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Production of pigments by *Monascus purpureus* in solid culture

Michael R. Johns and Deidre M. Stuart

Department of Chemical Engineering, The University of Queensland, Brisbane, Australia

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SUMMARY

The effect of physical and nutritional factors on the production of pigments by *Monascus purpureus* FRR 2190 was studied using cultures grown on both rice and a synthetic medium that was solidified with carrageenan and extruded into rice-like particles. Pigment yield was highly sensitive to physical parameters. Optimal pigment formation in rice cultures occurred at an initial pH of 6 and an initial moisture content of 56%. Lower moisture contents led to a large decrease in pigment concentration. Red and yellow pigment production on solidified gel media was increased up to three-fold compared to that of liquid cultures of the same medium composition, particularly when peptone was used as the sole nitrogen source. Solid state rice cultures gave the highest pigment yields.

INTRODUCTION

The fungus *Monascus purpureus* produces six, closely-related polyketide pigments ranging in colour from bright yellow to deep red. The organism has attracted continuing attention as a microbial source of natural red pigment to replace synthetic dyes, particularly erythrosine (FD&C Red No.3) [12,17]. The pigments and the fungus have been used to colour food in Asia for several centuries, but have yet to gain regulatory approval in the West.

Solid culture of *M. purpureus* on steamed rice, bread, brans or cereal meals is the traditional method of pigment production [9,13]. Some authors have described the development of mutant strains of *M. purpureus* giving high pigment yields on solid culture [9,13], but the effect of cultural parameters on yields has not been reported.

In contrast, extensive submerged culture studies of red pigment synthesis by various *M. purpureus* strains have revealed that the yield is affected markedly by many factors including medium composition, pH and agitation [2–5,12,14,18]. Comparisons between the red pigment yield of submerged and solid cultures have suggested that the yields are superior in the latter [7,13], although the conditions and the media used have differed. The fundamental reasons for the better results observed in solid culture have been explored [7], but the results were equivocal.

This work examined the effect of pH and the initial moisture content of rice on red pigment production in solid culture by *M. purpureus* and used an artificial gel medium to compare pigment productivity in solid culture with that of submerged cultures grown on medium of identical composition.

MATERIALS AND METHODS

Organism and media

Monascus purpureus FRR 2190 was obtained from CSIRO Food Research Laboratories, North Ryde, Sydney and maintained on potato dextrose agar (PDA) slopes (Difco Lab., Detroit, U.S.A.) at 4 °C. A distilled water suspension from a 6-day old PDA slope of *M. purpureus* grown at 30 °C was used for inoculation.

The glucose-peptone medium of Johns et al. [10] was used with a glucose concentration of 50 g/l. Where required, maltose (50 g/l) or sodium nitrate (3 g/l) was substituted for glucose and peptone respectively. To achieve solidification, 50 g/l of carrageenan (Sigma, St.Louis, U.S.A.) was added, the pH was adjusted to 6 and the medium was boiled. On cooling, the medium was extruded through a sterile, 10 ml syringe into a sterile petri-dish to form solid, rice-like pellets.

Calrose short-grain white rice (Sunrice, Australia) was used as substrate where indicated after sterilisation at 121 °C for 15 min. For use in pH experiments, 40 g lots of

Correspondence: M.R. Johns, Department of Chemical Engineering, The University of Queensland, Brisbane, 4072 Australia.

121 °C for 15 min. For use in pH experiments, 40 g lots or rice were soaked in distilled water (20 g) for 5 h, the pH was adjusted using 1 M HCl or 1 M NaOH and the rice dispensed as 8 g lots into conical flasks. After sterilisation, each flask was inoculated with 2.5 ml of a spore suspension and incubated at 30 °C.

Controlled humidity experiments

Sealed glass bottles (560 ml) containing 25 ml of solution and a stainless steel support (Fig. 1) for three rice cultures were used to provide environments in which the relative humidity of the gaseous phase was controlled during growth. Filter paper was included to increase the surface area between the solution and the atmosphere.

The bottles contained one of nine salt solutions, chosen to provide a range of equilibrium relative humidities between 0.751 and 1.00 at 30 °C according to Rao and Rizvi [15]. These were added aseptically to the bottles, which had been sterilised at 121 °C for 15 min. Solutions of water activity below 0.950 were not sterilised [11], whereas solutions with higher water activity were either filtered through a sterile 0.2 μm filter (NaCl solutions) or, the salt was added to sterile distilled water (saturated K_2SO_4).

Rice samples (1 g dry rice soaked in 1 ml water) were sterilised as above and were added to the bottles, which were incubated at 30 °C for 3 days. The incubation resulted in a gradation of the water contents of the rice samples, according to the water activity of the solution present in the chamber (Table 1). Inoculation of the rice was performed using fungal spores taken from a 6-day old

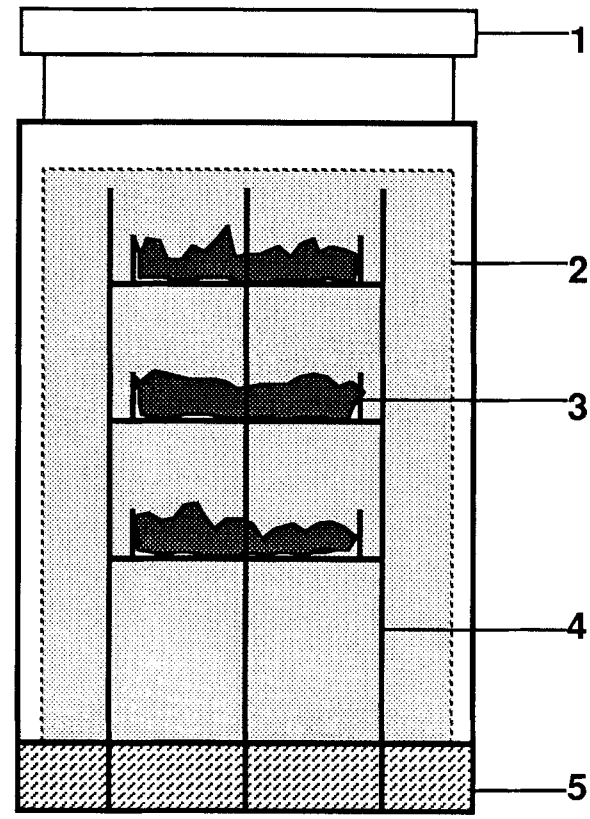

Fig. 1. Controlled humidity chambers. 1, Glass bottle with water-tight lid; 2, filter paper; 3, rice samples; 4, stainless steel sample support; 5, water activity control solution.

TABLE 1

Solutions used to control equilibrium relative humidity (ERH) and corresponding rice moisture content after 3 days incubation at 30 °C

Solution	ERH	Rice moisture content		Rice moisture content[a] (w.b.)
		w.b. (%)	SE (%)	
Distilled water	1.00	56.7	0.4	–
1.7% (w/w) NaCl	0.99	51.8	0.7	55.0
Satd K_2SO_4	0.970	45.8	2.3	41.2
8.0% (w/w) NaCl	0.950	48.2	1.4	35.0
Satd KNO_3	0.923	39.5	0.5	30.1
17.0% (w/w) NaCl	0.873	38.0	3.0	24.8
Satd KCl	0.836	19.0	4.0	22.3
Satd $(NH_4)_2SO_4$	0.806	20.0	5.0	20.7
Satd NaCl	0.751	15.0	2.0	18.4

Satd, saturated; S.E., standard error.

[a] Values calculated from a correlation given by Bason and Gras [1]. The correlation is strictly valid only between 40–90% d.b., but has been extrapolated for comparison.

PDA slope. At intervals of up to 16 days, one bottle containing three replicate rice cultures, was sampled for each value of water activity.

Analyses

Solid cultures were homogenised for 3 min after dilution to 10% (w/v) with distilled water and measured for pH using a calibrated pH electrode. The supernatant was used to determine carbohydrate, nitrate and pigment concentrations. Carbohydrate concentrations were determined by HPLC using an Aminex HPX 87H$^+$ (300 mm × 7.8 mm) column (Biorad., Hornsby, Australia) operated at 65 °C with a differential RI detector. The mobile phase consisted of 0.008 N H_2SO_4 with a flow rate of 0.6 ml/min.

Nitrate concentration was measured by suppressed ion chromatography using a Dionex Ion Chromatograph (201 Oi) equipped with HPIC AS4 Anion column, AG4 Guard column, and an anion fibre suppressor (Dionex Corp., U.S.A.). Detection was achieved using a Dionex conductivity detector with a sensitivity of 100 μS and a temperature coefficient of 1.7 at ambient temperature. An injection volume of 50 μl was used. The mobile phase comprised 1.7 mM $NaHCO_3$, 1.8 mM Na_2CO_3 and 25 mM H_2SO_4 as regenerants at a flow rate of 3 ml/min and a back pressure of 1100–1200 psi.

Pigment concentration was measured by spectrophotometry at 400, 470 and 500 nm for yellow, orange and red pigments respectively. Pigment in culture samples corresponding to 1 g of initial substrate (rice or gel) was extracted with 5 ml of 95% ethanol for 2 h with shaking. The extract was then centrifuged for 15 min to remove suspended solids and the supernatant was analyzed by spectrophotometry against a 95% ethanol blank.

The moisture content of rice samples was determined by heating the rice in a hot air oven for 24 h at 105 °C and measuring weight loss.

RESULTS

Effect of initial pH

The initial pH of rice inoculated with *M. purpureus* had a significant effect on pigment production in static flask cultures (Table 2), despite the convergence of all the cultures to a pH of 3–4 after 7 days. An initial pH of 6.0 gave the highest yields of all three colours of pigment. This is close to the natural pH of the rice samples.

The initial pH also influenced the proportion of each colour produced. In our experience, this particular strain of *M. purpureus* characteristically produces more yellow than red pigments under most culture conditions. In the first 6 days of culture, however, there was a striking dependence of rice colour on initial pH, with the colour being yellow, orange and red for initial pH values of 3.4, 6.0 and 7.0 respectively. After 16 days of incubation, however, the reverse was observed and the proportion of red pigment to yellow ($A_{500\ nm}/A_{400\ nm}$) increased with a decrease in the initial pH (Table 2) although visually the cultures were all a reddish colour. The ratio of red to orange pigment concentration was relatively constant. The pH of uninoculated flasks of rice was constant at 6.3 throughout the experiment.

Effect of initial moisture content

The influence of the initial moisture content of rice on pigment production was examined by inoculating triplicate, 1 g samples of rice, which had been held in a humidity chamber for three days prior to inoculation to permit equilibration of moisture content between the rice and the gaseous phase in the chamber. The samples were then incubated in the chamber for the duration of the experiment. A number of humidity chambers was used to give rice substrates with initial moisture contents ranging from 15% w.b. to greater than 55% w.b..

The humidity chambers achieved a gradation in the moisture content of the rice samples after three days

TABLE 2

The effect of initial pH on pigment concentrations in rice cultures of *M. purpureus*

Initial pH	Culture age (days)	Absorbance[a]			Ratio of absorbance		
		400 nm	470 nm	500 nm	500/400	470/400	500/470
3.4	7	6.32	1.76	1.40	0.22	0.28	0.79
	16	4.88	8.43	13.34	2.73	1.73	1.58
6.0	7	8.80	4.02	4.27	0.48	0.46	1.06
	16	14.57	12.05	18.46	1.27	0.83	1.53
7.0	7	6.63	2.44	2.76	0.42	0.37	1.13
	16	13.35	9.66	12.34	0.92	0.72	1.28

[a] Absorbance at 400, 470, and 500 nm is indicative of yellow, orange and red pigment concentrations respectively.

incubation, although the expected relationship with the water activity of the solution was not observed (Table 1). This appears to be due to variability in the wetness of the rice samples after autoclaving. For comparison, equilibrium moisture contents have been calculated using a correlation developed by Bason and Gras [1] from water desorption isotherms for uncooked Australian Calrose rice at 30 °C.

The rice appeared progressively drier with decreasing solution water activity and no condensation of water from growth was observed in bottles of water activity less than 1.00.

The initial moisture content (moisture content after 3 days incubation of the rice prior to inoculation) had a large effect on the growth and pigmentation of the fungus. Below an initial moisture content of 38% there was no fungal mycelium visible on the rice after 2 weeks of incubation. At initial moisture contents of 38–39.5%, fungal growth was limited to the outer layers of the rice grains and a dull pink colour was apparent, whereas at the highest initial moisture content, the fungus had penetrated the whole rice grain and the pigmentation was an orange-red colour.

The pattern of yellow ($A_{400\,nm}$) and red ($A_{500\,nm}$) pigment production followed the same trend with time at all initial moisture contents. The maximum pigment concentrations occurred after 8 days.

Production of pigments by *M. purpureus* displayed a strong correlation with initial moisture content (Fig. 2). The data plotted in Fig. 2 are the maximum pigment concentration (mean of three samples) observed. The ratios of red to yellow, and red to orange pigment concentrations

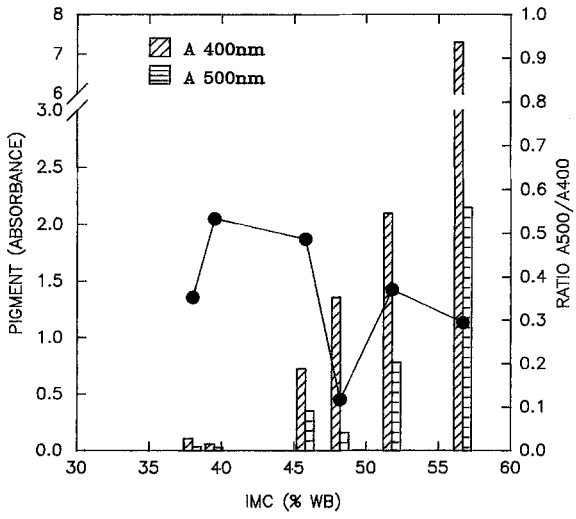

Fig. 2. Effect of initial moisture content of rice on pigment production by *M. purpureus*. Cultures were incubated at 30 °C in triplicate. (•), ratio of red to yellow pigment production.

exhibited no clear dependence on the initial water content of the rice. The former ratio was maximum at an IMC of 39.5%, whereas the latter was approximately 1 at all values of initial moisture content.

The maximum pigment concentrations, especially the red pigment, were considerably less than those observed in rice cultures incubated under similar conditions in the pH experiments (Table 2; pH 6, 16 days). It is possible that fungal respiration in the sealed humidity chambers reduced oxygen concentrations sufficiently to inhibit further growth and/or pigment production. Lin has suggested that gaseous oxygen is essential for red pigment production [12]. Nevertheless, it is unlikely that this affected the trends observed in Fig. 2.

Effect of carbon and nitrogen source

Chen and Johns [6] studied the production of pigment by *M. purpureus* FRR 2190 in shake flask culture. To compare the productivity of liquid and solid culture, the fungus was grown on the same media solidified with carrageenan incubated at 32 °C.

The carbon source in the solid gel media made little difference to the amount of pigment produced, or the ratio of red to yellow pigments. This is probably due to the rapid hydrolysis of maltose to glucose in these cultures (Fig. 3). In contrast, the nitrogen source had a significant effect on the production of red pigment. When nitrate was used, red pigment concentrations were less than half those of peptone media, and large quantities of glucose remained unutilised by the end of the culture.

Comparison of the data from the solid media experiments with those from liquid shake flask cultures [6], using identical medium composition and incubation temperature (Table 3), reveals that the production of both red and yellow pigments was greater in the solid cultures, particularly when peptone was the sole source of nitrogen.

DISCUSSION

The results demonstrate that pigment production by *M. purpureus* in solid culture is highly sensitive to cultural conditions, as is the case in submerged culture. The effect of initial pH on pigment production in solid rice cultures is similar to that observed by Carels and Shepherd [4,5] and Lin [12] in submerged cultures of *Monascus* species grown on carbohydrate media. They found that the initial pH of the culture determined pigment yield and colour, regardless of subsequent pH change and they suggested that this is due to the effect of pH on nutrient uptake.

The water content of the substrate is one of the most important factors determining the yield of secondary metabolites in solid substrate processes, but no systematic study of the effect of this parameter on *Monascus*

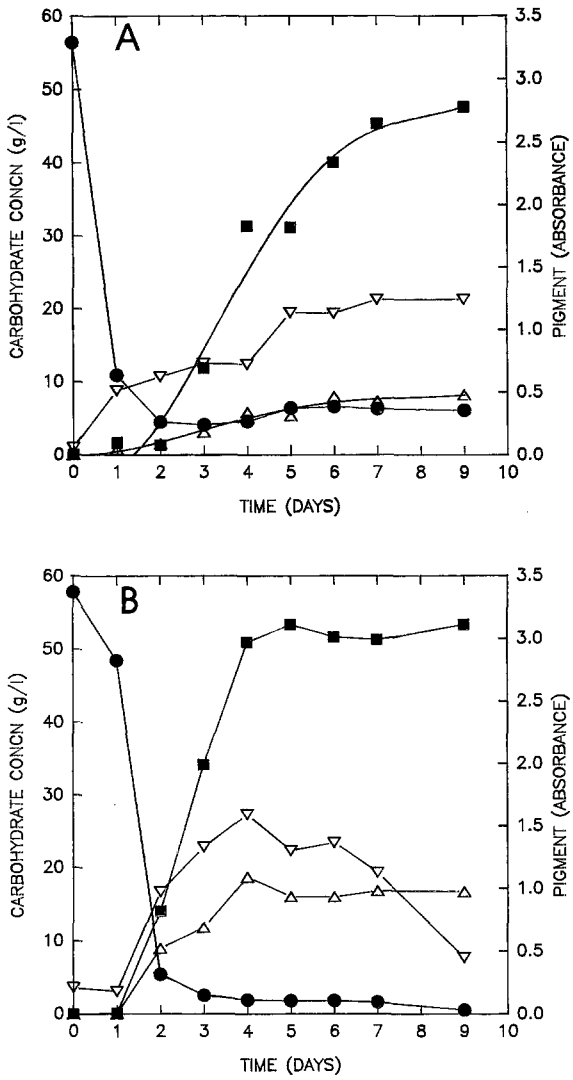

Fig. 3. Pigment production by *M. purpureus* on solidified gel media. A, maltose-nitrate medium; B, maltose-peptone medium. Symbols: (●), maltose; (▽), glucose; (■), absorbance at $A_{400\ nm}$ (yellow); (△), absorbance at 500 nm (red).

pigments has been published previously. Pigment production in solid culture on rice was favoured by a high initial moisture content (57% w.b.). This is consistent with the procedures reported in the literature for the production of ang-kak which maintain high humidities and add water to the solid mass during culture [16]. The result is also similar to those found for the production of other fungal metabolites in solid state processes. Optimal initial moisture contents reported for the formation of cephalosporin C by *Acremonium chysogenum* and citric acid by *Aspergillus niger* were 49–51 and 50–75% respectively [8].

Differences in pigment production in experiments using identical medium in both the liquid and solid form confirms previous observations that solid culture is superior to liquid culture for red pigment production by *M. purpureus* [7,12]. This result has been attributed to the derepression of pigment synthesis in solid systems due to the diffusion of intracellular pigments into the surrounding solid matrix [7,13]. In submerged culture, the pigments normally remain in the mycelium due to their low solubility in the usually acidic medium. Evans and Wang [7] also suggested that pigment production may be enhanced by the immobilisation of the fungus to the solid substrate.

Contrasting the pigment production per gram of initial substrate in rice (Table 2) and gel (Table 3) cultures, reveals that rice is a greatly superior substrate for pigment production as noted by previous authors [12,13]. The superiority of rice may result from its complex composition, which may derepress pigment formation, or its microscopic structure, which may differ from the model gel solid used in these experiments and may permit superior hyphal penetration or pigment diffusion. Evans and Wang [7] suggested that there was significant limitation of pigment diffusion in hydrogels.

Alternatively, the improved result on rice may reflect the extremely high concentration of utilisable substrate available in solid rice (e.g. glucose as starch) compared to

TABLE 3

Comparison of maximum pigment concentration in solid and liquid cultures grown on identical media

Medium	Shake flask			Solid culture		
	$A_{400\ nm}$	$A_{500\ nm}$	pH	$A_{400\ nm}$	$A_{500\ nm}$	pH
Glucose-nitrate	0.28	0.10	5.2	0.57	0.07	5.79
Glucose-peptone	0.19	0.09	2.7	0.69	0.18	4.38
Maltose-nitrate	–	–	–	0.61	0.10	5.74
Maltose-peptone	0.17	0.10	3.6	0.69	0.20	4.37

Data are given on the basis of the extraction of the same quantity of medium.

either the gel or liquid culture. Further studies are in progress to address these issues.

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