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Inoculum Sizes of Locally Isolated Phototrophic Bacterium on the Utilization of Palm Oil Mill Effluent

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Authors' contributions

This work was carried out in collaboration between both authors. Author ASA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author SRMS managed the literature searches and make comments on manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: To produce *Rhodobacter sphaeroides* strain UMSPSB3 biomass with the reduction of chemical oxygen demand (COD) from palm oil mill effluent.
Study Design: Locally isolated phototrophic bacterium with different inoculum levels were used in Palm Oil Mill effluent (POME). Collected POME was characterized before used as substrate. Inoculum of bacterium was grown in synthetic media and 48 hours inoculum was used to utilize the substrate.
Place and Duration of Study: Biotechnological laboratory, Borneo Marine Research Institute, University Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia, between February 2014 to April 2014.
Methodology: Growth characteristics of bacterium *Rhodobacter sphaeroides* strain UMSPSB3 was monitored at different light intensities. Later phototrophic bacterium *Rhodobacter sphaeroides* strain UMSPSB3 was grown in settled non-sterilized Palm Oil Mill effluent (POME). The growth characteristics of bacterium in term of dry cell weight and total carotenoids production, and reduction of COD were compared using 10%, 20% and 30% (v/v) levels of inoculum developed in synthetic 112 media.

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Results: The optimum light intensity for the growth of *Rhodobacter sphaeroides* strain UMSPSB3 was 2.5 klux. The highest bacterial biomass (X_{max}) of 6.5 g/L (dry weight) and 72% reduction of COD were obtained after 96-h culture with 20% (v/v) inoculum level. The reduction of COD (%) and cell yield ($Y_{x/y}$, g cell/g COD) in POME were 82% and 0.98 respectively, after 96-h culture with 30% (v/v) inoculum. Production of carotenoids was comparatively low in bacterium using POME as substrate. Inoculum levels of 20-30% (v/v) developed in synthetic 112 media supported the growth of phototrophic bacterium in settled POME, but higher level of inoculum was required for faster removal COD from effluent. A 10% (v/v) level of inoculum in POME did not support the isolate to grow.

Conclusion: Production of bacterial biomass with bioremediation of effluent could be achieved using POME as substrate with locally isolated *Rhodobacter sphaeroides* strain.

Keywords: Phototroph; inoculum size; palm oil mill effluent; growth; bioremediation.

1. INTRODUCTION

The palm oil industry generates the largest amount of waste in the oil industry. In the process of oil production it generates many by-product and wastes, which may have significant effect on the environment [1]. The palm oil mill effluent is the by-product of industry that also has profitable and viable biotechnological applications [2]. The effluent consists of a significant amount of solid wastes and wastewater, commonly termed as Palm Oil Mill Effluent (POME). POME is a thick brownish liquid that contains high amount of total solids (40,500 mg/L), oil and grease (4000 mg/L), chemical oxygen demand (50,000 mg/L), and biochemical oxygen demand (25,000 mg/L) [3], requires to be treated, because of potential environmental damage. An average of about 53 million tons of POME is being produced annually in Malaysia based on palm oil production in 2005. POME is required to be treated at acceptable level regulated in the Environmental Quality Act 1974 [4]. Indeed it has been identified as one of the most toxic and major sources of aquatic pollution in Malaysia and also the most harmful waste if discharged untreated [5]. The balance between high economic profitable and sustainable development of palm oil industry urge to find a way to preserve the environment while keeping the economy growing [6]. The traditional methods in treating palm oil mill effluent generally include tank digestion and facultative digestion, the decanter-drier system, aerobic and facultative pond and distillation ponds [7]. Facultative digestion that relies upon microorganisms that plays vital roles in the breakdown of organic particles to inorganic forms.

Purple non-sulfur bacteria (PNSB) are one of the potential microbes used by various researchers in the wastewater treatment process [8]. The unique character of the PNSB is their diverse metabolic activity and the ability to grow in different cultural conditions, both in synthetic and naturally available substrates [9]. PNSB has advantages as it can grow in wide range of substrate as well as in aerobic and anaerobic culture condition. Production of single cell protein (SCP) using industrial wastewater as substrate while simultaneous reducing of chemical oxygen demand (COD) [10] as a consequence of PNBS have been used in the wastewater treatment of sago effluent [11], SCP from anaerobic wastewater treatment [12], utilization and treatment of sardine processing wastewater and as feed additive for aquaculture [13] and utilization and treatment of tuna condensate by photosynthetic bacteria [14]. Besides reducing the chemical oxygen demand (COD) level in the wastes, purple non-sulphur bacteria also act as a bio-fertilizer and feed stock supplement. The commercial products of purple non-sulphur bacteria are rich in protein, amino acids, and vitamins [15]. In particular the success of utilization and treatment has been shown to be dependent on inoclulum sizes [13].

Further the inoculum sizes of PNSB play significant roles in the utilization of the wastewater [16,14]. An inoculum of *Rhodocyclus gelatinosus* R7 prepared in synthetic media (G5 medium) at 10% (v/v) was used for the utilization and treatment of diluted tuna condensate gave the highest biomass of 3.96 g/L [14]. On the other hand, an inoculum of *Rhodopseudomonas gelatinosa* grown in GMM at a 13% (v/v) level was used for the production of single cell protein in cassava starch media [17]. Inoculum size of 10% (v/v) of *Rhodovulum sulfidophilum* has been reported to utilize undiluted sardine processing wastewater (SPW) under aerobic dark condition and yielded SCP, but 30% (v/v) level of inoculum

is essential in the reduction of COD from undiluted sardine processing wastewater [18]. However, there is little information on the growth of purple non-sulfur bacteria with regards to the inoculum sizes for the utilization of palm oil mill effluent. The objectives of this study were: (i) to evaluate the efficacy of inoculum sizes from locally isolated purple non-sulfur bacterium on the growth characteristics using palm oil mill effluent (POME) as substrate, and (ii) to reduce organic load from POME.

2. METHODOLOGY

2.1 Bacteria and Substrate

Palm Oil Mill Effluent (POME) was collected from Beaufort Palm Oil Mill Sdn Bhd located at 5°18'29''N 115°42'16''E in Sabah. On spot 5mL of collected POME was poured into 30mL universal bottles, containing previously autoclaved 112 media. Media 112 was prepared by mixing 10.0 g of yeast extract, 0.5 g of magnesium sulfate, 1.0 g of di-potassium hydrogen phosphate and dissolved in 1 liter of distilled water. The mixture of the chemicals was dispensed into several 25 mL Mc. Cartney bottles and was autoclaved at 121°C for 15 minutes. Bottles were incubated under anaerobic light conditions at temperature of 30±2°C and 2500 lux illumination intensity under laboratory conditions. The development of the purple non-sulfur bacterial (PNSB) growth was monitored every day for change in color, normally pink to red or reddish in 112 media. The pure culture of PNSB was obtained after repeated streaking of single colony in agar plate with 112 media. Preliminary identification was done bio-chemically in pure isolate, finally the selected isolate was identified as *Rhodobacter sphaeroides* based on 16s rDNA sequence.

Collected POME was immediately characterized before utilizing as substrate. The settled and non-sterilized POME after adjusting pH at 7.0 was used as substrate.

2.2 Inoculum and Media

Pure PNSB *Rhodobacter sphaeroides* strain UMSPSB3 was used to prepare inoculum in sterilized 112 media. A loop full of *Rhodobacter sphaeroides* strain UMSPSB3 from a four-day-old pure culture plate was aseptically inoculated into universal bottle containing autoclaved 112 media. The bottles were incubated in anaerobic light condition at 2500 lux illumination and at a temperature of 30±2°C. A 48 h culture was used as inoculum.

2.3 Growth Characteristics of *Rhodobacter sphaeroides* strain UMSPSB3 in synthetic 112 media

A volume of 5 ml of 48-h inoculum was incubated in Mc. Corney bottles containing 20 ml of freshly prepared sterile 112 media. The bottles were incubated under anaerobic at 2.0 k-lux, 2.5 k-lux and 3.0 k-lux light intensity at 30±2°C for 120-h. After 0, 24, 48, 72, 96 and 120 h, three bottles from each of light intensity were randomly selected to monitor the growth of *Rhodobacter sphaeroides* strain UMSPSB3. The growth of bacterium was evaluated in the term of dry cell weight (g/L) and the total carotenoids (mg/g dry cell weight).

2.4 Studies on Growth and Reduction in COD

Settled and non-sterilized POME was dispensed into each of several 100 ml Schott bottles. The different concentration such as 10%, 20% and 30% (v/v) of 48 hours inoculum of *Rhodobacter sphaeroides* was used in 100 mL Schott's bottles. The bottle caps were hand tightened to maintain anaerobic condition. The bottle was incubated at 30±2°C temperature and 2500 lux illumination intensity. Destructive sampling was carried out every day during experimental period. Growth profiles of *Rhodobacter sphaeroides* strain UMSPSB3 in POME and reduction in COD were studied with 10%, 20% and 30% (v/v) levels of inoculum. The biomass produced in term of dry cell weight (g/L) and reduction in chemical oxygen demand (%) was monitored at 24 h intervals.

2.5 Data Analysis

The growth of the bacterium (X_{max}) was determined everyday by using dry cell weight (g/L) according to method of Sawada et al. [19]. Total carotenoids in bacterial cells were determined by acetone-methanol extraction method [20] using following formula:

$$C = \frac{\text{D. v. f. (10/2500)}}{\text{Dry weight of sample (g)}}$$

Where,

C = Total carotenoids (mg/g dry cell)
D = Absorbance at 480 nm.
V = Total volume sample used (mL)
F = Dilution factor of sample (only if absorbance is greater than 0.8)

Level of COD (mg/L) reduction was determined with the standard method [21].The parameters of oil and grease (mg/L), total solids (mg/L) total suspended solids (mg/L), volatile suspended solids (mg/L) and total nitrogen (mg/L) were determine according to the standard methods [21] (APHA 1998). The specific growth rate (μ_{max} per h) was determined according to Sasaki and Nagai [22] as follows:

$$\mu_{max} = \frac{\text{Log}_e W_2 - \text{Log}_e W_1}{T_2 - T_1} \times 100$$

(μ_{max}: specific growth rate, e: base of natural logarithms, W_2: cell dry biomass at time T_2 and W_1: initial cell dry biomass at time T_1)

One way ANOVA were used to test significant differences in the growth characteristics of *Rhodobacter sphaeroides* strain UMSPSB3 at different light intensities. Paired samples T-test in SPSS were used to determine significant level in dry cell biomass and reduction in COD by *Rhodobacter sphaeroides* with inoculums sizes.

3. RESULTS

The value of pH of both settled and non-settled remained constant, but COD was reduced to 63% upon settled POME in laboratory condition (Table 1). Oil and grease, total suspended solid and total volatile solid of POME shows differences after POME allowed to settle. The values of total solids, total suspended solids and total nitrogen in settled POME were reduced to 48%, 67% and 57% respectively. However, the nutrients in settled POME are enough to support the growth and survival of purple non-sulfur bacteria under anaerobic light culture system.

3.1 Growth Characteristics of *Rhodobacter sphaeroides* strain UMSPSB3 in Synthetic 112 media

The highest dry cell weight of 5.69 g/L was achieved by bacterium cultured under 2.5 klux light intensity illumination at 72 hours (Fig. 1). The growth of the bacterium at 2.0 klux, 2.5 klux and 3.0 k lux light intensities were observed to increase from 0 hour to 72 hours and then declined.

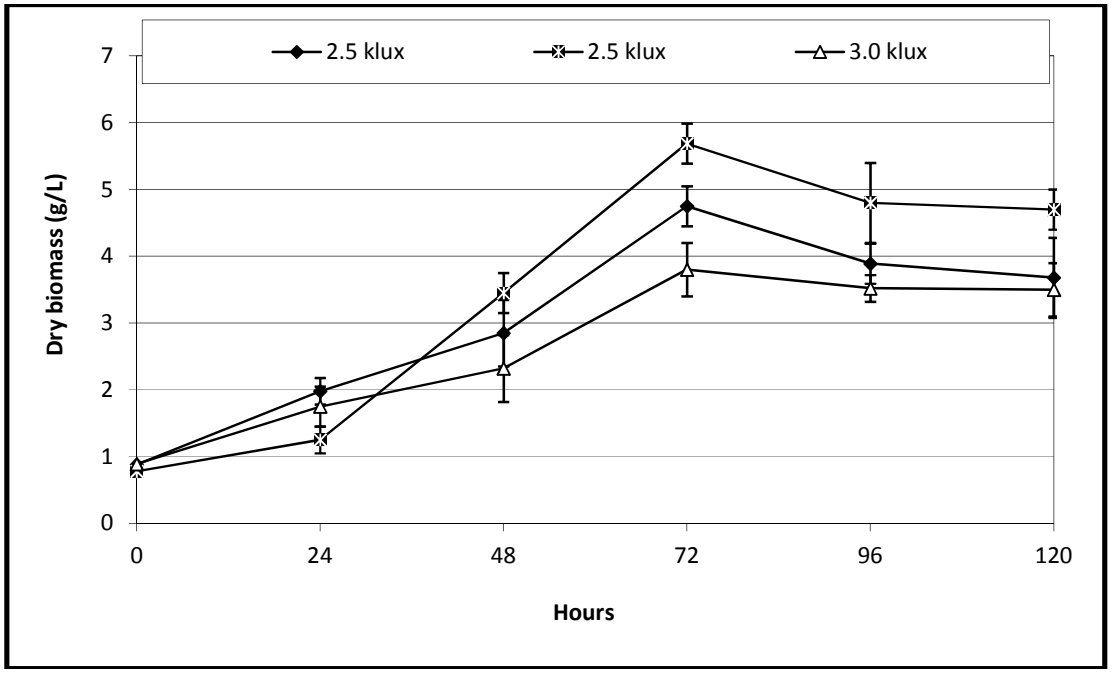

Fig. 1. The effects light intensities on the production of dry cell weight (g/L) by strain *Rhodobacter sphaeroides* strain UMSPSB3 using synthetic 112 media

Statistical analysis using one-way ANOVA shows significance differences (p=0.04) between the three different light intensities to the dry cell weight.

The highest total carotenoids of 1.43 mg/g dry cell weight was determined at 48-h culture with 2.5 klux light intensity (Fig. 2). Pattern in the proiduction of carotenoids were observed similar inrepective of light intensities.

However, no significant differences (p=0.64) was observed between the production of total carotenoids with three level of light intensities.

Bacterial cell did not grow well with 10% (v/v) inoculums. The highest bacterial biomass of 6.5 g/L was obtained with 20% (v/v) inoculum level after 96-h culture, which was not significantly different (p=0.075) in dry cell biomass (Table 2), obtained with 30% (v/v) inoculum level (Fig. 3). On the other hand, the specific growth rate (μ_{max} per hour) was the highest of 0.52 with 20% inoculum after 96-h culture, but at the same time the lowest μ_{max} of 0.47 obtained with 30% inoculum. No significant different values (p=0.68) were found in specific growth (Table 2) among the two level of inoculum sizes.

Table 1. Characteristic of settled and non-settled POME collected for experiment

Parameters	Unsettled	Settled
pH	4.55	4.55
Oil and Grease (mg/L)	0.61±0.019	0.24±0.021
Total solid(mg/L)	7.67±0.98	4.03±0.52
Total suspended solid(mg/L)	5.79±0.75	1.91±0.20
Total volatile solid (mg/L)	9.18±0.88	3.63±0.05
Chemical oxygen demand (mg/L)	24000±80	15000±36
Total nitrogen (mg/L)	69.61±1.52	40.21±0.98

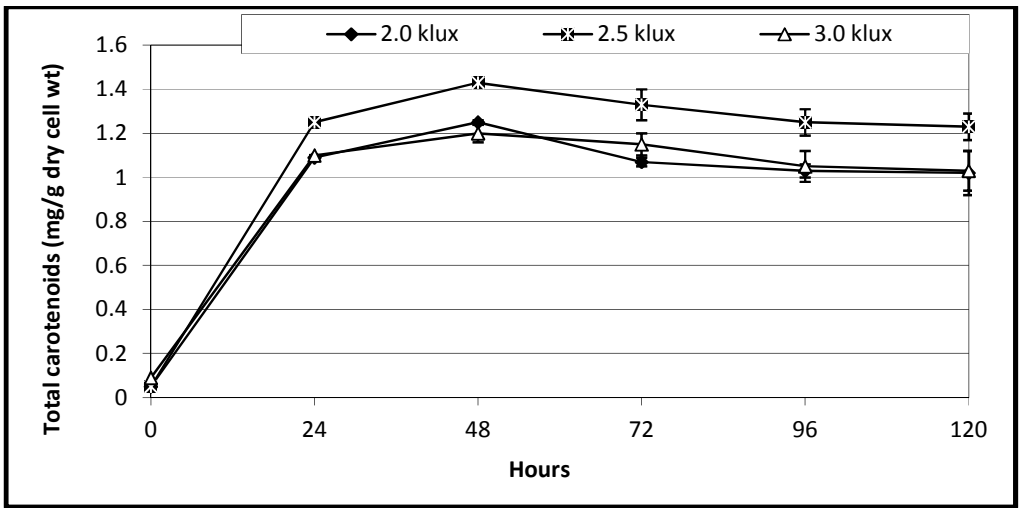

Fig. 2. The effects of and light intensities on the production of total carotenoids (mg/g dry cell weight) in strain *Rhodbacter sphaeroides* strain UMSPSB3 using synthetic 112 media as substrate

Table 2. Growth characteristics of bacterium *Rhodobacter sphaeroides* strain UMSPSB3 cultured in settled POME after 96-h culture under anaerobic light condition

Inoculum in % (v/v)	X_{max} (g/L)	μ_{max} (per h)	$Y_{x/y}$ (g/cell/g COD)	COD reduction (%)
10	0.1	not determined	not determined	not determined
20	[a]6.5±0.02	[a]0.52±0.003	[a]0.53 ± 0.006	[a]72± 2.6
30	[a]5.8±0.05	[a]0.47±0.003	[b]0.98±0.010	[b]82±3.0

X_{max} is production in maximum cell (g/L) and μ_{max} is specific growth rate (per h)
Different superscript shows significant differences

The highest carotenoids of 1.11 mg/g dry cell weight in *Rhodobacter sphaeroides* strain UMSPSB3 strain was obtained with 20% (v/v) level of inoculum at 48-h culture, which is significantly different (p= 0.045) than the total carotenoids production at the same time with 10% and 30% inoculum level (Fig. 4).

The production of total carotenoids in the POME observed comparatively low than the carotenoids obtained in *Rhodobacter sphaeroides* strain UMSPSB3 using synthetic 112 media as substrate.

Reduction of COD was the highest of 82% with 30% (v/v) level of inoculum was determined after 96-h culture and was significantly different (p=0.03) from the reduction of COD with 20% (v/v) inoculum level (Fig. 5, Table 2)

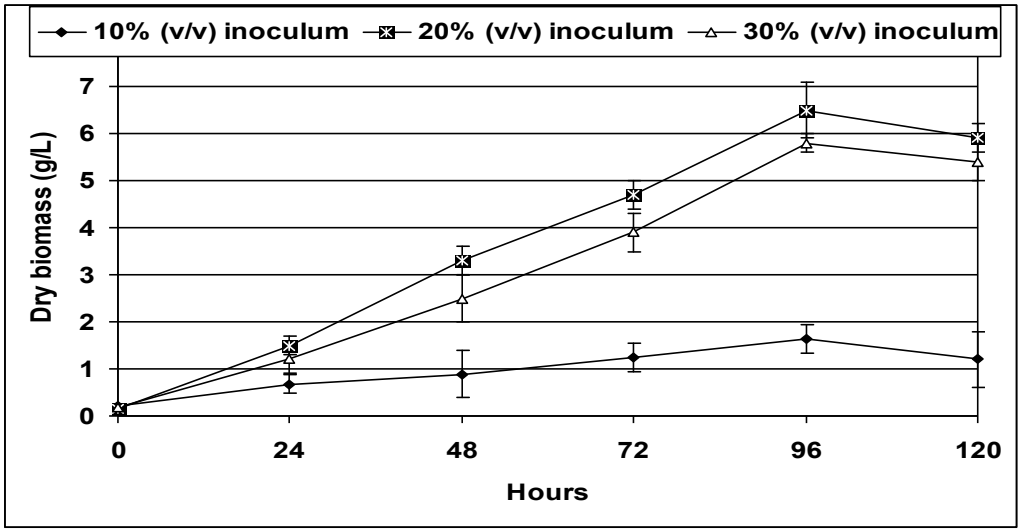

Fig. 3. Dry cell biomass (g/L) of isolate cultured in settled and non-sterilized POME with 10%, 20% and 30% (v/v) level of inoculum

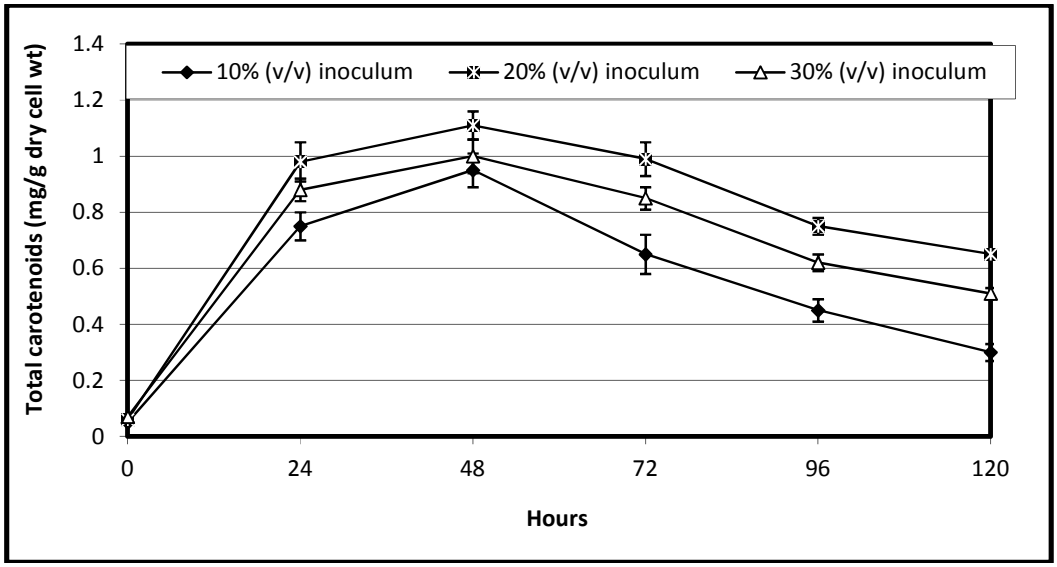

Fig. 4. Production of total carotenoids in *Rhodobacter sphaeroides* strain UMSPSB3 with 10%, 20% and 30% inoculum levels using POME as substrate

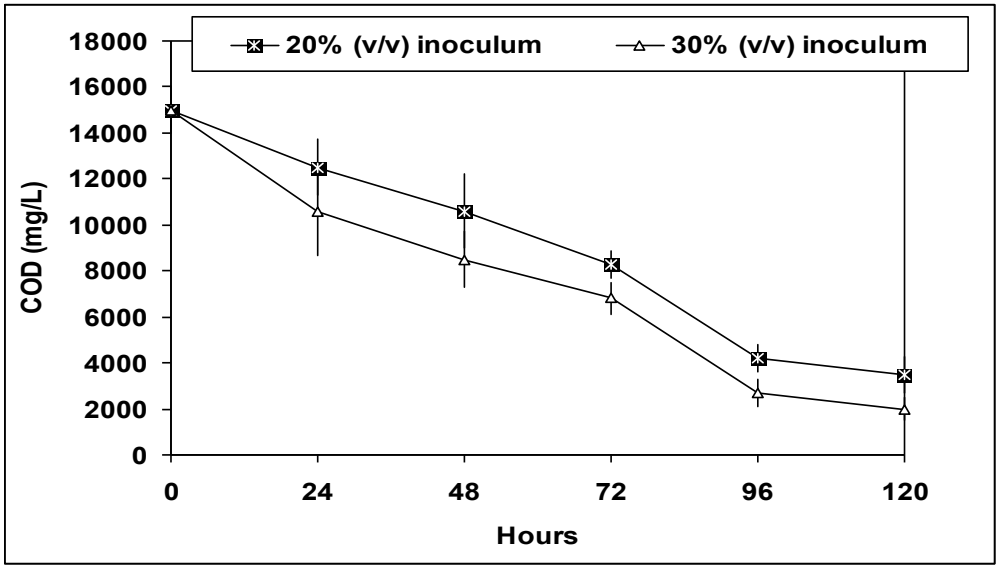

Fig. 5. Reduction in COD (mg/L) of isolate cultured in settled and non-sterilized POME with 20% and 30% (v/v) level of inoculum

4. DISCUSSION

The optimum incubation time for maximum dry cell weight (3.89 to 5.69 g/L) and carotenoids (1.25 to 1.43 mg/g dry cell weight) production in *Rhodobacter sphaeroides* strain UMSPSB3 in this study were recorded at 72h and 48h of culture, respectively. The optimum dry cell weight of 1.1 g/L and carotenoids production of 0.5 mg/g dry cell weight were observed in *Rhodomicrobium vannielii* at 72h culture and 48h culture respectively under anaerobic light condition (2 k lux) [23]. *Rhodovulum sulfidophilum* inoculated in sardine processing wastewater produced 2.8 g/L dry biomass at 72 h culture under anaerobic light condition (2.5 k lux) [16]. These results suggest that the production of dry cell weight and carotenoids in purple non-sulfur bacteria are optimum within 48 h to 72 h of culture. Purple non-sulfur bacteria prefer to grow as photoheterotrophs under anaerobic light condition (2 k lux to 3 k lux) [9]. This indicates light as an important factor to promote the growth and sustain life of purple non-sulfur bacteria [14]. The dry cell weight and production of carotenoids obtained in this study were comparatively higher than the maximum dry cell weight (4.8 g/l) and carotenoids (1.04 mg/g) of *Rhodovulum sulfidophilum* obtained in SPW [16] at the same light intensity of 2.5 k lux. However, these values were relatively higher than that in maximum dry cell weight and carotenoids production of many other purple non-sulfur bacteria. *Rhodomicrobium vannielii* grown in GM media produced dry cell weight of 1.1 g/l and total carotenoids of 0.5 mg/g dry cell weight [23]. *Rhodopseudomonas acidophila* incubated in modified synthetic medium recorded dry cell weight of 2.75 g/l under anaerobic light condition at light intensity of 3 k lux. Increments in total carotenoids production were also observed under anaerobic light condition. Light stimulates the production of carotenoids in purple non-sulfur bacteria under anaerobic condition [24]. Optimum carotenoids production of 0.782±0.02 mg/g dry cell weight was observed under anaerobic light condition at light intensity of 2.5k lux and a temperature of 30±2°C [14]. The biosynthesis of carotenoids was too sensitive to light intensity. Light intensity that slightly scattered from optimum light intensity (2.5 k lux) resulting in drastic decreased in total carotenoids production. Too low or too high light intensity can decrease the biosynthesis of carotenoids [14].

Purple non sulfur bacteria (PNSB) have the capability to utilize the carbon and nitrogen sources, under anaerobic light condition. The removal of the solid particles from wastewater did not affect the nutrients essential for PNSB growth [16]. The total nitrogen value of the POME sample may be limiting factor for the growth of some species of microorganisms [25]. However, the trend of the growth of purple non-sulfur bacteria during the bioremediation of POME indicates that the strain can survive in a

nitrogen-limited environment. The unique of PNSB is it can grow well diverse environmental and cultural conditions. In fact, purple non-sulfur bacteria prefer highly polluted areas than unpolluted areas in the nature [26]. Purple non-sulhur bacteria are the most adaptable prokaryotes, metabolically they can grow as photoautotrophs or photoorganotrophs under anaerobic or micro-aerobic light conditions, as well as chemoorganotrophs under aerobic dark conditions [27]. In the dark, purple non-sulphur bacteria also go through fermentation processes anaerobically or respiration aerobically [28]. The competition between purple non-sulfur bacteria and other microorganisms for the nutrients in the POME samples may decrease of dry cell biomass of purple non-sulfur bacteria [29], allowing other opportunistic microbes to grow which can be suppress with higher levels of inoculum [16].

The optimum pH values for the growth of PNSB are within the range of 6.5-7.5. In this experiment pH of the POME was adjusted at 7.0 to get optimum growth. Most of the purple non-sulfur bacteria grow well at optimum pH of 7.0. *Rhodocyclus gelatinosus* cultured in tuna condensate the optimum pH was 7.0 [14]. The increase of dry cell biomass with 20 and 30% (v/v) inoculums level that have used in the treatment shows that the bacteria growing in the POME, but 10% inoculum level was not supportive to suppress other opportunistic bacteria in on-sterilized POME. The highest biomass was observed with 20 and 30% ((v/v) inoculums in POME from a 96 h culture. These levels of inoculums might provide a high number of bacteria to fully utilize the nutrients that are available in the substrate, in addition to suppress the growth of other bacteria [16]. To suppress the growth of heterotrophic microbes in wastewater the inoculum must be of good quality, fast growing and of sufficient quantity to suppress the growth of contaminants [17]. Purple non-sulfur bacterium, *Rhodocyclus gelatinosus* was prepared in synthetic G5 media from whom 10% (v/v) inoculum was used in cultures of diluted tune condensate and dry cell biomass of 5.7 g/L with 86% reduction in COD were obtained [14]. *Rhodocyclus gelatinosus* cultured in diluted tuna condensate wastewater at 3000 lux intensities in anaerobic light produced 5.6 g/L of dry cell biomass that contained 50% protein. An 86% reduction in COD was observed, but SGR of 0.03/h, cell yield of 0.33 g cell/g COD and carotenoids of 1.31 mg/g cell [14] was comparatively less than obtained when *Rv. sulfidophilum* was cultured in Sardine Processing Wastewater [16].

The competition for nutrients in POME as substrate with a 10% (v/v) level of inoculum, however, is comparatively less than that of 30% (v/v) level of inoculum. In cassava starch, 13% (v/v) inoculum of *Rhodopseudomonas gelatinosa* was used for single cell production. Under anaerobic light condition maximum biomass of 1.8 g/L was obtained in experimentation [17]. Under different culture conditions PNSB was observed to reduce the chemical oxygen demand of wastewater. The growth of the PNSB for high cell yield for single cell protein (SCP) using industrial wastewater as substrate with simultaneous reduction of chemical oxygen demand (COD) are well documented [10]. PNSB strain *Rhodovulum sulfidophilum* cultured in settled and undiluted sardine processing wastewater with a 15% (v/v) inoculum had shown a reduction of 76%and 68% of COD under aerobic dark and anaerobic light conditions respectively. On the other hand, under aerobic dark condition with 15% (v/v) inoculum at 200, 300 and 400 rev/min agitation speed COD reduction was 78%, 69% and 73% respectively [16]. Purple non-sulfur bacteria are used in the treatments of wastewater, as the usage of the bacteria in the bioremediation of wastewater is one of the most cost-effective methods. It is due to the fact that purple non-sulfur bacteria do not only improve effluent quality, but also uses as products of commercial interest. They are used as feed additive and aquaculture supplementary diet due to their high nutritive values of protein, amino acid, and vitamins [15]. The PNSB can grow in POME although their growth rates are slower than cultured synthetic 112 media, but the bacterial biomass generated from POME has better nutritional quality in term of essential fatty acid composition [30]. In addition extracellular polymeric substances, like enzyme produced by purple non-sulfur bacteria can give additional supplement in diet for digestion of larval rearing in aquaculture species. *Afifella marina* strain ME (KC205142) produces protease under anaerobic light conditions at temperature of 30°C±2°C. The proteolytic activity was positively correlated with the dry cell weight in *A. marina* [31]. Other than that, carotenoids produced by purple non-sulfur bacteria are used as pigmenting additives to developed pigmentation on different parts of animal bodies, such as muscles, fat, skin, feather, legs, ovaries, and eggs [32].

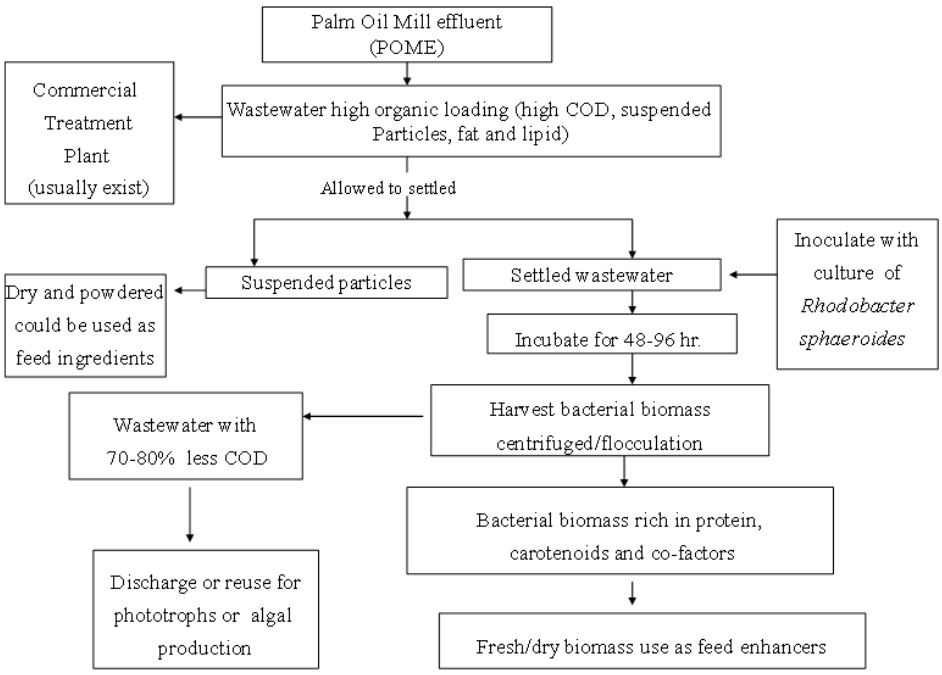

Fig. 6. Conceptual model for the utilization of Palm Oil Mill Effluent (POME) with *Rhodobacter sphaeroides* strain UMSPSB3

POME media also contained suspended particles. During incubation in an anaerobic light condition the transmission of light might not have been uniform in the culture bottles. This may have resulted in a lower specific growth rate of *Rhodobacter sphaeroides* culture in POME. Further, due to these suspended particles initial biomass in culture bottles was observed to be higher, but number of bacterial cell might have been less. As inoculum was calculated in volume-by-volume basis, 20% or 30% (v/v) of inoculum in media might have contained higher number of bacterial cells than that of 10% (v/v) inoculums [16]. So, growth rate was accelerated with higher inoculum sizes in *Rhodobacter sphaeroides* was cultured in POME using inoculum developed in GMM. Based on the experiment the following conceptual design could be one of the proposed models in POME utilization (Fig. 6, Above)

5. CONCLUSION

Based on the present study, the optimum light intensity for the production of bacterium biomass and total carotenoids was 2 klux. On the other hand the optimum size of inoculum for biomass production lies between 20–30% (v/v), but for the reduction of COD a 30% inoculation are necessary for the utilization POME as substrate for the growth of *Rhodobacter sphaeroides* strain UMSPSB3. The conceptual design was developed based on the utilization of POME *Rhodobacter sphaeroides* strain UMSPSB3 to achieved dual benefits: bioremediation of wastewater and production of beneficial bacterium biomass.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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