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Improvement of red-pigment-producing fungal strain (*Monascus purpureus* FTC 5391*)* using monospore isolation technique

[Peningkatan strain fungus penghasil pigmen merah (*Monascus purpureus* FTC 5391) menggunakan teknik pengasingan monospora]

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Key words: *Monascus purpureus*, monospore isolation technique, red-pigment

Abstract

Monospore isolation technique was performed to obtain the improved strain that has high and consistent ability to produce red pigment. The ability of *Monascus purpureus* FTC 5391 wild strain in producing red pigment was successfully improved using monospore isolation technique. By using this approach of improvement, three different monospore isolates of *M. purpureus* FTC 5391 (MP 3, MP 4 and MP 5) were obtained as the best red pigment producers when glucose, potato starch and rice starch were used as carbon source, respectively.

Introduction

One of the important aspects in the development of fermentation process is the ability and suitability of the microbial strain to be employed. Great care has to be taken to ensure that the strain is pure and produces the desired product at optimal level. The strain has to be preserved using the right technique to maintain its viability, stability and activity for as long as possible.

Bacteria, yeast and fungi have been used in industrial fermentation for production of various products. The fungi are more evolutionarily advanced forms of microorganisms, as compared to the prokaryotes (prions, viruses, bacteria). They are classified as eukaryotes, i.e., they have a diploid number of chromosomes and a nuclear membrane and have sterols in their plasma membrane. Most fungi occur in the hyphae form as branching, threadlike tubular filamentous. These filamentous structures either lack cross walls (coenocytic) or have cross walls (septate) depending on species. A mass of hyphal elements is termed the mycelium. Aerial hyphae often produce asexual reproduction propagules termed conidia (synonymous with spores).

Most of higher fungi exist as heterokaryon when genetically dissimilar nuclei share the same mycelia. On the other hand, a fungus is known as homokaryotic when the colonies contain genetically identical nuclei. Those dual phenomena were later identified as heterokaryosis, which cause variation in filamentous fungi and unstability or lost of metabolites during fermentation process (Kale and Bennet 1992). *Monascus purpureus* is a member of the genus *Monascus* which includes a set of

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fungi that are generally considered asexual, although the perfect forms (forms that reproduce sexually) have been found. As the case of many fungi, the taxonomy of *M. purpureus* is primarily based on morphological features, rather than the physiological, biochemical features and genetic characteristics often used to classify the bacteria.

Since this fungus is a source of various secondary metabolites of polyketide structure producers, monospore isolation of *M. purpureus* FTC 5391 was very important in order to achieve stable homokaryon cell, which has the ability to produce high red pigment consistently. In this study, monospore isolation step was performed as a technique to improve the wild strain of *M. purpureus* FTC 5391 in producing red pigment. This wild strain may exist in the form of unstable heterokaryon, in which, monopsore isolation may be used to isolate a stable monokaryon with high ability to produce red pigment. The ability of the monospore isolated in producing red pigment using different term of carbon sources was also investigated.

Materials and methods

Strain

The wild strain of *M. purpureus* FTC 5391, isolated at MARDI was used in this study. The fungus was maintained on potato dextrose agar (PDA) plate for 7 days at 37 °C. The culture was placed in a vial and freeze dried using a laboratory freeze drier according to the standard method. The freeze-dried stock culture in a vial was stored at –80 °C prior to use in spore production.

Monospore isolation

The isolation of monospores from strain *M. purpureus* FTC 5391 was carried out using spread plate technique. Monospore isolation was performed by serially diluting the fungal spore from 10^1 to 10^9. A 100 µl of each serially diluted spore suspension was plated onto potato dextrose agar (PDA) and incubated at 37 °C for 7 days. The clearly observed single colony was isolated from the plates and subcultured by 16-streak dilution for three passages. The spore was then transferred to slants and incubated at 37 °C for 7 days. After 7 days this fungus was used as an inoculum for fermentation process. From the monospore isolation step, five monospore isolates of *M. purpureus* FTC 5391 which showed high ability in producing red pigment were further tested. The monospore isolates were coded as MP 1 (monospore isolation 1), MP 2 (monospore isolation 2), MP 3 (monospore isolation 3), MP 4 (monospore isolation 4) and MP 5 (monospore isolation 5).

Media composition, inoculum preparation and fermentation

Two types of medium, production medium and inoculum medium were used in this study. The production medium consisted of 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. Three different carbon sources, rice starch, potato starch and glucose were tested in this study. The initial pH of the medium was adjusted to 6.5 by using either 1 M HCl or 1 M NaOH. The starch used in this study was in the form of dry powder, without any pretreatments and it was gelatinized by heating the starch slurry to slightly above 80 °C. The glucose solution was prepared and autoclaved separately from basal medium and was added together after they were cooled to room temperature in order to avoid mailard reaction.

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 25 ml (10%, v/v) inoculum culture was then inoculated into 500 ml flasks containing 250 ml of production medium. The flasks were incubated in orbital shaker incubator at 37 °C, agitated at 250 rpm for 7 days. All fermentations in shake flask were performed at least in triplicate.

Analytical determination

During the fermentation process, 3 ml of culture sample was taken at time intervals for chemical analysis. The sample was centrifuged at 3,000 rpm for 10 min. The cell pellet was used for the determination of dry cell weight and starch concentration. On the other hand, the supernatant was used for the analysis of sugars, amylolytic enzyme activity and red pigment concentration.

Determination of cell concentration

Cell concentration in the culture broth for fermentation using glucose as the sole carbon source was determined using filtration and oven dry method. A known volume of culture sample (3–5 ml) withdrawn from the shake flask was filtered through a pre-weighed filter paper (Whatman No. 1) by using vacuum pump. After drying period of more than 24 h in the 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 (X) was calculated by difference.

On the other hand, the cell concentration in culture broth using starch as the sole carbon source was determined by using oven dry method expressed as dry cell weight concentration (Soni et al. 1987). In this method, 0.1 ml α-amylase (Termamyl 120 litres, supplied by NOVO) was added to 1 ml culture sample and then incubated at 100 °C for 20 min to hydrolyse starch presence in the culture sample to soluble sugar. Sample was centrifuged at 3,000 rpm for 30 min and the supernatant was decanted. The cells, free from starchy materials were filtered onto preweighed filter paper (Whatman No. 1); washed twice with distilled water and dried in an oven at 95 °C for 24 h. Fresh medium was used as a blank for deduction of starch impurities in samples.

Determination of starch

The concentration of starch was determined colorimetrically on the basis of iodine starch complex colour (Smith and Roe 1948). An aliquot of sample was prepared by mixing 100 µl of supernatant from a culture broth with 60 µl of 0.1 M phosphate buffer (pH 7.2), 200 µl of 5 M NaCl, 50 µl of 1 M HCl and 50 µl of iodine reagent (0.3% w/v I_2 in 1% w/v KI). An amount of 10 ml of distilled water was added and the absorbance was read at 625 nm on a spectrophotometer. The starch in culture filtrate was quantified according to standard curve of starch which was linear for starch concentration ranging from 0–50 mg/ml.

Determination of glucose

Glucose concentration in the culture broth for fermentation using glucose as the sole carbon source was measured by using Glucose Analyzer (YSI 2700 Select Biochemistry Analyzer). Samples were prepared by filtering the supernatant through sep-pack in order to get clear supernatant prior to determination using glucose analyzer.

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% (v/v) 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.

Determination of α-amylase activity

Determination of α-amylase activity was conducted according to the method developed by Bhella and Altosaar (1984). The assay was based on the reduction of blue colour, resulting from enzymatic hydrolysis of starch. Each reaction mixture consisted of 30 µg (30 µl) enzyme protein in 3 ml of 0.05 M acetate buffer, pH 5.3, containing 0.4% (w/v) soluble starch. After 10 min of incubation at 37 °C, 0.2 ml/litre of reaction medium was added to 1 ml of iodine solution (1% (w/v) KI + 0.3% (w/v) I_2). The resulting solution was then diluted to 5 ml with distilled water and the colour intensity was analysed at 550 nm. One unit of α-amylase was defined as the quantity of protein producing a difference optical density of 0.1.

Determination of glucoamylase activity

Glucoamylase was assayed using the method as described by Ariff and Webb (1996) using maltose as a substrate. The procedure of glucoamylase determination was conducted as follows: 60 mM maltose was prepared in 0.1 M sodium acetate buffer pH 4.4. Substrate of 18 ml was incubated in a test tube at 40 °C for 10 min. A 2 ml sample of diluted broth supernatant was added and time was taken as 0 minute of reaction. At time intervals (10, 20 and 30 min), 0.5 ml of sample was collected for the measurement of glucose concentration and therefore the degree of substrate hydrolysis. The enzyme reaction in the sample was stopped by adding 0.5 ml of 0.05 M Tris/HCL buffer at pH 9 and then kept in the ice batch until the glucose concentration measurement. Glucoamylase activity was determined by measuring the initial rates of glucose production and express as µmole of glucose liberated per minute per ml broth supernatant (µmole.min ml) or unit per ml (U/ml). The method for the calculation of glucoamylase activity is described as below:

Blank calculation

Glucose concentration in 2 ml samples:
Glucose concentration in 18 ml of 60 mM Maltose:
Total glucose content in the reaction mixture = Blank

Kinetic data and calculation of glucoamylase activity

Glucose analyzer reading (g/litre) = A
Correlation for samples dilution (g/litre) = (A x 2) = B
g of glucose in 20 ml of reaction mixture = (B x 20) = C
Blank discount (mg glucose/20 ml) = (C – Blank) = D
U mole glucose in 20 ml = (D x 5.5) = E
Glucoamylase activity (U/ml) =

$$\frac{\text{(E x Dilution factor)}}{\text{(Time x Volume sample)}}$$

Calculation of growth kinetic parameter values

The growth of microbial cells is autocatalytic, that is, the rate of increase in cell dry weight is proportional to the concentration of cells initially present. Growth of microorganism during the fermentation can be expressed as;

$$dX/dt = \mu X \quad (1)$$

The differential coefficient (dX/dt) expresses the population growth rate. Thus, the specific growth rate (μ), which is regarded

as basic measure of growth, could be determined as;

$$\mu = (1/X)(dX/dt) \quad (2)$$

or

$$u = \ln (X_t/X_0) / t_t - t_0 \quad (3)$$

The relation between doubling time, t_d (h) with specific growth rate (μ) can be expressed as;

$$t_d = \ln 2 / \mu \quad (4)$$

Substrates are consumed not only to provide the necessary carbon, energy and structural components for cell growth but for the maintenance of cell viability (cell repair system, substrate transport processes, maintenance of membrane potential, etc.) and for product formation. The growth yield ($Y_{x/s}$) can be defined as dry cell weight obtained per amount of substrate consumed, that is

$$Y_{x/s} = (X_{max} - X_0) / (S_{max} - S_0) \quad (5)$$

Productivity, P (g/L.h), was determined by measuring the total amount of biomass formed over a period of fermentation time, which is taken as the time from inoculation to time where maximum concentration of pigment was achieved.

$$P = (X_{max} - X_0) / t \quad (6)$$

The rate of substrate utilization, dS/dt (g/L.h), is determined using equation (7);

$$dS / dt = \mu X / Y_{x/s} \quad (7)$$

Abbreviation

- X_0 = initial cell concentration (g/litre)
- S_0 = initial substrate concentration (g/litre)
- S_{max} = substrate concentration at X_{max} (g/litre)
- X_{max} = maximum concentration of cell (g/litre)
- X = cell concentration (g/litre)
- μ = specific growth rate (per h)
- t = fermentation time (h)

Results

A typical time courses of pigment fermentation by several monospores of *M. purpureus* FTC 5391, isolated from monospore isolation step, in a shake flask culture using different types of carbon source (glucose, rice starch and potato starch) are shown in *Figures 1* and *2*. Comparison of the performance and the kinetic parameter values of the fermentation are summarised in *Table 1*. In all cases the rate of pigment production was low during

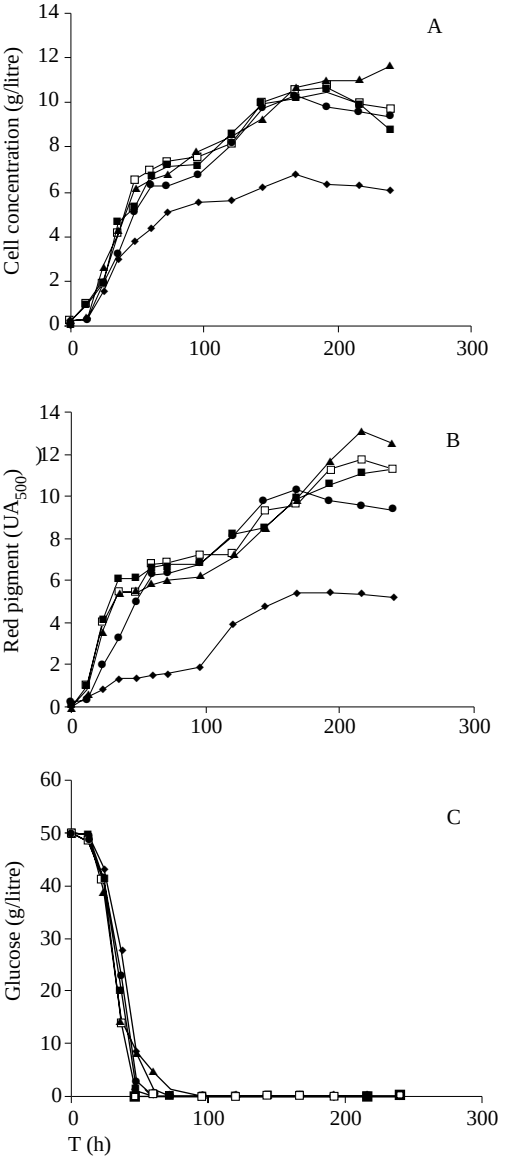

*Figure 1. Growth and pigment production by 5 monospore isolates of **Monascus purpureus** FTC 5391 using glucose as carbon source. A) cell concentration, B) red pigment production C) substrate consumption. ◆ = MP 1, ■ = MP 2, ▲ = MP 3, ● = MP 4, □ = MP 5*

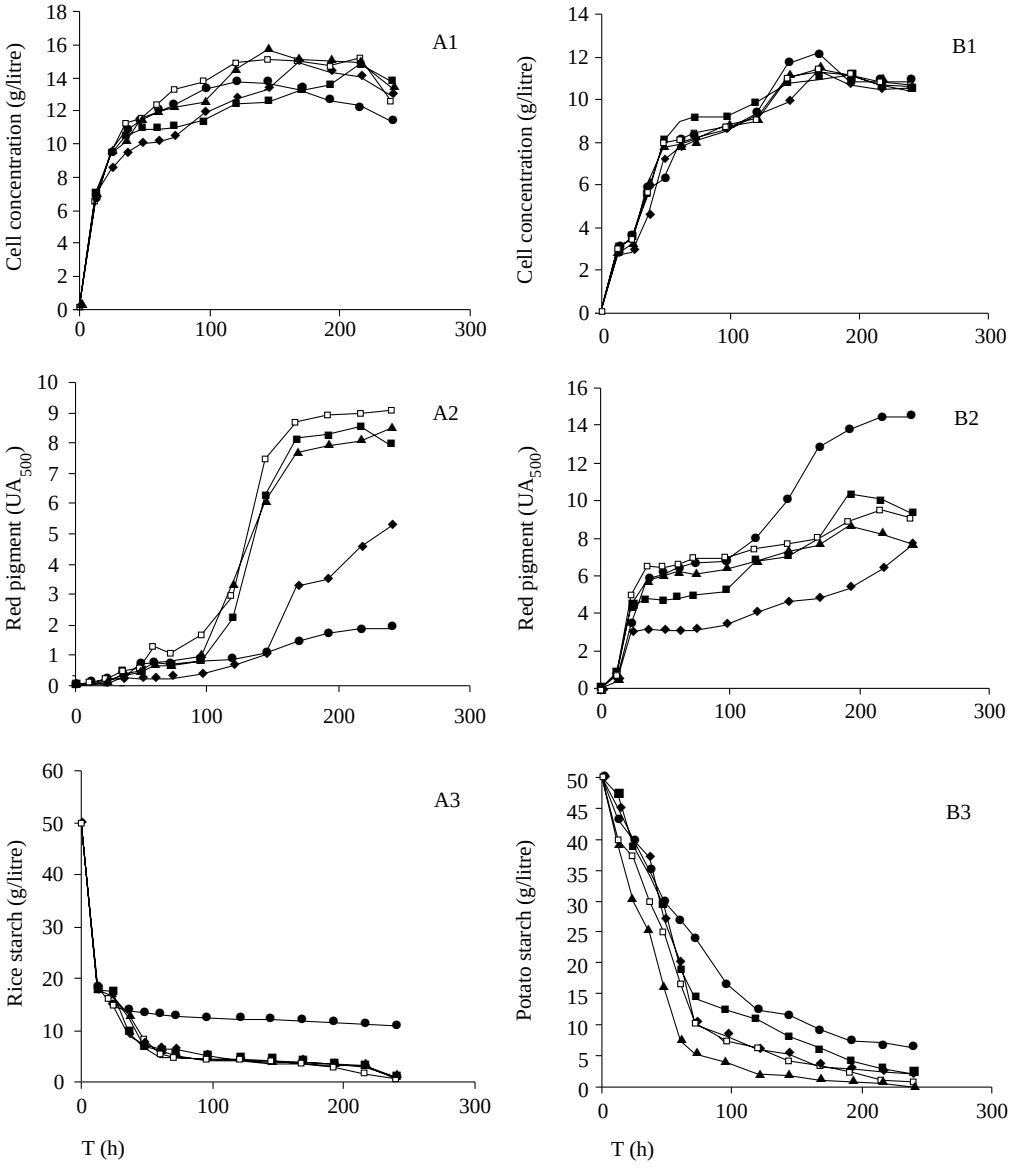

Figure 2. Growth and pigment production by 5 monospore isolates of ***Monascus purpureus*** *FTC 5391 using starch as carbon source. (A), Rice starch; (B), Potato starch. (A1 and B1), cell concentration; (A2 and B2), red pigment concentration; (A3 and B3), substrate consumption. ◆ = MP 1, ■ = MP 2, ▲ = MP 3, ● = MP 4, □ = MP 5*

the initial stages of fermentation. As fermentation progressed, pigment production was increased exponentially after 3 days. The production of red pigment reached a maximum concentration after 5 days and then a decrease in production was observed.

Fermentation using glucose as a carbon source

The different monospore isolates have different abilities in producing red pigment. In addition, the ability of the monospore isolates to produce red pigment was greatly influenced by different types of carbon

Table 1. Comparison of the performance and the kinetic parameter values of red pigment fermentation by monospores isolates of *Monascus purpureus* FTC 5391 and parent strain

Kinetic parameter values	Monospore isolation					Parent strain
	MP 1	MP 2	MP 3	MP 4	MP 5	
	Glucose as carbon source					
X_{max} (g/litre)	8.05	10.48	11.63	10.33	10.68	8.95
Red pigment concentration $(UA_{500})_{max}$	5.35	11.28	13.12	10.33	11.74	5.45
Maximum Glucoamylase Activity (U/ml))	–	–	–	–	–	–
Maximum α-amylase Activity (U/ml)	–	–	–	–	–	–
μ_{max} (h^{-1})	0.050	0.050	0.055	0.050	0.050	0.035
Yield *(Yx/s)* (g/g)	0.156	0.205	0.228	0.202	0.209	0.09
Productivity (g/litre/h)	0.046	0.053	0.047	0.06	0.054	0.025
t_d (h)	12.60	12.60	10.66	11.09	10.66	10.55
	Rice starch as carbon source					
X_{max} (g/litre)	14.95	14.85	15.65	13.73	15.18	10.76
Red pigment concentration $(UA_{500})_{max}$	5.27	8.56	8.51	1.92	9.15	5.25
Maximum Glucoamylase Activity (U/ml))	11.178	13.451	12.763	1.992	14.793	8.786
Maximum α-amylase Activity (U/ml)	0.219	0.204	0.312	0.203	0.215	0.097
μ_{max} (h^{-1})	0.085	0.0875	0.15	0.085	0.095	0.065
Yield *(Yx/s)* (g/g)	0.325	0.314	0.332	0.363	0.308	0.137
Productivity (g/litre/h)	0.129	0.068	0.114	0.111	0.069	0.045
t_d (h)	8.15	7.92	4.62	8.15	7.29	4.78
	Potato starch as carbon source					
X_{max} (g/litre)	11.35	11.18	11.33	12.23	11.48	10.39
Red pigment concentration $(UA_{500})_{max}$	7.65	10.36	8.65	14.46	9.53	7.85
Maximum Glucoamylase Activity (U/ml)	10.329	9.435	10.169	2.641	10.327	8.128
Maximum α-amylase Activity (U/ml)	0.882	0.844	0.814	0.844	0.714	0.657
μ_{max} (h^{-1})	0.085	0.085	0.085	0.085	0.085	0.060
Yield *(Yx/s)* (g/g)	0.222	0.224	0.332	0.241	0.225	0.152
Productivity (g/litre/h)	0.066	0.057	0.115	0.071	0.069	0.049
t_d (h)	8.15	8.15	4.62	8.15	8.15	5.69

μ_{max} = Maximum or initial specific growth rate (h^{-1})
X_{max} = Maximum cell concentration (g/litre)
(Yx/s) = Yield of cell concentration based on substrate consumed (g/g)
t_d = Doubling time (h)

source used. When glucose was used as a carbon source, the highest production of pigment was obtained in fermentation of *M. purpureus* MP 3 and the lowest production was obtained in fermentation of MP 1. During cultivation of *M. purpureus* MP 3 using glucose, the maximum cell concentration obtained was 11.63 g/litre. The maximum specific growth rate *(μ_{max})* with an average value of 0.05/h was not

significantly different with other monospore isolates tested.

Fermentation using starch as a carbon source

In fermentation using rice starch, the highest production of red pigment was obtained by MP 5. However, the production was not significantly different for those obtained in fermentation of MP 3 and MP 2. Very low red pigment production was obtained in fermentation of MP 4. In general, MP 1 can be classified as poor red pigment producer, since the production was very low in all types of carbon source tested.

On the other hand, when potato starch was used as a carbon source, the highest pigment production was obtained by MP 4 and still MP 1 gave the lowest production as observed in fermentation using glucose. It is interesting to note that the pigment production by MP 3 in fermentation using potato starch was about two times lower than MP 4.

The ability of *M. purpureus* FTC 5391 in producing amylolytic enzymes such as glucoamylase and α-amylase was also investigated. *Figure 3* shows the profile of glucoamylase and α-amylase activity by the 5 monospore isolates of *M. purpureus* FTC 5391 using rice and potato starch as carbon sources. In fermentation using rice or potato starch, substantially high glucoamylase activity was detected for monospore isolates MP 1, MP 2, MP 3 and MP 5. The profile of glucoamylase activity during the fermentation, using rice and potato starch, was not significantly different with the different monospore isolates investigated except MP 4, where very low glucoamylase activity was detected.

In fermentation using all monospore isolates investigated, α-amylase activity was also detected. However, the α-amylase activity was increased to high level during the initial stages of the fermentation, reduced drastically as fermentation proceeded and the activity was still maintained at certain value until the end of fermentation. It is worth to note that the α-amylase activity detected in fermentation using MP 3 was significantly higher than other monopsore isolates.

Discussion

From this study, it was found that *M. purpureus* FTC 5391 from five monospore isolates were able to utilise a number of carbon sources. The highest carbon consumption was observed in cultivation using glucose followed by rice starch and potato starch. About 50% of the selected carbon sources were used within 84 h of cultivation. The rate and degree of rice starch consumption during cultivation was slightly lower than glucose. The rate of potato starch consumption by all monospore isolates was very slow, but almost 80% of the total potato starch added to the medium was consumed at the end of the cultivation.

The cell concentration increased throughout the course of the cultivation in a rather linear fashion. The near-linear growth of the culture was limited by the supply of substrate available to the culture, possibly the glucose produced by the hydrolysis of the starch. Indeed, the estimated volume of the slurry starch in the culture system appeared to decrease almost linearly after 50 h of incubation and the residual soluble starch and glucose concentrations in the culture medium remained low after 50 h. The glucose concentration was negligible during the batch period, suggesting that the uptake of glucose was faster than the release of residual glucose from the hydrolysis of the starch. Thus, in theory the productivity of the *Monascus* culture could be improved by using high amylase producing strain of *Monascus*, or a system, which provides a larger solid-liquid interface, such as by coating the linear surface of the fermentation flasks with gelatinized starch (Lee et al. 1995).

Rice starch and potato starch, on the other hand, are ideal alternative substrates for *M. purpureus* fermentation, for they are affordable, colourless and readily digested

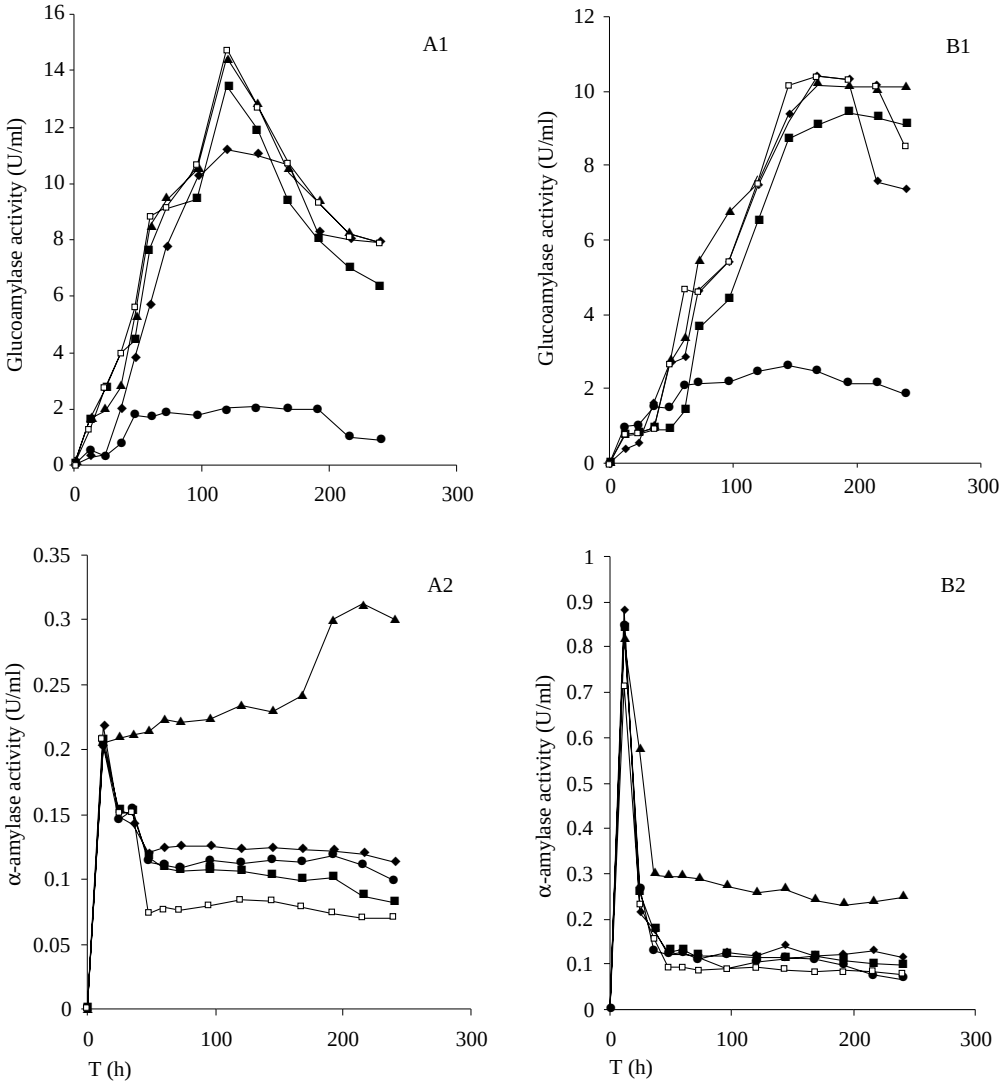

Figure 3. Profiles of glucoamylase activity (A1 and B1) and a-amylase activity (A2 and B2) during growth of 5 monospore isolates of ***Monascus purpureus*** *FTC 5391 using starch as carbon source. (A), Rice starch; (B), Potato starch. ◆ = MP 1, ■ = MP 2, ▲ = MP 3, ● = MP 4, □ = MP 5*

and utilized by the fungus. However, there are a few technical difficulties in the cultivation of *M. purpureus* in submerged culture using rice and potato starch. The high viscosity of gelatinized crude starch prevents practical use of a high initial starch concentration in the culture medium, resulting in relatively low final cell and pigment concentrations when fermentation was carried out using a stirred tank fermenter. Moreover, the low mass transfer rate in a viscous culture does not permit sufficient supply of oxygen to maintain high *Monascus* pigment productivity. For the same reason, the initial starch concentration in batch culture cannot be very high, which results in relatively low final cell and pigment concentrations at the end of fermentation.

Some of the technical difficulties caused by the rheological properties of crude starch could be overcome by pre-digesting

the substrate using α-amylase and glucoamylase prior to be used in fermentation. However, this entails additional manipulation, resulting in increased production cost and possibility of microbial fermentation (Lee et al. 1995). It is a common knowledge that a gelatinized starch cake is not easily dissolved in water. Thus addition of one starch block to a culture system will not greatly influence the rheological property of the liquid phase; however the hydrolysis of the starch by enzymes produced by the *Monascus* culture would result in a continuous supply of glucose as carbon source for the growth and pigment production (Lee et al. 1995).

It is important to note that *M. purpureus* FTC 5391 has high ability to produce amylolytic enzymes during growth on starch, with comparable production of red pigment as compared to growth on glucose. The ability of *M. purpureus* in producing α-amylase and glucoamylase during red pigment production has also been reported by Yongsmith et al. (1994) and Yongsmith et al. (2000). The ability of the red-pigment producing fungus to hydrolyse starch can be exploited for the use in direct fermentation of starches to red pigment.

Conclusion

The ability of the wild strain *M. purpureus* FTC 5391, in producing red pigment was successfully improved using monospore isolation technique. By using this approach of improvement, several monospore isolates that have different abilities in producing red pigment in different carbon sources was obtained. Monospore isolate MP 3 can be chosen as the highest red pigment producer when glucose was used as a carbon source. MP 4 was the best pigment producer when grown on potato starch, while MP 5 gave the highest pigment production when grown on rice starch. The result from the study showed that the wild strain of *M. purpureus* FTC 5391 present as heterokaryons, consisted of several unstable monokaryons. As a result from monospore isolation step, a stable pure monokaryon, which has high ability in producing red pigment, was obtained for used in subsequent study.

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Abstrak

Teknik pengasingan monospora telah dibangunkan bagi mendapatkan strain yang mempunyai keupayaan yang tinggi untuk penghasilan pigmen merah. Teknik pengasingan spora ini telah berjaya mempertingkatkan keupayaan strain liar *Monascus purpureus* FTC 5391 dalam penghasilan pigmen merah. Dengan kaedah ini, tiga jenis monospora (MP 3, MP 4 dan MP 5) telah berjaya diasingkan sebagai penghasil pigmen merah terbaik apabila glukosa, kanji kentang dan kanji beras digunakan sebagai sumber karbon utama.

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