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Biomass and Pigment Production by *Monascus* during Miniaturized Submerged Culture on Adlay

Supavej Maniyom, Gerard H. Markx

Abstract**—**Three reactor types were explored and successfully used for pigment production by *Monascus*: shake flasks, and shaken and stirred miniaturized reactors. Also, the use of dielectric spectroscopy for the on-line measurement of biomass levels was explored. Shake flasks gave good pigment yields, but scale up is difficult, and they cannot be automated. Shaken bioreactors were less successful with pigment production than stirred reactors. Experiments with different impeller speeds in different volumes of liquid in the reactor confirmed that this is most likely due oxygen availability. The availability of oxygen appeared to affect biomass levels less than pigment production; red pigment production in particular needed very high oxygen levels. Dielectric spectroscopy was effectively used to continuously measure biomass levels during the submerged fungal fermentation in the shaken and stirred miniaturized bioreactors, despite the presence of the solid substrate particles. Also, the capacitance signal gave useful information about the viability of the cells in the culture.

Keywords**—**Chinese pearl barley, miniature submerged culture, *Monascus* pigment, biomass, capacitance.

I. Introduction

ANGKAK as a natural red colorant which is usually made by culturing *Monascus spp.* on cooked rice. *Monascus* can produce colour pigments which are yellow (monascin, ankaflavin), orange (rubropunctatin, monascorubrin) and red (rubropunctamine, monascorubramine) [1]. Angkak has been used as a natural pigment in fish products, Chinese cheese, red wine, tomato ketchup and meat products [2]. Some concerns exist over the use of angkak because the fungus produces a mycotoxin, citrinin. One method that is being explored to reduce citrinin production is a change from rice to other substrates. Chinese pearl barley (adlay) is potentially an interesting raw material which can be used to make angkak adlay with a very low citrinin content [3]. Moreover, Chinese pearl barley in itself may have beneficial effects as it has been used in traditional medicine to treat warts, chapped skin, rheumatism and neuralgia.

In general, pigment production can be divided into two processes: solid state and submerge culture [2, 4, 5]. Pigment production in submerged culture with mechanical aeration can be more uniform than in solid state culture but product concentration is much lower and there is a significant problem with a large amount of wastewater [6, 7].

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Culture times are often long, typically several weeks, and optimization of a production process can therefore take a long time. To reduce the time needed for optimization, the use of Miniature Bioreactors (MBRs) was explored.

MBRs can be classified into shaken devices (flask, microtiter and spin tubes), stirred fermentors, bubble columns and other miniatures devices [8–13]. These bioreactors typically have a small volume, ranging from ca. 0.1 mL to approx. 100 mL. Their use can reduce labour and material costs. In addition many cell cultures can be performed in parallel and high efficiencies be achieved in agitation and aeration. This allows rapidly metabolizing, high-cell density microbial cell cultivations to be supported and increases the amount of the product that oxygen-dependent bioprocesses can yield. It can be expected that growth kinetics and product formation at miniature-scale can be scaled up quantitatively.

Currently, high power monitoring tools are available that enable biochemical engineers to understand and explain quantitatively the presence and activity of cells and the production of their metabolites during cultivation [14]. Such methods can be of considerable help during scale up and scale down of fermentation processes. The presence of fungal biomass is a requisite for pigment production, and strong relation can therefore be expected between biomass levels and pigment production. Measurement of biomass is difficult, especially on-line and in media with high concentrations of particulates which can interfere. Capacitance measurements are one of the best methods for measuring cell concentrations. The success of the method depends on the large difference in the electrical properties of the membrane (dielectric constant or permittivity and conductivity), the cytoplasm and the suspending medium [15,16,17,18]. This results in a change in the capacitance from low to high frequencies in the frequency range 100 kHz-100 MHz, which can be related directly to the microbial biomass concentration. Measurement of the capacitance of a cell suspensions at a single low frequency (typically around 400 kHz), or better the difference between a low and high frequency, can be used to overcome the need for other off line and often time-consuming methods for biomass measurement. An added advantage is that it only measures the volume fraction of biomass that is enclosed by a cellular membrane. Thus, the presence of non-cellular material without a membrane (such rice or adlay) does not have a major influence the measurement, and cell concentrations can be measured in the presence of high concentrations of other materials [17].

II. Materials And Methods

A. Microorganism and medium

Monascus ruber (strain TISTR3006, from Chiang Mai

University, Thailand) was used to produce *Monascus* pigment. Potato dextrose agar (PDA) was used for strain preservation and spore formation. Spore suspensions were prepared with 10^5 CFU/mL as seed cultures. This solution was added to a liquid medium solution in 2% by volume.

The liquid medium for pigment fermentation consisted of D-glucose or adlay powder in a mineral solution and was sterilized at 121°C for 15 minutes. The mineral solution contained KH_2PO_4 0.25%, $NaNO_3$ 0.3% and $MgSO_4.7H_2O$ 0.4% in distilled water (w/v) (modified from Lian [19] and Silveira [20].

B. Adlay Submerged Culture in shake flasks

The liquid medium was prepared by adding 7% (by volume) adlay powder to a 250 mL shake flask with 100 mL mineral solution and sterilizing it at 121°C for 15 minutes. It was then inoculated with 1 mL spore suspension with 10^4-10^5 spores/mL. The flask was incubated on an orbital rotary shaker at 150 rpm and at a controlled temperate of 25°C. Samples were taken to determine the pigment and glucosamine concentration and capacitance every week for 4 weeks.

C. Adlay submerged culture in polycarbonate miniaturized bioreactors.

Miniaturised bioreactors were made from 8 cm high sections of 3.5 cm ID polycarbonate tube and 1 cm thick polycarbonate sheet as shown in Figure 1. The stirred reactor had 4 stainless steel baffles, and was stirred with 3 2cm wide 6 mm high paddle-shaped stirrers. The Biomass sensor probe was inserted through the bottom to measure the capacitance on-line.

Liquid medium with 7% adlay powder in a mineral solution was added to both the shaken and stirred reactors (see figure 1.) The reactors were sterilized at 121^{O}C for 15 minutes and inoculated with a spore solution. The shaken reactor with 20 mL medium was incubated in an orbital rotary shaker at 150 rpm. The stirred reactor, with 10, 20 or 30 mL was mixed at 400, 800 or 1200 rpm. Samples were taken to determine the pigment and glucosamine concentrations.

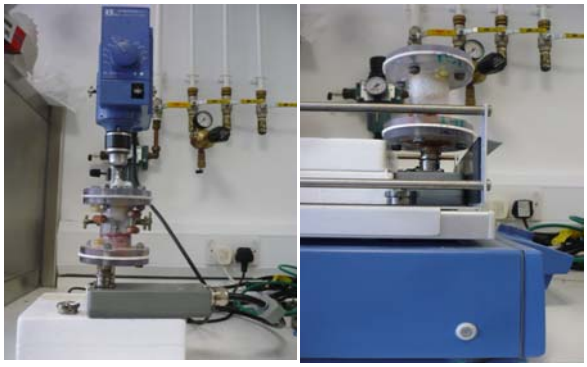


Fig. 1 Experimental setups for *Monascus* submerged culture in stirred (left) and shaken (right) miniaturized reactors

D. Analytical Methods

Fermented broths were dried in the oven at 55°C overnight until the weight was constant. 0.5 g pigment powder was dissolved with 5 mL 70% (v/v) ethyl alcohol and shaken for 8 hours on a reciprocal shaker at 150 rpm. Supernatants were obtained by centrifuge. Optical densities of pigments were measured with a spectrophotometer (Biochem Ltd., WPA spectrophotometer, England) at 400, 470 and 500 nm respectively as yellow, orange and red (modified from Kim [21], Pattangul [22] and Yongsmith [23]).

The growth of fungal culture was estimated off-line using two methods. Dry weights were determined by drying a known volume of cells at 60°C overnight. Also, the amount of N-acetyl glucosamine released during hydrolysis was used as a measure of the fungal cell concentration in food material. Glucosamine is found in chitin present in the fungal cell wall. First, a 0.1 g of sample was subjected to acid hydrolysis by dissolving it in 5 mL 2 M HCl 16-18 hours until clear. The acid hydrolysis sample was then mixed with 1 mL acetylacetone reagent and incubated in a boiling water bath for 20 minutes. After cooling, 1.5 mL ethanol was added followed by the addition of 1 mL of Ehrlich reagent. After that, the optical density was read at 530 nm against a reagent blank. N-acetyl glucosamine obtained from Sigma-Aldrich was used as the standard (modified from Babitha [24]).

To determine the biomass concentration on-line and offline, an Aber Instruments model 220 Biomass Monitor was used (see Figure 2). The capacitance signal at the probe was measured by taking capacitance data at 25 different frequencies in the range 100 kHz-20 MHz. The data were then averaged (minimally 30 datapoints at a given frequency).

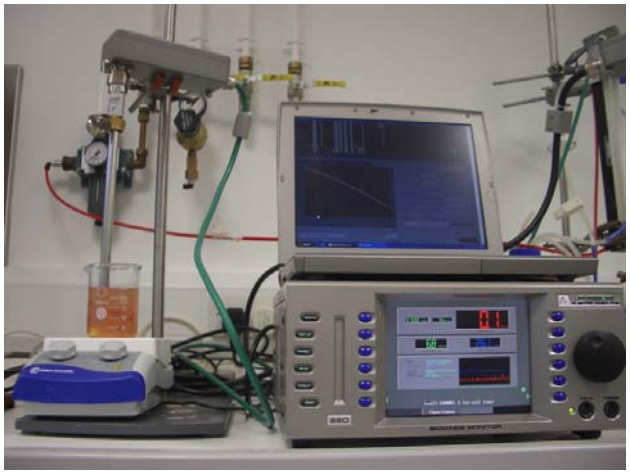


Fig. 2 The Aber Instruments Biomass Monitor

III. Results and Discussion

A. Calibration of the relation between fungal biomass, glucosamine concentration and capacitance signal

The relationship between capacitance and the cell concentration was determined first. To calibrate the Biomass Monitor, *Monascus* was cultured in 5 magnetically stirred Erlenmeyer flasks with 100 mL 4% D-glucose solution for 7 days.

The samples were harvested by centrifugation to obtain a suspension with a high concentration of fungal biomass. A 100 mL mineral solution was prepared. The concentrated fungal biomass was stepwise added to the stirred beaker with the mineral solution and the capacitance signal measured in the range 100 kHz-20 MHz. Samples were also taken from beaker to obtain the dry weight, and the glucosamine content of the samples.

The correlation between the dry weight of cells and glucosamine content is shown in figure 3(a). A linear relation between the fungal dry biomass weight and glucosamine content can be observed described by the equation: Y= 0.37x. The relation between the dry weight and the capacitance at 465 kHz-2MHz is shown in figure 3(b). A linear relation can be seen between dry weight and capacitance described by the equation: Y= 0.80 +1.01.

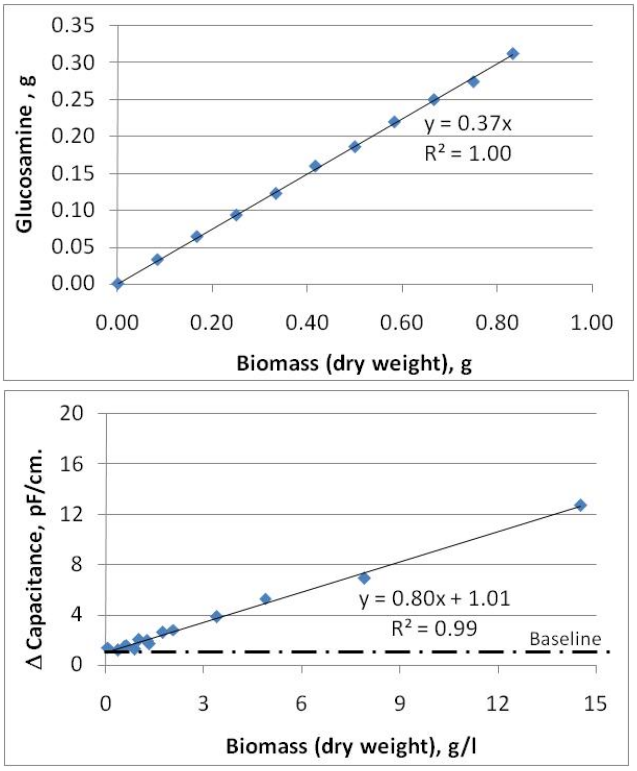


Fig. 3 Biomass and substrate calibration
(a) Relation between dry weight and glucosamine content.
(b) Cell dry weight and ΔCapacitance between 465 kHz-2MHz

B. Adlay submerged culture in shake flasks.

Figure 3 shows the changes in the glucosamine and pigment concentration and capacitance during a shaken flask culture during 4 weeks of fermentation. The capacitance value reached the maximum value of around 6 pF/cm in two weeks; however the glucosamine concentration kept increasing until week 3. Pigment production was small in the first week and reached its maximum after three weeks. A drop in the capacitance at the later stages of batch fermentations is commonly found and is thought to be caused by drop in the viability of the cells, the capacitance of nonviable cells being much lower than that of viable cells [15-18]. Glucosamine content, however, is indicative of the presence of cell wall material only, and not dependent on viability. Even though less viable, the cells still continued pigment production. The pigment content therefore kept increasing even though the capacitance declined. Interestingly, the decline in capacitance coincided with a decrease in the rate of pigment production, indicating a possible role of capacitance measurements in indicating when the rate of pigment production will decline.

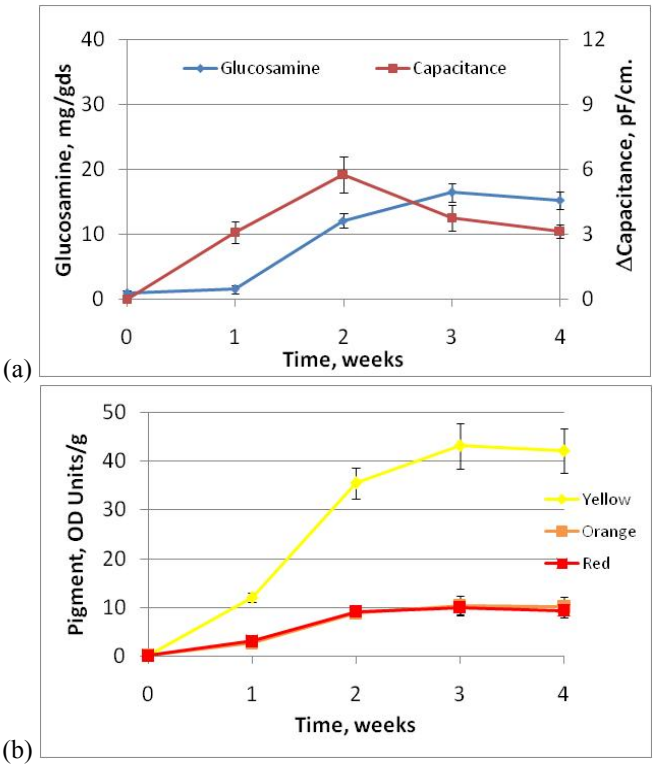


Fig. 4 Submerged fermentation in shake flasks
(a) Capacitance and glucosamine concentration
(b) Pigment production

C. A Comparison of Monascus growth and pigment production in shaken and stirred reactors

In the next set of experiments a comparison was made of the culture of *Monascus* in a shaken (150 rpm) and a stirred (800 rpm) miniaturized fermenter. In both types of reactors the biomass was monitored on-line using capacitance measurements. After the culture had finished, samples were taken to measure the pigment and glucosamine concentrations. It was not possible to take samples during culture.

Changes in the capacitance at a low and high frequencies, and the difference between the two frequencies, during the fermentation in the two reactor types are shown in Figure 5. In the shaken miniaturized bioreactor in the first 3 days the Δcapacitance signal only increased slightly. After that, it increased rapidly to reach a maximum value of 9.5 pF/cm in 12 days. After 12 days, the signal started to drop off. In the stirred miniaturized bioreactor the lag phase was much shorter (a day) and the increase in capacitance much faster than in the shaken reactor.

A maximum in the Δcapacitance was reached after a period of only 6 days and decreased after 8 days.

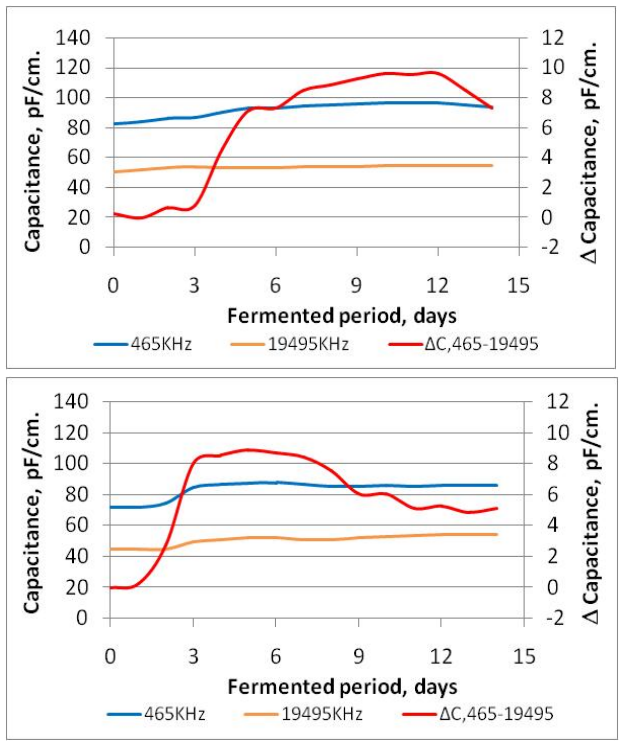


Fig. 5 Capacitance signal during submerged culture of *Monascus* (a) Shaken bioreactor (b) Stirred bioreactor

In figure 6, the results of the chemical analysis of the product of the fermentations are shown. When comparing both of the miniaturized systems, the total pigment and glucosamine content of the material is found to be higher in the stirred reactor in the shaken reactor column. The pigment composition in the shaken reactor is also different from that in the stirred reactor, with more orange and red pigment being produced in the stirred reactor than in the shaken reactor. The appearance of extracted pigment from the shaken reactor was distinctly yellow, whereas from the stirred reactor it was red.

The difference in the results is arguably because there is a greater transfer of oxygen in the stirred reactor than in the shaken reactor [25, 26]. The glucosamine concentration and the capacitance levels in the two systems were not significantly different. This means that oxygen content in miniature scale reactors may be sufficient to create biomass, but in the case of the shaken reactor not sufficient enough to realise *Monascus* fullest potential in pigment products, presumable because production of secondary metabolites has a higher oxygen demand than the primary metabolism needed for biomass production.

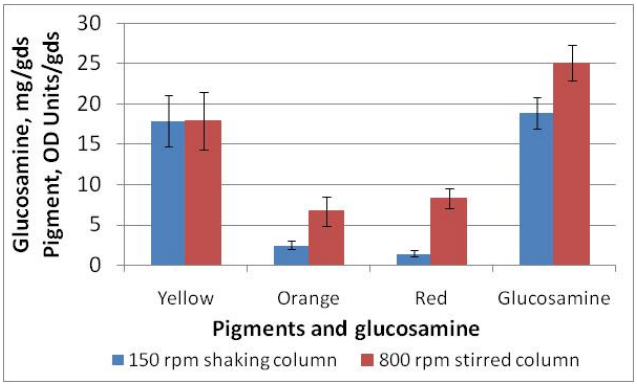


Fig. 6 Comparison of *Monascus* pigment and glucosamine concentration in ground adlay after 2 weeks culture in shaken and stirred bioreactors

D. Effect of the rotating speed of the impeller and the volume of liquid on the culture of Monascus in a miniaturized bioreactor.

To investigate this further, the effect of a rotating speed of impeller and volume of liquid in the reactor on biomass and pigment production was studied. It can be expected that an increasing rotating speed increases oxygen dispersal, and that an increasing volume increases oxygen demand. Rotating speeds were varied between 400, 800 and 1200 rpm, and the volume of liquid in the reactor medium from 10 to 30 mL.

Figure 7 shows the effect of the impeller speed and the volume of the liquid in the reactor on glucosamine production. The expectation mentioned above were confirmed, i.e. a higher stirred speed led to a higher glucosamine concentration, and an increasing volume in the reactor decreased it. Interestingly, at higher rotating speeds the effect of volume of liquid in the reactor on the glucosamine production was less than at lower rotating speed. This may indicate that mixing is more highly efficient at higher speeds.

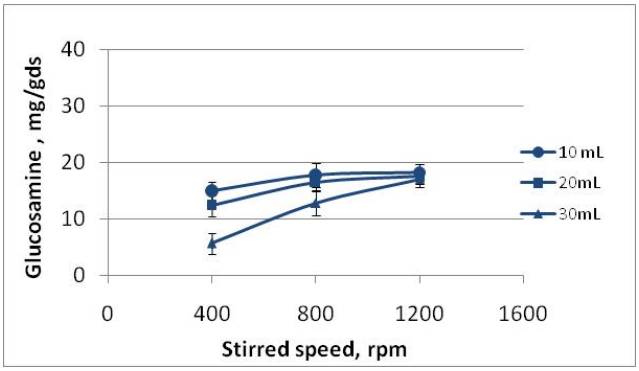


Fig. 7 Glucosamine production in a stirred miniature submerged culture of *Monascus* on ground adlay

Figure 8 shows the effect of the impeller speed and the volume of liquid medium in the reactor on the Δcapacitance during a 7 day fermentation. As expected, the data show a positive effect of the rotation speed of the impeller and a negative effect of the volume of liquid medium in the reactor. However, unlike the previous results obtained with glucosamine, the Δcapacitance signal at the higher impeller speed of 1200 rpm was different for different volumes in the

reactor. This may arguably be because the shear in the bioreactors is different. Shear affects cell viability and hence capacitance [27]. Shear is known to damage *Monascus* filaments and change their morphology, and affect pigment production [28]. The higher shear stress at higher impeller rates may have affected the cell viability in different ways at different volumes in the reactor.

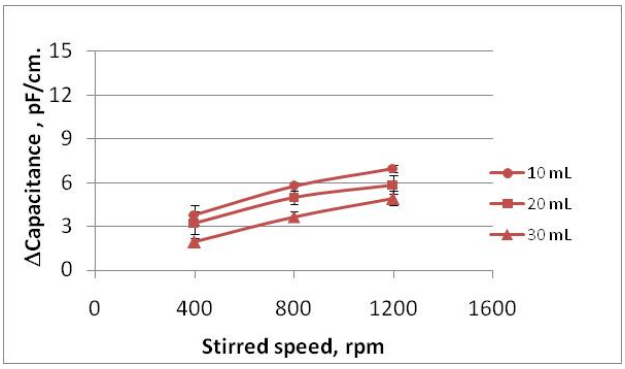
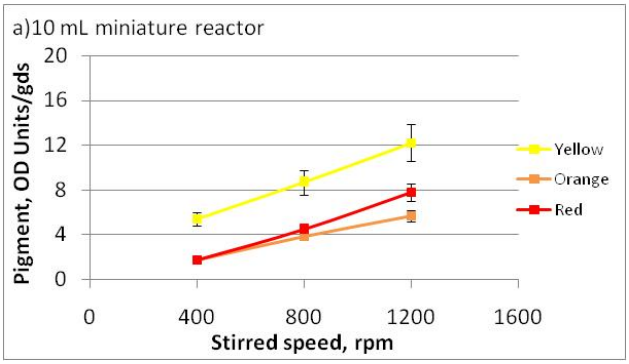


Fig. 8 Capacitance signal in a stirred miniature submerged culture of *Monascus* on ground adlay

Figure 8 show the effect of the impeller speed and the volume of liquid in the reactor on pigment yield in the miniaturized bioreactor. As expected, rotating speed had a positive effect and liquid volume a negative effect. In the 10 mL fermenter, the pigment production increased more than 2.5 times within the 7 days of culture when the rotating speed was increased from 400 rpm to 1200 rpm. In the 20 mL fermenter, the pigment concentration increased less than in the 10 mL fermenter when operated with the same rotating speed. Finally, in the 30 mL fermenter, the pigment yield increase with impeller speed was similar to that in the 20 mL fermenter. The highest pigment yields were achieved in the smallest volume and at highest impeller speed. Significantly more red pigment was produced in the smaller volume and at the higher impeller speed, again indicating that more oxygen is needed for red pigment production



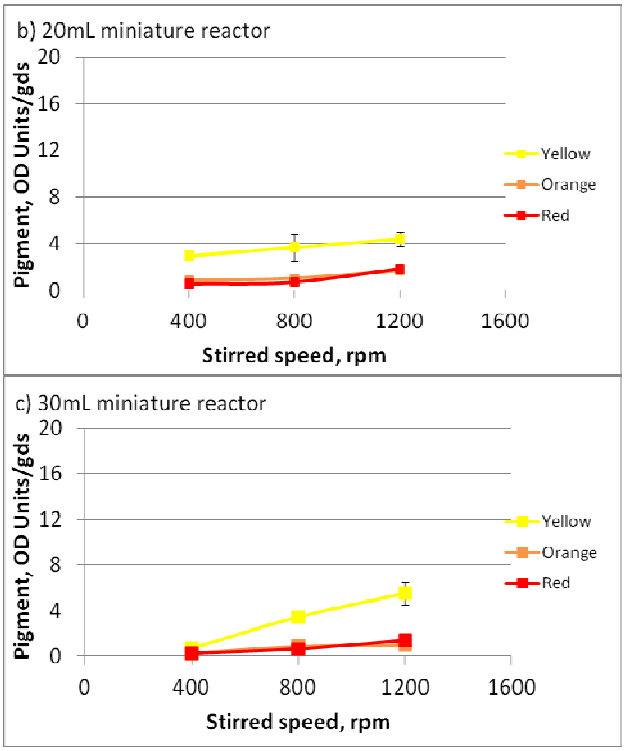


Fig. 9 *Monascus* pigment production in a stirred miniature submerged culture on ground adlay

IV. Conclusion

Miniaturized bioreactors were successfully used to investigate pigment and biomass production during submerged fermentation of *Monascus* on adlay. Shake flasks gave the high pigment concentrations, but on-line measurements were not possible, and scale up is difficult. Miniaturized shaken bioreactors were developed in which continuous biomass measurement using dielectric spectroscopy was possible, but pigment production was less successful, most likely due to poor mixing and oxygen transfer in the shaken column being lower. Stirred bioreactors proved better, with better pigment yields at smaller volumes of liquid in the fermenter at higher impeller speeds again indicating that the availability of oxygen is a determining factor in pigment production.

Dielectric spectroscopy proved to be an effective method for measuring biomass continuously in the miniaturized reactors. It also gave useful information about cell viability. Extension of the number of sensed parameters during the miniaturized submerged culture of *Monascus*, for example by the use for sensors for oxygen or pigment concentration, could increase the amount of information available even further

Acknowledgment

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REFERENCES

[1] S.-W. Chiu, "Submerge production of Monascus pigments," Mycologia, vol. 85, 214-218, 1993.

[2] L. Dufossé, P. Galaup, A. Yaron, S. M. Arad, P. B. Kotamalli, K. N. C. Murthy and G. A. Ravishankar, "Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality," Trends in Food Science & Technology vol. 16, pp. 389-406, 2005.

[3] J. H. Yang, Y. Tseng, H. Chang, Y. Lee and J. Mau, "Storage stability of monascal adlay," Food Chemistry, vol. 90, pp. 303-309, 2004.

[4] P. Jůzlová, L. Martínková and V. Křen, "Secondary metabolites of the fungus Monascus: review," Journal of Industrial Microbiology, vol. 16, pp. 163-170, 1996.

[5] T. H. Wang, and T. F. Lin, "Monascus rice products," Advances in Food and Nutrition Research, vol. 53, pp.123-159, 2007.

[6] U. Hölker, M. Höfer and J. Lenz, "Biotechnological advantages of laboratory-scale solid-state fermentation with fungi," Applied Microbiology Biotechnology, vol. 64, pp. 175-186, 2004.

[7] R. R. Singhania, A. K. Patel, C. R. Soccol and A. Pandey, "Recent advance in solid state fermentation," Biochemical Engineering Journal, vol. 44, pp.13-18, 2009.

[8] J. I. Betts and F. Baganz, "Miniature bioreactors: current practices and future opportunities," Microbiol Cell Factories, vol. 5, pp. 1-14, 2006.

[9] W.A. Duetz, "Microtiter plates as mini-bioreactors: miniaturization of fermentation methods," Trends in Microbiology, vol. 15, pp. 469 – 475, 2007.

[10] V. Hessel, C. Knobloch and H. Löwe, "Review on patents in microreactor and micro process engineering," Recent Patents on Chemical Engineering, vol. 1, pp. 1-16. , 2008.

[11] S. R. Lamping, H. Zhang, B. Allen and P. A. Shamlou, "Design of a prototype miniature bioreactor for high throughput automated bioprocessing," Chemical Engineering Science, vol. 58, pp. 747-758, 2003.

[12] M. Micheletti, and G. J. Lye, "Microscale bioprocess optimisation, "Current Opinion in Biotechnology, vol. 17, pp. 611-618, 2006.

[13] D. Weuster-Botz, S. Stevensb and A. Hawrylenkoc, "Parallel-operated stirred-columns for microbial process development," Biochemical Engineering Journal, vol. 11, pp. 69-72, 2002.

[14] U. v. Stockar, S. Valentinotti, I. Marison, C. Cannizzaro and C. Herwig, "Know-how and know-why in biochemical engineering," Biotechnology Advance, vol. 21, 417-430, 2003.

[15] R. Fehrenbach, M. Comberbach and J. O. Pêtre, "On-line biomass monitor by capacitance measurement," Journal of Biotechnology vol. 23, pp. 303-314, 1992.

[16] R. M. Matanguihan and K. B. Konstantinov and T. Yoshida, "Dielectric measurement to monitor the growth and physiological states of biological cells," Bioprocess Engineering, vol. 11, pp. 213-222, 1994.

[17] W. Penaloza, C. L. Davey, J. N. Hedger and D. B. Kell, "Stimulation by potassium of the growth of Rhizopus oligosporus during liquid- and solid-substrate fermentations," World Journal of Microbiology and Biotechnolpgy, vol 7, pp. 260-268, 1991.

[18] K. Mishima, A. Mimura, Y. Takahara, K. Asami and T. Hanai, "On-line monitor of cell concentrations by dielectric measurements," Journal of Fermentation and Bioengineering, vol. 72, pp. 291-295, 1991.

[19] X. Lian, C. Wang and K. Guo, "Identification of new red pigments produced by Monascus ruber," Dyes and Pigments, vol. 73, pp. 121-125, 2007.

[20] S. T. Silveira, D. J. Daroit, and A. Brandelli , "Pigment production by Monascus purpureus in grape waste using factorial design," LWT-Food Science and Technology, vol. 41, pp.170-174, 2007.

[21] H. J. Kim, J. H. Kim, H. J. Oh and C. S. Shin, "Morphology control of Monascus cells and scale-up of pigment fermentation," Process Biochemistry, vol.38 pp. 649-655, 2002.

[22] P. Pattangul, R. Pinthong, A. Phianmongkhol and S. Tharatha, "Mevinolin, citrinin and pigments of adlay angkak fermented by Monascus sp.," International Journal of Food Microbiology, vol. 126, pp. 20-23, 2008.

[23] B. Yongsmith, V. Kitprechavanich, L. Chitradon, C. Chaisrisook, and N. Budda, "Color mutants of Monascus sp. KB9 and their comparative glucosamylases on rice solid culture," Journal of Molecular Catalysis B: Enzymatic, vol. 10, pp.263-272, 2000.

[24] S. Babitha, C. R. Soccol, and A. Pandey, "Solid-state fermentation for the production of Monascus pigments from jackfruit seed," Bioresource and Technology, vol. 98, pp. 1554-1560, 2007.

[25] W. Kloeckner, and J. Buchs, "Advances in shaking technologies," Trends in Microbiology, vol. 30, pp.-307-313, 2012.

[26] M. Micheletti, and G. J. Lye, "Microscale bioprocess optimization." Current Opinion in Biotechnology, vol. 17, pp.611-618, 2006.

[27] G.H. Markx, H. J. G. ten Hoopen, J. J. Meijer, and K. L. Vinke, "Dielectric spectroscopy as a novel and convenient tool for the study of the shear sensitivity of plant cells in suspension culture," Journal of Biotechnology, vol. 19, pp. 145-158, 1991.

[28] H. J. Kim, J.H. Kim, H. J. Oh, and C. S. Shin, "Morphology control of Monascus cells and scale-up of pigment fermentation," Process Biochemistry vol. 38, pp 649-655, 2002.