A PRELIMINARY STUDY ON CULTIVATION OF *MUCOR PLUMBEUS* FOR MICROBIAL OIL PRODUCTION USING MOLASSES

By

Z ZHANG, G CAI, J TE'O, HENG-HO WONG, I O'HARA
Centre for Tropical Crops and Biocommodities,
Queensland University of Technology, Brisbane, Australia
jan.zhang@qut.edu.au

KEYWORDS: *Mucor plumbeus*, Microbial Oils, Molasses, Morphology, Stirred Tank Reactor.

Abstract

SUGARCANE MOLASSES, SUGARCANE trash and bagasse are the major by-products generated in cane sugar production process. Conversion of these by-products into valuable products has the potential to improve the profitability of the sugarcane industry. Biofuels are one of the value-added products. However, the profits from the production of low value biofuels such as bioethanol and biodiesel are marginal under current market conditions. In recent years, production of high value advanced drop-in biofuels from renewable carbohydrate feedstocks has gained increasing interests worldwide. The research team at QUT is working together with industrial partners on advanced biofuels production from sugarcane processing by-products through a two-stage process. In the first stage, microbial oils are produced by oleaginous microorganisms. In the second stage, advanced biofuels are produced through hydrodeoxygenation of either microbial oils extracted from microbial biomass or microbial oils obtained from hydrothermal liquefaction of microbial biomass. In this study, microbial oil production by an oleaginous filamentous fungus, *Mucor plumbeus,* was carried out using molasses as a carbon source. Morphology control strategy and nutrient optimisation were firstly developed to improve biomass and microbial oil production. Furthermore, microbial oil production by *M. plumbeus* was scaled up from shake flasks to laboratory scale stirred tank reactors. Inoculation of crushed fungal pellet biomass led to the formation of dispersed short hyphae in reactors and improved biomass and oil production. Finally, the inoculation strategy was demonstrated in a 1 000 L reactor at Mackay Renewable Biocommodity Pilot Plant.

Introduction

Sugarcane industry generates large quantities of low-value by-products like molasses, sugarcane bagasse and trash. Conversion of these by-products into high-value products will bring additional revenue to the industry. Since most of these by-products are rich in carbohydrates, they can be used as carbon sources for biofuels production through microbial fermentation processes.

Currently, fuel ethanol from sugarcane juice and molasses is produced in leading sugarcane producing countries such as Brazil and Thailand. However, the disadvantages of fuel ethanol including is low-heating value and difficulty in blending with fossil-derived fuels have limited its application. In addition, the low crude oil price at current market conditions makes ethanol not competitive as fuel compared with fossil-derived fuels.

In recent years, production of higher value advanced drop-in biofuels has attracted increasing research interests worldwide. Advanced biofuels with higher heating value can be produced from a number of carbon sources through a variety of biological and thermochemical processes. Oils accumulated by oleaginous microorganisms under nutrient limitation conditions are a promising feedstock for advanced biofuels production, which are converted to drop-in fuels through a hydrodeoxygenation process.

Microalgae have been widely studied for microbial oil production while oleaginous yeast and filamentous fungi are emerging oil producers. Compared with microalgae and yeast, biomass of filamentous fungi can be harvested easily for downstream processing. However, it is challenging to control the morphological forms of filamentous fungi for process control and scale-up as they vary depending on cultivation conditions. The variation of morphological forms can significantly affect the product yield and productivity. Although morphology control of filamentous fungi has been studied for the production of organic acids, proteins and enzymes, related research on oleaginous filamentous fungi is very limited.

Microbial oil production from sugarcane molasses has been previously using oleaginous yeast and algae (Vieira *et al.*, 2016; Yan *et al.*, 2011). In this study, microbial oil production from molasses using an oleaginous filamentous *Mucor plumbeus* was studied in terms of nutrient optimisation, morphological control and process scale-up. This study provides an option for microbial oil production using oleaginous filamentous fungi.

Materials and methods

Materials

M. plumbeus FRR no. 2412 purchased from FRR Culture Collection (Australia) was used as a model fungus for microbial oil production from molasses. Fungal spores were produced by cultivation of *M. plumbeus* on potato dextrose agar (PDA) plates at 28 °C for 5 days. Afterwards, the PDA plates were stored at 4 °C for later use. Chemicals of analytical grade were purchased from Sigma-Aldrich (Australia). Molasses was collected from Racecourse Sugar Mill in Mackay. The composition of molasses is shown in Table 1.

Table 1—Composition of molasses.

Composition	Content, g/kg	
	Original molasses	Diluted molasses (30 g/L glucose equivalent)
Sucrose	435.6	23.8
Glucose	45.9	2.5
Fructose	44.4	2.4
Composition	Content, mg/kg	
	Original molasses	Diluted molasses (30 g/L glucose equivalent)
N	3500	191.3
P	290	15.9
Mg	2410	131.7
Ca	3900	213.2
Fe	116	6.34
Zn	3.6	0.20
Cu	1.4	0.08
Co	0.8	0.04
Mn	18.8	1.03

Preculture

Preculture was conducted in a 250 mL shake flask containing 50 mL preculture medium consisting of 2.0 g/L glucose, 8.0 g/L sucrose, 1.0 g/L $(NH_4)_2SO_4$, 7.0 g/L KH_2PO_4, 2.0 g/L Na_2HPO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$ 0.1, 0.008 g/L $FeCl_3 \cdot 6H_2O$, 0.001 g/L $ZnSO_4 \cdot 7H_2O$, 0.0001 g/L $CuSO_4 \cdot 5H_2O$, 0.0001 g/L $CoCl_2 \cdot H_2O$, and 0.0001 g/L $MnSO_4 \cdot 5H_2O$ 0.0001 g/L. This medium composition was referred to a previous study (Kavadia *et al.*, 2001). 1 mL of spores solution containing 1×10^7 spores was transferred to the shake flask to start the preculture. Preculture was conducted in a rotary shaker at 28 °C with an agitation speed of 180 rpm for 24 h.

Repeated preculture

5 mL of 1st preculture was transferred to a 2nd 250 mL shake flask containing 50 mL fresh preculture medium, followed by 24 h cultivation at the same conditions. At the end of 2nd preculture, 5 mL of the preculture was transferred to a 3rd 250 mL shake flask containing 50 mL fresh medium.

Preculture of the 3rd preculture was also conducted for 24 h. After each preculture, biomass was collected, washed by tap water and filtrated under vacuum.

Preparation of crushed pellet biomass

5 mL of 1st preculture was transferred to a 2nd 250 mL shake flask containing 50 mL synthetic preculture medium (same as the 1st preculture media) and a 2nd 250 mL shake flask containing 50 mL molasses preculture medium (0.8 g/L glucose, 0.8 g/L fructose, 7.9 g/L sucrose and the additional nutrients concentrations same as those for 1st preculture medium). The 2nd precultures were cultivated for 24 h with the conditions same as those for 1st preculture. The 2nd preculture was transferred to an 80 mL container and crushed for 60 s using a Hamilton Beach® Blender. Crushed pellet biomasses from the synthetic preculture and molasses preculture were used as the seed cultures to inoculate in synthetic molasses and real molasses media, respectively.

Growth in synthetic molasses and real molasses production media in shake flasks

Synthetic molasses production medium consisted of 2.5 g/L glucose, 2.5 g/L fructose, 24.0 g/L sucrose and other nutrients with the concentrations same as the preculture medium. Real molasses production medium consisted of 30 g/L glucose equivalent (2.5 g/L glucose, 2.4 g/L fructose and 23.8 g/L sucrose), 1.0 g/L $(NH_4)_2SO_4$, 7 g/L KH_2PO_4 and 2 g/L Na_2HPO_4. Crushed preculture biomass was inoculated to 250 mL shake flasks containing 50 mL production media at inoculum sizes of 2%, 6%, 10% and 20% (preculture volume/medium volume).

Nutrient optimisation

Nitrogen

Effect of nitrogen level on cultivation of *M. plumbeus* was carried out in the real molasses medium in the presence of 7.0 g/L KH_2PO_4 and 2.0 g/L Na_2HPO_4. Different amounts of $(NH_4)_2SO_4$ were added in the molasses medium to achieve $(NH_4)_2SO_4$ concentrations of 0, 0.25, 0.5 and 1.0 g/L. The cultivation was carried out in a 250 shake flask containing 50 mL medium and started with the inoculation of fungal spores (2×10^5 spores/mL medium). After 48 h cultivation, the fungal biomass was collected, washed and dried at 45 °C under vacuum.

Phosphorus

Effect of phosphorus level on cultivation of *M. plumbeus* was carried out in the real molasses medium in the presence of 0.5 g/L $(NH_4)_2SO_4$ g/L. 4 phosphorus levels were used, including (1) 0 g/L KH_2PO_4 and 0 g/L Na_2HPO_4, (2) 0.175 g/L KH_2PO_4 and 0.05 g/L Na_2HPO_4, (3) 0.35 g/L KH_2PO_4 and 0.1 g/L Na_2HPO_4, and (4) 0.7 g/L KH_2PO_4 and 0.2 g/L Na_2HPO_4. After 48 h cultivation, the fungal biomass was collected, washed and dried at 45 °C under vacuum.

Microbial oil production in reactors

Microbial oil production was conducted in the real molasses medium in a 1.4 L stirred tank reactor (STR) (a working volume of 0.9 L) and a 14 L STR (a working volume of 8 L) located at QUT Gardens Point Campus, respectively, and a 1000 L STR (a working volume of 750 L) located at QUT Mackay Renewable Biocommodities Pilot Plant. The molasses medium contained ~30 g/L sugars (glucose equivalent) with the addition of 0.5 g/L $(NH_4)_2SO_4$ and 0.25 g/L KH_2PO_4 (based on the nutrient optimisation results). The 1.4 L and 14 L STRs were inoculated with crushed pellet biomass from 2nd precultures prepared in shake flasks. The inoculum size was 2.5%. The cultivation was maintained at pH 6.0 controlled by addition of 2 M NaOH and 28 °C. The DO level was maintained at above 20% by controlling the agitation speed at 200 – 600 rpm and the aeration rate at 0.6 – 2.0 vvm. Samples of 20–30 mL were withdrawn every day during cultivation. At the end of cultivation, the total volume of fermentation broth was measured, followed by collection of fungal biomass. The fungal biomass and the liquid were separated by filtration. The biomass was washed and dried at 45 °C under vacuum. The liquid was stored at –20 °C for later analysis.

For the cultivation using the 1000 L STR, a two-step preculture process was used to prepare the seed culture. The 1st preculture was carried out using the synthetic preculture medium in 8 × 2.0 L shake flasks containing 8 × 500 mL synthetic preculture medium.

After 24 h cultivation at 28 °C and 180 rpm, the 1st precultures were transferred to a 15 L STR containing 10 L of the real molasses medium (~30 g/L glucose equivalent with the addition of 0.5 g/L $(NH_4)_2SO_4$ and 0.25 g/L KH_2PO_4) to start the 2nd preculture. After 48 h cultivation, the 2nd preculture was collected and the fungal pellets were settled down. The fungal pellets were transferred to a 4 L Hamilton Beach Blender.

The pellets were crushed for 60 s, followed by inoculation of the crushed biomass into the 1000 L STR containing 750 L real molasses medium (~30 g/L glucose equivalent with the addition of 0.5 g/L $(NH_4)_2SO_4$ and 0.25 g/L HK_2PO_4) to start the cultivation. The cultivation was carried out with an aeration rate of 1.0 vvm at 28 °C for 6 days.

After 6 days cultivation, the fungal biomass was collected, washed by tap water and pressed through a juice presser. The fungal biomass was firstly air-dried under sunlight and further dried in a vacuum oven at 45 °C.

Analysis

Microbial oil content in the dried fungal biomass was determined by the Bligh and Dyer method (Bligh an Dyer, 1959) with modification. Briefly, dried fungal biomass of 40.0 mg was transferred to a 2.0 mL centrifuge tube containing 4 stainless steel beads (1 bead with a diameter of 5.0 mm and 3 beads with a diameter of 2.5 mm). The tube was sealed with a screw-on lid and fungal biomass was lysed for 4 min by a tissue lyser (QIAGEN, US). Following lysis, 0.4 mL water, 0.3 mL chloroform and 0.6 mL methanol were added into the centrifuge tube, respectively.

The mixture was further homogenised for 2.0 min, followed by centrifugation at 12,000 rpm for 2 min. After centrifugation, 0.3 mL chloroform was added to the centrifuge tube, followed by 0.5 min homogenisation and 2 min centrifugation at 12,000 rpm, respectively. The bottom layer was removed by a 1 mL syringe with a stainless steel needle and transferred to a pre-weighed HPLC vial. Another 0.3 mL chloroform was added to the centrifuge tube to repeat the extraction steps. The extraction was repeated 3 times (a total extraction times of 4) and the bottom layer solutions were transferred to the same HPLC vial. The solvents in the HPLC vial were evaporated out at 45 °C under vacuum. The amount of oils left in the vial was determined.

Sugars in molasses and fermentation media were detected by a high performance liquid chromatography (HPLC) system (Waters, US) equipped with a Dionex CarboPac™ PA-1 column (4 × 250 mm, Thermo Scientific, US), an electrochemical detector (Waters 2465) and the pump and autosampling system (Waters e2695, US).

The mobile phase was 200 mM NaOH with a flow rate of 1.0 mL/min. Fermentation by-products like ethanol was analysed by HPLC equipped with a Bio-Rad 87H column (8 × 300 mm, US) and refractive index (RI) detector (Waters 410). The mobile phase for the Bio-Rad 87H column was 5 mM H_2SO_4 with a flow rate of 0.5 mL/min.

Morphological pictures of *M. plumbeus* were taken by a digital camera after transferring fungal biomass solutions to 9 cm Petri dishes.

Results

Repeated cultures with pellets leading to reduced biomass yield

As shown in Figure 1, small uniform pellets with an average dimeter of ~1 mm were readily formed with the inoculation of spores at a final concentration of 2×10^5 spores/mL. Repeated cultivation of pellets in the 2nd and 3rd led to a significant increase in pellet size (Figure 1).

Although the biomass concentration increased from 1.7 g/l after 24 h 1st preculture to 2.0 g/L after 24 h 2nd preculture, further increase in pellet size in the 3rd preculture led to a significantly decreased biomass concentration to 1.4 g/L.

A preliminary scale-up test (from shake flasks to a 100 L bubble column reactor) using fungal pellets as the seed culture showed that the final biomass concentration was only 1.6 g/L and the pellet size increased to >5 mm after 60 h cultivation at an initial sugar concentration (glucose equivalent) of ~30 g/L (data not shown).

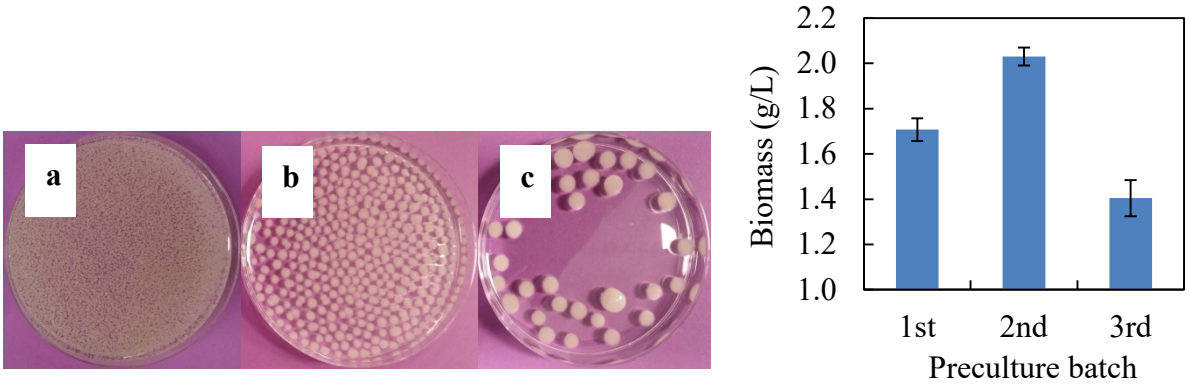

Fig. 1—Effects of repeated cultivations on fungal pellet size and biomass concentration. a, 1st preculture; b, 2nd preculture; c, 3rd preculture; d, biomass concentration.

Morphology control in shake flasks

Previous studies also show that it is possible to form pellets from crushed pellet biomass (Lu *et al.*, 2015).

In this study, this method was firstly tested for the cultivation of oleaginous filamentous fungus, *M. plumbeus,* in shake flasks with two media: synthetic molasses medium and real molasses medium.

Figure 2 shows that with the inoculation in range from 2% to 20% crushed fungal biomass, *M. plumbeus* grew in pellet forms and the pellet size reduced with the increasing inoculum size.

At an inoculum size of 20%, the small uniform pellets and a large fungal biomass clump formed in duplicate flasks.

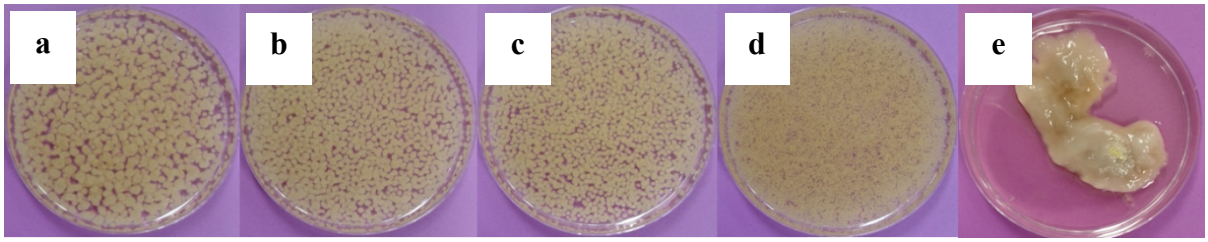

Fig. 2—Effect of inoculum size on morphological forms of *M. plumbeus* in synthetic molasses media. Crushed pellet biomass was used for inoculation. a, 2%; b, 6%; c, 10%, d and e, 20%.

Furthermore, the effect of inoculum size on morphological forms of *M. plumbeus* in the real molasses media was investigated.

As shown in Figure 3, different morphological forms were observed in the real molasses media. With an inoculum size of 2%, pellets formed.

When the inoculum size increased to 6% and above, dispersed hyphae-like fungal biomasses were observed.

The results from the trials with the synthetic molasses and real molasses media show that it is possible to control the fungal morphological forms in either pellets or dispersed hyphae-like fungal biomass by inoculation of crushed pellet biomass, depending on the inoculum size and medium composition.

Optimisation of nitrogen and phosphorus levels

Nitrogen

Nitrogen has a significant effect on microbial oil production and nitrogen limitation is a commonly used strategy to improve microbial oil production (Fakas *et al.*, 2009). Figure 4 shows that increasing nitrogen concentration led to increased biomass concentration. However, the oil

content at the highest $(NH_4)_2SO_4$ concentration of 1.0 g/L was the lowest. As a result, the maximum oil concentration was achieved at 0.5 g/L $(NH_4)_2SO_4$.

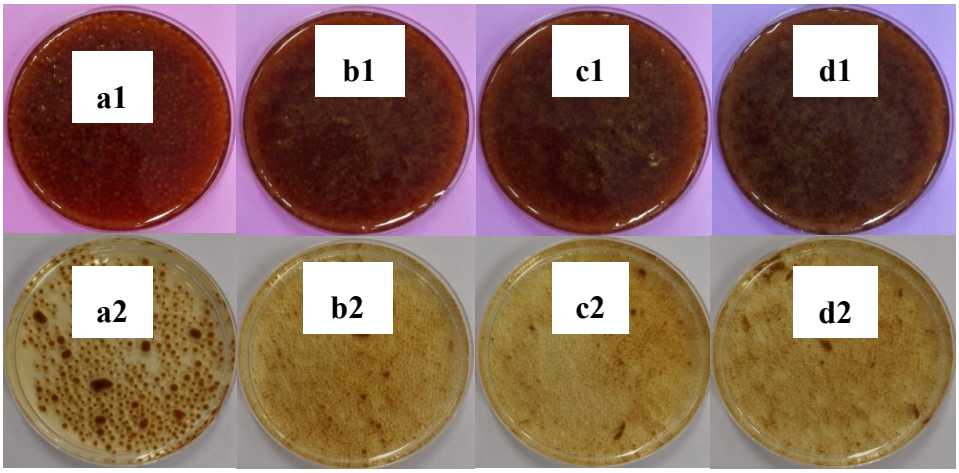

Fig. 3—Effect of inoculum size on morphological forms of *M. plumbeus* in real molasses media. Crushed pellet biomass was used for inoculation. a, 2%; b, 6%; c, 10%, d and e, 20%. Top, original fermentation broth; bottom, fermentation broth diluted 10 times.

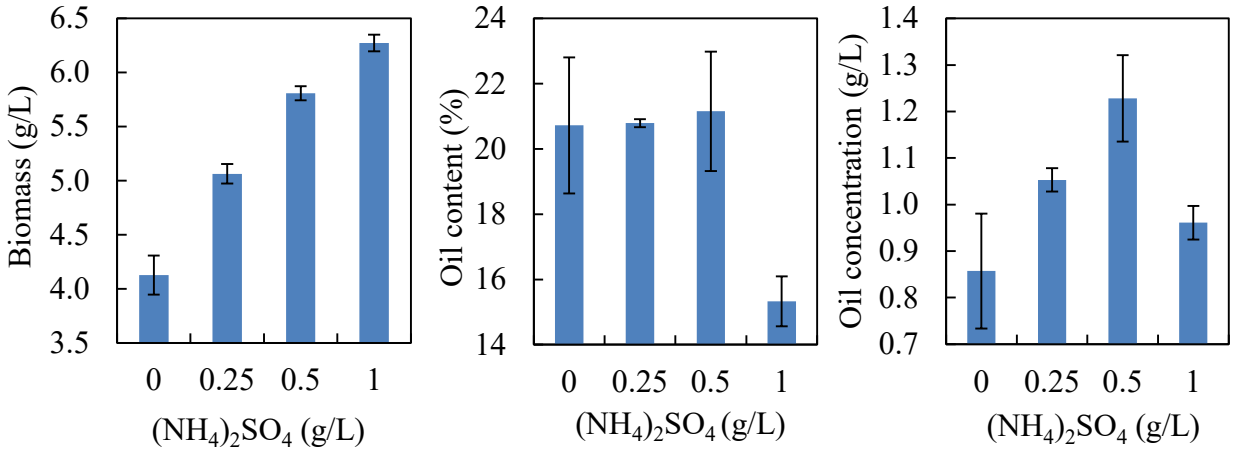

Fig. 4—Effect of $(NH_4)_2SO_4$ concentration on fungal biomass and oil production

Phosphorus

Phosphorus is an expensive nutrient. The effect of phosphate concentrations on biomass and microbial oil production is shown in Figure 5. The phosphate types were based on a previous study (Kavadia *et al.*, 2001).

As can be seen from Figure 5, addition of 0.175 g/L KH_2PO_4 and 0.05 g/L Na_2HPO_4 increased biomass concentration and oil production. However, further increase in phosphate concentrations did not improve biomass and oil production.

Microbial oil production in the 1.4 L, 14 L and 1 000 L STRs

Following the development of inoculation strategy and nutrient optimisation, microbial oil production was conducted in the STRs. The molasses medium was added with 0.5 g/L $(NH_4)_2SO_4$ and 0.25 g/L KH_2PO_4 and inoculated with crushed pellet biomass at 2.5%. Initially, the cultivations were conducted in a 1.4 L STR. As shown in Figure 6, *M. plumbeus* did not grow in pellets in the STR. Instead, it grew as free dispersed hyphae. The hyphae were short and did not entangle to form large biomass clumps. When the fermentation broth containing the fungal biomass was diluted, the hyphae were freely dispersed.

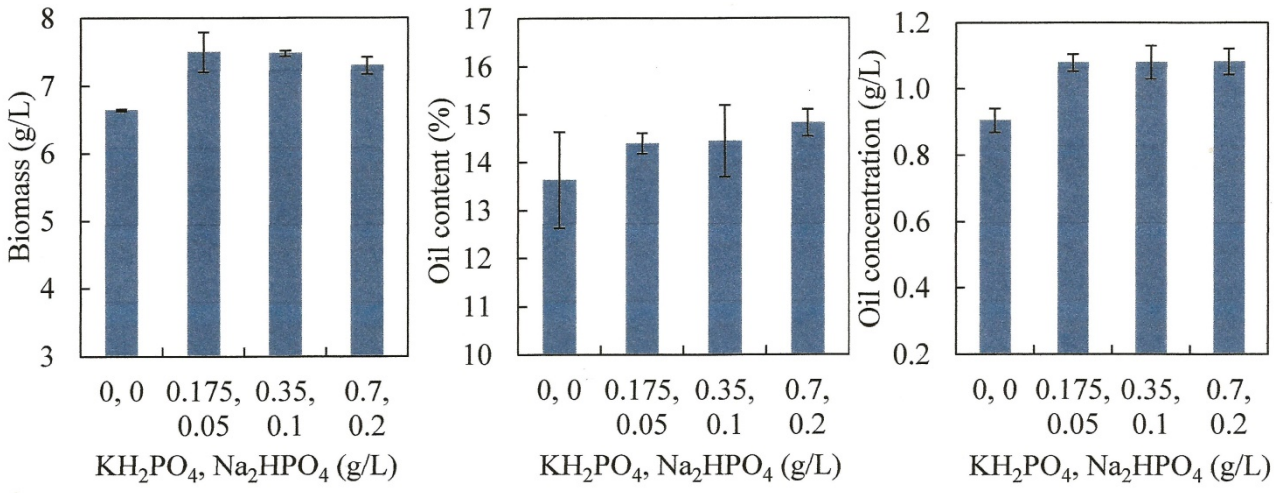

Fig. 5—Effect of phosphate concentration on fungal biomass and oil production.

Fig. 6—Morphological forms of *M. plumbeus* in the 1.4 L reactor.

Further laboratory cultivation of *M. plumbeus* was conducted in the 14 L reactor because the 1.4 L reactor was too small and not suitable for continuous sampling. In the 14 L reactor, similar morphology of *M. plumbeus* to that in the 1.4 L reactor was observed. Figure 7 shows the kinetics of sugars consumption during the cultivation process.

The initial sugars contained 2.6 g/L glucose, 3.3 g/L fructose and 21.4 g/L sucrose. After 2 days of cultivation, sucrose was hydrolysed almost completely to glucose and fructose. The former had a concentration of 2.9 g/L while the later was 11.5 g/L because glucose was firstly consumed by *M. plumbeus* prior to fructose. The total sugars concentration was reduced to only 1.0 g/L after 7 days cultivation. During cultivation, the maximum ethanol concentration was only 0.01 g/L, indicating oxygen supply was sufficient for the growth of biomass and oil production.

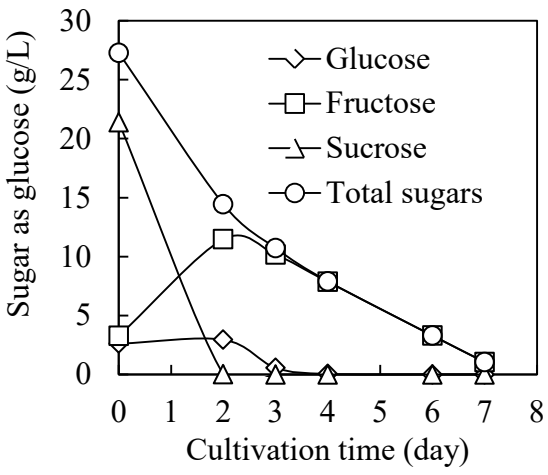

Fig. 7—Kinetics of sugars consumption.

Figure 8 shows the change of oil contents during cultivation. The oil content was 7.4% on day 1, but significantly increased to 17.5% on day 2 and further increased to 22.6% on day 6. The oil content on day 7 had a similar level to that at day 6. It is well known that nitrogen limitation leads to microbial oil accumulation. The low oil content on day 1 was likely due to the high nitrogen concentration and the increased oil accumulation afterwards was due to the depletion of nitrogen.

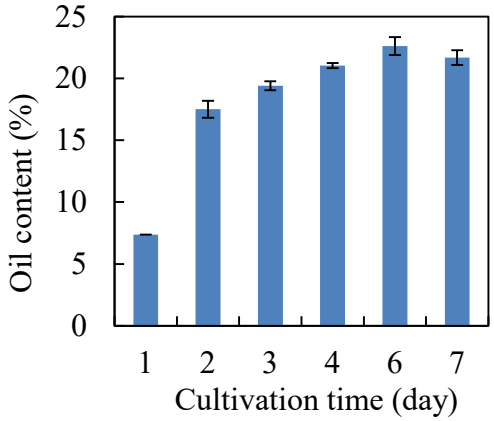

Fig. 8—Microbial oil content during cultivation.

Table 2 summarises the results of microbial oil production by *M. plumbeus* in the 14 L and 1000 L STRs. With the 14 L STR, the biomass concentration after 7 days cultivation reached 9.7 g/L with a biomass yield 0.37 g/g consumed sugars. The oil concentration was 2.1 g/L with an oil content of 21.7% and an oil yield of 0.08 g/g consumed sugars.

In contrast, biomass concentration in the 1000 L reactor reached 7.3 g/L after 6 days cultivation with a biomass yield of 0.43 g/g consumed sugars. The oil concentration was 1.9 g/L with an oil content of 26.0% and an oil yield of 0.11 g/g consumed sugars. In summary, oil content and oil yield on consumed sugars increased with the increase in cultivation scale, possibly due to improved mixing and high C/N ratio.

Table 2—Results of microbial oil production by *M. plumbeus* in the 14 L and 1 000 L STRs.

Working volume (L)	Initial sugars, g/L	Biomass, g/L	Biomass yield, g/g consumed sugars	Oil content, %	Oil, g/L	Oil yield, g/g consumed sugars
8	27.3	9.7	0.37	21.7	2.1	0.08
750	28.4	7.3	0.43	26.0	1.9	0.11

The microbial oil production was also demonstrated in the 1 000 L reactor at QUT Mackay Renewable Biocommodities Pilot Plant. Figure 9 shows the fermentation broth and fungal biomass cake after washing and pressing after 6 days cultivation. Again, fungal biomass pellets did not form in the 1 000 L STR with the inoculation of crushed pellet biomass.

It is worth noting that the cultivation of *M. plumbeus* in the 1 000 L reactor was not at the optimised conditions as the dissolved oxygen level and pH were not controlled during the cultivation. In addition, the reactor has a marine-blade impeller, which is different from the 6-bladed Rushton-type impeller used in the 14 L reactor. As a result, about 7.5 g/L of fungal biomass was collected at the end of cultivation.

Discussion

The fungus (*M. plumbeus* FRR 2412) used in this study is an oleaginous microorganism identified previously by Ahmad *et al.* (2015). In this study, cultivation of this fungus for microbial oil production using molasses was investigated in terms of morphology control, nutrient optimisation and scale-up.

Fig. 9—Fungal biomass broth and biomass cake.

Small uniform pellets generally are preferred morphological forms for cultivation of filamentous fungi as they favours mass and oxygen transfer. However, as shown in this study, repeated cultivation of these biomass pellets during multi-step scale-up process could lead to a significant decrease in biomass concentration (as well as oil concentration).

In order to grow filamentous fungi in small uniform pellet forms (e.g., <1.0 mm) of filamentous fungi, a number of methods have been developed. However, most of these methods are only applied with the inoculation of fungal spores. Control of spores concentration is one of the most commonly used methods to form small uniform fungal pellets from spores (Liu et al., 2008; Papagianni and Mattey, 2006).

In addition, addition of solid particles like silicate is also used to induce the formation of small pellets (Driouch *et al.*, 2010; Gao *et al.*, 2014). However, as shown in this study, the pellets growing from spores will become larger and larger during a multi-step scale-up process. Although direct inoculation of spores can lead to the production of small pellets, large quantities of spores will be required for an industrial scale reactor.

For example, for a 1 000 L reactor, it will require spores number of 2×10^{11} to achieve an initial spores concentration of 2×10^{5} spore/mL, 20 000 times of that used for a 250 mL shake flask containing 50 mL medium. Therefore, it is not realistic to directly inoculate spores into industrial scale reactors.

It is also possible to grow filamentous fungi in pellet forms with the inoculation of crushed biomass (Lu *et al.*, 2015). However, this study shows that inoculation of crushed pellet biomass could lead to the formation of small pellets as well as dispersed short hyphae, depending on the cultivation conditions. In the STRs, only dispersed short hyphae were observed but they did not cause problems associated with mixing and oxygen supply. Growth of *M. plumbeus* at different scale reactors demonstrated that it is a reliable method to control the morphology as freely dispersed short hyphae.

However, the cultivation process of using 1000 L reactor needs to be better controlled to achieve biomass and microbial oil production similar to that of using laboratory scale reactor. It is also expected that this morphology control method is applicable for the cultivation of other oleaginous filamentous fungi like *Mortierella isabellina* for microbial oil production.

Molasses is a nutrient rich carbon source. For microbial oil production by *M. plumbeus*, low levels of nitrogen and phosphorus are required. At a sugars concentration of 30 g/L (glucose equivalent) although molasses media contained a nitrogen level similar to that of synthetic culture media, addition of 0.5 g/L $(NH_4)_2SO_4$ is still required, possibly because the nitrogen in molasses is present in the form of Maillard reaction products, which are not readily utilised by *M. plumbeus*. Molasses contained very low levels of phosphorus and addition of phosphate is also necessary for improved biomass and oil production.

Compared with the phosphorus levels used in the synthetic culture medium in previous studies (Gao *et al.*, 2014; Kavadia *et al.*, 2001), only 0.25 g/L KH_2PO_4 was added in the molasses medium in this study. Since phosphate is an expensive nutrient, the use of low levels of phosphate will significantly save the production cost.

Although filamentous fungi has the advantages in terms of biomass harvest, the biomass and oil yields on consumed sugars in this study are low compared with previous studies by oleaginous yeast and algae (Vieira *et al.*, 2016; Yan *et al.*, 2011). This will be improved through improved process control and the use of more efficient oleaginous filamentous fungi such as *M. isabellina* (Ruan *et al.*, 2014; Zheng *et al.*, 2012).

Conclusions

In this study, cultivation of oleaginous filamentous fungi – *M. plumbeus* for microbial oil production was firstly studied using sugarcane molasses as a carbon source. Inoculation of crushed fungal pellet biomass to STRs was a suitable method for cultivation of *M. plumbeus* on molasses, which led to freely dispersed short hyphae. Only 0.5 g/L $(NH_4)_2SO_4$ and 0.25 g/L KH_2PO_4 were required for the cultivation of *M. plumbeus* using molasses at an initial sugars concentration of 30 g/L (glucose equivalent).

Cultivation of *M. plumbeus* in the 14 L STR led to a production of 9.7 g/L biomass and 2.1 g/L microbial oils at an initial sugars concentration of 27.3 g/L. It is expected that fungal biomass and oil production can be further improved through process control and the use of more efficient oleaginous filamentous fungi.

Acknowledgements

This project is supported by Sugar Research Australia, through funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit program, the Queensland Government Department of Agriculture and Fisheries and project partners including Cotton Research and Development Corporation, Forest and Wood Products Australia, Australian Pork Ltd, Southern Oil Refining, NSW Department of Primary Industries and Queensland University of Technology. The authors also thank Mr Darant Longford and Mr Vincent Barfield for demonstration of microbial oil production at QUT Mackay Renewable Biocommodities Pilot Plant.

REFERENCES

Ahmad FB, Zhang ZY, Doherty WOS, O'Hara IM (2015) A multi-criteria analysis approach for ranking and selection of microorganisms for the production of oils for biodiesel production. *Bioresource Technology* **190**, 264–273.

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.

Driouch H, Sommer B, Wittmann C (2010) Morphology engineering of *Aspergillus niger* for improved enzyme production. *Biotechnology and Bioengineering* **105**, 1058–1068.

Fakas S, Papanikolaou S, Batsos A, Galiotou-Panayotou M, Mallouchos A, Aggelis G (2009) Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina*. *Biomass & Bioenergy* **33**, 573–580.

Gao DF, Zeng JJ, Yu XC, Dong T, Chen SL (2014) Improved lipid accumulation by morphology engineering of oleaginous fungus *Mortierella isabellina*. *Biotechnology and Bioengineering* **111**, 1758–1766.

Kavadia A, Komaitis M, Chevalot I, Blanchard F, Marc I, Aggelis G (2001) Lipid and gamma-linolenic acid accumulation in strains of zygomycetes growing on glucose. *Journal of the American Oil Chemists Society* **78**, 341–346.

Liu Y, Liao W, Chen SL (2008) Study of pellet formation of filamentous fungi *Rhizopus oryzae* using a multiple logistic regression model. *Biotechnology and Bioengineering* **99**, 117–128.

Lu T, Zhang Q, Yao S (2015) Mycelia pellet formation of marine-derived fungus: new fomation pathway directly from hyphae. *Research and Reviews: Journal of Microbiology and Biotechnology* **4**, 18–25.

Papagianni M, Mattey M (2006) Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level. Application of neural network and cluster analysis for characterisation of mycelial morphology. *Microbial Cell Factories* **5**, 3.

Ruan ZH, Zanotti M, Archer S, Liao W, Liu Y (2014) Oleaginous fungal lipid fermentation on combined acid- and alkali-pretreated corn stover hydrolysate for advanced biofuel production. *Bioresource Technology* **163**, 12–17.

Vieira JPF, Ienczak JL, Costa PS, Rossell CEV, Franco TT, Pradella JGC (2016) Single cell oil production integrated to a sugarcane-mill: Conceptual design, process specifications and economic analysis using molasses as raw material. *Industrial Crops and Products* **89**, 478–485.

Yan D, Lu Y, Chen YF, Wu QY (2011) Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production. *Bioresource Technology* **102**, 6487–6493.

Zheng YB, Yu XC, Zeng JJ, Chen SL (2012) Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw. *Biotechnology for Biofuels* **5,** 50.