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Screening of endophytic fungi for biofuel feedstock production using palm oil mill effluent as a carbon source

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ABSTRACT

Aims: Palm oil mill effluent (POME) is a major agricultural waste product of Malaysia. The aim of this study was to identify endophytic fungi capable of producing biofuel feedstock utilizing POME.
Methodology and results: Endophytes were isolated from the Nipah palm tree, *Nypa fruticans*, and exposed to different POME concentrations (25%, 50% and 75%), with and without the addition of nutrients. The utilization of glucose was measured using the Dinitrosalicylic Acid assay whereas the lipid content in the fungal cells was extracted using the Bligh and Dyer method with slight modifications. Three endophytic fungi that displayed the highest growth on POME were identified using ITS 1 and 4 primers and found to be related to *Pestalotiopsis* sp., *Lasiodiplodia theobromae* and *Rhizoctonia bataticola*. Nutrient addition caused an average increase of 8 times in biomass, indicating nitrogen requirement for cell proliferation. The highest POME concentration (75%) resulted in lower biomass yield. Furthermore, all fungal samples in high POME concentration and nutrient conditions showed a decrease in lipids accumulated per milligram of biomass whereby lipid synthesis was enhanced under nitrogen limitation (25% without nutrients).
Conclusion, significance and impact of study: In conclusion, all fungal samples can be classified as oleaginous microorganisms with *Pestalotipsis* sp. being the most efficient (up to 70% of its biomass). This is to our knowledge the first study that shows the potential use of *Pestalotiopsis* sp., *L. theobromae* and *R. bataticola* for the utilisation of POME as biofuel feedstock and could in the future potentially provide an alternative approach to the treatment of POME with value-added effect.

Keywords: biofuel feedstock, endophytic fungi, oleaginous fungi, *Nypa fruticans,* palm oil mill effluent

INTRODUCTION

Since the mid-1950s, petroleum has been the world's major source of energy. However, due to the fact that fossil fuels are limited and also the impacts of global warming (Fargione *et al.*, 2008), it is crucial to find alternative cleaner energy sources. In an effort to produce biodiesel, it was shown that endophytic fungi have the ability to accumulate high lipid content of more than 70% (Certik *et al.*, 1999) with a high ratio of monounsaturated fatty acids to polyunsaturated fatty acids (Dey *et al.*, 2011). This is generally preferable as low amounts of polyunsaturated fatty acids (PUFAs) contribute to oxidative stability and also operability at low temperatures (Liu *et al.*, 2013). In this study, endophytic fungi were isolated from the Nipah palm which can be found in mangrove wetlands. According to Khot *et al.* (2012), fungi and mangrove plays an important role in decomposition, nutrient cycling and energy flow of the marine web which justifies the usage of endophytic fungi for the degradation of POME. Also, endophytic fungi possess enzymes such as cellulases (Peng and Chen, 2007) which are able to degrade wastes such as sugarcane (Robl *et al.*, 2013) and would be essential for degrading the components of the POME.

Current trends of producing biofuels using fungi include using wastewaters from the treatment of animal fat and olive oil mill as their alternative carbon source and have shown to produce promising results (Rossi *et al.*, 2011). Furthermore, other oleaginous microorganisms such as microalgae, yeast, fungi and bacteria were also studied for their lipid accumulating capabilities (Meng *et al.*, 2009) where microalgae have been grown in POME (Putri *et al.*, 2011). POME was used in this study as the fermentable media for the endophytic fungi isolated because the discharge of POME into rivers and the

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ponding system implemented by most palm oil mills (Rupani *et al.*, 2010) poses detrimental effects to the environment due to their high biochemical oxygen demand, chemical oxygen demand, acidity and the release of methane gas to the environment (Lim and Low, 2013).

The aim of this study is to screen the ability of fungi isolated from mangroves to be able to (1) grow solely in POME and obtain its dry biomass and (2) subsequently measure the fatty acids produced per biomass produced for biofuel feedstock.

MATERIALS AND METHODS

Initial screening and growth conditions for the screening of endophytic fungi

Eight strains of isolated fungi from the *Nypa fruticans* plant strains obtained from Choo *et al.* (2015) were grown on 25% POME agar which was prepared by mixing 25% POME and general bacteriological agar. The three selected fungi from the screening which were seen to be able to grow on the agar with the addition of 25% POME were cultured in conical flasks with different conditions; 25%, 50% and 75% POME concentrations, both with and without nutrients. Nutrients added included 0.125 g of yeast extract, 0.125 g of peptone water and 0.03 g of potassium dihydrogen phosphate for each 100 mL broth (Taoka *et al.*, 2008). Fungi growth in different conditions was observed by growth of mycelia and total biomass measured after 6 days.

Quantitative determination of growth and yield of endophytic fungi with and without the addition of nutrients

Glucose concentrations in POME which indicate POME degradation by fungi were monitored using the Dinitrosalicylic Method (Miller, 1959) at day 0 before and after inoculation of fungi, as well as day 1, 4 and 6. The pH was measured daily using pH paper. This was first done by vacuum-filtering the contents of the inoculation flasks to obtain the biomass on a pre-dried filter paper (Whatman, Nucleopore, 45 mm diameter, pore size 0.47 µm). The filter paper and biomass were then dried in an oven overnight at 90 °C and the lipid content in POME (control) and fungi was measured using the Bligh and Dyer (1959) method on the sixth day of inoculation.

Genomic DNA extraction

DNA of the three fungi was extracted using thermolysis methods (Zhang *et al.*, 2010) with slight modifications. The pure colonies of the fungal isolates were picked out and placed into a 1.5 mL microcentrifuge tube with 100 µL of sterilized water. The tubes were then centrifuged at 10,000 rpm for 1 min for the fungal pellet to be accumulated at the bottom of the tube. After centrifugation, the supernatant was discarded and the tubes were then added with 100 µL of lysis buffer (50 mM potassium phosphate, 1 mM EDTA, and 1% glycerol). Then, the tubes were placed in a water bath at 85 °C for 30 min and subsequently used for DNA amplification.

DNA amplification

The amplification of fungal ITS region was performed according to White *et al.* (1990) using 0.6 µL fungal primers Internal Transcribed Spacer 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and Internal Transcribed Spacer 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), 15 µL of 2× MyTaq™ Red Mix (Bioline, UK), 12.8 µL deionized distilled water and 1 µL of DNA template. The Polymerase Chain Reaction (PCR) conditions were set up using instructions provided by Bioline and the PCR tubes containing the MasterMix and DNA template were amplified using a Thermofisher PCR Thermal Cycler. The conditions for the DNA amplification are: Initial denaturation at 95 °C for 3 min, followed by 30 cycles of: 3 sec denaturation at 95 °C, 30 sec annealing at 45 °C and 2 min elongation at 75 °C. This was then followed by a final elongation for 5 min at 75 °C and cooled at 4 °C. The PCR products were run in 1% agarose gel electrophoresis (AGE) using 1× TAE buffer at 100 V for 30 min and visualized under the UV transilluminator.

DNA sequencing and phylogenetic tree construction

The PCR products sequences obtained were sent to Beijing Genomic Institute (BGI) for nucleotide sequencing. The sequencing results were then subjected to BLAST analysis, with sequences aligned and compared to the other fungal sequences in the GenBank Database NCBI (National Centre for Biotechnology Information, http://www.ncbi.nml.nih.gov/BLAST/Blast.cgi).

Phylogenetic analyses were conducted using the MEGA5 software (Tamura *et al.,* 2011*)*. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model. Next, initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join algorithm to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together was calculated by the bootstrap test (1000 replicates).

RESULTS

The selected fungi identified using ITS 1 and 4 were found to be *Pestalotiopsis* sp. (Fungi 1), *L. theobromae* (Fungi 2) and *R. bataticola* (Fungi 3) with a percentage similarity of 99%, 98% and 99% respectively (Table 1) when compared with other taxa obtained from the GenBank directory.

The three sequences from the amplified ITS 1 and ITS 4 region clusters into their related taxa and the tree with

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Table 1: Taxa with the highest percentage similarities that were selected for the construction of the phylogenetic tree.

No.	Taxa with the Highest Percentage Similarities	Accession Number	Identity
1	Pestalotiopsis mangiferae isolate As1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	JX157863	480/483 base pairs (99%)
2	Pestalotiopsis microspora isolate MLSC-PL1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	KF155295	472/474 base pairs (99%)
3	Fungal endophyte strain CM35 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region	JX156058	476/477 base pairs (99%)
4	Lasiodiplodia theobromae isolate RSGV/T09 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	HM466956	459/464 base pairs (99%)
5	Rhizoctonia bataticola isolate VRF 25/07 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	EF446282	420/426 base pairs (99%)

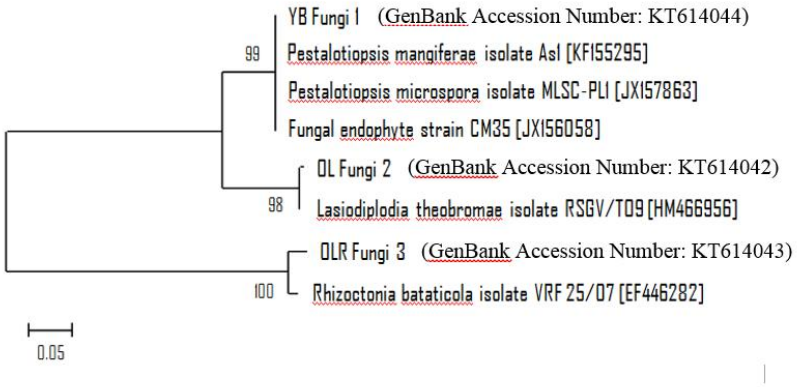

Figure 1: Phylogenetic tree giving genetic distance and bootstrap values based on the nucleotide sequence data from the ITS1 and ITS4 region of the rDNA of Fungi 1 (YB) (GenBank accession number: KT614044), Fungi 2 (OL) (GenBank Accession Number: KT614042) and Fungi 3 (OLR) (GenBank Accession Number: KT614043) respectively. The phylogenetic tree was generated with Maximum Likelihood method and sequence distances were estimated with the neighbor-joining method. Bootstrap values are shown and the scale bar represents a difference of 0.05 substitution per site.

the highest log likelihood from the Maximum Likelihood analysis is as shown in Figure 1. The numbers next to the nodes respresent the bootstrap values (in percentage) which are based on 1000 bootstrap resamplings in which the associated taxa clustered together is shown next to the branches (99%, 98% and 100% respectively).

Based on Table 2, in the presence of nutrients (addition of yeast extract, peptone water and potassium dihydrogen phosphate), at 25%, 50% and 75% POME concentrations in the presence of nutrients, a general downward trend in the reduction of glucose for *Pestalotiopsis* sp. was observed where it decreased from 0.041 g/L to 0.035 g/L, 0.047 g/L to 0.044 g/L and 0.068 g/L to 0.062 g/L respectively.

On the other hand, without the presence of nutrients, all conditions (25%, 50% and 75% POME) showed a general increase in glucose concentration for *Pestalotiopsis* sp.. At 25%, the glucose concentration increased from 0.024 g/L to 0.027 g/L. Similarly, at 50%, the glucose concentration increased from 0.034 g/L to 0.035 g/L. For 75% POME concentration, the glucose concentration also increased from 0.055 g/L to 0.060 g/L on the sixth day.

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Table 2: Glucose concentration in POME with Fungi 1, 2 or 3 at 25%, 50% and 75% POME concentrations respectively with or without the presence of nutrient. The bold numbers indicate that the values increased or maintained instead of decrease.

	Glucose Concentration (gL^{-1})											
	Nutrients						No Nutrients					
	25% POME		50% POME		75% POME		25% POME		50% POME		75% POME	
	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6
Fungi 1	0.041	0.035	0.047	0.044	0.068	0.062	0.024	0.027	0.034	0.035	0.055	0.060
Fungi 2	0.037	0.036	0.046	0.044	0.063	0.065	0.028	0.028	0.038	0.035	0.049	0.054
Fungi 3	0.038	0.034	0.040	0.051	0.062	0.061	0.026	0.024	0.036	0.036	0.055	0.052

Furthermore, *L. theobromae* showed a general downward trend in the reduction of glucose in the presence of nutrients at all conditions (25%, 50% and 75% POME respectively) where at 25% and 50%, the glucose concentration decreased from 0.037 g/L to 0.036 g/L and 0.046 g/L to 0.044 g/L respectively. On the other hand, at 75%, the glucose concentration of POME increased from 0.063 g/L to 0.065 g/L on the sixth day.

Glucose reduction of *L. theobromae* also showed a general downward trend at POME concentrations of 25%, 50% and 75% without the presence of nutrients. At 25%, the glucose concentration maintained at 0.028 g/L whereas at 50%, glucose concentration decreased from 0.038 g/L to 0.035 g/L. For 75% POME, glucose concentration had a slight increase from 0.049 g/L to 0.054 g/L.

Moreover, at 25%, 50% and 75% POME concentrations in the presence of nutrients, 25% and 75% POME concentrations showed a general downward trend in the reduction of glucose for *R. bataticola*, with 50% POME concentration showing a slight upward trend. At 25% and 75%, glucose concentration decreased from 0.038 g/L to 0.034 g/L and 0.062 g/L to 0.061 g/L respectively. On the other hand, at 50%, glucose concentration had a slight increase from 0.040 g/L to 0.051 g/L.

Similarly, without the presence of nutrients at 25%, 50% and 75% POME concentrations, Fungi 3 showed a general increase in the glucose concentration. At 25%, glucose concentration increased from 0.026 g/L to 0.029 g/L. Similarly, at 50% POME, the glucose concentration maintained at 0.036 g/L to 0.036 g/L at the sixth day. At 75%, the glucose concentration reduced from 0.055 g/L to 0.052 g/L.

It can be seen from Figure 2 that the production of biomass for *Pestalotiopsis* sp., *L. theobromae* and *R. bataticola* at 25%, 50% and 75% POME concentration in the presence of nutrients to be 0.047 ± 0.007 g, 0.060 ± 0.001 g and 0.064 ± 0.007 g for *Pestalotiopsis* sp., 0.075 ± 0.005 g, 0.044 ± 0.005 g and 0.079 ± 0.001 g for *L. theobromae* and 0.021 ± 0.009 g, 0.063 ± 0.001 g and 0.069 ± 0.005 g for *R. bataticola* respectively. This is in contrast to the POME media without the addition of nutrients for 25%, 50% and 75%, whereby *Pestalotiopsis* sp. produced 0.008 ± 0.001 g, 0.007 ± 0.001 g and 0.010 ± 0.001 g whereas *L. theobromae* produced 0.006 ± 0.001 g, 0.006 ± 0.003 g and 0.007 ± 0.002 g and *R. bataticola* produced 0.007 ± 0.001 g, 0.008 ± 0.002 g and 0.006 ± 0.002 g respectively.

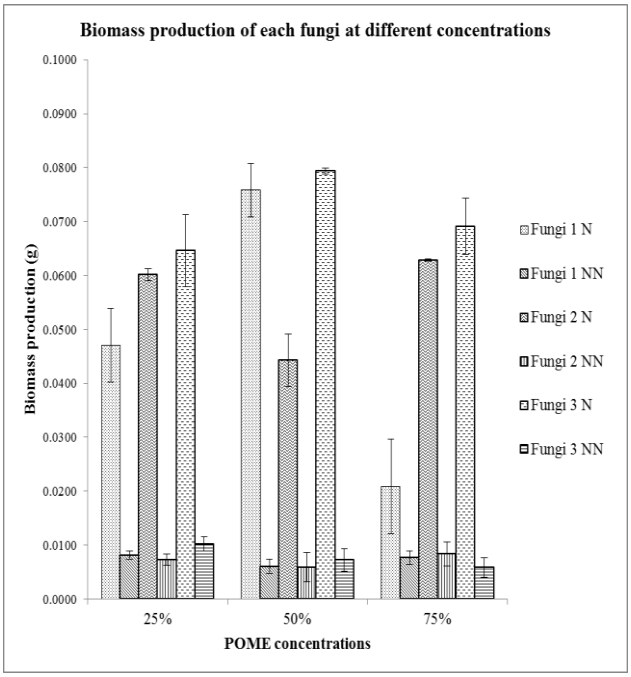

Figure 2: Biomass production of Fungi 1, 2 and 3 at 25%, 50% and 75% POME concentration with and without nutrients respectively.

In the presence of nutrients, the determination of the percentage of lipids per biomass produced can only be done for 25% and 50% POME for *Pestalotiopsis* sp., whereby the percentage were found to be 10.741 ± 0.045% and 3.548 ± 0.000% respectively whereas *L. theobromae* only showed 7.192 ± 0.060% and 3.928 ± 0.007% for 25% and 50% respectively whereas *R. bataticola* produced 3.371 ± 0.037%, 2.955 ± 0.000% and 5.654 ± 0.000% for Fungi 3 in 25%, 50% and 75% POME respectively (as shown in Figure 3). On the other hand, in the media without the addition of nutrients, *Pestalotiopsis* sp. showed a percentage of lipid per biomass produced to be 72.301 ± 0.299%, 34.837 ± 0.188% and 6.610 ± 0.048% respectively. Furthermore, *L. theobromae* produced 51.356 ± 0.218% and 30.303 ± 0.082% for 25% and 50% only whereas in *R. bataticola*, it produces 26.357 ± 0.226%, 17.766 ± 0.059% and 6.383 ± 0.000% for 25%, 50% and 75% respectively.

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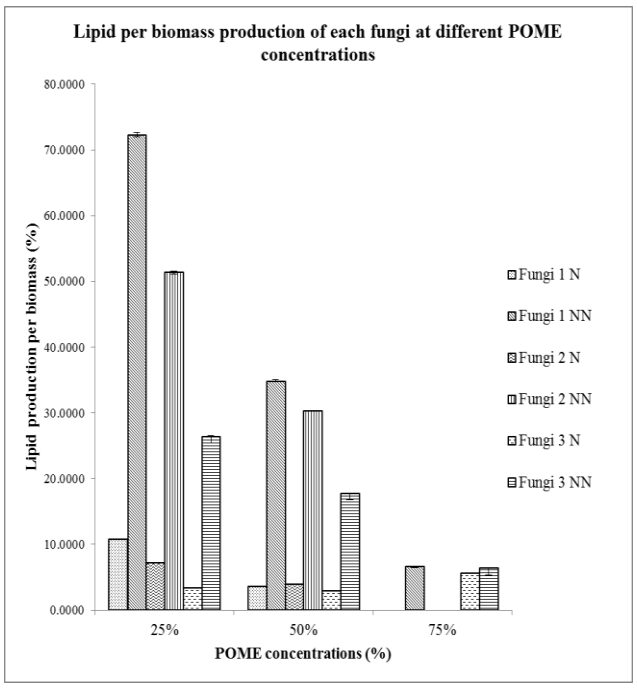

Figure 3: Lipid per biomass production of Fungi 1, 2 and 3 at 25%, 50% and 75% POME concentration with and without nutrients respectively.

DISCUSSION

The fungal isolates have been identified. Based on Table 2, the uptake of glucose by all fungal isolates in both conditions – with nutrients (N) and without nutrients (NN), is indicated by the reduction, increase or maintaining of glucose content in POME for all percentages of POME content (25%, 50% and 75% respectively). This is with the exception of *L. theobromae* in 75% POME without the addition of nutrients and Fungi 3 in 50% POME with the addition of nutrients which showed an increase in glucose concentration which may indicate an outlier. One of the possible explanations for the gradual increase in glucose content over time may be due to the auto-hydrolysis of the polymeric bonds of the polysaccharides under extremely low sulphuric acid and high temperature conditions (Ojumu *et al.*, 2003). Nevertheless, the POME utilized was not subjected to low pH conditions but only a single exposure to high thermal condition when sterilizing the POME for use as media. Thus, in the case where the concentration of glucose in POME maintained (based on Table 2) it may only be explained that although there is uptake of glucose into the fungal cells, the rate of glucose increase for unknown reasons may be greater than that of the uptake of glucose. This increasing glucose content could potentially be explained by the fact that the fungi may produce exoenzymes such as cellulases (Amadioha, 1998) that can break down the polysaccharide polymers into glucose units. Subsequently, the observed reduction of glucose in the POME may be more substantial than measured as there could be a constant breakdown of polysaccharides within the POME by the secretion of exoenzymes from the fungi (Umezurike, 1979; Amadioha, 1998; Arfi *et al*., 2013).

The greater increase in biomass in the samples that were added with nutrients (Figure 2) was due to the higher amount of nitrogen which is required for cell proliferation (Ajdari *et al.*, 2011). Additionally, a trend can be seen by which the samples with nutrients added show that the optimal concentration for the highest biomass obtained for *Pestalotiopsis* sp. and *R. bataticola* was 50% POME and 75% for *L. theobromae* (Figure 2). The increase of POME to 75% however resulted in a lower yield of biomass for both *Pestalotiopsis* sp. and *R. bataticola*. Thus, it can be deduced that each fungi has varying optimal C:N ratio for biomass yield with the addition of nutrients where the optimum concentration for *Pestalotiopsis* sp. and 3 was at 50% of POME and nitrogen becomes limiting when additional carbon is added (75% POME, Figure 2). On the other hand, in the samples without the addition of nutrients, a different progression can be seen whereby *Pestalotiopsis* sp. and *L. theobromae* had varying amounts of biomass obtained at different concentrations with the optimal concentration for biomass accumulation of *Pestalotiopsis* sp. is at 25% POME and *L. theobromae* at 75% POME. Moreover, the increasing concentration of POME resulted in a decrease in biomass for *R. bataticola*. Our findings are supported by Beopoulos *et al.* (2009) who also observed that the biomass of the fungal cultures was inhibited in the presence of high concentrations of carbon source.

To assess the efficiency of lipid accumulation in fungal cells, the percentage of lipids per biomass was obtained from total lipids extracted from the fungal mycelia and their dry biomass. In order to drive lipid synthesis instead of cell proliferation, starvation is required. This supports the notion whereby lipid accumulation is due to an excess of carbon source found in POME that is being continuously assimilated into the fungal cells. This carbon flux was switched into lipid synthesis as the limited nitrogen source in POME was required for protein and nucleic synthesis for the fungal cells, hence impeding cell proliferation (Beopoulos *et al.*, 2009). Based on Figure 3, there is an inclination when fungal isolates that were inoculated in media with additional nutrients (additional nitrogen source), the lipids/mg of biomass becomes significantly less than that of the media without the addition of nutrients as the low C:N ratio becomes a limiting factor for accumulating lipids. Figure 3 further show a trend whereby the increasing percentage of POME resulted in a decrease in lipids accumulated within the fungal cells, reflecting that although excess POME is required for lipid synthesis, the individual optimal C:N ratio must be kept in order to maintain the fungal cell's efficiency in accumulating lipids. From Figure 3, it can be seen that all three fungal isolates are oleaginous fungi as the percentage of lipid accumulation under nitrogen limiting environments (at 25% POME) were more than 20%, the criteria to be classified as oleaginous microorganism (Ageitos *et al.*, 2011) which is further

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supported by the fact that there was no significant increase in biomass (Figure 2) as they store lipids within the cells, thus indicating the optimal C:N ratio for was at 25% POME. The fungi which is the most efficient in accumulating lipids under nitrogen limited conditions per biomass biosynthesized is *Pestalotiopsis* sp. inoculated in 25% POME, whereby the percentage of lipids accumulated was 71.1% as compared to when it was inoculated under additional nitrogen content. This was supported by Certik *et al.* (1999), who observed that oleaginous fungi are able to accumulate up to or more than 70% of their biomass under nitrogen limiting conditions, comparable to the fungi *Mortierella isabellina* ATHUM 2935 utilized by Vicente *et al.* (2009), which was shown to be able to accumulate 50.4% oil when grown on glucose. *Pestalotiopsis* sp. showed a high ability to accumulate lipids when compared to other studies utilizing wastewater such as the usage of *Rhodotula glutinis* in POME with the addition of yeast extract at 32.6% (Saenge *et al.*, 2011), *R. glutinis* in corn starch wastewater at 35.0% (Xue *et al.*, 2010) and lastly *Aspergillus oryzae* in potato processing waste water at 40.0% (Muniraj *et al.*, 2013), indicating its potential for the simultaneous reduction of POME and production of biodiesel feedstock. However, this is based only on the lipids per biomass produced as its lipids per substrate consumed cannot be determined due to the anomalies observed in the determination of glucose concentration.

CONCLUSION

In conclusion, the closest matches of Fungi 1, 2 and 3 were *Pestalotiopsis* sp., *L. theobromae* and *R. bataticola* respectively with all three isolates having a percentage similarity of 99%. All three isolates were able to utilize POME and accumulate lipids in their cells whereby Fungi 1 – *Pestalotiopsis* sp. is the most promising fungi for biodiesel production with 71.1% lipid content. To our knowledge, this is the first time that members of all three genera were utilized for lipid production from POME and highlights the potential of endophytic fungi for bioremediation purposes.

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