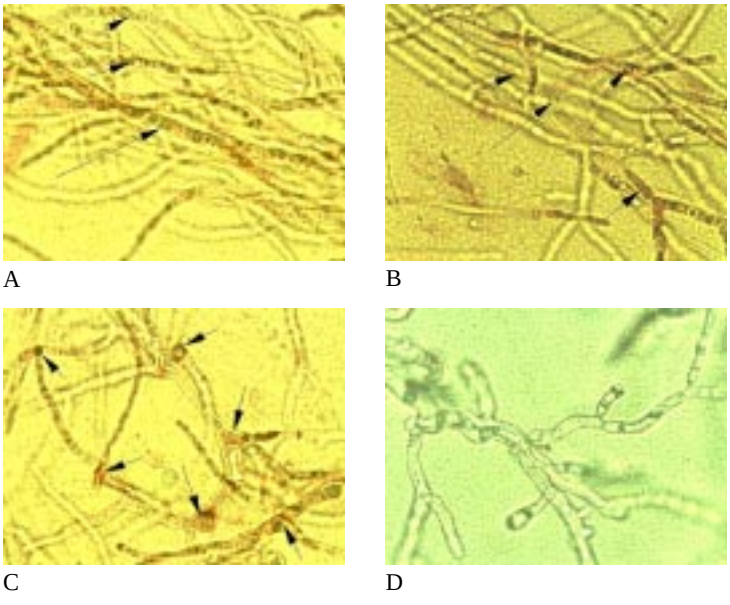

*Plate 2. The morphological of **Monascus purpureus** FTC 5391 in a 2-litre stirred tank fermenter. Observation (x100) of 6-day-old culture. Arrows show the vacuolated cells. DOT levels: (A) 90%, (B) 80%, (C) 50% and (D) 30%*

change in morphology during fungal growth affects nutrient composition and oxygen uptake rate in submerged cultures (Schugerl et al. 1983). *Plate 2* shows the different growth morphologies of *M. purpureus* FTC 5391 in stirred tank fermenter at different degrees of aeration. *Plate 2A* shows the fungal morphologies at DOT level of 90% saturation, which was the favourable condition for red pigment production and mycelial growth.

The cells were typically highly branched and the largest hyphal lengths were observed at the late exponential growth (day 6). More highly branched cells appeared with highly vacuolated cell morphology was observed at 90% saturation, with increasing red pigment production. Branching of mycelium is the mechanism by which new apical compartments are formed, and it occurs at certain branching points of hyphae. When the cell age increases, the cell becomes more and more vacuolated, and eventually, the cell does not contribute to the overall growth process. These vacuolated cells have a metabolism completely different from the actively growing apical and subapical cells, and they are believed to be

involved in the synthesis of some secondary metabolites like red pigment (Nielsen 1993).

Relationship between red pigment production and ethanol accumulation

The profile of ethanol production during *M. purpureus* FTC 5391 fermentation at different agitation speeds and dissolved oxygen is shown in *Figures 1F* and *2F*, respectively. In all cases, it can be seen that rapid consumption of glucose, concomitantly with rapid reduced in DOT were occurred during the active growth. The on-set of ethanol production was observed when either glucose in the culture became depleted and/or DOT level dropped to very low level i.e. almost zero. Red pigment production occurred during growth on carbon source supplied in the medium and also during growth on ethanol accumulated in the culture (i.e. after exhaustion of carbon source supplied). However, higher red pigment production with reduced growth rate, in some cases the growth ceased, occurred during utilization of ethanol as carbon source.

The ability of *Monascus* species to produce ethanol is well known, having been extensively used in Asia for alcoholic beverage manufacture (Lin and Iizuka 1982). Ethanol production was enhanced when mass transfer limits the oxygen consumption and when anoxic conditions occurred in the cultures (dissolved oxygen concentration close to zero) *(Figures 1E* and *2E).* In the presence of high glucose concentrations, as for certain *Monascus* species, ethanol is also produced which inhibits pigment production (Pastrana et al. 1995). Ethanol production also leads to a corresponding decrease in cell yield (Chen and Johns 1994).

Conclusion

Degree of aeration and agitation greatly influenced the production of red pigment by *M. purpureus* FTC 5391 during fermentation in stirred tank fermenter. Cell morphology during cultivation of *M. purpureus* FTC 5391 affected the final pigment yield. Mycelia morphologies could be controlled by the agitational shear stress. Mycelia cells with short branches were best for red pigment production because cultures were not highly viscous and high DOT level could be maintained with sufficient air supply and agitation. However, under conditions of excessively strong agitation, cell activities and oxygen uptake rate were diminished due to mechanical damage of the cells. Hence, increased overall productivity was observed.

Among the agitation and aeration control strategies investigated in this study, the highest red pigment production was obtained in fermentation where the agitation speed was controlled at 600 rpm and DOT level at 90% saturation. A simple model employing Logistic and Luedeking-Piret equations was found sufficient to describe growth of *M. purpureus* FTC 5391 and red pigment production. The production of pigments in *M. purpureus* FTC 5391 cultures appears to be non-growth associated process.

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Abstrak

Kesan kadar pemutaran dan kadar pengudaraan terhadap morfologi miselium dan penghasilan pigmen merah *Monascus purpureus* FTC 5391 telah dikaji di dalam fermenter berpengaduk 2 liter. Pada kadar kelajuan pemutaran 400 rpm atau kurang, didapati pengumpulan miselium yang serius dan pembentukan pelet yang gebu dengan kadar penghasilan menghampiri kira-kira di bawah 0.27 UA_{500}/ g.liter. Penghasilan sel-sel miselium yang berbulu panjang oleh *Monascus purpureus* FTC 5391 telah menyebabkan pembentukan kultur yang likat. Nilai K_La meningkat secara beransur-ansur apabila kadar kelajuan pemutaran meningkat daripada 200 kepada 1,000 rpm. Pengambilan oksigen spesifik (QO_2) pada kadar 800 dan 1,000 rpm didapati lebih rendah berbanding pada kadar 600 rpm.

Miselium mempunyai cabang pendek dan dirosakkan oleh daya ricih mekanikal akibat daripada kadar pemutaran yang tinggi. Aras kepekatan oksigen terlarut (DOT) berubah-ubah antara ketepuan 30–100% apabila proses fermentasi dijalankan pada kadar pemutaran yang tetap iaitu 600 rpm. Kepekatan sel maksimum dan penghasilan pigmen merah adalah pada kadar 13.2 g/liter dan 0.411 UA_{500}/g.liter masing-masing yang dicapai pada ketepuan 100%. Morfologi sel yang bercabang dan bervakuol dapat diperhatikan pada kadar DOT yang tinggi (100% tepu). Proses fermentasi dalam fermenter berpengaduk 2 liter telah berjaya dibangunkan pada kadar kelajuan pemutaran yang tetap iaitu 600 rpm dan kadar DOT lebih daripada ketepuan 100% yang merupakan keadaan terbaik bagi penghasilan pigmen merah dan pembentukan morfologi sel.

Appendix. List of abbreviations

α	Growth-associated rate constant for glucose consumption (g glucose/g cell)
β	Non-growth-associated rate constant for glucose consumption (g glucose/g cell.h)
μ_{max}	Maximum or initial specific growth rate (h^{-1})
m	Growth associated rate constant for red pigment production (UA_{500}/g cell)
n	Non-growth-associated constant for red pigment production (UA_{500}/g cell.h)
P	Productivity
P_0	Initial red pigment concentration (UA_{500})
P_{max}	Maximum red pigment concentration (UA_{500})
S_0	Initial substrate concentration (g/litre)
S_{max}	Substrate concentration (g/litre)
X_0	Initial cell concentration (g/litre)
X_{max}	Maximum cell concentration (g/litre)
$Y_{p/s}$	Yield of red pigment based on glucose consumed (UA_{500}/g.litre)

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