CHEMICAL ENGINEERING TRANSACTIONS
VOL. 37, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Eliseo Ranzi, Katharina Kohse- Höinghaus

ISBN 978-88-95608-28-0; **ISSN** 2283-9216
DOI: 10.3303/CET1437123

Enhancement of Lipid Content in Isochrysis Galbana and Pavlova Lutheri Using Palm Oil Mill Effluent as an Alternative Medium

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In this study, the influence of different concentrations of filtered and centrifuged palm oil mill effluent (POME) in sea water (1, 5, 10, 15 and 20 %) were investigated as an alternative medium for microalgal cell growth and lipid yield. Both *I. galbana* and *P. lutheri* had enhanced cell growth and lipid accumulation at 15 % level with maximum specific growth rate (0.16 d^{-1} and 0.14 d^{-1}) and lipid content (26.3 ± 0.31 % and 34.5 ± 0.82 %), respectively, after 16 days of cultivation. The microalgal cell growth was not only enhanced with POME feeding, the COD (74.5-80.1 %), BOD (68.3-82.5 %), TOC (43.1-62.3 %), TN (48.7-59.9 %) and oil and grease (59.1-68.8 %), were also efficiently removed from POME.

1. Introduction

Microalgae is seen as a source of transportation fuels mainly because of its potential to produce up to 10 times more oil per acre than conventional biofuel crops (Chisti, 2007). However, a microalgae-based fuel production technology still has a long way to go. The main limitations are the selection of strains which can grow and produce high quantities of lipids, and the high energy inputs necessary for pumping, mixing or harvesting the produced biomass and extraction of lipids (Norsker et al. 2011).
POME is considered as one of the most polluting agro-industrial effluent due to its high values of COD and BOD concentrations ranging from 50,000 to 90,000 mg L^{-1} (Damayanti et al. 2010). POME release into the waterways and ecosystems remain a major concern by the authorities overseeing the public health and food sectors (Foo and Hameed 2010). If discharged without effective treatment, considerable environmental problems such as land and water pollution and destruction of aquatic biota could occur (Singh et al. 2011). The use of microalgae is increasingly seen as a sustainable and inexpensive alternative method to treat wastewater and for CO_2 and NO_x removal with high capacity of nutrient uptake (Jin et al. 2008) and to breakdown the organic complexes (Chen et al. 2011). Microalgae can utilize the nitrogen and phosphorus compounds present in wastewater to generate biomass for lipid synthesis and conversion to biofuel (Gao et al., 2010).
Isochrysis galbana is a haptophyceae which is of substantial interest in aquaculture, principally to feed mollusk larvae, as well as fish and crustaceans in the early stages of growth (Wikfors and Patterson 1994). *Pavlova lutheri*, a phytoflagellate, is a good example of high lipid producing marine microalgae. It is a member of the Pavlovophyceae (Haptophyta), often used as food source for aquatic filter-feeders and cultured to produce high levels of polyunsaturated fatty acids (PUFAs). The size of 4-6 µm, cell density of 0.4-1.5 g L^{-1} and 15-36 % lipid content has been reported, with doubling time of 3-6 days (and Guihéneuf et al. 2011 and Shah et al. 2014).
The aim of this work was to explore the use of filtered and centrifuged POME at different compositions with sea water for the culturing of brown marine microalgae *I. galbana* and *P. lutheri***.** The biomass production and lipid content were evaluated together with the removal of the nutrients, BOD, COD and oil and grease from POME.

Please cite this article as: Shah S., Ahmad A., Othman M., Abdullah M., 2014, Enhancement of lipid content in isochrysis galbana and pavlova lutheri using palm oil mill effluent as an alternative medium, Chemical Engineering Transactions, 37, 733-738
DOI: 10.3303/CET1437123

2. Material and Methods

2.1 Culture of microalgae and harvesting

I. galbana and *P. lutheri* were obtained from Fisheries Research Institute, Pulau Sayak, Sungai Petani, Kedah, Malaysia. Microalgae from the stock culture (with density of 50.6 x 10^6 cells mL^{-1}) was inoculated into 250 mL Erlenmeyer flask and 10% (v/v) inoculum density was used in the subsequent experiments. Conway media was used for control culture. All chemicals and solvents were obtained from Merck (Darmstadt, Germany). The standard conditions for culture were 30 ppt NaCl and initial pH 8, under 24 h illumination from fluorescence white light (Phillips) of 90 µmol photons $m^{-2}s^{-1}$ intensity. The culture flasks were grown on an orbital shaker at 80 rpm, at 28 ± 2^oC for 16 days. All the glass-wares used in the experiment were sterilized by autoclaving at 121^oC, 20 min and all media constituents were added aseptically after sterilization. Three replications were used both for the culture and control media.
Algal growth and lipid content were recorded every alternate day. For harvesting, the algae were centrifuged at 3,000 rpm for 10 minutes to separate the media from microalgae. The supernatant was analyzed.

2.2 POME medium preparation

Raw POME was collected from FELCRA Nasaruddin Oil Palm Mill in Bota, Perak, Malaysia and stored in plastic container at 4 ^oC in order to avoid contamination and biodegradation.
POME was filtered to remove sand and dust particles and then centrifuged (Avanti J-251 Centrifuge). The supernatant of the effluent which contained nutrient was taken for algal culture and the pellet kept for future use. The supernatant was diluted with sea water to make 1, 5, 10 and 15 % concentration and autoclaved at 121^oC for 30 min to eliminate bacterial and other contaminations. pH level of POME medium was adjusted to pH 7-8 and filtered again before use.

2.3 Chemical analyses of POME

Biological oxygen demand (BOD_5) was analyzed using Standard Methods by HACH (HACH, USA). COD measurement was carried out by using spectrophotometer DR 5000, according to 8000-Reactor Digestion Methods (HACH). Total Organic Carbon (TOC) and Total Nitrogen (TN) were analyzed by using TOC Analyzer (TOC-$V_{CSH\ SHIMADZU,}$ Japan). TN is the sum of organic nitrogen, ammonia (NH_3), and ammonium (NH_4^+) in the chemical analysis of wastewater. Oil and grease was analyzed by using InfraCal TOG Model HATR-T2 analyzer. pH of POME was measured by using Mettler Toledo-320 pH probe.

2.4 Measurement of cell growth and dry weight (DW)

Cell growth was measured by haemocytometer (Hirschmann/Germany) by removing 10 µL of sample. Cells were harvested in triplicates after 16 days by removing 100 mL samples and later centrifuged at 3000 rpm (Avanti J-251 Centrifuge). Algal suspension was filtered through a pre-dried and pre-weighed glass-fiber filter (Whatman GF/C). The biomass were washed with deionized water, dried at 80°C in an oven until constant weight, cooled in a desiccator and weighed.

2.5 Lipid extraction

Lipid content was determined based on modified method adapted from Bligh and Dyer (1959). This method extracted lipids from the algal cells by using a mixture of methanol, chloroform, and water. Algal sample was centrifuged at 3500 rpm for 10 min and the pellet was mixed with water, methanol, and chloroform. After overnight stay, the mixture was re-centrifuged and the lower layer that contained lipid and chloroform was extracted and dispensed into pre-weighed vials. All vials were placed in a water bath at 65 °C for 8 h or kept in an oven at 80 °C for 4 h to evaporate the chloroform and lipids, before weighing.

2.6 Statistical analysis

The data were analyzed by one-way Analysis of Variance (ANOVA) using Statgraphic Version 5 (Rockville, USA) to determine significant difference among the treatment means.

3. Results and Discussion

3.1 Cell growth and lipid content

Figure 1 shows the growth curve of *I. galbana* and *P. lutheri* cultivated under control, 1, 5, 10, 15 and 20% POME media. The highest cell density of 15.4 x 10^6 cells mL^{-1} and 14.2 x 10^6 cells mL^{-1} was obtained at 15% POME level for *I. galbana* and *P. Lutheri*, respectively. As shown in Table 1, this corresponds, respectively, to the maximum biomass formation rate of 0.14 g L^{-1} d^{-1} and 0.13 g L^{-1} d^{-1}, the

doubling time of 4.3 and 4.95 days, μ_{max} of 0.16 d^{-1} and 0.14 d^{-1}, and lipid content of 26.3 ± 0.31 % and 34.5 ± 0.82 % for *I. galbana* and *P. lutheri*. These were almost similar to control. There is a statistically significant difference between the mean dry weight and lipid content from one level of POME level to another, at 95% confidence for both species ($P < 0.05$).

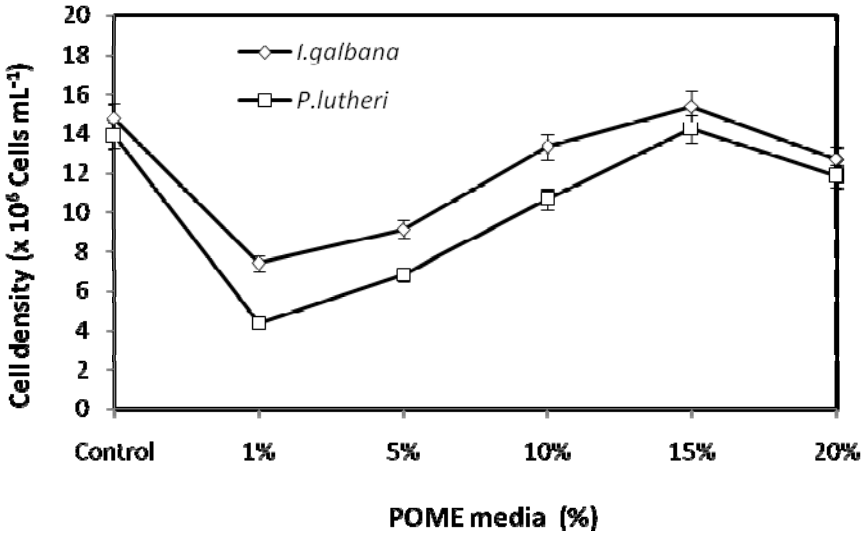

Figure 1. Growth curve of I. galbana and P. lutheri cultivated under control and POME media

Table 1. Kinetics of cell growth and lipid production of I. galbana and P. lutheri cultivated under control and POME media

Media conditions		Maximum biomass formation rate, X'_{max} ($gL^{-1}d^{-1}$)	Maximum specific growth rate, μ_{max} (d^{-1})	Doubling time, t_d (day)	Lipid Content (%)
I. galbana	Control	0.152	0.15	4.66	22.3 ± 1.94
	1 %	0.104	0.11	6.07	15.4 ± 1.20
	5 %	0.108	0.12	5.77	17.3 ± 0.95
	10 %	0.128	0.15	4.71	19.7 ± 0.97
	15 %	0.142	0.16	4.26	26.3 ± 0.31
	20 %	0.094	0.13	5.21	19.8 ± 1.10
P. lutheri	Control	0.137	0.15	4.74	29.7 ± 1.45
	1 %	0.097	0.12	5.77	19.5 ± 3.05
	5 %	0.110	0.12	5.63	22.8 ± 1.83
	10 %	0.122	0.14	4.95	27.5 ± 0.84
	15 %	0.130	0.14	4.95	34.5 ± 0.82
	20 %	0.117	0.13	5.37	26.3 ± 0.96

The POME composition at 5-15 % showed better growth performance and productivity for *I. galbana* and *P. lutheri*. The higher biomass and lipid production for the 15% level was likely due to higher and balanced nutrient concentrations. This is in agreement with a study on mixture of green algae and diatoms which has the biomass concentrations increased from 0.5 g L^{-1} to 0.9 g L^{-1} and lipid content from 14 % to 29 % when the composition of waste water is increased from 10 to 25 % (Woertz et al 2009). The maximum specific growth rates (0.14−0.16 d^{-1}) in our study with *I. galbana* and *P. lutheri* were lower than those reported values of 0.27 g $L^{-1}d^{-1}$ and μ_{max} of 0.49 d^{-1} with *Auxenochlorella protothecoides* UMN 280. However, the lipid accumulation at 26−34 % were relatively higher than the reported 28.9 % total lipid content for *A. protothecoides* UMN 280 cultured in concentrated municipal wastewater (Zhou et al. 2012). The higher biomass accumulation has also been reported for *Chlorella* sp. grown on concentrated municipal wastewater (Li et al 2011).

Due to the nature of POME and its dark colour, the suitable percentage to be used in the formulation for optimum growth and lipid content of the marine *I. galbana* and *P. lutheri* was only 15 %. A study on marine *Isochrysis* sp. utilizing 5 % POME-fortified medium achieves maximum biomass of 91.7 mg m^{-2} day^{-1} and lipid content of 52.8 ± 2.4 % under 10 L outdoor culture system (Vairappan and Yen 2008). Another report suggests that algae can grow optimally at 14% POME and lowest at 30% (Anton et al. 1994).

3.2 Chemical analyses of POME

3.2.1 COD, BOD and TOC

As shown in Table 2, raw POME had high concentrations of chemical components. These inorganic components are suitable to be used as nutrients for the culturing of microalgae. There was a decreasing trend of pH (7 to 3.8) with increasing POME ratio from 1−20 % before inoculation of microalgal species. On day 16, pH became more basic around 7.9-8.4. These were most probably due to the uptake of nitrogen and lack of CO_2 sparging. The highest growth rate of *Scenedesmus obliquus* is reportedly achieved at a constant pH of 7 (Hodaifa et al. 2009). In our study, the BOD removal of 68.3-82.5 % was achieved for 1–20 % POME after microalgal addition. Higher removal of COD (74.5-77.4 %) and TOC (43.1-57.9 %) were achieved for 1–20 % POME dilution with addition of *I. galbana*, while removal of COD (77.6-80.1 %) and TOC (45.2-62.3 %) were achieved with *P. lutheri*. The COD removal was enhanced when the POME concentration was increased to 5 % and 20 %, which was in agreement with the 76% COD removal from piggery wastewater associated with microbe in high rate algal ponds (Godos et al., 2009). A study with *A. protothecoides* UMN280 achieves the removal efficiency of 88% COD and 96% TOC when the algae is grown in concentrated municipal wastewater (Zhou et al. 2012).This may suggest that different algal strains could utilize the different organic compounds as carbon sources with different efficiency. Algae could utilize the amount of dissolved oxygen to break down organic material. The algae-based sewage treatment plant (STP) has reportedly achieved total BOD removal of 82% (Mahapatra et al., 2013), while the use of *Synechocystis* sp achieves 98% BOD removal efficiency from treated wastewater under hydraulic residence time of 24 h (Sekaran et al., 2013).

Table 2. Chemical characteristics of POME (mg L^{-1}) and removal efficiency (%) in different dilutions with sea water before and after inoculation of I. galbana and P. lutheri

POME: Sea Water (v/v)	pH	COD	BOD	TOC	TN	Oil and grease
1. Raw						
1 %	6.5-7	627	183	33.4	5.4	30.3
5 %	6.2	2974	843	153.1	28.6	144.5
10 %	5.5	5839	1642	285.5	56.7	285.1
15 %	4.7	8947	2448	456.8	80.3	364.5
20 %	3.8	11129	2985	618.4	98.5	438.2
2. *I. galbana*						
1 %	7.3	148 (76.4%)	58 (68.3%)	21.3 (43.1%)	2.8 (48.7%)	12.4
5 %	7.5	716 (75.9%)	251 (70.2%)	78.4 (48.8%)	13.3 (53.5%)	55.7
10 %	7.8	1394 (76%)	440 (73.2%)	130.2 (54.4%)	25.5 (55%)	112.2
15 %	8.4	2018 (77.4%)	605 (75.3%)	197.5 (56.8%)	34.2 (57.4%)	143.8
20 %	8.8	2833 (74.5%)	753 (74.8%)	260.3 (57.9%)	41.9 (57.5%)	171.2
3. *P. lutheri*						
1 %	6.8	128 (79.6%)	47 (74.3%)	18.3 (45.2%)	2.5 (53.7%)	10.2
5 %	7.2	666 (77.6%)	213 (74.7%)	73.1 (52.2%)	12.4 (56.6%)	51.8
10 %	7.5	1274 (78.2%)	337 (79.5%)	121.2 (57.5%)	22.7 (59.9%)	94.3
15 %	7.9	1784 (80.1%)	484 (80.2%)	172.3 (62.3%)	31.1 (61.3%)	113.7
20 %	8.4	2451 (78%)	523 (82.5%)	236.3 (61.8%)	39.8 (59.6%)	138.5

3.2.2 TN

The highest average TN of 98.5 mg L^{-1} and lowest average TN of 5.4 mg L^{-1} was recorded in 20% and 1 % POME, respectively, before microalgal inoculation (Table 2). The highest removal of 57.5% was achieved in 20% POME with *I. galbana*, while the highest removal with *P. lutheri* was 61.3%. Nitrogen is an essential ingredient for cell growth. Total Kjeldahl nitrogen (TKN) removal of 36%, ammonium N (NH_4 -N) removal of 18 %, nitrate (NO_3 -N) removal of 22 %, and nitrite (NO_2 -N) removal of 57.8 % have been reported for algae-based STP where the predominant algae are euglenoides and chlorophycean (Mahapatra et al. 2013). The TN removal of 50.8 %– 82.8 % have also been reported for

green algae *Chlorella* sp. when grown in different wastewaters from municipal wastewater treatment plant (Wang et al. 2010).

3.2.3 Oil and grease

POME contains 95–96 % water, 0.6–0.7 % oil and grease and 4–5 % total solids (Industrial Processes & The Environment 1999). Typically, the oil and grease mean value is 6000 mg L^{-1}. The highest average oil and grease of 438.2 mg L^{-1} and the lowest of 30.3 mg L^{-1} was recorded in 20% and 1% POME, respectively, with microalgae (Table 2). The highest removal of 60.9 % was achieved in 20% POME with *I. galbana*, while the highest removal of 68.8 % was in 15% POME with *P. lutheri*. The high removal of oil and grease content by algae can be considered for handling and treatment of the waste material for disposal. Free Gram-negative bacteria (*Pseudomonas* sp., *P. diminuta* and *P. pseudoalcaligenes*) are able to degrade the palm oil completely, utilizing the free fatty acids (FFA) as a carbon source (El-Bestawy et al. 2005), making these good candidates for co-cultivation with microalgae for oil and grease removal from contaminated industrial effluents.

4. Conclusion

The influences of different concentrations of POME in sea water (1, 5, 10, 15 and 20 %) on microalgal cell growth and lipid yield were successfully established. The better cell growth and lipid content for *I. galbana* and *P. lutheri* were observed at 15 % level with maximum specific growth rate of 0.16 d^{-1} and 0.14 d^{-1}; and lipid content of 26.3 ± 0.31 % and 34.5 ± 0.82, respectively. The maximum removal efficiencies of COD (77.4 % and 80.1 %), BOD (75.3 % and 82.5 %), TOC (57.9 % and 62.3 %), TN (57. 5 % and 61.3 %) and oil and grease (60.9 % and 68.8 %) for *I. galbana* and *P. lutheri*, respectively, were achieved for different dilutions of POME. This study suggested that cultivation of microalgae in POME may be a practical and economical alternative to efficiently enhance the nutrients removal from POME, coupled with biomass and lipid enhancement.

Acknowledgments

The authors are grateful to the Universiti Teknologi PETRONAS for providing facilities and for the scholarship to Syed Muhammad Usman Shah. Funding through the Fundamental Research Grant Scheme (FRGS/2/2010/TK/UTP/02/14) is acknowledged.

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