MICROBIAL LIPID EXTRACTION BY ELECTROPORATION TECHNIQUE CULTURING LIPOMYCES STARKEYI ON PALM OIL MILL EFFLUENT (POME)

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Abstract - Microbial lipid is considered as a substitute of traditional edible oils like plant oil, animal fat, cooking oil etcetera as feedstock of biodiesel. Oleaginous yeast strain Lipomyces starkeyi is a potential candidate can play a key role for simultaneous bioremediation as well as lipid accumulation from hydrocarbon enriched palm oil mill effluent (POME). The main obstacle to obtain higher lipid extraction from microbial biomass is the cell wall breakup. Therefore, the present study is intended to develop a novel cell wall disruption technique for the extraction of biomolecules from microbial biomass within a short time as well as eco-friendly. In this study, the electroporation (EP) technique was employed to disrupt yeast cell where voltage, pulse frequency and pulse width were 4kV, 100 Hz and 0.01 s respectively. In addition, different treatment time (2, 4, 8 min) were employed to observe the effect of EP treatment on cell inactivation as well as lipid extraction. Our experimental results show that the maximum lipid extraction was achieved by employing 8 minutes EP treatment. These results suggest that EP would be an attractive technique alternative to the traditional microbial cell disruption that leads to significant lipid extraction.

Keywords - Palm oil mill effluent; Bioremediation;Lipomyces starkeyi; Electroporation; Microbial lipid.

I. INTRODUCTION

The increasing demand for more sustainable and carbon-neutral energy resources has resulted in searching of bio fuel production from biomass. Biomass is likely to have a great contribution to future sustainable energy systems [1, 2]. Now a days, the biological sources such as vegetable oils and animal fats and oils are considered as the main raw materials for biodiesel production[3]. However, the cost of various food-stuffs has increased due to the increasing demand of biodiesel and this situation has led to the necessity of finding of non-conventional sources of oils that can be subsequently converted into biodiesel [4]. Therefore, there has been redirected theattention in searching for more renewable and abundant oil sources. Due to the high biomass and lipid production rates of microbial oilsand minimal competition with the food supply for human society compared with traditional lipid feedstocks, microbial lipid is considered as a potential alternative for large scale production of non-petroleum based transportation fuel [5-7]. The cultivation of oleaginous microorganisms for production of oil sources is more advantageous than production of vegetable oilssince it is not affected by seasons and climates. Moreover, yeasts are beneficial compared to molds and algae among the oleaginous microorganisms, because those have faster growth rate and are easy to be cultivated in large scale [8]. Oleaginous microorganisms including bacteria [9], yeasts [10] , moulds [11] and algae [12] can produce lipids up to more than 20% of their dried biomass. The ability of oleaginous yeasts to accumulate high lipid content by assimilating nutritional residues from agriculture and industry makes them superior to other microorganisms[13]. Consequently, it is likely to be an interesting approach to harvestsignificant microbial lipids as oil sources for biodiesel production by using abundant industrial wastes as fermentation media.

Palm oil mill effluent (POME) is a wastewater generated from palm oil milling industries. The production of palm oil is abundant in Southeast Asia, especially in Thailand, Malaysia, and Indonesia. It is reported that the 0.65 tons of POME is producing from the processing of every ton of palm oil [14].However, POME is a high strength pollutant with a low pH (due to the organic and free fatty acids), arising from partial degradation of palm fruits before processing which has large amounts of total solids (40,500 mg/L), oil & grease (4000 mg/L), biochemical oxygen demand (25,000 mg/L) and chemical oxygen demand (50,000 mg/L) [15]. Several reports have shown that these values are 100 times higher than those of municipal sewage [16, 17]. Therefore, the discharge of untreated POME is objectionable since the by-products and waste generated by palm oil mill may have a significant impact on the environment. On the other hand, POME contains high amount of nutrients such as carbohydrates, protein and mineral salts that could stimulate the growth of yeast.[18, 19].

In this study, oleaginous yeast strain Lipomyces starkeyi has been used as a potential candidate for their lipid producing capability using POME as a low-cost cultivation medium. Compared to other microorganisms, the yeast species have a rigid cell

wall [20]with high chemical resistance and mechanical strength[21]. Thus, the cell wall disruption is the most challenging part of lipid extraction process from yeast cells, since lipids are located in inner of cell membrane[20, 22, 23]. However, many cell wall disruption methods including physical, chemical and enzymatic have been developed to achieve selective release of biomolecules [24-26]. Several methods are often limited to small scale, usually result in low intracellular product release, low process efficiency. Additionally, the use of chemicals and enzymes in some other methods may lead to higher degree of complexity to the environment. Though high-pressure homogenization [27], ultrasonication [28], osmotic shocks and bead-beating [7, 29]are extensively used to disrupt microbial cell in order to release intracellular products [24, 26], they are associated with high energy and time consuming [20]. Therefore,electroporation (EP) is a microbiological technique that has been applied in this study in order to increase the permeability of the cell membrane by applying a short burst of high voltage electrical field to a sample placed between two electrodes[30], and extract intracellular biomolecules from the cell [5, 31].

The aim of this study was to harvest lipids from L. starkeyi by utilizing the carbon sources and nutrients of POME. Moreover, a unique cell disruption technique calledirreversible EP was applied to disrupt the yeast cell wall to extract lipids. The efficiency of EP was evaluated by calculating cell inactivation rates and measuring extracted lipid from wet biomass of L. starkeyi. Furthermore, the performance of lipid extraction by EP technique was compared with other methods to validate its applicability. The capability of this oleaginous yeast to accumulate lipid by valorisingPOME and its probable applicability in direct transesterification process, would have great contribution to cost-effective and eco-friendly biodiesel production.

II. MATERIALS AND METHODS

2.1. Collection and preparation of POME medium

The POME was obtained from LKPP Corporation Snd. Bhd., Gambang, Pahang and stored at 4 °C to prevent the deterioration of ingredients. The submerged solids and debris were removed through a Whatman No.1 filter paper and subsequently the wastewater was sterilized by autoclaving at 121 °C for 20 min before its use as the culture medium. The whole experiment was conducted using POME without adding additional nutrients. POME samples were prepared by 50 % dilution of the raw POME.

2.2. Cell culture and biomass collection

The oleaginous yeast strain L. starkeyi (ATCC 56304) was collected from the laboratory of Biochemical Engineering, University of Naples Federico II, Italy. The strain was cultured in YPD agar (m/v: yeast extract 1%, peptone 2%, dextrose/glucose 2%, agar 2%) slants to maintain a stock culture. The subculture was done on petri plates to grow new cells. The primary inoculum was prepared by dissolving 10 loops of microbes from the sub-cultured petri plates in 10 mL of sterilized water. The 150 mL of POME medium was taken in 500 mL Erlenmeyer flasks and sterilized at 121 °C for 20 min. Thereafter, the medium was inoculated with 1mL of primary inoculum mm and incubated at 25 °C for 5 days with a rotation speed of 150 rpm. The biomass was separated using centrifugation technique (at 10000 rpm for 10 min). The weight of collected biomass was measured by analytical balance. In case of dry biomass, the wet biomass was oven dried at 40 °C until getting constant weight.

2.3. POME treatment analysis

Several parameters of POME such as chemical oxygen demand, biological oxygen demand, total solids, total suspended solids, total organic carbon, total phenolic content, ammoniacal nitrogen, nitrate nitrogen, oil and grease and total dissolved solids were examined before treatmentand after treatment of POME by standard APHA methods[32]. The organic compounds were extracted from POME by liquid–liquid extraction using n-hexane. Fixed volumes (1 µL) of extracted samples were investigated by Gas Chromatography–Mass Spectrometry (GC-MS). Pure Helium gas (99.999%) was used as carrier gas at a flow rate of 1 mL/min. To adopt in the separation system, a DB-35MS capillary column with inner diameter of 0.25 mm and length of 30 m was used. The temperature of gasification compartment was 40 °C for 5 min; then increased to 280 °C at a rate of 3 °C /min.

2.4. EP treatment

The 200 mg of wet biomass was mixed with 10 mL of sterile water and were taken in the EP reactor. The cell concentrations were adjusted by OD at 600 nm (Shimadzu model UV-160A) for each time to maintain equal concentration of cell at initial. The high voltage (4 kV), with the frequency of 100 Hz was employed to the yeast cell through two circular stainless steel plates used as electrode in EP reactor. All experiments were triplicated to confirm the observation of each treatment.

2.5. Effect of EP on cell viability

To evaluate the viability of cells under EP treatment spread plate technique was used. The 20 µL of sample was taken from reactor before and after treatment (2, 4, 8 min) EP treatment and mixed with 980 µL sterile water. Each sample (20 µL + 980 µL = 1 mL) was then spreaded onto the petri-plates (YPD agar media) and incubated at 25 °C for 48 h. After that, the colony forming units (CFU) was counted to calculate cell inactivation rate.

Cell inactivation rate= (Initial CFU at t_i – Final CFU at t_n) *100/ Initial CFU at t_i
t_i= No. of CFU before treatment (i=0 min)
t_n= No. of CFU after treatment (n= 2, 4, 8 min)

2.6. FESEM analysis

The disruption of L. starkeyi cells was observed by using FESEM (JEOL JSM-7800F, Japan). The samples were collected from EP reactors before and after treatment (8 min) and taken onto a slide. The samples were then dried with a critical-point dryer and coated with platinum (using an ion-sputter) to a thickness of 10 nm. Finally, the cell wall breakup was visualized in FESEM image.

2.7. Lipid extraction and quantification

To extract microbial lipid, the treated samples were obtained at the end of the electroporation process and followed by mixed with 10 mL solvent (chloroform: methanol = 2:1). Then the liquid part of treated biomass was separated and collected by centrifugation (at 4000 rpm, 10 min). Thereafter, the biomass was again mixed with 5 mL solvent and shaken for mixing properly followed by centrifugation for 10 minutes (at 4000 rpm). After centrifugation, the lower liquid phase was withdrawn by Pasteur pipette and taken into a watch glass. The liquid was evaporated using an oven drier at 40 °C until getting a constant weight and calculated the extracted lipid. A different batch of EP was conducted for 8 min to compare the lipid extraction performance of EP with other techniques. To this purpose the 50 mg of dry biomass (L. starkeyi) was mixed with 10 mL of sterile water and EP treatment was followed as stated before in EP treatment section. The lipid extraction and recovery method was followed as described above.

III. RESULTS AND DISCUSSION

3.1. Characterization of POME

POME is a brownish colour colloidal suspension,which contains 95–96% water, 4–5% of total solids, 0.6–0.7% of oil and grease and 4–5% of total solids and acidic in nature with a pH value of 4.0–5.0[33, 34]. High biological and chemical oxygen demands of POME are the main obstacle to its agricultural uses [35-37]. On the other hand, POME contains diverse kind of sugar like arabinose, xylose, glucose, galactose and mannose; which can serve as potential carbon sources to the microbial community. POME is not only rich in organic carbon content but also have high nutrients or essential minerals such as K, N, Mg, Ca, P, Fe, B, Zn, Mn and Cu which support plant growth [37] as well as microbial biomass growth [38].

The nitrogen, COD, BOD, TOC, TPC, AN, NN, TDS, TS and TSS were investigated in order to assess the bioremediation of POME by oleaginous yeast. The data obtained from waste water analysis of untreated and treated has been shown in Table 1. There was anoutstandingreduction in COD (73.15%) after120 h retention time as presented in Table 1, indicating that L. starkeyi utilized and hydrolysed most of the organic compounds.The decrease of BOD by 24.39% suggests that L. starkeyican reduce the organic load from the POME. The variations in ammonia content might br due to the conversion of organic nitrogen into inorganic nitrogen. Macro-molecular organic matter was significantly degraded to small-molecule organic matter in this aerobic digestion process, hence AN and NN reduced by 68.57% and 47.19%respectively.

	50% POME		
Parameter, mg/L	Before treatment (After autoclaved)	Aftertreatment	Removal efficiency, %
Chemical oxygen demand (COD)	30,683±40	8,238±8	73.15
Biochemical oxygen demand (BOD)	13,165±25	9,953±13	24.39
Total organic content (TOC)	5,684±31	2,149±16	62.19
Total phenolic content (TPC)	2,650±19	1,287±13	51.43
Ammoniacal nitrogen (AN)	35+3	11+2	68.57
Nitrite nitrogen (NN)	89+7	47+5	47.19
Total solid (TS)	9,453±29	5,678±14	39.93
Total dissolve solid (TDS)	14,603±33	7,157±17	50.98
Total suspended solid (TSS)	3,542±16	1493±7	57.84
Oil and Grease	8,419+28	4,274+21	49.23

Table 1:Wastewater analysis of 50% POME (the removal efficiency was calculated only for average value)

3.2. Bioremediation of inhibitors

The huge amounts of organic matter in the form TSS, volatile suspended solids (VSS), TS, oil and grease of POME increase BOD and COD of POME if discharged directly without treatment [39].Three major components such as COD, TOC and TPC were analyzed to evaluate the ability of oleaginous yeast to reduce and digest organic load from POME.

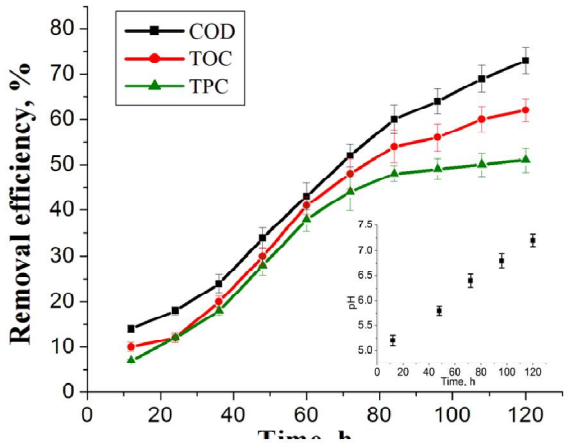


Figure 1: Removal efficiency of inhibitors presence in POME

As shown in Fig. 1, the reduction of COD was attained up to ~73% with the treatment of L. starkeyi. Louhasakul et al. [18]found that the COD removals from POME by oleaginous yeast remained at 66–73%. The COD elimination by Yarrowia lipolyticastood at maximum 72.9 ± 2.5% in that study. In biological treatment of POME, the mixed

cultures of Bacillus cereus and Bacillus subtilis were the utmost effective bacterial combination since its ability to lessen the COD and BOD of 90.64% and 93.11% respectively reported byBala et al. [39].

The TOC must be reduced forfurther use of any industrial wastewater. In this study, the TOC removal was achieved up to ~62% (Fig. 1).Since the yeasts consume sugars and other carbon compounds (proteins and phenols) as nutrient, microbial biomass growth is directly related to TOC reduction [38]. At the same time, the reduction trend of TPC was almost similar to that of TOC, which indicated that L. starkeyi is a suitable species to consume or reduce the phytotoxic compounds (mainly phenols). The removal of phenolic compounds was further confirmed by the increment of pH with time (Fig. 1 inset). The value of pH was increased since theL. starkeyiconsumed some compounds of the effluent that led to the formation of hydroxide ions [18]. Nwuche et al. [40] have been reported similar trends of results, where they observed the increment of pH from 3.98 to 7.79, while POME was treated with mixed culture of bacteria. This study, therefore, provides an alternative way of valorization of POME as a cheap growth media to produce microbial lipids suitable for biodiesel production and simultaneously a strategy to manage wastewater pollution loads.

3.3. Cell inactivation

Cells are the main functional unit of all living organisms which show fundamental properties of life. Like other cells, yeast cells are also enclosed by the cell membrane and segmented into various partitions. EP can interact with the cell membrane and other cellular components, and able to show strong effect on cell viability and cytoplasmic membrane permeabilization. Culture-based technique was used to test the viability of cells under different EP treatment time. Inactivation was calculated by colony counting.

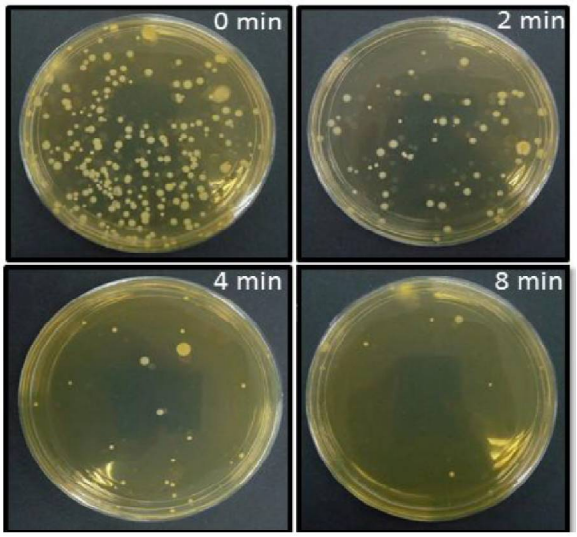


Figure 2: Decreasing of cell viability with increasing of EP treatment time

The effect of EP on cell viability is presented in Fig. 2 which illustrates that the cell inactivation (Table 2) was gradually increased until 8 min. At 2 min of EP treatment the cells were inactivated sharply to almost 72%, then cell inactivation was gradually increased up to 95% at 8 min. The higher cell inactivation at 8 min treatment time demonstrates that the high pulse electric field on the cell would have damaged the cell wall of microbes thus enhanced more dead cells in the reactor, since the high-voltage electrical pulses of short duration (0.01 s) were applied to induce irreversible permeabilization of the cell membrane.

Treatment time, min	Colony forming units (CFU), Number	Cell inactivation rate, %
0	210±8	-
2	59±5	71.90
4	23±3	88.09
8	9±2	95.23

Table 2:The cell inactivation rate with different treatment time (the cell inactivation rate was calculated only for average value)

Related study also stated that the EP treatment has strong effect on cell viability and cytoplasmic membrane permeabilization[41].More than 90% of Pseudomonas fluorescens cells were inactivated by applying electrical fields of 15 kV/cm for 30 min [42]. Similar effects wereobserved while the P. fluorescens were exposed to fields of5 and 10 kV/cm for 2 h reported by Yao et al. [42].

3.4. Cell disruption visualization

To detect cell wall break- up, L. starkeyi cells were visualized by FESEM as presented in Fig. 3. The undisrupted cell in absence of EP treatment is displayed in Fig. 3a, while, ruptured cells (red circles) are clearly observed after 8 min of EP treatment as shown in the Fig. 3b.The dramatic increase in mass permeabilityas a result of electrical fieldmay cause mechanical rupture of the plant, animal or microbial cells [30, 31] which was clearly observed by FESEM image.

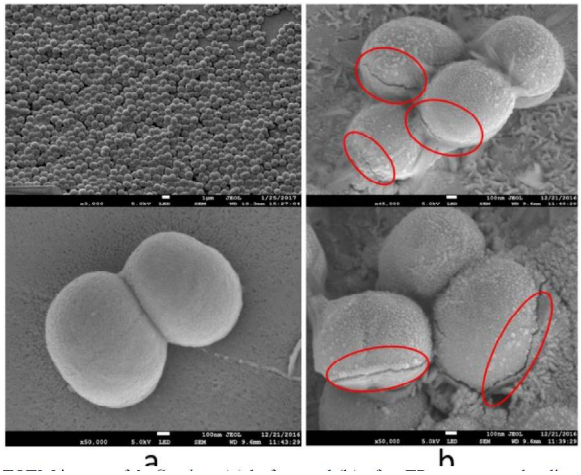


Figure 3: FESEM image of L. Starkeyi (a) before and (b) after EP treatment, the disrupted cells are indicated by red circles

When external electric field with a high intensity is applied to cells, a critical electric potential tempted across the cell membrane, which leads to the rapid electrical breakdown and local structural changes of the cell membrane and the cell wall [43]. However, the higherelectric pulses could directly attack the basic building blocks of microbial cell walls (the phospholipids and the peptidoglycan) thus inactivate the microbial cells and enhances the release of intracellular biomolecules [44]. Similar observation found by Sheng et al. [5], while pulsed electric field (PEF) was applied to Synechocystis PCC 6803 in order to disrupt the cell before microbial lipid extraction. Therefore, EP can be a vibrant technique to extract intracellular biomolecules since the cell breakup is the most challenging task in microbial lipid extraction.

3.5. Lipid Extraction and Quantification

Generally, yeast cell contains many valuable productssuch as proteins, cytoplasmic enzymes, polysaccharides etc. which are stored inside the cell and the extraction of these products involves a cell disintegration step. Yeast cells have strong cell walls, making cell disintegration and extraction rather difficult and energy intensive[45]. Therefore, the efficient cell disruption technique is required in order to retrieve these intracellular compounds especially for lipid. To study the lipid extraction efficiency of EP treatment, high voltage electrical pulses were applied to the L. starkeyifor time of 2, 4 and 8 min.

The extracted lipid obtained by applying EP to the L. starkeyiwere 17, 36 and 57 mg/g for of 2, 4 and 8 min timeof treatmentrespectively. The lower lipid extraction was achieved at 2 min treatment time might be due to less cell disruption efficiency by EP. The exposure of biological cells to high intensity electric field pulses can alter the structure of the cell membrane. The external field provokes a charging of the membrane[46]. At a sufficiently high transmembrane voltage the arrangement of the phospholipid molecules changes[47]. As a result, the membrane loses its barrier function and becomes permeable. Thus, this membrane rupture promotes the release of intracellular matter and facilitates solvent access into the cell[48].

No.	Cell disruptionmethods used	Organisms used	Lipid content (%)	Time (min)	Reference
1	Microwaves	*Chlorella vulgaris*	11.00	5	[49]
2	Ultrasound	*Rhodosporidium toruloides*	11.66	30	[50]
3	Osmotic shock	*Scenedesmus sp*	8.00	288	[49]
4	Beads beating	*Yarrowia lipolytica*	28.87	144	[18]
5	Bligh and Dyer method	*Candida silvae*	15.81	60	[15]
6	Electroporation	*Lipomyce starkeyi*	28.84	8	This Study

Table 3:Performance of lipid extraction using different cell disruption methods

The lipid extraction performance of several methods has been compared with the present study in Table 3 which shows the better performance (28.84%) of EP technique over others. It clearly shows that the maximum lipid (28.84%, wt%) was extracted for EP treatment at 8 min treatment time whereas minimum amount (8.00%) was obtained for osmotic shock.Although,Louhasakul et al. [18]achieved quite higher lipid content (28.87%) compare to our study, the process was time intensive and required 1440 min to achieve maximum lipid content which is 180 times higher than the present study. In another study Lee et al. [49], only 5 min of treatment time was required to extract maximum lipid (11%) using microwave oven from microalgae (Chlorella vulgaris). Though the lipid was extracted within shorter time, however, the lipid yield was about 3 times lower than EP. Moreover, the method is less feasible due to high temperature (100 °C) and more energy (2450 MHz) consumption. Comparably EP is a gentle cell disintegration process, since it is usually performed at ambient temperatures and does not introduce additional impurities into the process. Hence, it helps to prevent undesirable changes in the target material [45].

CONCLUSION

In this article, it is demonstrated that bioremediation of POME and lipid accumulation by L. starkeyi can be attained simultaneously. Moreover, it has been verifiedthat the irreversible electroporation can be an exciting technique to disrupt microbial cell wall, which is a rate limiting factor for the lipid extraction step in biodiesel synthesis. Therefore, wastewater management combined with microbial lipid extractionby EP for biodiesel production can be one of the interesting approach for a more sustainable future.Pursuing both environmental resilience and an eco-friendly method for biofuel production, further engineering design, research and optimization studies are required to enhance biomass production rates to satisfactory levels for economic and sustainable lipid production through the remediation of POME.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Higher Education, Malaysia through Universiti Malaysia Pahang (RDU 160150). The authors stated that there is no conflict of interest involved.

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