Effect of carbon source on ethanol and pigment production by *Monascus purpureus*

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The effect of glucose and its substitution by maltose on pigment production by Monascus purpureus *192F were studied in pH-controlled fermenter cultures using HPLC analysis to determine individual pigment concentrations. A maximum of five pigments were detected in fungal extracts. These were the yellow pigments, monascin and ankaflavin, the orange pigments, rubropunctatin and monascorubrin, and the red pigment, monascorubramine. Monascorubramine was the major product in all cases. The use of maltose (50 g l^{-1}) improved the production of monascorubramine, compared to glucose cultures, especially when peptone was the nitrogren source. At a carbon source concentration of 50 g l^{-1}, large amounts of ethanol were produced by the fungus, despite aeration, which suggested that respirofermentative metabolism was occurring. Subsequent use of a low initial glucose concentration (20 g l^{-1}) in batch culture, or fed-batch culture with glucose feeding, resulted in minimal production of ethanol and high monascorubramine concentrations. These conditions also favored production of the orange analogue, monascorubrin, whereas ankaflavin was favored at high glucose concentrations.*

Keywords: *Monascus purpureus*; pigments; monascorubramine; Crabtree effect; respirofermentation

Introduction

There is continuing interest in the production of polyketide pigments by the fungus *Monascus purpureus*, particularly the red pigments, for use as food and cosmetic colorants.[1-4] However, fundamental knowledge concerning the biochemical synthesis of polyketides and the regulation of the pathways remains rudimentary, despite progress with simpler polyketides, such as 6-methyl salicylic acid[5] and actinorhodin from *Streptomyces*.[6,7] Consequently, optimal conditions for pigment synthesis by *M. purpureus* remain to be established.

Considerable contradiction exists in the published work as to the best carbon source for red pigment production. In addition to the traditional rice culture, a number of starch substrates have been proposed for pigment production in liquid and solid-state fermentations. These include corn,[8] oats,[9] wheat and barley,[10] cassava,[11] and processed products such as bread and bran.[12] Maltose,[3,13] soluble starch,[14] ethanol, and glucose[15] have been claimed to be superior to other carbon sources in liquid cultures. A possible reason for the inconsistency in reported results may be the fact that the influence of the carbon source on individual pigment concentrations has not been determined and systematic analysis for by-products of carbohydrate metabolism has been neglected.

Curiously, previous work has also reported poor biomass yields, typically 0.32 g g^{-1} or less in aerobic, submerged cultures of *M. purpureus*.[16] Furthermore, organic acids and ethanol have been detected in these cultures, often in considerable quantity,[1,17,18] although their production has not been systematically studied. It is possible, therefore, that the carbon source may be metabolized to other products in pigment-producing *Monascus* strains.

The objective of the work described in this paper was to study the effect of glucose and maltose on the production of pigments, particularly the red monascorubramine, and ethanol by *M. purpureus* 192F in liquid culture.

Materials and methods

Microorganism

Monascus purpureus UQM 192F (FRR 2190) was obtained from CSIRO Food Resarch Laboratories (North Ryde, NSW, Australia) and was maintained on potato dextrose agar (PDA) slopes (Difco Lab., Detroit, MI) at 4°C.

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Inoculum and media

One milliliter of a distilled water (10 ml) suspension from a 6-day-old PDA slope of *M. purpureus* grown at 30°C was used to inoculate shake-flask cultures. For 2-l fermentations, 100 ml of actively growing shake flask culture, grown on identical medium, was used. The culture medium was composed of ($g l^{-1}$): glucose or maltose, 50; NH_4Cl, 2, or bacto-peptone, 5 (Difco, Detroit, MI); KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; and trace metals.[19] Sterilization of the medium was performed at 121°C for 15 min. All medium components were analytical grade and used with distilled water.

Cultivation

Shake-flask cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml medium (initial pH of 6.5) incubated at 30°C in an orbital water bath (150 rev min^{-1}) in darkness. Duplicate flasks were run for all conditions.

Batch fermenter cultures were grown in a 2-l glass vessel (B. Braun, Sydney, NSW) sealed with a stainless steel headplate. The working volume was 1.3 l. Agitation was provided at 500 rev min^{-1} by a flat-bladed impeller and sterile air was supplied at 1.3 l min^{-1}. Automatic temperature (30°C) and pH control were performed, the latter using a pH control module (LH Fermentation Ltd., Stoke Poges, UK) equipped with a steam-sterilizable pH electrode (Ingold AG, Urdorf, Switzerland) and sterile solutions of 1 M NaOH or 1 M HCl. A foam controller (LH Fermentation Ltd., Stoke Poges, UK) added silicon antifoam (Dow Corning, Sydney, NSW) as required. The glass vessel was autoclaved at 121°C for 15 min and sterile medium was added aseptically upon cooling. After inoculation (7.5% v v^{-1}), the fermenter was maintained in darkness.

For fed-batch culture, a sterile glucose solution (200 $g l^{-1}$) was fed to the fermenter at a constant rate (0.24 g h^{-1} per liter fermenter volume), 48 h after inoculation. There was negligible volume change in the fermenter due to this feed.

Analysis

Fermentation samples (20 ml) were analyzed for sugar and nitrogen concentration as described previously.[20]

Ethanol concentration was determined using a Varian 3700 gas chromatograph (Varian, Palo Alto, CA). Sample filtrates were mixed 1:1 by volume with a solution which contained isopropyl alcohol (10 $g l^{-1}$) as internal standard. A 3-μl sample was injected onto a 2 m × 3 mm glass column packed with Carbowax 1500 on Graphpac GC 80/100 (Alltech Assoc., Sydney, NSW) using helium carrier gas at a flow rate of 25 ml min^{-1} and 90°C column temperature. Detection was achieved by a flame ionization detector at 200°C, with an injector temperature of 150°C. Sample peak areas were determined by comparison with an ethanol standard (15.8 $g l^{-1}$).

Pigment concentration was determined by both spectrophotometric and high-performance liquid chromatography (HPLC) analysis as described previously.[20]

Unaccounted carbon was calculated by a carbon balance as the difference between glucose carbon (0.40) consumed and that distributed to cells (0.50), ethanol (0.522), and CO_2 (0.273) associated with ethanol production (on an equimolar basis). The numbers in parentheses represent the carbon mass fraction used in calculations.

Results

Effect of maltose

The time course of the maltose-ammonium and glucose-ammonium fermentations, conducted at pH 4, are compared in *Figure 1*. In the maltose culture, *M. purpureus* grew as filamentous mycelium and the broth became red after 1.5 days. In contrast, the fungus formed dense pellets (1–2 mm diameter) in the glucose culture, and the broth was less red. Both the final biomass dry weight concentration and the observed growth yield from the maltose culture were higher than those for the glucose culture (*Table 1*), the latter by a large margin.

Maltose was hydrolyzed more rapidly than it was utilized, resulting in the transient accumulation of a high concentration of glucose, which was consumed once maltose was exhausted. In both cultures, exhaustion of nitrogen resulted in residual concentrations of glucose when the fermentation was stopped.

The rates of sugar consumption in the two cultures are contrasted in *Figure 2*. Although the gross volumetric rate of maltose hydrolysis in the maltose culture exceeded that of glucose consumption in the glucose-ammonium culture, the net rate of sugar consumption by the fungus was considerably slower in the former. The same was also true for specific rates of substrate consumption (*Table 1*).

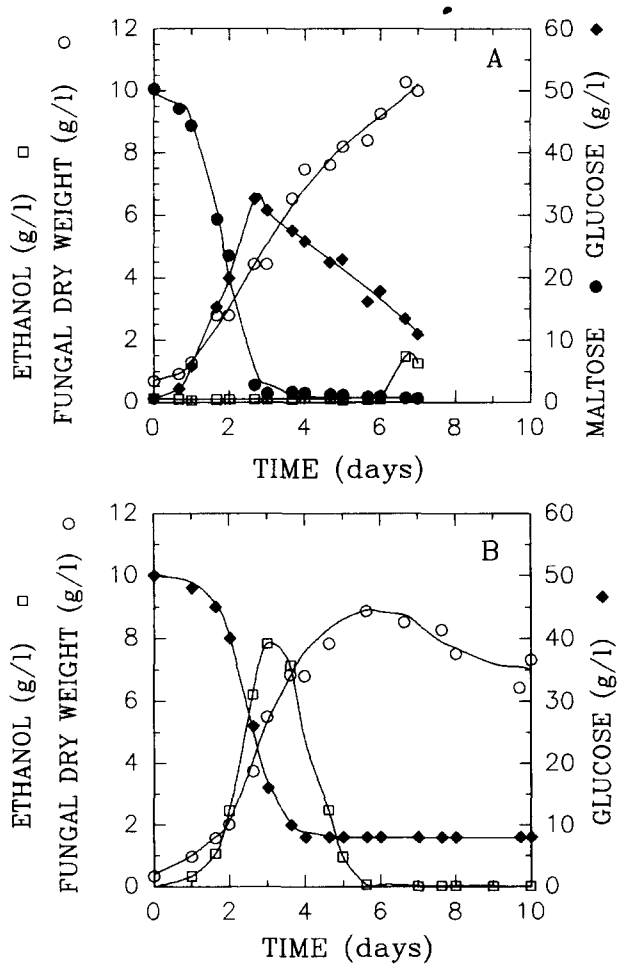

Figure 1 Time course of batch fermenter cultures of *M. purpureus* 192F on maltose-ammonium (A) and glucose-ammonium (B) media at pH 4, 30°C

Table 1 Effect of carbon source on growth and product formation in batch fermenter cultures of *M. purpureus* 192F

Carbon source	Maltose	Glucose
$Y_{x/s}$ (g g^{-1})	0.25	0.15
$Y_{p/s}$ (mg g^{-1})	8.2	3.0
q_s (g g^{-1}d^{-1})	32.0	8.0
r_e (g l^{-1}d^{-1})	0.0	5.3
q_e (g g^{-1}d^{-1})	0.0	1.5
r_p (mg l^{-1}d^{-1})	76.7	40.0
q_p (mg g^{-1}d^{-1})	18.7	12.6
μ_{max} (h^{-1})	0.044	0.036

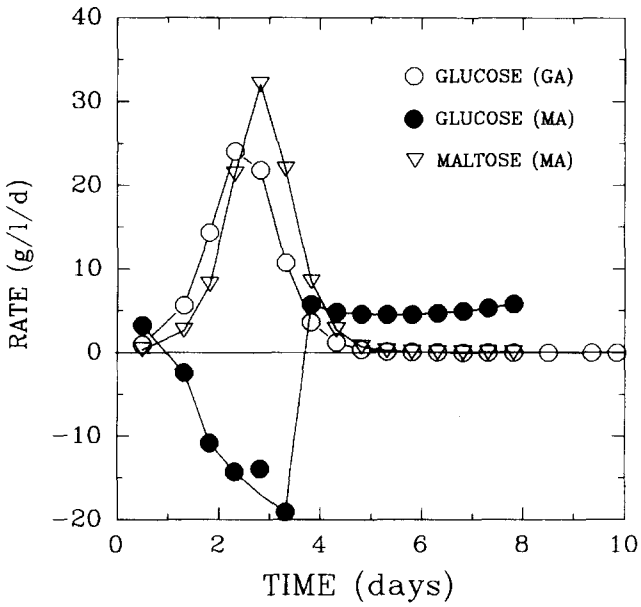

Figure 2 Volumetric rates of maltose and glucose consumption by *M. purpureus* 192F in batch fermenter culture on glucose- (GA) and maltose-ammonium (MA) media at pH 4, 30°C

The two cultures differed markedly with respect to product formation. Whereas high concentrations of ethanol accumulated during active fungal growth on glucose, little ethanol was formed in the maltose culture (*Figure 1*). The production of pigments, as measured by HPLC, is presented in *Figure 3* for both fermentations. The maltose-ammonium culture produced primarily three pigments, monascorubramine, monascin, and monascorubrin, and only trace amounts of ankaflavin and rubropunctatin. In contrast, monascorubrin was not detected in the glucose-ammonium culture, which produced larger quantities of ankaflavin and rubropunctatin. In maltose culture, monascorubramine (red) concentrations were three times those of the glucose culture, and higher maximum volumetric and specific rates of monascorubramine production were obtained (*Table 1*).

Effect of peptone

Peptone was found to give superior yields of monascorubramine compared to ammonium when glucose was the carbon source.[20] The effect of substituting peptone for ammonium was therefore studied using maltose at pH 4.0. The resulting intracellular pigment concentrations are compared with the corresponding glucose-peptone culture[20] in *Figure 4*. The superiority of maltose as a carbon source is pronounced, with a maximum monascorubramine concentration of 1.6 g l^{-1} after 5 days, compared to only 180 mg l^{-1} in the glucose-peptone culture after an equivalent time. This superiority is also reflected in the rate and yield of monascorubramine production by the maltose culture (*Table 2*), which are at least almost an order of magnitude higher than those for glucose.

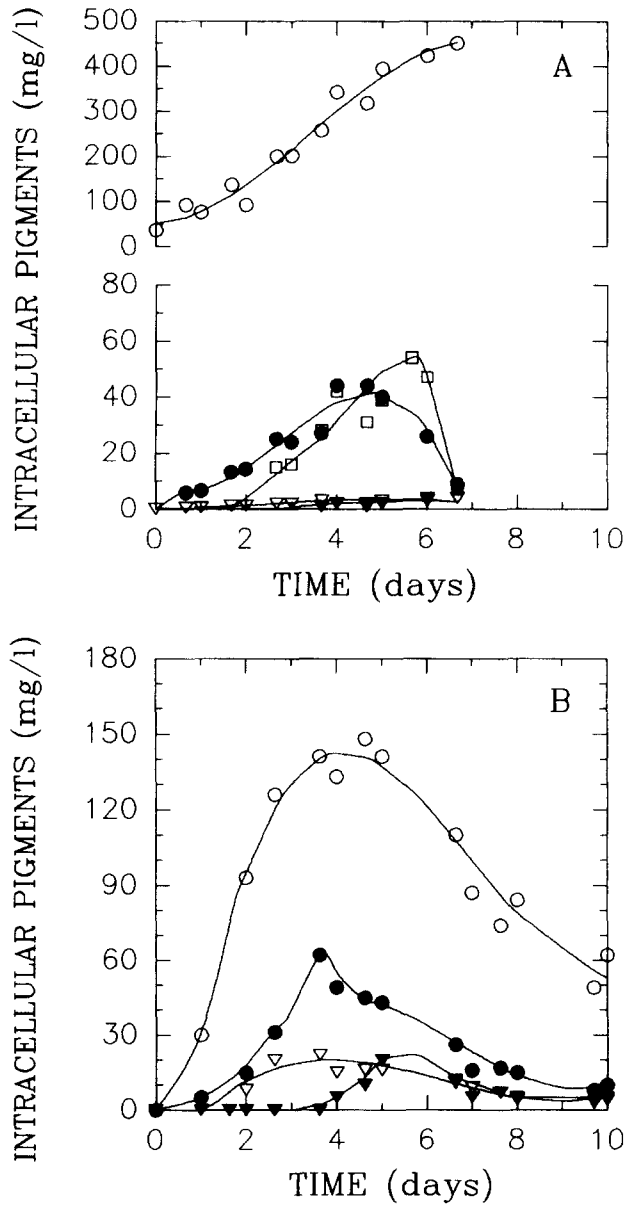

Figure 3 Intracellular pigment production by *M. purpureus* 192F in batch fermenter culture on maltose-ammonium (A) and glucose-ammonium (B) media at pH 4, 30°C. (○) Monascorubramine; (●) monascin; (■) monascorubrin; (▽) rubropunctatin; (▼) ankaflavin

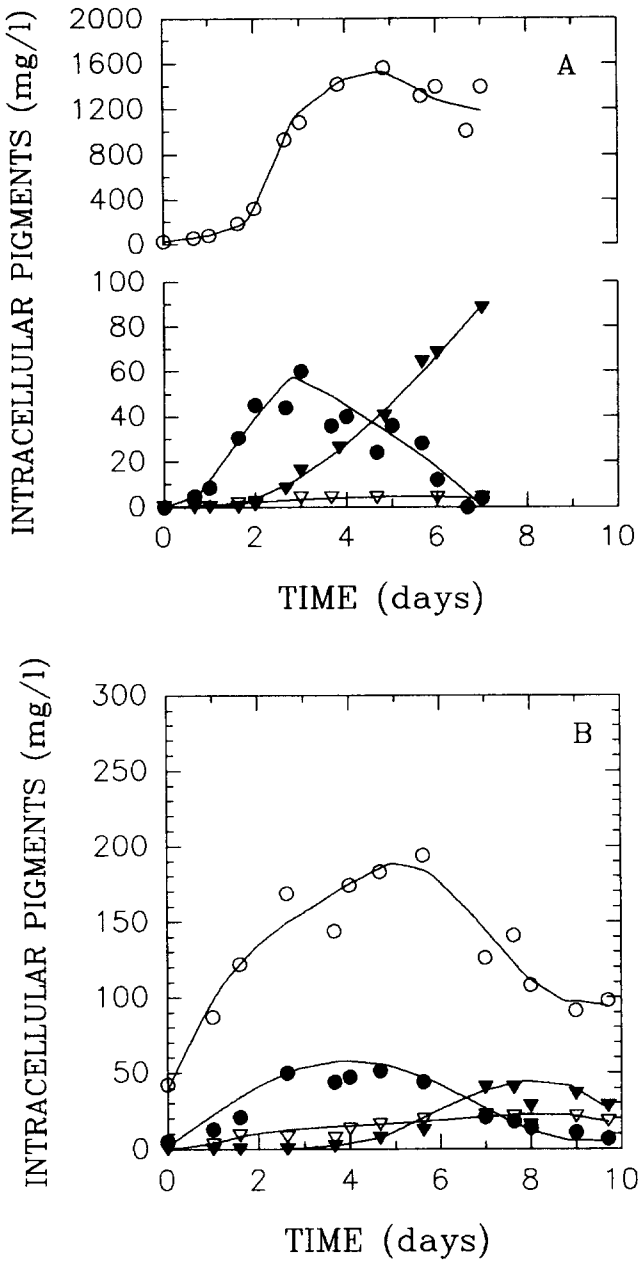

Figure 4 Intracellular pigment production by *M. purpureus* 192F in batch fermenter culture on maltose-peptone (A) and glucose-peptone (B) media at pH 4, 30°C. Symbols as in *Figure 3*

The concentrations of other pigments and their partitioning between the aqueous medium and the mycelia were similar for both cultures and relatively independent of carbon source, except for the concentration of ankaflavin, which was synthesized late in the maltose-peptone fermentation, corresponding to a decrease in monascin.

Other variables for both fermentations are summarized in *Table 2*. Use of maltose-peptone medium gave a higher final biomass concentration (14 g l^{-1}) and yield, and a lower ethanol concentration (4.0 g l^{-1}) than the glucose culture (6.7 g l^{-1} biomass), in which large quantities of ethanol (13.4 g l^{-1}) were produced. No sugar remained in the maltose culture, and less than 5 g l^{-1} in glucose culture, after 5 days.

Effect of glucose concentration on ethanol production

Experimental data from shake-flask cultures of *M. purpureus* are summarized in *Table 3*. All flasks exhibited rapid growth of the fungus as small pellets (3–4 mm diameter), after a lag of 2–4 days, with a correspondingly rapid decline in the pH of the culture to final values below pH 2.3. Pigmentation of the biomass became evident after 3 or 4 days, and the final color of the broth was yellow-orange at all glucose concentrations. Large glucose residuals remained at higher initial glucose concentrations.

There was no ethanol production at 10 g l^{-1} initial glucose concentration or less. Above this concentration, however, significant ethanol production occurred, the amount of ethanol increasing almost monotonically with glucose concentration (*Figure 5*). The ethanol yield ($Y_{e/s}$) at 20 g l^{-1} initial glucose was 74.5% of the theoretical maximum yield from glucose (0.51 g g^{-1}). Ethanol production led to a corresponding decrease in cell yield (*Figure 5*).

Table 2 Effect of peptone on growth, substrate consumption, and product formation by *M. purpureus* 192F in batch fermenter cultures at pH 4.0

Carbon source	Maltose	Glucose[a]
$Y_{x/s}$ (g g^{-1})	0.29	0.14
$Y_{p/s}$ (mg g^{-1})	33.3	3.2
r_s (g $l^{-1}d^{-1}$)	42.0	25.0
Net r_s (g $l^{-1}d^{-1}$)[b]	19.0	—
q_s (g $g^{-1}d^{-1}$)	32.0	8.0
r_e (g $l^{-1}d^{-1}$)	1.9	9.7
q_e (g $g^{-1}d^{-1}$)	0.3	3.4
r_p (mg $l^{-1}d^{-1}$)	820	39.0
q_p (mg $g^{-1}d^{-1}$)	99.4	11.1
μ_{max} (h^{-1})	0.065	0.035

[a] Chen and Johns[20]
[b] Net r_s is the rate of maltose consumption less the rate of glucose formation

Table 3 Effect of initial glucose concentration on *M. purpureus* in shake-flask culture.

G_i (g l^{-1})	X (g l^{-1})	G_f (g l^{-1})	$Y_{e/s}$ (g g^{-1})	r_e (g $l^{-1}h^{-1}$)	C (g l^{-1})	CO_2/G (mol mol^{-1})
4.8	1.4	0.2	0.03	0.01	1.9	6.0
9.7	2.3	0.1	0.07	0.02	3.8	5.9
20.5	3.5	0.6	0.38	0.15	4.2	3.2
44.4	6.6	9.8	0.32	0.28	8.6	3.7
90.9	6.7	25.1	0.34	0.47	18.6	4.2

G_i, G_f, Initial and final glucose concentrations; X, maximum cell dry weight; C, unaccounted for carbon; CO_2/G, ratio of non-ethanol-associated CO_2 produced (assuming unaccounted for carbon was liberated as CO_2) to glucose consumed

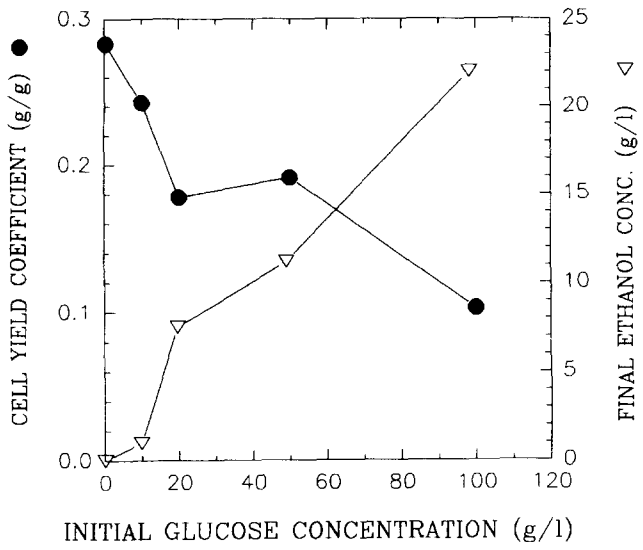

Figure 5 Effect of initial glucose concentration on cell yield and ethanol concentration in shake flasks of *M. purpureus* 192F using glucose-ammonium medium at 30°C

The molar ratio of CO_2/glucose was calculated by assuming that carbon, unaccounted for in the carbon balance, was released as respiration-associated CO_2. The ratio of 6 for low glucose concentration flasks corresponds to the expected molar ratio for consumption of glucose by respiration only. The ratio decreased markedly as ethanol appeared in flask cultures containing higher initial glucose levels. The increase in the CO_2/glucose ratio for higher glucose concentrations was probably due to the evaporation of ethanol from the flasks over the 8 days of cultivation.

Effect of glucose concentration on pigment production

A batch fermentation was performed at pH 4 using a medium containing 20 g l^{-1} glucose and ammonium chloride as the nitrogen source. The fungus grew rapidly (μ = 0.087 h^{-1}) as filamentous mycelia, and the broth became orange-red after 36 h. Growth ceased after 48 h with a maximum fungal dry weight of 5 g l^{-1} ($Y_{x/s}$ = 0.34 g g^{-1}) and a residual glucose concentration of 5.3 g l^{-1}. Ethanol formation was negligible. The volumetric and specific rates of glucose consumption were 14 g $l^{-1}d^{-1}$ and 4 g $g^{-1}d^{-1}$, respectively.

Five pigments were identified by HPLC analysis, although rubropunctatin and ankaflavin were present only at very low concentrations (< 5 mg l^{-1}). After 54 h, the main pigments produced were monascorubramine (430 mg l^{-1}) and monascorubrin (45 mg l^{-1}). The concentration of monascin rose to a maximum of 20 mg l^{-1} but decreased after 18 h. This disappearance was not observed with the other pigments. The volumetric and specific rates of monascorubramine production were 205 mg $l^{-1}d^{-1}$ and 68 mg $g^{-1}d^{-1}$, respectively, and the yield ($Y_{p/s}$) was 25 mg g^{-1}. These rates and the yield were at least fivefold those of the 50 g l^{-1} glucose culture conducted under equivalent conditions (*Table 1*).

Since the use of low initial glucose concentrations led to greatly improved monascorubramine production and minimal ethanol formation, the use of a fed-batch system with glucose feeding was attempted to ascertain whether the additional glucose would improve monascorubramine concentrations. The feed rate was calculated to maintain low glucose levels in the fermenter to avoid ethanol formation and was begun after 48 h, when glucose concentrations in the fermenter were low.

The results from the fermentation are presented in *Figure 6*. Fungal growth and glucose consumption occurred at rates similar to the 20 g l^{-1} glucose-ammonium batch fermentation. However, a high fungal dry weight was obtained at an observed cell yield of 0.48 g g^{-1}. Small amounts of ethanol (< 1 g l^{-1}) were formed toward the end of the fermentation, as glucose concentrations in the fermenter rose.

Glucose feeding enhanced the final concentration of all three pigments detected in amounts greater than 10 mg l^{-1}, namely, monascorubramine (950 mg l^{-1}), monascorubrin (130 mg l^{-1}), and monascin (65 mg l^{-1} after 3 days), compared to the 20 g l^{-1} glucose-ammonium batch fermentation. Pigment synthesis was affected by some unknown factor after 3 days, and recovery of pigment synthesis required a further 3 days for monascorubramine and did not occur

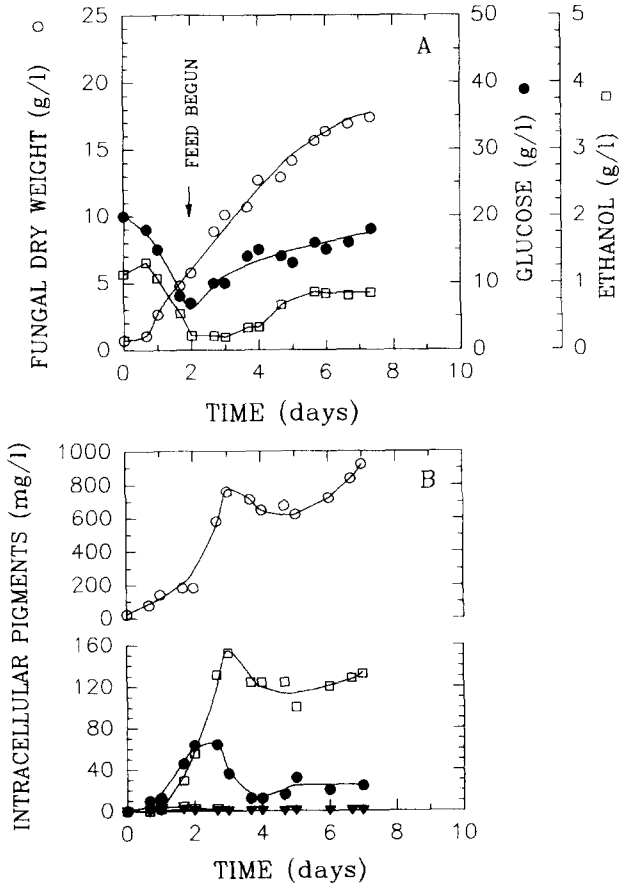

Figure 6 Growth, glucose consumption, and intracellular pigment production by *M. purpureus* 192F in fed-batch culture on glucose-ammonium media at pH 4, 30°C. Symbols as in *Figure 3*

for other pigments. Nevertheless, high volumetric (490 mg $l^{-1}d^{-1}$) and specific (65.3 mg $g^{-1}d^{-1}$) rates of monascorubramine production were obtained, and the final yield on glucose consumed was 47 mg g^{-1}, the highest obtained in these studies.

Discussion

The production of the red pigment monascorubramine, by *M. purpureus* is distinctly affected by glucose concentration. Regardless of whether nitrogen was provided as ammonium or peptone in batch fermenter cultures of the fungus, initial glucose concentrations of 50 g l^{-1} led to poor monascorubramine yields, low rates of pigment synthesis, low growth rates and cell yield (about 0.15 g g^{-1}), and considerable ethanol production. This result confirms a previous report that a high glucose concentration limits pigment production.[21]

Ultimately, the best results, with ammonium as nitrogen source, were achieved by maintaining glucose levels in the medium at less than 20 g l^{-1}. This resulted in a marked increase in cell growth and yield and a substantial improvement in monascorubramine yield and rate of production. It also correlated with negligible ethanol production and a lower specific rate of glucose consumption. Since the specific rate of monascorubramine production was almost identical in the 20 g l^{-1} glucose batch and fed-batch fermentations, the benefit of glucose feeding was largely due to higher biomass concentrations and yield of pigment from glucose, leading to a higher final pigment concentration.

The induction of respirofermentative metabolism (i.e., the Crabtree effect) in submerged, aerobic cultures of *M. purpureus* by high glucose concentrations ($>$ 20 g l^{-1}) would explain the appearance of ethanol and the poor observed growth yields of the fungus (typically 0.30 g g^{-1} or less) reported both in this work and by previous researchers.[1,17,18] Many yeast and fungi, including *Ascomycetes*, produce ethanol under aerobic and high sugar conditions.[22] This effect may arise for several reasons, depending on the organism.[23] Since *M. purpureus* is incapable of anaerobic growth on glucose and produced ethanol under conditions of excess dissolved oxygen concentration, it would appear to fall into the category of a Crabtree-negative, respiration-limited microorganism.[23]

The ratio of CO_2 produced per mole glucose consumed in shake-flask cultures of *M. purpureus* provides strong evidence that only respiration occurs at initial glucose concentrations of less than 20 g l^{-1}, whereas above this level, respirofermentative metabolism results in the concomitant generation of ethanol, lower cell yields, and a CO_2/glucose ratio less than 6.

In ammonium cultures, conditions leading to ethanol formation also resulted in greatly reduced monascorubramine concentrations and lower rates of monascorubramine synthesis. This is presumably due to both diversion of substrate carbon to ethanol via fermentative metabolism, which would reduce the quantity of acetyl-CoA and malonyl-CoA available as precursors for pigment synthesis via the polyketide pathway, and the reduced yield of energy available for pigment synthesis per mole of glucose metabolized. Consequently, conditions that lead to ethanol formation must be avoided to obtain high pigment yields in short fermentation times.

A further possible reason for low pigment production at high glucose concentrations may be catabolite regulation effects of glucose. However, recent work by Lin and Demain[3] found no catabolite regulation of pigment production by glucose with their strain of *M. purpureus*.

In addition to either low initial glucose concentrations or the use of controlled glucose feeding in fed-batch systems, the use of slowly metabolized carbon and energy substrates may be used to minimize ethanol formation or glucose regulatory effects. This may be the reason that many authors have reported that starchy substrates give enhanced red pigment production in both liquid and solid cultures,[14,24] although starch hydrolysis by *M. purpureus* appears to exceed the rate of glucose utilization, leading to finite glucose concentrations.[25]

The use of a 50 g l^{-1} maltose-ammonium medium gave significantly improved monascorubramine yields and rates of production compared to the corresponding glucose-ammonium culture, despite the presence of glucose concentrations in the medium of 32 g l^{-1} due to the very rapid hydrolysis of maltose. This improvement agrees with previous reports that the use of maltose typically gives higher yields of red pigments than glucose.[3] The beneficial influence of maltose may relate to the fact that net substrate consumption by the fungus was lower than that for glucose cultures. Consequently, ethanol production was negligible, which presumably released carbon and energy for pigment synthesis. However, the monascorubramine yields and rates of production achieved in the maltose-ammonium culture were at least three times lower than those attained in glucose-ammonium cultures in which the glucose concentration was maintained below 20 g l^{-1}. This fact highlights the importance of controlling glucose concentrations, whether the substrate is glucose or maltose.

Glucose concentration also influenced the synthesis of other pigments when ammonium was used. Low glucose concentrations (i.e., in fed-batch culture or the use of maltose) favored the synthesis of relatively large quantities of monascorubrin, the orange analogue of monascorubramine, whereas high glucose concentrations favored the synthesis of ankaflavin, the yellow analogue. In contrast, the synthesis of the yellow pigment, monascin, was largely independent of culture conditions. A stationary phase decline in its concentration invariably corresponded to ankaflavin synthesis, particularly when peptone was the nitrogen source. This may be due to the conversion of monascin to ankaflavin.

The use of peptone, rather than ammonium, as nitrogen source made little difference to fungal growth or pigment synthesis in glucose (50 g l^{-1}) batch cultures, although more ethanol was generated with peptone. However, with maltose as carbon and energy source, there was an immense difference between the two nitrogen sources with respect to pigment synthesis. The use of peptone increased the yield of monascorubramine fourfold and the rate of production by almost an order of magnitude compared to ammonium cultures, despite the presence of reasonably high glucose concentrations in the culture and concomitant ethanol production. Lin and Demain[3] reported an identical response

for *M. purpureus* grown on a defined medium using monosodium glutamate or ammonium salts, with maltose, in shake-flask cultures.

The difference was not due to increased partitioning of pigments in the aqueous culture broth, which remained low at the pH used. The synergistic effect of peptone with maltose, but not glucose, on pigment synthesis is difficult to explain. The fact that the nitrogen is in essentially the same form in both peptone and ammonium suggests that the benefit of peptone may lie in its ability to be utilized also as a carbon and energy source. Alternatively, pigment synthesis may be subject to some form of nitrogen (i.e., ammonium) regulation, which is relieved when an organic form of nitrogen is used. The failure of peptone to enhance monascorubramine synthesis in cultures containing high glucose levels suggests that the detrimental effect of high glucose concentrations on red pigment synthesis overrides the benefit gained by using peptone.

In conclusion, these results highlight the complexity of the red pigment fermentation using *M. purpureus* and clearly reveal the importance of controlling the type and concentration of the carbon source to obtain improved monascorubramine synthesis.

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Nomenclature

q_e	maximum specific rate of ethanol production (g $g^{-1}d^{-1}$)
q_p	maximum specific rate of monascorubramine production (mg $g^{-1}d^{-1}$)
q_s	maximum specific rate of glucose consumption (g $g^{-1}d^{-1}$)
r_e	maximum rate of ethanol production (g $l^{-1}d^{-1}$)
r_p	maximum rate of monascorubramine production (mg $l^{-1}d^{-1}$)
r_s	maximum rate of substrate consumption (g $l^{-1}d^{-1}$)
$Y_{e/s}$	observed yield of ethanol on glucose consumed (g g^{-1})
$Y_{p/s}$	observed yield of monascorubramine on substrate consumed (mg g^{-1})
$Y_{x/s}$	observed growth yield (g g^{-1})
μ_{max}	maximum specific growth rate (h^{-1})

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