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# Development of *Rhodotorula mucilaginosa* strain via random mutagenesis for improved lipid production

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## ABSTRACT

**Aims:** Oleaginous yeasts are widely used for the production of biodiesel feedstocks because of their high lipid content. This research was aimed to conduct random mutagenesis of *Rhodotorula mucilaginosa* using ethyl methane sulfonate (EMS) and identify the mutants with improved lipid production.
**Methodology and results:** A total of twenty-two mutant isolates prescreened with cerulenin were produced and further characterized via M13 PCR fingerprinting to determine their polymorphism and genetic distances. Eight strains, namely M1, M2, M3, M4, M7, M10, M11 and M18, were chosen based on their genetic distances from the parental strain for biomass production. Six mutants (M1, M2, M3, M4, M7 and M18) showing the highest dry cell weights were further selected for evaluation of lipid production in a laboratory-scale bioreactor using glucose as a carbon source. Results indicated that parental strain exhibited lipid content of 1.83 g/L, while strains M1, M2, M3, M7 and M18 generated 2.37 g/L, 2.27 g/L, 2.27 g/L, 3.10 g/L and 3.83 g/L of intracellular lipid, respectively. These five mutants were identified to have significant increase in lipid production compared to the parental strain.
**Conclusion, significance and impact of study:** This study demonstrated enhanced lipid production in *R. mucilaginosa* by random mutagenesis. New generated strains had higher lipid productivity compared to parental strain and application of these strains in industry may reduce the overall cost of biodiesel production.

*Keywords:* Oleaginous yeasts, *Rhodotorula mucilaginosa*, random mutagenesis, cerulenin, lipid productivity

## INTRODUCTION

The limited petroleum reserves and serious environmental pollutions have triggered the necessity to exploit alternative renewable energy sources, such as bioethanol and biodiesel (Huang *et al*., 2018). Biodiesel is an outstanding renewable fuel that can supplement petroleum fuel (Vincent *et al*., 2018). It is produced from the transesterification of triacylglycerides (TAGs) and alcohol in the presence of a catalyst such as NaOH or KOH (Moser *et al.*, 2009). Biodiesel is highly degradable, non-toxic and could reduce the emission of harmful gases (Sitepu *et al.*, 2013; Vincent *et al*., 2018). Most currently produced biodiesel is made from plant oils such as sunflower, rapeseed, peanut and palm oils (Atabani *et al.*, 2012; Demirbas *et al.*, 2016). Besides the high feedstock cost, the usage of edible plant oils for biodiesel production causes the issue of food-fuel competition, therefore a cheaper raw material with high oil productivity is crucial to enhance biodiesel production (Chhetri *et al.*, 2008; Atabani *et al.*, 2012; Shikha and Rita, 2012). Third-generation biodiesel produced from microbial oils has the potential as an excellent alternative source for biodiesel production (Soccol *et al.*, 2017; Vincent *et al*., 2018).

Microbial oil, also known as single cell oil (SCO), is produced by oleaginous microorganisms. Oleaginous microorganisms are microbes that are able to accumulate more than 20% of its dry biomass as storage lipid (Ochsenreither *et al.*, 2016). There are different types of oleaginous microorganisms that include yeasts, microalgae, bacteria and fungi (Steensels *et al.*, 2014). Compared to plant oils, microbial oil production does not use large arable lands, have higher lipid yield and production rate, and is independent of climate and geographical locations (Gientka *et al.*, 2017). Despite the extensive use of oleaginous microorganisms in industrial fermentation, further improvements are needed to enhance the metabolic capacity through genetic modifications and optimization of fermentation conditions.

Genetic engineering is frequently practiced to generate novel strains with improved productivity and better utilization of low-cost substrates in the development of biodiesel (Ochsenreither *et al.*, 2016). It involves the insertion of foreign genes into another species to modify the fatty acid profile or increase the lipid content

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(Beacham *et al.*, 2015). For instance, some of the modifications made to enhance the strains include the overexpression of enzymes in fatty acids biosynthetic pathway, repression of competing pathways and introduction of new genes into heterologous species (Sitepu *et al.*, 2014). However, these techniques require costly materials and complex equipments that are not available in many research laboratories (Harlander, 1992). The conventional method of strain improvement through random mutagenesis, therefore, remains practical in inducing genetic variations in many organisms. This method has been applied on plants especially crops, where more than 2,500 varieties have been generated from different species such as cotton, sunflower, rice and wheat (Schouten and Jacobsen, 2007).

In the current study, strain improvement was conducted on the oleaginous yeast *Rhodotorula mucilaginosa*. It is a member of the genus *Rhodotorula* and phylum Basiodiomycota. *R. mucilaginosa* is commonly found in humans, animals, food and beverages (Wirth and Goldani, 2012; Deligios *et al.*, 2015). Over the years, this species has been widely studied for its carotenoids production (Aksu and Eren, 2005; Maldonade *et al.*, 2012). The ability of this yeast to accumulate high oil content has also been reported, but most of these studies only highlighted its substrate utilization (Li *et al.*, 2010; Reyna-Martinez *et al.*, 2015; Gientka *et al.*, 2017). No attempt on strain improvement of *R. mucilaginosa* to improve its lipid production has been documented. Therefore, the present study was aimed to employ random mutagenesis using ethyl methane sulfonate (EMS), followed by mutant selection through cerulenin screening. Selected mutants were then evaluated in fermentation studies to identify strains with improved lipid production.

## MATERIALS AND METHODS

### Culture preparation

The parental strain of *R. mucilaginosa* used in this study was obtained from Microbiology Lab 2, Faculty of Resource Science and Technology (FRST), UNIMAS. For long term storage, the strain was preserved in Yeast Malt Broth (YMB) containing 20% glycerol and stored at –80 °C. For instant use, the stock culture was revived by growing it on Dichloran Rose Bengal Chloramphenicol (DRBC) agar plates. The single colony was inoculated into YMB and incubated at 25 °C, 150 rpm on shaker for 2 days. The ingredients of YMB are: 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract and 10 g/L dextrose.

### EMS mutagenesis

Mutagenesis treatment was conducted as described previously by Winston (2008), with minor modifications. An amount of culture was transferred to microcentrifuge tube and the pellet was harvested via centrifugation at 13000 rpm for 1 min. The pellet was washed two times with distilled water and resuspended in 1 mL of 0.1 M sodium phosphate buffer, pH 7.0. Approximately 30 µL of EMS (3% v/v) was added into the cell suspension and vortexed vigorously. The culture was left incubation on shaker at 25 °C for 2 h (Hashimoto *et al.*, 2005). An extra tube without EMS was prepared as well as the control. After 2 h, culture was washed with 5% sodium thiosulphate to inactivate the EMS function followed by resuspension in sterile distilled water. Next, the mutagenized cell was diluted (serial dilution of 1000-fold) and inoculated on malt extract agar (MEA) plates supplemented with cerulenin. The final concentration of cerulenin within agar plates was 10 µg/mL (Tapia *et al.*, 2012). Non-mutagenized cell was grown on both the cerulenin supplemented and non-supplemented agar plate as control. The plates were left incubation for 3-4 days until the colony forms.

### Molecular characterization of mutants

DNA extraction of yeast strains was performed using DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany). Then, PCR mixtures were prepared as follows: 2.5 µL of 10X PCR buffer, 1.0 µL of 10 mM dNTP, 2.5 µL of 50 mM $MgCl_2$, 2.0 µL of M-13 primer (5' GAGGGTGGCGGTTCT 3'), 3.0 µL of DNA template, 0.3 µL of *Taq* polymerase and certain volume of sterile distilled water. The final volume of PCR mixture was added to 25 µL using sterile distilled water. The thermo cycling condition was based on the protocol of Silva *et al*. (2008), with minor modifications. The cycle condition was as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min; optimal annealing of primer at 66.3 °C for 1 min, extension of DNA at 72 °C for 1 min; and final extension at 72 °C for 10 min. The PCR product was loaded on gel and electrophoresis was conducted at 70V for 90 min. Then, the gel was stained with SYBR-Green and visualized under blue light transilluminator. A dendrogram was constructed by Unweighted Pair Group Method with Arithmetic mean (UPGMA) linkages using software PyElph version 1.3.

### Determination of propagation curve

From grown culture in YMB, 16 mL of culture (1% v/v) was inoculated into a small-scale bioreactor containing 1.6 L of YMB supplied with aeration. The culture was harvested at every 24 h interval for a duration of 168 hours. The harvested sample was centrifuged at 4000 rpm for 10 min and the pellet was collected. The pellet was dried in oven (70 °C) overnight to obtain its dry cell weight (DCW). All the readings were recorded and a graph showing cell propagation was plotted.

### Submerged fermentation in bioreactor

A volume of 800 mL YMB with yeast strain (1% v/v) was prepared and incubated with aeration supplied for six days in which the nutrients within the medium is about to be consumed completely. The duration of propagation

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was set according to the growth curve of cells performed in previous steps. Taking day 6 as the example, 800 mL of glucose solution (5%) was prepared and added aseptically into the grown culture as substrate. The final total volume was 1.6 L and the glucose concentration was 2.5%. Daily sampling was conducted for 168 h and pellet was harvested through centrifugation. The steps were repeated for different mutants.

**Lipid extraction**

Lipid extraction was performed directly from wet biomass instead of dry biomass. Acid pre-treatment was done prior to lipid extraction by adding 3 mL of 4 M of hydrochloric acid (HCl) and incubated at 78 °C water bath for 2 h (Yu *et al.*, 2014). After the samples cooled down, 10 mL of hexane:isopropanol (HIP) with ratio 3:2 was added into sample mixtures. The samples were homogenized at maximum speed for 90 sec using hand held homogenizer (Hercuvan, Malaysia). After homogenization, the samples were centrifuged at 4000 rpm, 25 min for phase separation and two layers were observed. The upper layer containing hexane and lipid was transferred into a pre-weight tube and heated to evaporate the solvent. The sample tube was stored in oven overnight until a constant mass was obtained.

**Sudan III solution preparation and staining**

Sudan III solution was prepared by mixing 0.5 g of Sudan III powder in 50 mL of 70% ethanol. The mixture was heated and stirred to dissolve the powder. The solution was filtered to remove excess solid residue. The filtrate was collected and stored before staining. For culture staining, 100 µL of culture was mixed with 100 µL of Sudan III solution and leave it for one hour. After one hour, a small volume of mixture was applied on microscopic slide and observed under microscope to check the presence of lipids.

**Statistical analysis**

All the data were analyzed using Tukey post hoc test using software IBM SPSS Statistics version 22 to determine the significance difference. The significance level of parental strain and mutants was compared at 95% confidence interval.

**RESULTS**

EMS toxicity on *R. mucilaginosa* was determined by exposing the cells to 3% EMS for 240 min. The cell number was reduced to less than 5% of the total number in control plate as shown in Figure 1.

**Mutant selection by cerulenin**

After EMS treatment, the cells were spread onto agar plates containing cerulenin to screen for mutants. It was observed that the growth of the parental *R. mucilaginosa*

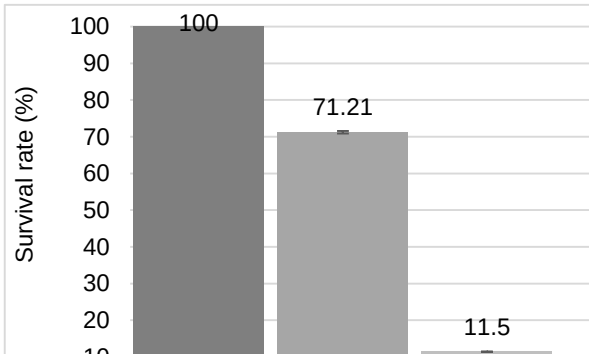


**Figure 1:** The survival rates of *R. mucilaginosa* cells after EMS treatment, based on the number of colonies observed in different period of exposition.

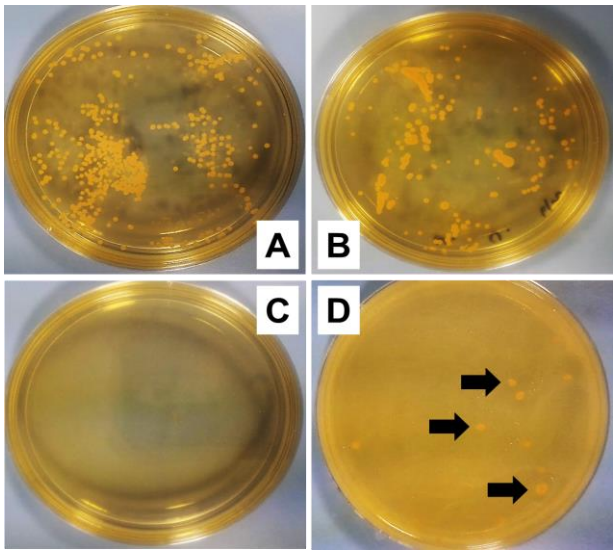


**Figure 2:** Mutant screening by cerulenin**.** A. Growth of *R. mucilaginosa* in non-supplemented MEA plate. B. Growth of mutagenized cell in non-supplemented MEA plate. C. Growth of *R. mucilaginosa* in cerulenin-supplemented MEA plate. D. Growth of mutagenized cell in cerulenin-supplemented MEA plate. Black arrow indicates the colony grown.

was inhibited in the presence of 10 µg/mL cerulenin (Figure 2C). Normal growth was observed in non-mutagenized and mutagenized *R. mucilaginosa* when cerulenin was not added (Figure 2A and 2B). A total of twenty-two isolates were obtained and each was further characterized using M-13 PCR fingerprinting to confirm mutagenesis (Figures 3 and 4).

**Propagation curve**

The propagation curves were generated to determine the growth of the yeast cells in the fermentation setup and to estimate the duration taken by each strain to consume the nutrients completely. From 22 isolates, eight strains (M1,

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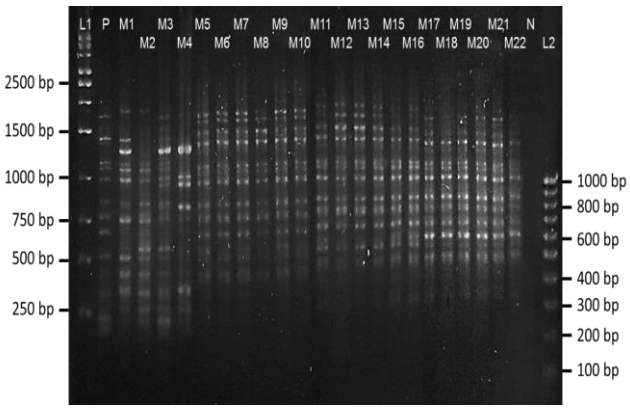


**Figure 3:** Electrophoresis gel of M-13 PCR fingerprinting for all 22 isolates and *R. mucilaginosa* parental strain. L1 and L2 represent 1kb ladder and 100 bp ladder; P represents parental strain; M1-M22 represent mutant strain 1 to 22; N represents negative control.

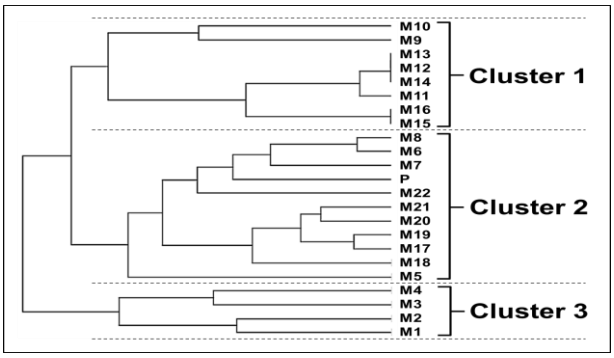


**Figure 4:** Dendrogram constructed using UPGMA and PyElph version 1.3 software. P indicates *R. mucilaginosa* parental strain.

M2, M3, M4, M7, M10, M11 and M18) were selected randomly from three clusters for the propagation stage. As shown in Figure 5, the highest dry cell weight of parental strain was 7.77 g/L at the 168$^{th}$ h. Out of all the mutant strains tested, the highest biomass of 8.63 g/L was recorded for M1 after 168 h of fermentation while M10 showed the lowest biomass of 7.53 g/L at the same time point. With the same amount of nutrients provided, the dry cell weight of M2 and M3 started to decrease after 144 h while those of the other strains continued to increase (Figure 5). This indicated a shorter log phase for these two strains and they entered the death phase earlier than the other mutants. At a 95% confidence interval, M1, M2, M3 and M18 showed a significant increase in dry cell weight when compared to parental strain while the rest of the mutants tested showed no significant difference.

### Lipid productivity

Six strains with the highest dry cell weight (M1, M2, M3, M4, M7 and M18) were selected for evaluation of lipid

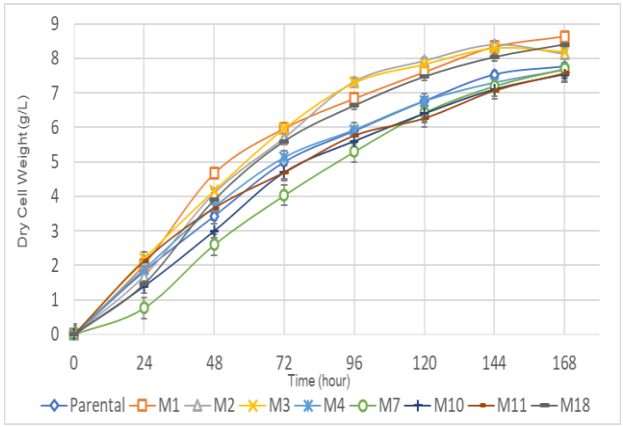


**Figure 5:** Growth of *R. mucilaginosa* parental and mutants strains for 168 h.

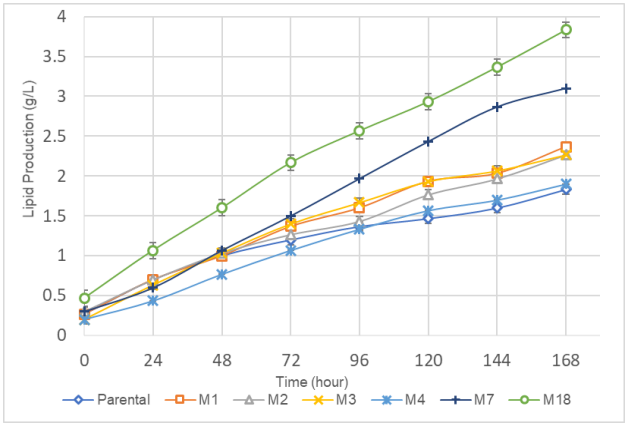


**Figure 6:** Lipid yield of *R. mucilaginosa* parental and selected strains at different time intervals.

production. After the initial cultivation, the cells were fed with glucose and incubated for 168 h for lipid accumulation. As shown in Figure 6, the highest lipid yields in all strains tested were recorded at the last sampling point (168-hour). The cells were stained with Sudan III solution to indicate the accumulation of lipids within cells (Figure 7). All the mutants exhibited higher lipid production than parental strain. M18 produced the highest amount of lipid at 3.83 g/L, which was 36.98% of its biomass (Table 1). Tukey post hoc analysis showed that, at 95% confidence interval, five mutants (M1, M2, M3, M7 and M18) exhibited significant increase in lipid production as compared to parental strain. Out of the six mutants tested, only M4 showed no significant difference from its parental strain.

## DISCUSSION

Industrial strain improvement aims to produce superior strains with increased metabolic and biosynthetic capacities. There are different approaches to modify microbial strains including mutation, selection and genetic


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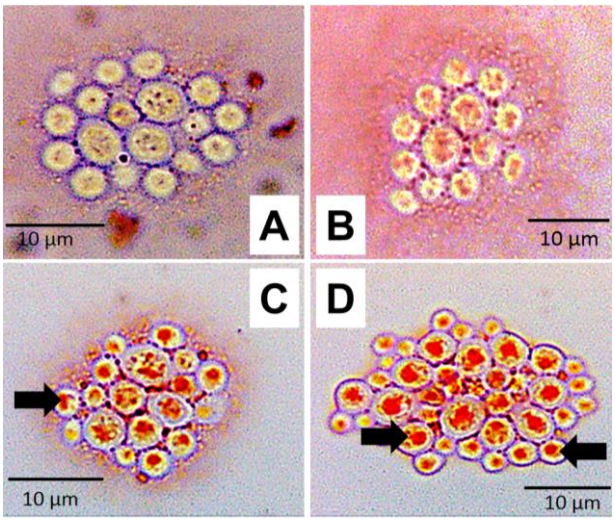


**Figure 7:** Microscopic images of lipid staining using Sudan III at different times. A. 0-h B. 24-h C. 48-h D. 96-h. The reddish orange regions (black arrow) within cells indicates intracellular lipid bodies.

recombination (Parekh *et al.*, 2000). Compared to genetic engineering, the classical method of random mutagenesis coupled with selection through specific screening is a cost-effective and time-saving strategy in the fermentation industry (Masurkar *et al.*, 2016). The combination of conventional mutation using ethyl methane sulfonate (EMS) and chemical screening enables the isolation of yeast strains with high metabolite-producing properties (Nishiuchi *et al.*, 2012). Thus, this study employed the use of the chemical mutagen EMS to induce random mutation in *R. mucilaginosa*, followed by cerulenin screening to isolate the potential strains with improved lipid production.

EMS is a very powerful mutagen and carcinogenic to human (Ariraman *et al.*, 2014). It causes high lethality of cell in which the survival rate was reduced to less than 5% after exposure of 180 min in *R. mucilaginosa* (Figure 1). Thus, this mutagen should be handled with care and deactivated before disposal. Cerulenin [(2S) (3R)2, 3-epoxy-4-oxo-7,10-dodecadienoylamide] is a type of antibiotic that inhibits fatty acid synthesis specifically the FAS enzyme by attaching covalently to the active site cysteine (Zhang and Miyake, 2007). Due to its inhibitory properties, cerulenin has been used in a large number of strain improvement researches. The present study also showed similar results in which the growth of *R. mucilaginosa* was inhibited or reduced after cerulenin screening (Figure 2A-2D). When cerulenin was not supplemented, many colonies were observed (Figure 2A and 2B) while only a few or no colonies were observed in the presence of cerulenin (Figure 2C and 2D). Previous studies showed that polyunsaturated fatty acids (PUFAs)-producing bacteria in cultures supplemented with cerulenin exhibited enhanced production of PUFAs (Morita *et al.*, 2005; Wan *et al.*, 2016). A similar strategy of cerulenin screening coupled with UV irradiation to induce mutation was also conducted on *Lipomyces starkeyi* DSM 70296, resulting in elevated lipid production (Tapia *et al.*, 2012). Cerulenin screening has proven to be a quick and cost-efficient method to isolate superior strains with enhanced enzyme activity in fatty acid biosynthesis (Wang *et al*., 2009). In this study, there were

**Table 1:** Optimum lipid productivity of *R. mucilaginosa* parental and selected mutant strains.

| Strains | Calculated dry biomass (g/L)* | Lipid Production (g/L) | Glucose Consumption (g/L/h) | Time (hour) | Lipid percentages (%) |
|---|---|---|---|---|---|
| Parental | 7.83 ± 0.30 | 1.83 ± 0.06 | 0.08 ± 0.01 | 168 | 23.40 |
| M1 | 8.12 ± 0.23 | 2.37 ± 0.06 | 0.11 ± 0.01 | 168 | 29.15 |
| M2 | 8.38 ± 0.09 | 2.27 ± 0.06 | 0.10 ± 0.01 | 168 | 27.05 |
| M3 | 8.81 ± 0.05 | 2.27 ± 0.06 | 0.10 ± 0.00 | 168 | 25.74 |
| M4 | 8.01 ± 0.06 | 1.90 ± 0.00 | 0.09 ± 0.01 | 168 | 23.71 |
| M7 | 8.77 ± 0.32 | 3.10 ± 0.00 | 0.12 ± 0.01 | 168 | 35.36 |
| M18 | 10.37 ± 0.20 | 3.83 ± 0.15 | 0.13 ± 0.00 | 168 | 36.98 |

Data are the mean of triplicates. ± S. D. *Dry biomass= Wet biomass × (20/100); Assuming wet biomass: 80% moisture content (Wahidin *et al.*, 2014).

twenty-two isolates survived after cerulenin screening and were considered as potential strain with enhanced lipid production (Figure 2D).

After cerulenin screening, twenty-two isolates were further analyzed through M-13 PCR fingerprinting to confirm the mutations. From Figure 3, the sizes of DNA fragment of *R. mucilaginosa* were in the range of 200 – 2500 bp. The parental strain had 15 DNA bands while the mutants had slightly varied number and pattern of bands. The image was analyzed using PyElph software through UPGMA and all isolates were found to be mutated as shown in dendrogram (Figure 4). The isolates were grouped into three main clusters, namely cluster 1, cluster 2 and cluster 3. Each cluster represents the grouping of different mutants according to the similarities of DNA bands and genetic distance. The strains within the same cluster were considered as closely related. For example, parental strain was placed in cluster 2, hence the strains (M5, M6, M7, M8, M17, M18, M19, M20, M21, M22) were closely related while strains from clusters 1 and 3 were substantially different from parental strain (Figure 4). Next, eight strains were randomly chosen from each

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cluster for further propagation. The propagation was conducted using laboratory-scale bioreactor with aeration supplied for 168 h. The results showed that mutagenized *R. muciginosa* strains after cerulenin screening had higher biomass than parental strain (Figure 5). These strains propagate faster to achieve high density culture and tends to accumulate more lipids. Six strains (M1, M2, M3, M4, M7 and M18) with the highest biomass were chosen and further evaluated for their ability in lipid accumulation (Figure 5).

Two-stage microbial lipid production was performed in this study with the first stage involving cultivation of *R. mucilaginosa* and second stage of substrate feeding with glucose. As shown in Figure 6, the lipid production of each strain increases throughout the fermentation time and all strains had the highest lipid production at 168$^{th}$ hour. The increasing trend indicates that there is possibility the lipid will still exhibit an increase after 168$^{th}$ hour. The lipid accumulation within cell was detected using Sudan III staining to indicate the presence of lipid. It was observed that the orange region was getting larger over the fermentation time (Figure 7). This is because glucose was continuously utilized by cell and accumulated as storage lipids within the cell. The lipid content of yeast will drop when the carbon sources is consumed completely (Jadhav *et al.*, 2012). Five strains (M1, M2, M3, M7 and M18) showed significant increase in lipid production compared to parental strain. It was observed that the higher the lipid yield, the higher the glucose consumption efficiency (Table 1). The enhanced lipid yield suggests that these mutant strains had improved FAS enzyme activities to overcome the inhibitory effect of cerulenin (Wang *et al.*, 2009). Since random mutations using EMS was performed in this study, it is difficult to predict the types of mutation generated and details on the specific genes affected were not within the scope of this project. Oleaginous microorganisms have different capabilities in lipid accumulation due to their respective genetic makeup, biochemistry and structural composition (Wynn and Ratledge, 2006; Nigam and Singh, 2014; Jiru *et al.*, 2016). Even the strains within the same species might have different abilities in accumulating lipid (Jiru *et al.*, 2016).

In this study, lipid was directly extracted from wet biomass instead of dry biomass to determine the yield. Dewatering technology is an expensive, time- and energy-consuming downstream process in lipid extraction of the industrial scale (Halim *et al.*, 2012; Sathish and Sims, 2012; Roux *et al.*, 2017). When lipid is extracted directly from wet biomass, the overall cost is reduced and the process is more energy efficient (Yang *et al.*, 2014). Previous studies also reported that the lipid yield from wet microalgae was encouraging when the drying step was removed after cell harvesting (Sathish and Sims, 2012; Taher *et al.*, 2014; Laurens *et al.*, 2015; Ansari *et al.*, 2017). In addition, dry biomass treated with water before the extraction step showed enhanced lipid recovery efficiency. The presence of water increases the polarity of the solvent mixture and helps weaken the links between polar lipids and proteins that are anchored into the cell membrane, making neutral lipid more available to non-polar solvent (Ren *et al.*, 2017). The direct extraction from wet biomass has the potential to reduce overall cost without affecting significantly on the quality and amount of lipids (Taher *et al.*, 2014). A direct extraction from wet biomass also requires less energy and could increase the Energy Returned on Energy Invested (EROEI), making the industrial process more economically favorable (Dong *et al.*, 2016).

However, efficient lipid extraction is often achieved with the combination of solvent extraction and pre-treatment steps that disrupt the cell structure (Forfang *et al.*, 2017). Acid hydrolysis prior to extraction weakens the yeast cell wall, causing monomeric sugars to be released into the aqueous phase, enabling the solvents to bind the lipids directly (Yu *et al.*, 2014; Dong *et al.*, 2016; Roux *et al.*, 2017). Up to 97% of total fatty acids was recovered through extraction with hexane after acid pre-treatment (Laurens *et al.*, 2015). Generally, both acid hydrolysis and homogenization function to disrupt the cells for better lipid accessibility and extraction efficiency (Halim *et al.*, 2012; Dong *et al.*, 2016; Roux *et al.*, 2017). The rupture of cells through pre-treatment steps reduces the amount of solvents used during extraction compared to untreated biomass (Mercer and Armenta, 2011; Naghdi *et al.*, 2016).

Overall, the lipid yield in this study was slightly lower than other previous studies, where lipid contents of more than 50% were achieved (Li *et al.*, 2010; Zhao *et al.*, 2010). This could be due to the lack of optimization on the fermentation conditions and lipid extraction methods, which has a significant impact on the lipid production. Thus, further research on optimizing lipid production and the use of industrial or agricultural waste as the substrate for strain improvement are recommended. A suitable lipid extraction method needs to be applied on different species as the lipid extraction efficiency is affected by the type of microbial biomass (Lee *et al*., 2010; Forfang *et al.*, 2017).

## CONCLUSION

The methods used in the current study provided a quick isolation of mutant strains with improved lipid yield in an oleaginous yeast after chemical mutagenesis. Pre-screening with cerulenin enabled the selection of potential strains. This is more efficient, and time-saving compared to random selection. Next, pre-treatment of the biomass enhanced the efficiency of lipid extraction and the direct extraction of wet biomass reduced the consumption of time and energy. The isopropanol and hexane used for lipid extraction are cost-efficient and less toxic than the conventional solvents chloroform and methanol. The lipid extraction should be analyzed individually for each strain as the extraction efficiency depends highly on the type of the microbial biomass. The mutagenesis procedure employed in the present study is feasible, easy and cost effective in generating improved strains compared to genetic engineering. Thus, the combination of random

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mutagenesis and cerulenin screening is practical for strain improvement and other biochemical studies.

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