

Kinetics and modeling of red pigment fermentation by *Monascus purpureus* FTC 5391 in 2-litre stirred tank fermenter using glucose as a carbon source

(Pemodelan dan kinetik bagi fermentasi pigmen merah oleh *Monascus purpureus* FTC 5391 di dalam fermenter berpengaduk 2 liter menggunakan glukosa sebagai sumber karbon)

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Key words: *Monascus purpureus*, red pigment, shake flask, fermenter, growth associated, non-growth associated

Abstract

The experimental data from batch fermentation were analysed to form a kinetic model of the process. The unstructured model, based on logistic and Leudeking-Piret equations, was suitable to describe growth, substrate consumption and red pigment production by *Monascus purpureus* FTC 5391. The maximum specific growth rate (μ_{max}) of 0.055/h and 0.065/h were obtained from simulated modeling of *M. purpureus* FTC 5391 during growth in shake flask and 2-litre stirred tank fermenter, respectively. The maximum red pigment, P_{max} and cell concentrations, X_{max} obtained in batch fermentation using 2-litre stirred tank fermenter (20.63 UA₅₀₀ and 13.2 g/litre) and using shake flask (9.26 UA₅₀₀ and 11.425 g/litre) with overall productivity (P) was 0.122 UA₅₀₀/h and 0.055 UA₅₀₀/h, respectively. The production of red-pigment by *M. purpureus* FTC 5391 appeared to be a non-growth associated process; whereby rapid red-pigment production occurred during non-growth phase after the depletion of glucose in the medium.

Introduction

It is true that traditional food colourants are more likely to obtain approval from regulatory authorities and acceptance of consumers. Thus, the main task of food biotechnologist is to apply modern biotechnological methods in improving the productivity and application of these traditional food colourants. Traditionally, the fungus *Monascus* has been cultivated on rice and other cereals by solid-state fermentation.

This method does not permit an easy control of the environmental parameter and require intensive space. There have been reports that *Monascus* could be cultured in submerged culture systems and the submerged fermentation approach could overcome the drawback of the former process. *Monascus* is usually produced accordingly to empirically found rules and so far, this fermentation has never been described completely using scientific parameters

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(Juzlova et al. 1998). Despite this, little work has been performed on synthetic medium (Wong et al. 1981; Lin and Demain 1991).

Although the cultural conditions for improvement of microbial pigment production by *Monascus* sp., in general, have been studied extensively, information on the kinetics of production and development of models to describe the process in more meaningful ways remained scarce. *Monascus purpureus* FTC 5391 is also a newly isolated strain and presently, no attempt has been made to study its growth kinetics. Furthermore, the culture characteristic of this fungal strain could be different from other fungus.

The objective of the present study was to develop simple models that can be used to describe the kinetics of growth of *M. purpureus* FTC 5391 and red pigment production. The experimental data from batch fermentations of *M. purpureus* FTC 5391 using shake flasks and 2-litre stirred tank fermenter were analysed to form the basis for a kinetic model of the process. The model may allow better understanding of the fermentation process, so that suitable fermentation technique and process control strategy may be developed for improvement of pigment production by *M. purpureus* FTC 5391.

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; $MgSO_4 \cdot 7H_2O$, 1.0 g/litre; KCl, 0.5 g/litre; $ZnSO_4 \cdot 7H_2O$, 0.01 g/litre; $FeSO_4 \cdot 7H_2O$, 0.01 g/litre and $MnSO_4 \cdot H_2O$, 0.03g/litre. The sugars solution was prepared and autoclaved separately from basal medium and added prior to start the fermentation process. It is often best to sterilize sugars solution separately because they may be reacted with ammonium ions and amino acids to form black nitrogen containing compounds which will partially inhibit the growth of many microorganisms (Stanbury and Whitaker 1984).

Fermentation

Batch submerged fermentations of *M. purpureus* FTC 5391 were carried out using shake flask and stirred tank fermenter. The 500 ml shake flasks containing 250 ml medium with an initial pH 6.5 was inoculated with a 10% (v/v) inoculum and incubated at 37 °C in a rotary shaker and agitated at 250 rpm (rev/min).

The 2-litre stirred tank fermenter (Biostat B. Braun, Germany) with a working volume of 1.5 litres was used in this study. A single six-bladed turbine impeller with a diameter (D) of 52 mm mounted on the agitator shaft was used for agitation. The fermenter was equipped with temperature and dissolved oxygen controllers. During the fermentation, agitation speed (N) was fixed at 600 rpm (impeller tip speed = $\eta ND = 1.64$ m/s). In all experiments, the dissolved oxygen tension (DOT) control level was set at 90% (v/v) 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. DOT was recorded continuously during the fermentation. The initial pH of the culture was adjusted to 6.5 and the culture pH was not controlled but recorded continuously during the fermentation. In all fermentations, the temperature within the fermenter was controlled at 37 °C. During

fermentation, samples were withdrawn at various time intervals for analysis.

Analytical methods

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 was used as follows; 10 ml of ethanol was added to 1 g wet cell in 20 ml 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.

Mathematical method

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 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:

$$\text{Cell growth} \quad dX/dt = [\mu_{\max}(1-X/X_{\max})]X \quad (1)$$

$$\text{Substrate consumption} \quad -dS/dt = \alpha(dX/dt) + \beta X \quad (2)$$

$$\text{Product formation} \quad dP/dt = m(dX/dt) + nX \quad (3)$$

The batch fermenter models (equations 1–3) were fitted to the experimental data by non-linear regression with MS EXCELL computer software. The model parameter values were evaluated by solving equation (1–3). The predicted values were then used to simulate the profiles of cell, substrate and product concentrations during the fermentation. In order 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. 1988).

Results and discussion

Comparison of batch fermentation of *M. purpureus* FTC 5391

Shake flask A typical time course of red pigment fermentation by *M. purpureus* FTC 5391 in a shake flask is shown in Figure 1. Cell grew in the form of mycelium and reached a stationary phase after about 144 h when the glucose in the culture was sharply decreased during active growth phase. The maximum concentration of cell material obtained was about 11.425 g/litre. Yield of cells based on glucose consumed ($Y_{x/s}$) and the overall productivity (P) was 0.224 g cell/g glucose and 0.0665 g cell/litre.h respectively. Red pigment production stopped when the glucose in the culture was depleted but the cell grew until the stationary phase was achieved. This result assumed that ethanol accumulated in the culture was used as the subsequent substrate after the depletion of glucose (data not shown). The maximum concentration of red pigment at 60 h fermentation was 9.26 UA_{500} and this represents the yield based on glucose consumed ($Y_{p/s}$) and the overall productivity of 0.184 $UA_{500}/g.litre$ and 0.0546 UA_{500}/h , respectively.

The production of red pigment in shake flask cultures appeared to be growth associated. Oxygen limitation and different hydrodynamic conditions may be the cause of the low growth rate of *M. purpureus* FTC 5391 in the shake flask compared to fermenter cultivation. In shake flask, pigment production was rapid during initial stages of rapid growth and the production was stopped even when growth was still in the late exponential phase. The growth was on the utilization of ethanol after depletion of glucose (data not shown). These observations suggested that growth of *M. purpureus* FTC 5391 on ethanol did not significantly produce red pigment. Another possible explanation of reduced productivity and yield for fermentation in shake flask

might be due to non-optimal aeration conditions which were not favourable for red pigment production.

Growth associated pigment production kinetics of *Monascus* have been reported by many researchers (Yoshimura et al. 1975; Han and Mudgett 1992; Lee et al. 1994). This agrees with general observations that accumulation of red pigments in a *Monascus* cultures is usually not observed during the initial lag period, and the production of the pigments levels off before growth reached a stationary phase.

2-litre stirred tank fermenter Figure 2 shows a typical time course of batch *M. purpureus* FTC 5391 fermentation in 2-litre stirred tank fermenter. Similar patterns of growth and glucose consumption as well as red pigment production to that fermentation in a shake flask were observed. However, in the fermenter, due to different hydrodynamic conditions, final cell concentration was slightly higher as compared to shake flasks cultivation. Growth reached a stationary phase after about 84 h and red pigment production was maximal after 120 h. In comparison with the shake flask, the rates of growth and red pigment production in the fermenter were substantially higher. It is interesting to note that red pigment production in fermentation using stirred tank fermenter became rapid during a stationary growth phase, indicating that the production was non-growth associated. The maximum concentrations of cells and red pigment obtained in the fermenter were 13.2 g/litre and 20.63 UA_{500} respectively. Yield of cells based on glucose consumed ($Y_{x/s}$) and the overall productivity (P) was 0.248 g cell/g glucose and 0.077 g/litre.h respectively. Yield of red pigment based on glucose consumed ($Y_{p/s}$) and the overall productivity (P) was 0.411 $UA_{500}/g.litre$ and 0.1222 UA_{500}/h respectively.

Better aeration and agitation conditions in the stirred tank fermenter are the possible explanation for the higher growth and red

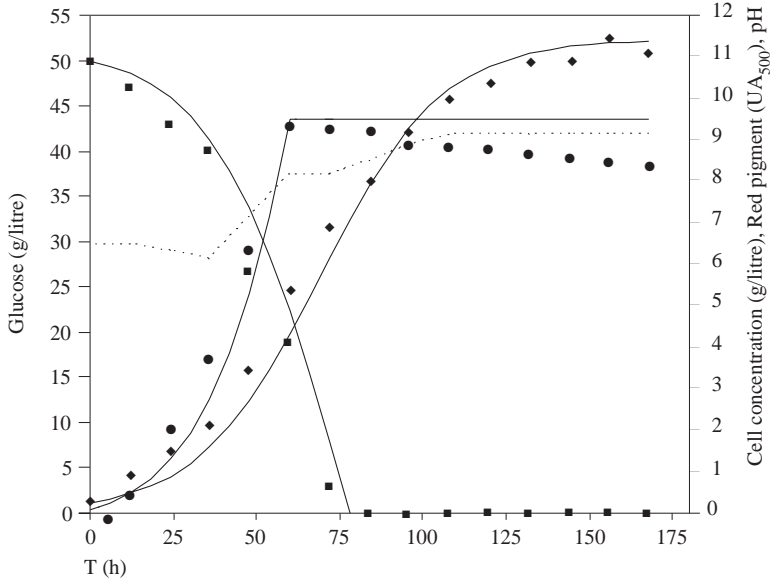


Figure 1. Comparison of calculated and experimental data for batch fermentation of red pigment using a shake flask: \blacklozenge = cell concentration; \bullet = red pigment; \blacksquare = glucose; ----- = pH; solid lines represent data according to equations 1–3

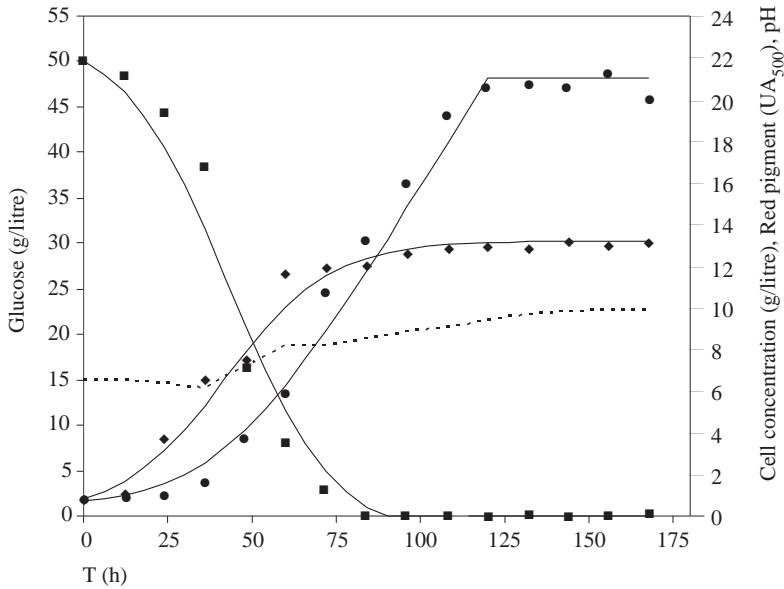


Figure 2. Comparison of calculated and experimental data for batch fermentation of red pigment using the 2 litre fermenter: \blacklozenge = cell concentration; \bullet = red pigment; \blacksquare = glucose; ----- = pH; solid lines represent data according to equations 1–3

pigment production by *M. purpureus* FTC 5391 as compared to shake flask. Juzlova et al. (1996) reported that *Monascus* pigments are polyketides and oxygen is an essential substrate for their biosynthesis. In order to understand how pigment production in *Monascus* cultures was regulated, the effects of dissolved oxygen on the growth of *Monascus* and its ability to take up the substrates to produce pigments were also studied.

The kinetic parameter values of batch fermentations in shake flask and in the fermenter is summarized in Table 1. These

kinetic parameter values can be used to verify the experimental data of red pigment production by *M. purpureus* FTC 5391. The maximum concentration of cell and the maximum specific growth rate (μ_{max}) obtained in fermentation were almost similar while the maximum red pigment in fermenter was substantially higher. The value of the growth associated rate constant for glucose consumption (α) and yield of cells over glucose consumed ($Y_{x/s}$) were almost similar for both batch fermentations. However, yield of red pigment based on glucose consumed ($Y_{p/s}$) was higher for

Table 1. Comparison of the performance and the kinetic parameter values of red pigment production in batch fermentation by *Monascus purpureus* FTC 5391 using a shake flask and the 2 litre stirred tank fermenter

Kinetic parameter values	Shake flask	Fermenter
X_{max} (g/litre)	11.4	13.2
X_0 (g/litre)	0.25	0.25
μ_{max} (unit/h)	0.055	0.065
P_0 (UA ₅₀₀)	0.085	0.085
P_{max} (UA ₅₀₀)	9.26	20.63
$Y_{x/s}$ (g cell/g glucose)	0.224	0.248
P (g cell/litre.h)	0.0665	0.077
$Y_{p/s}$ (UA ₅₀₀ /g.litre)	0.184	0.411
P (UA ₅₀₀ /h)	0.0546	0.122
α (g glucose/g cell)	4.5	4.0
β (g glucose/g cell.h)	0.1	0.005
m (UA ₅₀₀ red pigment/g cell)	0.1	0
n (UA ₅₀₀ red pigment/g cell.h)	0	0.02
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)	

fermentations using stirred tank fermenter than fermentation using a shake flask.

Conclusion

Unstructured models based on Logistic and Leudeking-Piret equations were proposed to describe growth, substrate consumption and red pigment production by *M. purpureus* FTC 5391. The results from the models strongly suggest that red pigment production in *M. purpureus* FTC 5391 in shake flask is growth associated and non-growth associated in the stirred tank fermenter. The maximum red pigment and cell concentration obtained in batch fermentation using 2-litre stirred tank fermenter was 20.63 UA₅₀₀ and 13.2 g/litre, respectively and 9.26 UA₅₀₀ and 11.42 g/litre, respectively, when fermentation was carried out using shake flask. The maximum specific growth rate (μ_{max}) in 2-litre stirred tank fermenter and shake flask was not significantly different (0.065/h and 0.055/h, respectively).

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Abstrak

Daripada data eksperimen proses fermentasi sesekelompok, satu model kinetik proses telah dibentuk. Model tidak berstruktur ini berdasarkan persamaan Logistik dan Leudeking-Piret didapati sesuai untuk menerangkan pertumbuhan, penggunaan substrat dan penghasilan pigmen merah dalam sistem sesekelompok oleh *Monascus purpureus* FTC 5391. Nilai kadar pertumbuhan spesifik maksimum (μ_{max}) iaitu 0.055/jam dan 0.065/jam diperoleh dari model pertumbuhan *M. purpureus* FTC 5391 dalam kelalang bergoncang dan fermenter berpengaduk 2 liter. Kepekatan maksimum pigmen merah, P_{max} dan sel, X_{max} diperoleh dalam fermentasi sesekelompok menggunakan fermenter berpengaduk 2 liter (20.63 UA₅₀₀ and 13.2 g/liter) dan menggunakan kelalang bergoncang (9.26 UA₅₀₀ dan 11.425 g/liter) dengan produktiviti keseluruhan (P) 0.122 UA₅₀₀/jam dan 0.055 UA₅₀₀/jam, masing-masing. Penghasilan pigmen merah oleh *M. purpureus* FTC 5391 ditunjukkan sebagai proses pertumbuhan tidak berkait, di mana penghasilan pigmen merah lebih pantas semasa fasa tiada pertumbuhan iaitu selepas glukosa di dalam kultur semakin berkurangan.