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Turbo thin film continuous flow production of biodiesel from fungal biomass

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Abstract: Direct biodiesel production from wet fungal biomass may significantly reduce production costs, but there is a lack of fast and cost-effective processing technology. A novel thin film continuous flow process has been applied to study the effects of its operational parameters on fatty acid (FA) extraction and FA to fatty acid methyl ester (FAME) conversion efficiencies. Single factor experiments evaluated the effects of catalyst concentration and water content of biomass, while factorial experimental designs determined the interactions between catalyst concentration and biomass to methanol ratio, flow rate, and rotational speed. Direct transesterification (DT) of wet *Mucor plumbeus* biomass at ambient temperature and pressure achieved a FA to FAME conversion efficiency of > 90% using 3 wt./v % NaOH concentration, if the water content was ≤ 50% (w/w). In comparison with existing DT methods, this continuous flow processing technology has an estimated 90-94% reduction in energy consumption, showing promise for up-scaling.

Keywords: direct transesterification, *Mucor plumbeus*, conversion efficiency, continuous processing, thin film microfluidics.
1. Introduction

Given the depletion of petroleum-derived fuels and increasing environmental concerns regarding pollution, research in renewable energy has escalated and much effort has been devoted to the production of biodiesel (Faried et al., 2017). Semi-refined and refined vegetable oils are very often used as starting materials for biodiesel production, which is not sustainable due to the diversion of valuable food resources. Therefore the current emphasis is placed on developing 2nd and 3rd generation biofuels from non-food source feedstock, such as waste cooking oil and animal fats, Jatropha and microalgae (Islam et al., 2013). Conventional biodiesel production is costly, as the transesterification reaction requires heating, and, if biomass is used, drying and extraction, add additional costs (Cui & Liang, 2014). The techno-economic outcomes for biodiesel production can be significantly improved using direct transesterification (DT) of wet biomass feedstock and developing less expensive raw material supply chains (Kumar, 2017).

Promising oil-rich feedstock are oleaginous microorganisms such as microalgae, fungi, and yeast, which have several advantages, including (i) year-round, rather than seasonal production on non-arable land, (ii) higher biomass productivities and (iii) higher lipid contents than food oil crops (Shuba & Kifle, 2018). Oleaginous microorganisms can grow in inexpensive media such as industrial waste waters (Deeba et al., 2016), glucose and acid hydrolysate of sugarcane bagasse (Brar et al., 2017), and dairy farm waste water (Sun et al., 2018). For example, Cryptococcus humicola, grown on glycerol as a carbon source, has a lipid content of ~71% (Souza et al., 2017).

Direct transesterification of oleaginous microorganisms can reduce processing time and restrict the use of harmful solvents to methanol only (Yousuf et al., 2017). Total production times can be further reduced using wet biomass; for example, DT of wet Pichia guilliermondii reduced the total production time by up to 8 h compared to the conventional
two-step method by avoiding the drying step (Chopra et al., 2016). High biodiesel conversion efficiencies, however, still required long reaction times (6 h). Overall production times can also be shortened using high energy input microwave- and ultrasonication-intensified DT of wet biomass, yielding conversion efficiencies of 92% and 94.3% with reaction times of 4 min from wet Cryptococcus curvatus and Yarrowia lipolytica biomass, respectively (Yellapu et al., 2017). To date, however, most studies on DT of fungal biomass have been conducted at high reaction temperatures of 60 - 100\(^\circ\)C, representing a significant energy penalty. Therefore, developing a rapid DT process with low energy consumption is necessary to increase the competitiveness of direct biodiesel production.

A remarkably versatile vortex fluidic device (VFD) was recently tested for rapid DT of wet microalgal biomass (Chloroparva pannonica, water content ~68%) to biodiesel, achieving rapid (~2 min residence time) and high (>96%) conversion efficiencies, when operated under continuous flow conditions at room temperatures (Sitepu et al., 2018a). The success of the VFD microfluidic platform, for which upscaling is challenging, led to the design of the high throughput, high shear turbo thin film device (T\(^2\)FD, Fig. 1), where the maximum thickness of the dynamic thin film can be adjusted from 100 to 200 \(\mu\)m. The DT of wet C. pannonica biomass with a conversion efficiency of ~98% was achieved under continuous flow operation with a residence time of ~ 2 min at ambient temperature and pressure (Sitepu et al., 2018b). The T\(^2\)FD consists of two main parts: a 3D-printed titanium-rotating blade, and a stainless steel base. When a biomass/reactant mixture enters the T\(^2\)FD, the internal fins on the rotating blade push the mixture into the base resulting in high shear stress within the thin film, which releases lipids that directly react with methanol in the presence of a catalyst to yield fatty acid methyl ester (FAME). The localised average shear stress (\(\overline{\chi}\)) in a film is described by equation 1 (eq. 1) (Schilde et al., 2011),

\[
\overline{\chi} = \frac{\Delta \nu}{d},
\]

(eq. 1)
where $\Delta v$ is the velocity difference across the stationary base and rotating surface fluid boundaries that are separated by the gap distance, $d$. Because of the conical shape of the rotor and base, which both have matching apex angles of $90^\circ$, the average shear stress increases as the fluid moves up the conical surface. Here the average shear is a function of the radial distance from the axis of rotation ($r$) and the rotational angular velocity ($\omega$),

$$\bar{\gamma}(r) = \frac{r\omega}{d}. \quad \text{(eq. 2)}$$

In this in-house developed system, the blade has been designed with periodic gaps that allow the fluid to flow into the rotor-base gap. Here, the atmosphere above the liquid can also be drawn under the blades, and into the thin film. After the mixture passes under the blade, the thin film relaxes, leading to significantly improved mass transfer across the large vapour-liquid interface. Observations resulting from this study confirmed that the centrifugal motion of the rotor drives the fluid outwards and up the conical surface of the base in a helical-like motion. Here, the conical shape of the base introduces a component of the gravitational force that opposes the outward motion of the fluid. As such, depending on the rotational speed and liquid flow rate, the fluid may (i) form a continuous turbulent film, (ii) experience viscous fingering or (iii) form droplets, streaking, and phase deformation (Jha et al., 2011). The shear stress that the fluid experiences through repeated contact with the rotating blade surfaces creates intense micro-mixing within the fluid, as the large shear rate typically exceeds the critical shear rate required for single phase formation, i.e. homogenization (Hashimoto et al., 1995). All these features create a novel hybrid chemical processing environment, whereby the fluid(s) injected into the device are subjected to a unique mix of high shear and efficient mass transfer.

This study reports the outcomes of $T^2$FD-intensified DT of dry and wet fungal biomass operated in continuous mode at ambient temperature and pressure, using methanol as a solvent and sodium hydroxide as the catalyst. Effects of processing parameters such as the
ratio of biomass to methanol, flow rate, rotation speed and the catalyst concentration were systematically investigated to optimize the process. Finally, energy requirements were estimated and compared to two step-dry biomass DT and single step wet biomass DT (Chopra et al., 2016).

2. Materials and Methods

2.1. Fungal biomass cultivation and validation of T\textsuperscript{2}FD-induced cell breakage

*Mucor plumbeus* biomass was cultivated using diluted molasses as the growth medium in a 1,000 L stirred tank fermentor at the Mackay Renewable Biocommodities Pilot Plant – a facility of Queensland University of Technology, Australia. The molasses medium contained a sugar concentration of \(\sim 30 \text{ g L}^{-1}\) (glucose equivalent), 0.5 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) and 0.25 g L\(^{-1}\) KH\(_2\)PO\(_4\). Fungal biomass was produced at 28 °C, pH 6.0 over a cultivation period of 6 days with oxygen levels maintained at above 20\% by aeration (Ahmad et al, 2017). Microbial oil production was performed under nitrogen-limiting condition. As a result, sugar consumption was slow, requiring longer cultivation time for the utilisation of sugars. At the end of the cultivation period, the fungal biomass was harvested on a filter cloth, and washed with tap water for removal of residual growth medium. The washed fungal biomass was subsequently pressed in a juice press to remove excess water, followed by air-drying. The dried fungal biomass was kept at 4 °C until use.

In order to investigate the cell disruption effect of the T\textsuperscript{2}FD, the fungal biomass was analysed by scanning electron microscopy (Inspect F50, Thermo Fisher Scientific, Australia) before and after T\textsuperscript{2}FD-processing. As expected, fungal hyphae initially had a tubular shape, with a relatively smooth surface and undamaged. After passage through the T\textsuperscript{2}FD, fungal hyphae were fully disrupted (data not shown). Consequently, T\textsuperscript{2}FD-induced cell breakage
should release stored and membrane lipids into the solution, making them accessible for catalytically conversion to biodiesel in the transesterification reaction.

2.2 Conventional extraction and transesterification of fungal biomass

To investigate the efficacy of the T²FD extraction and transesterification process, *M. plumbeus* dry biomass was processed following a protocol for *Rhodosporidium toruloides* (Thliveros et al., 2014). Briefly, 2 g dry biomass of *M. plumbeus* was mixed with methanol containing 4 wt./v % sodium hydroxide as a catalyst at a ratio of 1:20 and reacted at 50 °C for 10 h. Another control sample was prepared using sulphuric acid as the catalyst following a protocol for biodiesel production from *Pichia guilliermondii* (Chopra et al., 2016). Briefly, 2 g dry biomass of *M. plumbeus* was mixed 1:20 with methanol containing 4 v/v% sulphuric acid and reacted at 60 °C for 6 h. After cooling, 20 mL hexane was added to these samples to extract the FAME and the hexane phase was dried *in vacuo*. For $^1$H-NMR, the sample was reconstituted in 800 µL deuterio-chloroform (chloroform-d, Sigma-Aldrich, Castle Hill, NSW) and analysed by $^1$H-NMR on a 600 MHz Bruker spectrometer set at 64 scans and 1 second D1 delay, while the extract was directly analysed after drying by FT-IR.

2.3 Direct transesterification of fungal biomass using the T²FD

For biodiesel production, water was added to reconstitute the biomass to mimic the naturally occurring water content (Kim et al., 2015). The fungal biomass slurry was premixed with methanol in a modified 60 mL syringe containing a magnetic stir bar, which was located above a magnetic stirrer to achieve homogeneity of the mixture. The homogeneous fungal biomass-water-methanol mixture was pumped to the T²FD using a syringe pump (Adelab Scientific, 12VDC, Australia). The catalyst, sodium hydroxide in methanol, was delivered via another syringe pump at the investigated concentrations. In all experiments, the matched and quoted flow rates were used for both the biomass slurry and the catalyst in methanol, as high FA to FAME conversion efficiencies were achieved, allowing to minimise solvent use.
Biodiesel conversion was optimized by exploring different reaction conditions. For each experiment, 2 g of fungal biomass was prepared as a biomass/methanol mixture at biomass to methanol ratios of 1:6, 1:9, 1:12, 1:15, 1:18 and 1:25 wt./v. Flow rates (1, 2, 3, 4, 5, 8 and 10 mL/min), rotational speed of the turbo blade (2,000 to 6,000 rpm, with increments of 500 rpm), catalyst concentrations (0, 0.5, 1, 3, 5, 7, 9 and 12 wt./v %) and water content (5, 25, 50 and 75% of dry weight (DW)) were systematically varied. After collection of the methanol and catalyst-reacted cell lysate from the T²FD (~10 mL), the biodiesel was extracted with 10 mL of hexane. The hexane was removed in vacuo and the product stored in a desiccator prior to analysis. All experiments were performed in triplicate, with reported values and uncertainties being the average and standard errors, respectively.

2.4. Analyses of total lipid, fatty acid profile and FAME formation and FA to FAME conversion efficiency

Analytical procedures followed the methodology described in Sitepu et al. (2018a). Briefly, total lipids were extracted using the Folch method and quantified gravimetrically. Total fatty acid content and the FAME profile were established using GC/MS after conventional transesterification. FTIR and 1H-NMR analyses were used to confirm the formation of biodiesel and to calculate fatty acid (FA) extraction and FA to fatty acid methyl ester (FAME) conversion efficiencies, respectively.

2.5. Statistical analysis

Significance of the effect of catalyst concentration, biomass to methanol ratio, flow rate, rotational speed and water content was statistically analysed for independent replicates (n = 3) using Statistica v13.6 at a significance level set to α = 0.05. Since data did not conform to assumptions of ANOVA (i.e. normality and homogeneity of variances) despite log transformation, significance was validated using non-parametric statistics, Kruskal-Wallis ANOVA, Newman-Keuls and Tukey post hoc tests to determine extraction conditions.
responsible for significant effects, after verifying normal probability distributions of residuals using P-P plots.

3. Results and Discussion

The total fatty acid content of the *M. plumbeus* biomass was 25 ± 1.2% of its dry matter, composed of 41 ± 0.1 of saturated fatty acid (SFA), 43% of monounsaturated fatty acid (MUFA) and 16 ± 0.1% of polyunsaturated fatty acids (PUFA). Unsaponifiable compounds such as tocopherol and sterols were not detected in the fungal oil, while saponifiable compounds composed of free fatty acid (56%), monoacylglycerols (2%), diacylglycerols (18%), and triacylglycerols (24%) were obtained. Based on GC/MS analyses of FAMEs after conventional extraction and transesterification, the fatty acid profile was dominated by oleic acid (C18:1; 43 ± 0.03%), followed by stearic acid (C18:0; 24 ± 0.1%), the ω-6 fatty acid linoleic acid (C18:2; 15 ± 0.1%), and palmitic acid (C16:0; 13 ± 0.1). In contrast, α-linolenic acid (C18:3n-3) and myristic acid (C14:0) were presented at very low levels (0.02 ± 0.002 and 0.3%, respectively). Other fatty acids presented at <2% of the total fatty acids were C12:0, C15:0, C20:0, C16:1(n-7) and C18:1(n-7), and the long-chain fatty acids C22:0, C24:0, C20:1(n-9), C20:2, C22:4(n-6) and C22:6(n-3) (docosahexaenoic acid, DHA).

3.1. Effect of catalyst concentration on conventional DT and $T^2FD$-intensified DT of *Mucor plumbeus* biomass

Catalyst concentration had a significant effect on the extraction of C18:2, C18:1 and C16:0 (Fig. 2A). Extraction of C18:2 was significantly lower at 0 and 5 wt./v % of the catalyst, achieving extraction efficiencies of only 27 ± 5 and 87 ± 2 %, respectively, whilst high catalyst concentrations led to 100% extraction efficiencies. In contrast, C18:1 was completely extracted in the absence of the catalyst, and extraction efficiencies ranged
between 83 ± 2 and 90 ± 0.3 for the other catalyst concentrations, being lowest at catalyst concentrations of 9 wt./v %. Extraction efficiencies for C16:0 behaved similarly, and catalyst concentrations of 7 and 9 wt./v % resulted in reduced extraction efficiencies of 91 ± 2 and 88 ± 2, respectively. At catalyst concentrations < 5 wt./v %, the algorithm overestimated extractions of C16:0 by 3 to 58% (e.g. at catalyst concentrations of 0.5 and 1 wt./v % calculated extraction efficiencies were 159 ± 0.3 and 152 ± 2%, respectively). The amount of saturated fatty acids is derived by difference (e.g. 100% - amount of quantified unsaturated fatty acids), which is then multiplied by empirically established factors for C18:0 and C16:0 (Knothe & Kenar, 2004), which could explain these overestimations, being 27% for total saturated fatty acids compared to GC/MS quantified amounts. In addition, differences in the molecular weights of the fatty acids have been attributed to an overestimation of saturated fatty acids (mol%) compared to GC-derived wt% quantification (Sedman et al., 2010). As C16:0 was overestimated for all parameter settings, no further analyses or quantifications are shown.

A homogenous base catalyst has greater catalytic activity than that of an acid catalyst in a transesterification reaction (Ma & Hanna, 1999). However, soap formation spontaneously occurs when the oil/biomass contains free fatty acids. Soap formation reduces the FA to FAME conversion yields and increases the cost of downstream biodiesel processing (Kasim & Harvey, 2011). In fact, in a control experiment, no conversion of FA to FAME was detected when *M. plumbeus* was directly processed using a conventional method (Thliveros et al., 2014) with 4 wt./v % sodium hydroxide as a catalyst, a biomass to methanol ratio of 1:20, and reaction temperature of 50 ºC for a reaction time of 10 h. In contrast, in another control experiment using sulphuric acid at 4 v/v % at the same biomass to methanol ratio, a reaction time of 6 h at 60 ºC, quantification of $^1$H-NMR spectra integration yielded a FA to FAME conversion efficiency of ~98 ± 0.3. This suggests that the base catalyst under
conventional DT operation induced saponification of fatty acids, whilst this did not occur using the acid catalyst (Kakkad et al., 2015b). However, this phenomenon did not arise when using the T²FD, even at a base catalyst concentration of 12 wt./v % under continuous operation. Our previous studies using microalgal *Chloroparva pannonica* biomass containing 43% of free fatty acid, using either VFD (Sitepu et al., 2018a) or T²FD (Sitepu et al., 2018b) thin film microfluidic platforms, showed that saponification did not occur at low concentrations of sodium hydroxide. The FA to FAME conversion efficiencies for both studies decreased at high concentrations of the base catalyst, suggesting saponification did occur at excess sodium hydroxide concentrations (Sitepu et al., 2018a). Based on these studies, the effect of sodium hydroxide was analysed at concentrations from 0 to 12 wt./v % in the T²FD for dry *M. plumbeus* biomass, which also contained high free fatty acids. Sodium hydroxide concentrations of ≥ 1 wt./v % in methanol achieved ≥ 90% FA to FAME conversion efficiencies using T²FD-intensified DT of the fungal biomass, which were, however, significantly reduced at low catalyst concentrations. This is similar to T²FD-intensified DT of *C. pannonica* biomass (Sitepu et al., 2018b), while concentrations lower than 1 wt./v % base catalyst was not examined in the VFD-intensified DT of the algal biomass (Sitepu et al., 2018a). Based on these results, sodium hydroxide concentrations were set at 1 and 3 wt./v % in experiments investigating the interactive effects of both concentrations with the ratio of biomass to methanol, flow rate, and rotation speed (Figs. 3A-3C).

3.2. Effect of biomass to methanol ratio on T²FD-intensified DT of *Mucor plumbeus* biomass at catalyst concentrations of 1 and 3 wt./v %

The effect of biomass to methanol ratio on T²FD-intensified fatty acid extraction (Fig. 2B) and FA to FAME conversion efficiencies of *M. plumbeus* biomass (Fig. 3A) was investigated at five different ratios in the presence of 1 and 3 wt./v % of the base catalyst at
room temperature with a flow rate of 3 mL/min and a rotational speed of 4,000 rpm. Methanol is regarded to be a poor solvent for lipids, as the solubility of triglyceride in methanol is very low, i.e. an extraction of only 9% compared to hexane was demonstrated (Zeng et al., 2009). In DT, however, methanol acts both as an extraction solvent and a reactant (Kasim et al., 2010). Biomass to methanol ratio had no significant effect on C18:1 extraction, with efficiencies ranging from 84 ± 2 to 92 ± 3%, irrespective of the catalyst concentration. Biomass to methanol ratio did also not greatly affect the extraction of C18:2, but a significantly lowered the extraction efficiency from 100% for other conditions to 87 ± 5% for a ratio of 1:18 at a catalyst concentration of 3 wt./v.

As mentioned above, excess methanol is necessary to drive the extraction and FA to FAME conversion in DT applications. FA to FAME conversion efficiencies were >90%; except for a biomass to methanol ratio of 1:25 at a base catalyst concentration of 1 wt./v %, (Fig. 3A), demonstrating efficient conversion to biodiesel under continuous T²FD operation. This is similar to VFD- and T²FD-intensified FA to FAME conversion efficiencies of C. pannonica biomass for the similar biomass to methanol ratio range investigated (1:6 to 1:18) (Sitepu et al., 2018a; Sitepu et al., 2018b).

3.3. Effect of flow rate

One of the main factors affecting FA extraction and FA to FAME conversion efficiencies is reaction time (batch processing) or residence time (flow rate in continuous flow devices) (Britton et al., 2016). At flow rates of 1-10 mL/min and catalyst concentrations of 1 and 3 wt/v %, extraction yields for C18:2 and C18:1 ranged from 9 ± 1 to 17 ± 1 and 34 ±1 to 45 ± 1 mol% (Fig. 2C) with extraction efficiencies of 56 ± 9 to 100 and 80 ± 2 to 100%, respectively. A factorial ANOVA determined a significant effect of the catalyst concentration and flow rate on T²FD extraction of C18:2, C18:1 and C16:0 from M. plumbeus biomass, and a significant interaction of catalyst concentration with flow rate. A Tukey post hoc analysis
determined that the significance was driven by low extractions of C18:2 at flow rates of 8 and 10 mL/min at a catalyst concentration of 1 wt./v %, achieving extraction efficiencies of only $56 \pm 9$ and $66 \pm 3\%$. Likewise, the same parameters were the main drivers of significance of extraction of C18:1, but here highest extraction efficiencies of $107 \pm 2$ and $104 \pm 2\%$ were achieved. A catalyst concentration of 3% at a flow rate of 1 mL/min also had a significant effect on the extraction of C18:2 and C18:1, but resulted in the highest and lowest yields of $17 \pm 1$ and $34 \pm 1\%$, respectively.

Fluid dynamics are highly dependent on flow rate and rotational speed, when the T$_2$FD is operated in continuous processing mode. A fast flow rate may reduce the residence time (when a continuous film is formed). As exposure to high shear stress drives the DT process (Zhou et al., 2017), a reduced residence time in the T$_2$FD may influence conversion efficiency. In contrast, although a slow flow rate provides more residence time when a continuous film is formed at a fixed rotational speed, it could lead to lower FA to FAME conversion efficiencies. Here evaporative loss of methanol increases the base catalyst concentrations, which can potentially induce saponification (Niju et al., 2014). Despite these potentially adverse effects, using T$_2$FD-intensified DT of dry M. plumbeus biomass achieved conversion efficiencies between 92 and 96% at a rotational speed of 4,000 rpm, with a catalyst concentration of 3% and a biomass to methanol ratio of 1:12. No significant effect of flow rate was detected. This may suggest that the residence time under these low-flow conditions of the device is mainly driven by the rotational speed (Fig. 3B). As the T$_2$FD is a high throughput device, capable of operating at much larger flow volumes, this result is consistent with the residence time being independent of the flow rate, as for this combination of flow rate and rotational speed a minimal volume of fluid is retained in the device. In contrast, high flow rates (8 and 10 mL/min) significantly reduced FA to FAME conversion efficiencies from $\sim89$ (FR 1-5) to 75% at 1% catalyst concentration. Under these operational
parameters of the T²FD, FA to FAME conversion efficiencies at 1% catalyst concentration were in general, slightly but not significantly lower than that at 3% catalyst concentration (Fig. 3B). Previous studies on the T²FD-intensified DT of wet biomass of microalgal *C. pannonica* observed a negative impact on FA to FAME conversion efficiencies at higher rotational speeds (6,000 rpm) and a slow flow rate of 1 mL/min (Sitepu et al., 2018b); a result contrary to that observed in VFD-intensified processing which achieved conversion efficiencies of ~99% (Sitepu et al., 2018a). This result could be attributed to increased evaporative loss of methanol in the T²FD, exacerbating the effect of the water content of the wet microalgae biomass (Sitepu et al. 2018b). In contrast, the fungal biomass was dry and the negative effect of high flow rates on FA to FAME conversion efficiencies are likely the result of reduced reaction time. As flow rates of more than 5 mL/min were not investigated for T²FD-intensified DT of *C. pannonica*, the effects cannot be compared. Mathematical modelling of high flow rates, however, also predicted a decrease in FA to FAME conversion efficiencies in VFD-intensified DT of wet *C. pannonica* biomass operated in continuous mode, which is thought to be a consequence of reduced mechano-energy exposure of the reactants in thicker films of liquid (Sitepu et al., 2018a). The present results, in combination with the previous work (Sitepu et al., 2018b), reinforce the view that the fluid dynamics within the T²FD involves a complex interplay between rotational speed and fluid volume available (flow rate). In this respect, the biomass slurry will have a distinctly different viscosity from the methanol, which will further complicate the fluid dynamics and may impact on FA extraction and FA to FAME conversion efficiencies.

3.4. Effect of rotational speed

Rotational speed is one of the critical operating parameters of the T²FD, which can impact FA extraction and FA to FAME conversion efficiencies. Normally in VFD-generated
thin films, increased rotational speed can enhance reaction rates through providing higher shear stress and larger surface contact area between reactants (Britton et al., 2017; Luo et al., 2016). At rotational speeds of 2,000 to 6,000 rpm in the T^2FD and catalyst concentrations of 1 and 3 wt/v %, extraction yields for C18:2 and C18:1 ranged from 13 ± 0.3 to 16 ± 0.3 and 35 ±1 to 39 ± 0.6 mol% (Fig. 2D) with extraction efficiencies of 83 ± 5 to 100 and 83 ± 9 to 92 ± 2%, respectively. A major significant effect was only observed for C18:2 extraction at a catalyst concentration of 3 wt/v % and rotational speeds of 3,500, 4,500 and 5,000 rpm, which yielded the lowest extraction efficiencies of 83%. Similar extraction efficiencies of 83% for C18:1 at a catalyst concentration of 1% and 3,000 rpm contrasted with highest extraction efficiencies of > 91% at a catalyst concentration of 3 wt/v % at rotational speeds of 3,500, 4,500 and 5,000 rpm.

No significant effect of changing the rotational speed was observed for T^2FD-intensified FA to FAME conversion efficiencies (~96 - 97%) for a catalyst concentration of 3 wt/v % at a flow rate of 3 mL/min and a biomass to methanol ratio of 1:12 (wt/v) (Fig. 3C). In contrast, at a catalyst concentration of 1 wt/v % at the same operational settings, FA to FAME conversion efficiencies were generally lower (~74 - 90%) and significantly reduced at the highest rotational speeds of 5,500 and 6,000 rpm (~74 - 76%) (Fig. 3C). For a catalyst concentration of 1 wt/v %, increasing the rotational speed led to the same outcomes compared to increasing flow rate, while on both occasions these parameters had no significant effect at a catalyst concentration of 3 wt/v %. This strongly suggests that 1 wt/v % catalyst concentration is insufficient at reduced residence/reaction times of T^2FD-intensified DT of dry M. plumbeus biomass. For all parameter settings tested, the highest FA to FAME conversion efficiencies (97% ± 0.5) were achieved for a catalyst concentration of 3 wt/v %, a rotational speed of 4,500 rpm, a biomass to methanol ratio of 1:12 (wt/v) and a flow rate of 3 mL/min (Fig. 3C).
3.5. Effect of water content in fungal biomass

One of the main bottlenecks in biodiesel production is dewatering of the biomass, which requires high energy input (Salam et al., 2016). Even though air-drying is energy-efficient, the process is prohibitively time-consuming, and highly dependent on unpredictable sunlight radiation, and typically land area-intensive (Guldhe et al., 2014). Therefore, DT of wet biomass is desirable to overcome these limitations. To investigate the suitability of continuous mode-operated T²FD-intensified DT of wet fungal biomass, parameter settings were chosen based on single parameter best outcomes. Outcomes of a Box-Behnken model for continuous mode-operated VFD-intensified DT of wet algal biomass were also considered. Here the Box-Behnken model predicted a decrease in FA to FAME conversion efficiencies at high biomass to methanol ratios, increased water contents and increased flow rates (Sitepu et al., 2018a). Accordingly, a biomass to methanol ratio of 1:9 (wt./v), a flow rate of 2 mL/min and a rotational speed of 4,500 rpm at a catalyst concentration of 3 wt./v % were chosen for T²FD-intensified DT of M. plumbeus biomass of various water contents of 5, 20, 50, and 75% (w/w) water. Irrespective of biomass water content, extraction of C18:2 was complete, while an extraction efficiency of ~86% was achieved for C18:1 at these parameter settings. In contrast, Kruskal-Wallis ANOVA established a significant effect of water content on FA to FAME conversion efficiency, which was driven by significantly reduced efficiencies at highest water content of 75% (w/w) (46 ± 4% compared to 91 ± 1% to 94 ± 0.5% at the other water contents). This may indicate inhibition of DT through saponification of fatty acids by base catalysts in the presence of water (Ma & Hanna, 1999) and/or reduced concentration of the catalyst at high water contents. Taking all results into account, T²FD-intensified DT of wet or dry M. plumbeus biomass is feasible up to a water content of 50% (w/w). Energy requirements for biomass processing and biodiesel production should be lower than that for conventional transesterification or other DT processes requiring heating, as
T²FD-intensified DT of *M. plumbeus* biomass was achieved at room temperature with minimal dewatering requirements. Establishing an optimal water content, however, will be a trade-off between FA extraction, FA to FAME conversion efficiencies and energy cost for biomass drying.

3.6. Implications for biodiesel production from fungal biomass

Lipid content is likely to determine biodiesel production yields and is therefore a primary concern for economic feasibility assessment, but reported values vary widely even for the same species, e.g. for *Rhodosporidium toruloides* reported values range from as low as 10 to as high as 70 mg g\(^{-1}\) dry weight biomass (Liu & Zhao, 2007; Ling et al., 2016; Koutinas et al., 2014; Thliveros et al., 2014; Cao et al., 2012). Other very important processing variables ultimately determining biodiesel yields are FA extraction yields and FA to FAME conversion efficiencies. Most studies on the DT of yeast/fungal biomass reported FA yields but very few reports on FA to FAME conversion efficiencies and interestingly, other than this study, none report on outcomes for both parameters, or provide information on FA extraction efficiencies, which could be used to calculate FA yields. For example, FA to FAME conversion efficiencies in this study ranged from < 2% in the absence of a catalyst, ~25% at very low to ~97% at higher catalyst concentrations. Using this as an example of potential impact, a FA to FAME conversion efficiency of 95% would yield ~24 kg biodiesel per tonne dry biomass containing 25% total lipids (calculation based on best scenario from this study), whilst a similar biodiesel yield could be achieved for an organism with 50% total lipid content and a conversion efficiency of 50%.

The impact of water content on biodiesel production potential from fungal biomass has only been explored in four other studies for species with high lipid content (Ward et al., 2017; Yellapu et al., 2017; Cui & Liang, 2014; Cheirsilp & Louhasakul, 2013). Where reported, FA
yields were slightly lower for fungal biomass with higher water content compared to yields achieved for *M. plumbeus* biomass with 50% water content in this study. Reported FA to FAME conversion efficiency for *Yarrowia lipolytica* with a total lipid content of 61 mg g\(^{-1}\) dry weight was, however, only 72 compared to ~91% achieved for *M. plumbeus* biomass achieved in this study despite similar water content (Cheirsilp & Louhasakul, 2013). Placing these outcomes into context of the much lower total lipid content of *M. plumbeus*, biodiesel yields for *M. plumbeus* would only approach half of the yields for *Y. lipolytica* (23 vs 44 kg tonne\(^{-1}\) biomass dry weight).

Other than production parameters, time (reaction time, biomass drying and processing) and energy consumption (drying, reaction temperature, biomass processing) and reagent costs are also prime considerations for biodiesel production. Only one study was conducted with comparable FA yields at room temperature (Yellapu et al., 2017), while all other required reaction temperatures of between 50 to 100 °C to either assist in cell breakage and/or shorten the reaction time. In addition, except for one study reporting a reaction time of 4 min using *Cryptococcus curvatus* (Cui & Liang, 2014), typical reaction times were two orders of magnitude greater for the processing of dry fungal materials and at least 5 to 30 times longer for the processing of wet fungal biomass than reported here for *M. plumbeus*. The volumes of methanol used varied less, ranging from biomass to methanol ratios of 1:10 to 1:32 for the processing of dry fungal biomass (Katre et al., 2018; Carvalho et al., 2017; Ling et al., 2016; Chopra et al., 2016; Kakkad et al., 2015a; Koutinas et al., 2014; Thliveros et al., 2014; Zhang et al., 2014; Cao et al., 2012; Subhash & Mohan, 2011; Vicente et al., 2009; Liu & Zhao, 2007) and 1:9 (this study) to 1:50 for the processing of wet fungal biomass (Ward et al., 2017; Yellapu et al., 2017; Cui & Liang, 2014; Cheirsilp & Louhasakul, 2013).

3.7. Energy consumption consideration
For economic assessment of energy requirements of the T²FD-intensified DT of dry *M. plumbeus* biodiesel production at lab-scale, energy consumption of the T²FD and the syringe pumps were roughly determined based on the energy (kWh) consumed in these small-lab scale experiments (Table 1). Energy consumption of the T²FD increased with an increase of rotational speed. Thus, the energy consumption of the T²FD was based on a rotational speed of 4,000 rpm (38.4 W), the setting for most parameter assessment for the T²FD-intensified DT of dry *M. plumbeus* biomass. The energy consumption of the two syringe pumps was 20.4 W. As this study was conducted at room temperature, the total energy required for processing 2 g of biomass in T²FD at 4,000 rpm for 5 min was 2.44 kWh kg⁻¹ (Table 1), which is equivalent to 8.784 MJ kg⁻¹ dry biomass. The energy consumption of the T²FD was lower than for the magnetic stirrer hotplate, which is typically used for conventional heating processes at lab-scale. In comparison, biodiesel production from *Pichia guillermondii*, which used sonication for cell disruption prior to transesterification, using a magnetic stirrer hotplate in a two-step process (extraction and transesterification), requires 85.62 kWh kg⁻¹ dry biomass (Table 1). Biodiesel production from wet biomass is certainly more energy-wise, saving 46% using a single step method instead of the traditional two-step method. The T²FD-intensified DT, however, provides an energy saving of ~90% for the two step dry biomass approach and ~94% for processing of wet biomass (Table 1). Using wet biomass as the raw material could also reduce processing time by at least 12 h, the time usually taken for biomass drying.

4. Conclusion

T²FD-intensified DT combines oil extraction, cell disruption and transesterification of wet biomass into a single step process, operating at ambient temperature and pressure. This process is both energy- and time-efficient. Biodiesel production routinely occurred at >90%
conversion at a base catalyst concentration of 3 wt./v %. Importantly, no significant effect of water content up to 50% was evident on FA to FAME conversion efficiency. A preliminary energy consumption assessment demonstrated that the T²FD-intensified DT provides energy savings of >90%. Furthermore, the T²FD operates in a continuous mode, a process that is suitable for up-scaling for commercial production.

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Appendix A. Supplementary data

E-supplementary data for this work can be found in e-version of this paper online.
References


Figure Captions

**Figure 1** (A) An exploded diagram of the key components of the T²FD. (B) A cross-sectional segment of the device illustrating the assembled device and the fluid paths into and out of the device, with the inset showing the rotor base gap - d. Adapted from Sitepu et al., (2018b).

**Figure 2** Effect of (A) catalyst concentration (NaOH) on fatty acid extraction efficiency (mol%) of T²FD-intensified DT of *M. plumbeus* biomass operated in continuous mode. Fatty acid extraction was then investigated at catalyst concentrations of 1 and 3% (wt./v) for (B) variable biomass to methanol ratios, (C) flow rates, and (D) device rotational speed. Error bars represent SD; n = 3.

**Figure 3** Effect of (A) variable biomass to methanol ratios, (B) flow rates and (C) rotational speed on FA to FAME conversion efficiency (%) of T²FD-intensified DT of *M. plumbeus* biomass at catalyst concentrations of 1 and 3% operated in continuous mode. Error bars represent SD; n = 3.
Figure 1.

Figure 2.
Figure 3.
**Table 1.** Comparison of energy (kWh kg\(^{-1}\) biomass) consumed for different biodiesel production methods based on this study and Chopra et al. (2016).

<table>
<thead>
<tr>
<th>Unit Operation / Process</th>
<th>Methods</th>
<th>Dry Biomass</th>
<th>Wet Biomass</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Two Step</td>
<td>T(^2)FD</td>
<td>Single Step</td>
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<tr>
<td>Biomass drying</td>
<td>6</td>
<td>6</td>
<td>-</td>
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<tr>
<td>Cell Disruption</td>
<td>42.5</td>
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<td>Lipid Extraction</td>
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<td>-</td>
</tr>
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<td>Transesterification</td>
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<td>2.44</td>
<td>3.6</td>
</tr>
<tr>
<td>Total</td>
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<td>46.1</td>
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<tr>
<td>Cost of electricity(^a)</td>
<td>(AUS 43.67 c per KWh)</td>
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<td>$3.68</td>
</tr>
</tbody>
</table>

\(^a\) Average electricity cost per kWh in South Australia is AUS 43.6 cents (O'Neill, 2018).
Turbo thin film continuous flow production of biodiesel from fungal biomass

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Highlights:

- Develop a novel turbo thin film continuous flow platform.
- Direct biodiesel production in continuous flow system at room temperature in short residence time.
- Water content up to 50% did not affect the conversion efficiency.
- Offer a fast and easy to scale-up for biodiesel production.
Methanol
Solution

Biodiesel