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STUDIES ON THE GROWTH, MODELLING AND PIGMENT PRODUCTION BY THE YEAST *Phaffia rhodozyma* DURING FED-BATCH CULTIVATION

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SUMMARY

As *Phaffia rhodozyma* is a Crabtree positive yeast, its cell yield and pigment production are reduced at high sugar concentrations. A method for maintaining low growth medium sugar concentrations is fed-batch culture. Using a mass balance approach and Monod growth kinetics a model is presented which describes the fed-batch culture of *Phaffia rhodozyma* and enables the calculation of a feed regime to obtain the maximum yield of cells and pigment. Although developed on a glucose medium, the model was also applied successfully to a molasses-based medium.

INTRODUCTION

Astaxanthin (3,3'-dihydroxy-ß,ß,-carotene-4,4'-dione) is the principal carotenoid pigment responsible for the distinctive orange-red pigmentation of marine invertebrates (lobsters, crabs and shrimps), fish (salmon and trout) and birds (flamingoes) (Johnson *et al.* 1977; Christophersen *et al.* 1989). Since animals are unable to synthesize carotenoids, these pigments must be present in their food sources as consumer acceptance of cultivated salmonids and crustaceans is affected by the colour of their flesh. The chemical synthesis of astaxanthin is complex and costly due to the presence of chiral centers in its molecular structure. Considerable interest is being generated within the aquaculture industry in natural sources of astaxanthin for commercial purposes, such as its production by the yeast *Phaffia rhodozyma* (Johnson and An 1991).

In this paper, the application of fed-batch culture was investigated to overcome aerobic fermentation at high sugar concentrations, also known as the Crabtree effect (Reynders 1995). The development of a simple mathematical model, based on Monod growth kinetics and growth associated product accumulation, to predict microbial growth and pigmentation during fed-batch cultivation of *P. rhodozyma*, is presented.

MATERIALS AND METHODS

Yeast strain: *P. rhodozyma* mutant UCT-1N-3693, obtained by NTG mutagenesis according to An *et al.* (1989), was used for the evaluation of *P. rhodozyma* growth and pigment formation during continuous and fed-batch cultivation. This mutant is a strong-growing strain that produces approximately twice the amount of carotenoid as the wild-type *P.rhodozyma* (CSIR Y1194). Pre-inoculum and inoculum cultures were grown on YM broth consisting of (per liter): 10 g glucose, 5 g peptone, 3 g yeast extract and 3 g malt extract.

Bio-reactor: The aseptic bio-reactor system used for these investigations was designed in the Department of Chemical Engineering at the University of Cape Town and has been described previously (Reynders 1995). The experiments were conducted under mono-culture conditions in an agitated, aerated 2 l Quickfit culture vessel at a working volume of 1.5 l, an agitation rate of 540 r.p.m and a temperature of 22°C. The pH was controlled at pH 5.0 by the automatic addition of 2 M NaOH and 1 M HCl. The dissolved oxygen concentration (DO) was maintained above 2.0 mg/l by supplying sterile air at a flowrate equivalent to 2 v/v.m.

Continuous cultivation: The medium contained (per liter): 2 g $(NH4)_2SO_4$, 2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.1% silicone antifoam, 1 g yeast extract and 10 g glucose (Meyer and Du Preez 1994). In addition, 1 g/l potassium hydrogen phthalate and 0.25 g/l NaOH was added as an initial buffer (pH 5.0). For cultivation of *P. rhodozyma* on molasses, glucose was replaced by a molasses solution (10 g total sugars/l). After inoculation, the culture was allowed to grow in batch mode to stationary phase after which sterile medium was added for chemostat operation. A period of at least three residence times was allowed for culture conditions to reach steady state after a parameter had been changed (Pirt 1975).

Fed-batch cultivation: After inoculation, the culture was allowed to grow to stationary phase. Fed-batch operation was started prior to complete glucose exhaustion from the medium (at a residual glucose concentration of approximately 0.3 g/l). Nitrogen and phosphate concentrations in the feed medium were increased with increasing glucose concentration in order to ensure that carbon was the only limiting nutrient.

Analytical methods: Biomass formation was measured gravimetrically as dry cell mass. Residual sugar concentration was determined by enzymic analysis using Boeringer Mannheim analysis kits for glucose and molasses . The carotenoid content of the *P. rhodozyma* cell was determined by the dimethyl sulphoxide (DMSO) method (Sedmak *et al.* 1990).

MODEL DEVELOPMENT

The material balance for the growth-limiting substrate in fed-batch culture is given by:

$$\frac{dS}{dt} = \frac{\mu X}{Y} - \frac{F(t)}{V}(S_f - S) \qquad (1)$$

where S_f is the concentration of growth-limiting substrate in the feed stream, S is the residual substrate concentration in the culture, μ is the specific growth rate, X is the biomass concentration, Y is the biomass yield, F(t) is the substrate feed rate and V is the culture volume.

A fed-batch reactor may be operated at a constant feed rate (resulting in varying culture conditions) or at an exponential feed rate, resulting in a constant substrate concentration and hence constant growth rate in the reactor. In the latter, the substrate is fed at its rate of consumption ($^{ds}/dt = 0$) and may be maintained at a low level such that $S<<S_f$. The fed-batch system is then said to operating in a quasi-steady state, and Equation 1 becomes:

$$\frac{F(t)}{V}(S_f - S) = \frac{\mu X}{Y} \quad (2)$$

The Monod equation may be used to describe the functional relationship between the specific growth rate (μ) of a microorganism and the concentration of a single limiting nutrient (S) in the growth medium. It is given by:

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad (3)$$

where μ_{max} is the maximum achievable growth rate under specified culture conditions and K_s is the saturation constant. Substitution of Equation 3 into Equation 2 results in:

$$\frac{F(t)}{V} = \frac{(\mu_{max} S)X / (K_s + S)Y}{(S_f - S)} \quad (4)$$

Equation 4 can be used to calculate the feed rate required for a constant substrate concentration to be maintained while taking into account the volume changes in the reactor. Knowledge of the kinetic parameters and the substrate feed concentration are required.

At quasi-steady state, the total biomass formed (X^T) can be defined as $X^T = X_m V$, where X_m is the maximum biomass concentration and $X_m = YS_f$. The rate of change in total biomass concentration can then be written as:

$$\frac{dX^T}{dt} = F(t)\frac{X^T}{V} = X_m F(t) = F(t)YS_f \quad (5)$$

Integration of Equation 5 yields Equation 6 which may be used to predict a final biomass yield for a fed-batch reaction.

$$X^T = X_o^T + F(t)YS_f \quad (6)$$

Similarly, a microbial product profile at quasi-steady state was obtained by using the definition for the specific rate of product formation (q_p). For growth associated product formation, $q_p = Y_{(p/x)}\mu$ where $Y_{(p/x)}$ is the final product yield (g product formed/g biomass utilized). When q_p is constant then:

$$\frac{dP^T}{dt} = q_p X_m \left[V_o + F(t)t\right] \quad (7)$$

where P_T is the total amount of product in the culture ($P_T = PV$). Integration of Equation 7 gives:

$$P^T = P_o^T + q_p X_m \left[V_o + F(t)\, t/2)\right] t \quad (8)$$

Equation 8 may be used to predict the pigment formation during fed-batch cultivation of *P. rhodozyma.*

RESULTS AND DISCUSSION

The values for the kinetic parameters μ_{max} and K_s were determined during continuous cultivation of *P. rhodozyma.* For continuous operation at steady state, with a sterile feed stream, the biomass material balance shows that

$$\mu = D \qquad (9)$$

where D is the dilution rate. Following linearisation of Equation 3 and its substitution with Equation 9, a Lineweaver-Burk plot of [1/D] as a function of [1/S] may be constructed. Values of μ_{max} and K_s are determined from the slope and the intercept of the line respectively. The values obtained for μ_{max} and K_s for *P. rhodozyma* were 0.22 h^{-1} and 1.9 g/l respectively (Reynders 1995).

It has been shown that *Phaffia rhodozyma* is Crabtree positive (Reynders 1995) and hence both biomass yield and product yield decrease with increasing residual glucose concentration. In order to achieve high biomass concentrations without comprising yield, fed-batch operation was investigated.

To determine the feed regimes required for fed-batch cultivation of *P. rhodozyma* at various glucose concentrations, the values for μ_{max} and K_s were substituted into Equation 4. Values for S, X and Y in Equation 4 were estimated based on data obtained from the trial fed-batch cultures (data not shown). For a specified glucose concentration in the feed (S_f), the feed regime F(t) was calculated. Table 1 shows good agreement between the predicted values used in Equation 4 and actual experimental data obtained during fed-batch cultivation of *P. rhodozyma* at feed glucose concentrations of 27, 40 and 55 g/l respectively. Operation of the fed-batch system at quasi-steady was confirmed by the constant value observed for residual glucose concentration.

Figure 1 shows a comparison between the total biomass content of the culture at various glucose feed concentrations (27, 40 and 55 g/l) predicted theoretically by calculating the feeding profile and using Equation 6, and experimental total biomass values. Due to the manual adjustment of the

Table 1. Predicted data versus experimental data obtained using a mass balance approach for the calculation of a feed regime during fed-batch cultivation of *P. rhodozyma* at various concentrations of glucose in the feed.

Parameters	S_f = 27g/l		S_f = 40 g/l		S_f = 55 g/l	
	Predicted	Experiment	Predicted	Experiment	Predicted	Experiment
Residual substrate (S, g/l)	0.29	<0.10	0.33	<0.10	0.39	<0.10
Final biomass (X, g/l)	11.00	10.97	14.00	13.70	19.50	19.00
Yield coefficient (Y, g/g)	0.50	0.57	0.50	0.52	0.50	0.55

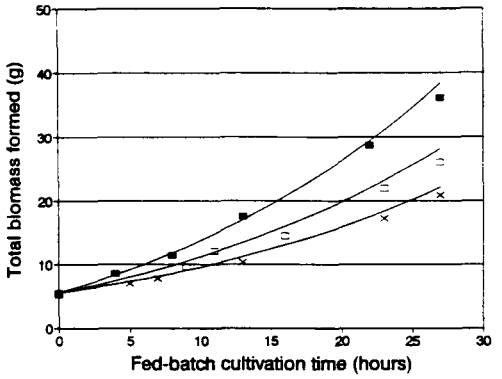

Figure 1.
Comparison of theoretical predictions and experimental data for biomass formation during fed-batch cultivation of *P. rhodozyma* at 22°C, pH 5.0, and glucose at 27 (X), 40 (□) and 55(□) g/l in the feed.

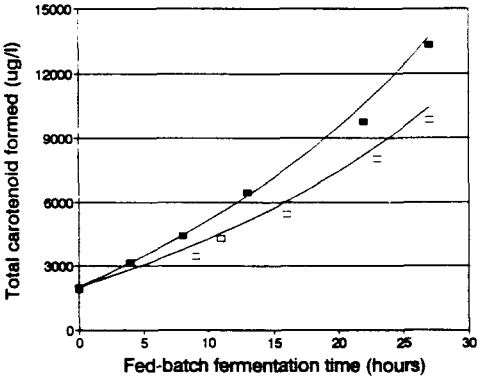

Figure 2.
Comparison of theoretical predictions and experimental data for carotenoid formation during fed-batch cultivation of *P. rhodozyma* at 22°C, pH 5.0, and glucose at 40 (□) and 55(□) g/l in the feed.

feed pump and culture volume losses from sampling, the actual experimental culture volumes and feed rates during cultivation varied slightly from the theoretical values predicted by the feed regime calculation. Similarly, Equation 7 was used to predict the total carotenoid formation profile of *P. rhodozyma* during fed-batch cultivation. Figure 2 shows a comparison between the predicted and experimental values of the total carotenoid content of *P. rhodozyma* obtained during fed-batch cultivation at glucose concentrations of 40 and 55 g/l in the feed. Values predicted theoretically (by calculating the feeding profile and using Equation 8) again show good comparison with the actual experimental data.

Fed-batch cultivation of *P. rhodozyma* on molasses medium, with a total sugar concentration of 40g/l, was also investigated. The nitrogen and phosphate composition of the molasses medium was adjusted in order to ensure carbon limitation. Using the same kinetic constants and the same approach as detailed above for glucose medium, the experimental data for *P. rhodozyma* grown on molasses medium showed good agreement with the predictions for total biomass and carotenoid concentrations (Figure 3). Furthermore, comparison of the biomass yields and pigment levels for *P. rhodozyma* grown on molasses medium with those for glucose medium at the same fermentable sugar concentration (40 g/l) also shows good agreement (Figure 4). The successful application of this model to fed-batch cultivation of *P. rhodozyma* on molasses medium indicates that similar growth and pigmentation yields are obtained when *P. rhodozyma* is grown on either glucose or molasses medium.

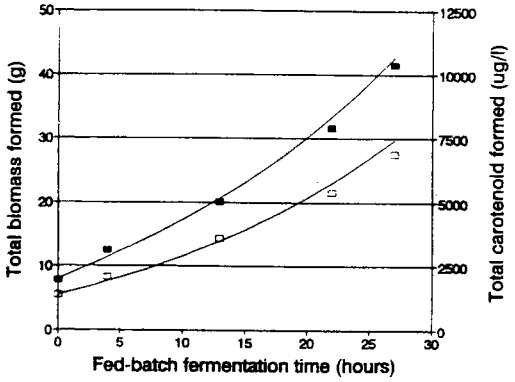

Figure 3.
Comparison of theoretical predictions and experimental data for biomass (□) and carotenoid (□) formation during fed-batch cultivation of *P. rhodozyma* at 22°C, pH 5.0, and molasses sugar of 40 g/l in the feed.

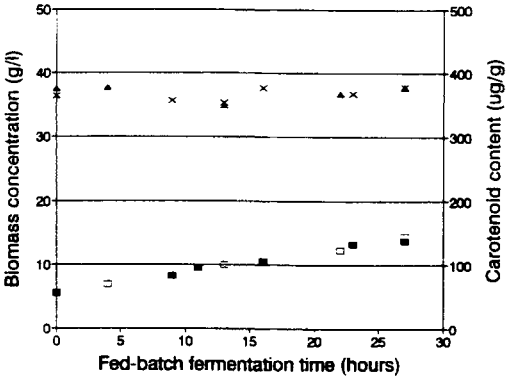

Figure 4.
Comparison of biomass and carotenoid formation on glucose and molasses substrates during fed-batch cultivation of *P. rhodozyma* at 22°C, pH 5.0 and 40 g sugar/l in the feed. (Glucose: biomass (□),carotenoid (X); Molasses: Biomass(□), carotenoid (Δ)).

CONCLUSIONS

Using continuous culture, kinetic parameters μ_{max} and K_s as well as yield coefficients have been evaluated for *P. rhodozyma.* Fed batch cultivation of *P. rhodozyma* has been shown to be an efficient method for growing *P.rhodozyma* cells. By use of a mass balance approach, Monod kinetics and a growth associated product expression, a model to calculate a suitable feeding regime for exponential fed batch culture was proposed. This enables the accurate prediction of biomass and pigment levels generated in the process. In addition, molasses was shown to be an equivalent carbon source to glucose for which the model generated is also applicable.

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