Scientific Research Publishing

Journal of Geoscience and Environment Protection, 2024, 12, 163-172
https://www.scirp.org/journal/gep
ISSN Online: 2327-4344
ISSN Print: 2327-4336

Growth Characteristics of Phototrophic Bacterium *Afifella marina* in Wastewater

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How to cite this paper: Al Azad, S., & Mohamad Lal, M. T. B. (2024). Growth Characteristics of Phototrophic Bacterium *Afifella marina* in Wastewater. *Journal of Geoscience and Environment Protection, 12,* 163-172.
https://doi.org/10.4236/gep.2024.1211009

Received: October 10, 2024
Accepted: November 19, 2024
Published: November 22, 2024

Abstract

Nutrients available in wastewater are supportive for the growth of phototrophic bacteria. Phototrophic bacterium, *Afifella marina* strain ME was grown in a characterized finfish hatchery wastewater. The effects of three light intensities (2000 lux, 2500 lux and 3000 lux) with 30% (v/v) inoculum on the growth, in terms of dry cell weight (g/L) and production of total carotenoids (mg/g dry cell weight) were observed in this study. Total nitrogen (mg/L) and phosphorus (mg/L) are the two major nutrients identified in wastewater. The highest bacterial cell weight of 0.37 g/L was obtained after 72 hours of culture at 2500 lux light intensity, whilst the highest total carotenoid production of 0.06 mg/g dry cell weight was determined in 24 hours of culture at same light intensity. Different light intensities affected the production of bacterial cell weight and total carotenoid production. However, statistical analysis indicated that there were no significant differences between bacterial dry cell weight and total carotenoid production due to the differing light intensities ($p > 0.05$). Poor growth (dry cell weight) and carotenoids production with low SGR, but efficient use of substrate. The 30% (v/v) inoculum level observed was not very supportive on the growth characteristics of bacterium. In addition, other opportunities for bacteria that remained in wastewater might suppressed the growth of *Afifella marina* strain ME, which need further investigation. Further, several other factors like, strain type, temperature of the culture substrate, nutrients and types of inoculum media, aerobic and anaerobic culture condition and agitation speed can alter and change the growth profile of bacterium, which need to be optimized. However, phototrophic bacterium *Afifella marina* strains ME is capable to grow in finfish in all these three light intensities but not at 30% (v/v) inoculum level.

Keywords

Afifella marina, Growth, Finfish Wastewater, Light Intensity

1. Introduction

Phototrophic bacteria are well known for their organic transformation properties and have a wide range of potential applications (Sasikala et al., 1995). These bacteria require anoxic conditions and are able to utilize light to assimilate various organic compounds for growth and pigment production (Madigan & Jung, 2009). Major groups of these bacteria have the potential for the utilization of wastewater nutrients both from carbon based or nitrogenous based substrate. Species of *Rhodopseudomonas palustris*, *Rhodovulum sulfidophilum, Rhodobacter sphaeroides* and *Afifella marina* are suitable candidates for utilization of wastewater as those bacteria are capable of conversion of waste material into value-added products (Sasikala et al., 1995). There is potential phototrophic bacterium in the utilization of finfish hatchery wastewater for the for-biomass production that could be used as aquaculture feed supplement (Al-Azad et al., 2020). Fish hatcheries are facilities for specific activities such as maintaining brood stock in captivity, carrying out artificial breeding, production of hatching, rearing early life stages of fish and raising fry and fingerlings (Crespi & Coche, 2008). Aquaculture activity including finfish hatcheries usually generates waste and wastewater. Finfish hatchery wastewater is composed of both organic and inorganic in dissolved or solid form. Solid wastes in wastewater contain 7% - 32% total nitrogen and 30-84% total phosphorus (Al-Azad et al., 2020). The wastewater content in these hatcheries is rich in nutrients like nitrogen and phosphorus that pose risks when discharged directly into aquatic ecosystems. The release of high-nutrient wastewater into aquatic ecosystems may increase eutrophication which leads to the growth of harmful algal blooms in coastal ecosystem (Wurtsbaugh et al., 2019). The best option is to manage hatchery wastewater with the beneficial ways, such as growing phototrophic bacteria to produce single call protein and, at the same time, reducing the organic loading load. Growth characteristics of phototrophic bacteria in terms of bacterial cell biomass (g/L) and total carotenoids production (mg/g) are influenced by certain factors like pH, Temperature, Nutrients, agitation and Light Intensity. In a comparison of all these factors, light intensity is the key factor that regulates the growth of bacterium. In essence, biomass production of bacteria reaches a maximal value at optimum light intensity; however, the highest production of total carotenoids production in terms of pigmentation is usually achieved under dimmer light intensities (Soon et al., 2014). This research focuses on the effects of light intensity on *Afifella marina* growth characteristics in terms of bacterial cell biomass and total carotenoid production in Universiti Malaysia Sabah Finfish Hatchery Wastewater as substrate.

2. Materials and Methods

The hatchery wastewater was collected from the initial drainage point, where the waste was generated from the brood stock tank of Hybrid Grouper (*Epinephelus lanceolatus*) and Napoleon Wrasse (*Cheilinus undulatus*).

The specific strain of *Afifella marina* strain ME (KC205142) was obtained from Borneo Marine Research Institute (BMRI) culture collection. The strain was isolated from the mangrove mud of Kota Kinabalu, Sabah, and identification of the isolate was done based on molecular characteristics of strain (Soon et al., 2014). The inoculum was developed in 112 culture media and 48-h inoculum was used. A 30% (v/v) inoculum was used in an experiment, where hatchery wastewater used as substrate. All the inoculated bottles were arranged layer by layer in front of the tungsten bulbs with specifically measured and drawn lines to ensure that three different light intensities, namely, 2000 lux, 2500 lux and 3000 lux. A lux meter was used to measure and ensure that the culture was under the right light intensity. All those bottles were incubated aerobically with specific light intensity at a temperature of 30˚C ± 2˚C for 5 days. Everyday three (3) bottles daily were selected randomly from the respective light intensities and determined the growth characteristics of bacterium in terms of dry cell weight (g/L) and production of total Carotenoid (mg/g dry cell weight).

Collected wastewater was characterized before being used as substrate for determining the growth bacterium. The parameters analyzed were: Total solids (mg/L), nitrate (mg/L), nitrite phosphate (mg/L), total ammonia nitrogen (mg/L) and COD (mg/L), based on APHA (1998).

The bacterial dry cell weight of *Afifella marina* in hatchery wastewater was determined by measuring the dry cell weight of the sample after repeated washing and centrifugation, finally dried in the oven at 105˚C overnight (Sawada et al., 1977). The total carotenoid production of *Afifella marina* was determined by measuring the absorbance value of the samples pigments at 480 nm using a spectrophotometer after centrifugation, re-suspension and addition of acetone-methanol (7:2 v/v) (Liaaen-Jensen & Jensen, 1971).

One-way ANOVA was used to analyze the significant difference between the bacterial dry cell weight (g/L) and total carotenoid production (mg/g dry cell weight) against the three different light intensities.

3. Results

The Characteristics of wastewater coming from the brood stock tank (mean values and ranges) are shown in **Table 1**. Values in the mean concentration of dissolved inorganic nutrients, such as total ammonia nitrogen of 2.38 mg/L, nitrate (3.00 mg/L) and phosphate of 7.55 mg/L, as well as chemical oxygen demand of 320 mg/L were determined from the collected samples.

Table 1. Characteristics of wastewater sample collected from UMS Hatchery.

Parameters (mg/L)	Mean value	Range
Total solids	60 ± 5	55 - 70
TAN	2.38 ± 1.3	0.25 - 6.5
Nitrite	0.68 ± 1.2	0.05 - 1.2
Nitrate	3.00 ± 1.3	0.04 - 4.2
Phosphate	7.55 ± 2.1	0.41 - 10.7
COD	320 ± 10	400 - 500

*Values expressed as mean ± SD.

During five days of experimentation, the *Afifella marina* indicated a rapid growth pattern until day three (72-h) in all light intensities with a major decrease starting from 96-h culture. The highest dry cell weight of 0.37 g/L was obtained in the sample culture at 2500 lux on 72-h of the experiment. The lowest value, which was 0.33 g/L bacterial cell biomass was obtained in the sample culture at 3000 lux on 72-h of the experiment (Figure 1). A one-way ANOVA analysis was performed to compare the effects of light intensity towards bacterial cell biomass (g/L) which indicated no statistically significant difference in mean between all three different light intensities to bacterial cell biomass ($p = 0.471$).

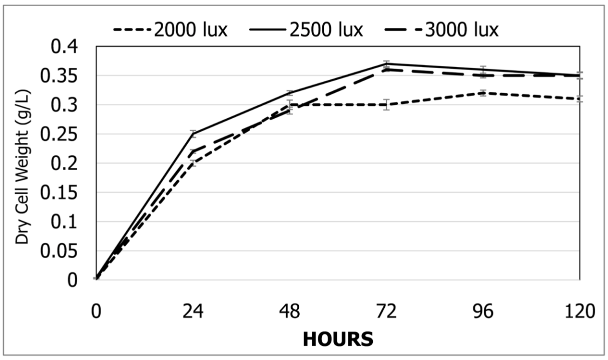

Figure 1. Mean values of bacterial cell biomass of *Afifella marina* at 2000 lux, 2500 lux and 3000 lux light intensities in hatchery wastewater.

Total carotenoid production of *Afifella marina* in hatchery wastewater is presented in Figure 2. The highest value 0.06 mg/g of total carotenoid production was obtained in the culture of 2500 lux on 24-h and the lowest value of 0.049 mg/g total carotenoid production was obtained in the culture at 3000 lux on 24-h (Figure 2). One-way ANOVA analysis was performed to compare the effects of light intensity towards total carotenoid production which indicated no statistically significant difference in mean between all three different light intensities to total carotenoid production ($p = 0.839$).

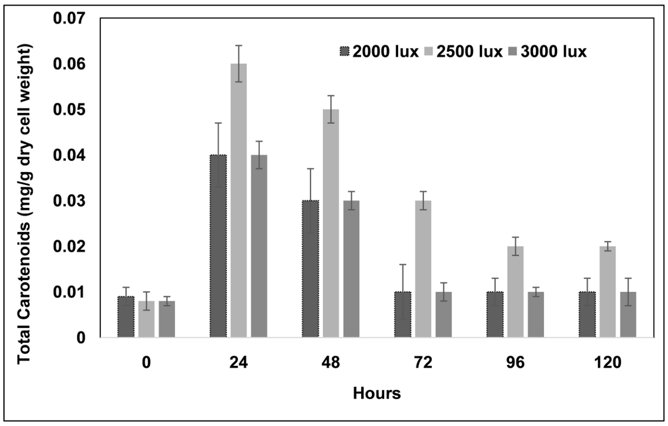

Figure 2. Mean values of total carotenoid production of *Afifella marina* at 2000 lux, 2500 lux and 3000 lux light intensities in hatchery wastewater.

The maximum dry cell weight 0.38 ± 0.006 of *Afifella marina* was obtained in hatchery wastewater with 2500 light intensity, as well as efficient utilization of substrate (Yx/s g cell/g subs). Although no significant differences were observed in the production of dry cell weight with 2000 lux and 3000 lux intensity, substrate utilization was observed better with 2000 lux light intensity (Table 2).

Table 2. Growth characteristics of *Afifella marina* strain ME in undiluted and non-sterilized UMS finfish rearing wastewater.

Light Intensity (lux)	X_{max} (g/L)	μ_{max} (per h)	Yx/s (g cell/g subs)	Total Carotenoids (mg/g DCW)
2000	0.36 ± 0.004^a	0.002 ± 0.001	0.141 ± 0.055^a	0.045 ± 0.01^b
2500	0.38 ± 0.006^a	0.005 ± 0.002	0.215 ± 0.061^b	0.062 ± 0.02^a
3000	0.36 ± 0.006^a	0.002 ± 0.001	0.115 ± 0.061^b	0.040 ± 0.014^b

a and b superscript indicate significant differences. Same symbol of superscripts are not significantly differences.

4. Discussion

4.1. Characteristics of Finfish Hatchery Wastewater

In general, the wastewater generated from aquaculture sectors is less than the wastewater generated from other agro-based industries. In this research the wastewater collected was generated from a brood stock tank. The quality and quantity in aquaculture hatchery wastewater depend on the feed and feeding habit of cultured fishes. The brood fish were generally fed with pelleted diets or fresh trash fishes. So, the waste generated from those brood fish contribute less environmental effects when discharged to nature. The results from this study indicated that the mean values of dissolved inorganic are lower than the values obtained from other studies. The concentration of DIN in this study, like ammonia of 2.38 mg/L, nitrate 3.00 mg/L and phosphate 7.55 mg/L are comparably less than that obtained the values of Ammonia (4.38 mg/L), nitrate (3.37 mg/L) and phosphate (8.55 mg/L) by researcher (Al-Azad et al., 2020). The values observed were higher than those determined by another researcher (Lin et al., 2002), where concentrations were in the range of 0.12 - 14.7 mg/L of NH_4-N, 0.02 - 1.5 NO_2-N mg/L, 0.01 - 5.3 mg/L of NO_3-N, and 3.1 - 17.7 PO_4-P mg/L. Those values are from the waste generated in the fishpond as the consequences of feed residue and fish excreta. The variation in the chemical composition of finfish effluent is influenced by certain factors, such as, types of rearing tank, culture techniques, types of species and sizes of species, feed types and feed management, dynamics of nutrient in circulation and utilization, species handling techniques and the other physical chemical environment of the culture areas (Mudrak, 1981). On the other hand, the total solids of 60mg/L obtained from this study are comparatively very low compared to the total solids generated from other sources like 40500mg/L total solids from palm oil mill (Ibrahim et al., 2012). Palm oil mill effluent has been used as a substrate for PNSB species *R. sphaeroides* (Azad et al., 2019) and has shown enough nutritional values to support growth of live feed like rotifers. Sardine processing water which was also used as a substrate for *R. sulfidophilum* cultivation recorded high nitrogenous waste materials and total solids, which included cooked sardine skin, tissues, soft bones and nitrogenous organic compounds which resulted in high organic loading supplementing the PNSB's growth. Characterization of wastewater is essential to provide further information on its ability of nutrients to sustain the growth of microbes. Few information is available on the growth characteristics of *Afifella marina* strain ME using hatchery wastewater as substrate.

4.2. *Afifella marina* Dry Cell Weight (g/L)

The isolated phototrophic bacterium *Afifella marina* strain ME (KC205142) growth with 30% inoculum with hatchery wastewater as a substrate demonstrated a growth pattern with close proximity of other phototrophic bacterial isolates. The bacterium growth profile of this species shows a sigmoid growth pattern for the parameter of dry cell weight. Various factors contributed to the difference in values of growth profiling parameter (e.g., light intensity, strain type, temperature, nutrients and medium composition). Light intensity is chosen to acquire maximum production of bacterial cells between species and even strains of the same species which is defined by each microorganism's photo-adaptation (Lazaro et al., 2015). The growth characteristics of the bacteria started with a negligible lag phase, proceeded with an exponential phase and finally ended with stationary phase. In general, within the first 24 hours of incubation, the bacteria culture will initiate extracellular enzymes production with quantitative enzyme activities increasing even until 72 hours of incubation (Willerding et al., 2011).

In the current study, isolate *Afifella marina* strain ME was found to be capable of bacterial biomass production, but dry cell weight obtained comparatively lower to past studies. The highest peak bacterial dry cell wight was observed under 2500 lux light intensity measuring 0.37 g/L with the lowest peak being 0.33 g/L under 3000 lux light intensity. The value of dry cell weight is determined very low compared with other researchers. A dry cell weight of 2.44 g/L was determined from the settled POME with 30% inoculum level with the phototrophic bacterium of *Rhodobacter sphaeroides* strain UMSFW1 (Azad & Shaleh, 2015). On the other hand, observed values of dry cell weight were almost similar to the dry cell weight of 0.42 g/L when *R. sphaeroides* was incubated in soybean wastewater for the production of bacterial biomass with 20% (v/v) inoculum level (Madukasi et al., 2011). Phototrophic bacterium of *Rubrivivax gelatinosus* with 1% (v/v) inoculum size in poultry wastewater resulted 0.57 g/L of dry cell weight (Ponsano et al., 2008). Based on the outcome of the current study, it indicates that 2500 lux is the most optimal light intensity despite recording a minor difference in peak values with the other two light intensities (2000 and 3000 lux). The current study also shows that there is no statistically significant difference in mean between light intensity and the production of bacterial cell biomass. This is probably due to the moderate difference in light intensity which results in a mild contrast of culture condition. At higher light intensity thresholds, however, the production of bacterial cell biomass decreased (Al-Azad et al., 2013) which may be attributed to the increase in temperature. The higher the light intensity, the higher the heat generation which resulted in bacterial death of the culture contributing to growth profile of bacterium. Carotenoids are a type of pigment found in cells that work as light-absorbing and photoprotective components when coupled to protein components on the cell membrane (Saejung & Ampornpat, 2019). Despite the significant influence of light intensity on the growth properties of *Afifella marina* strain ME, the bacteria still depends strongly on the organic matter present and the provision of necessary nutrients within the wastewater substrate for its optimal development in the production of bacterial biomass.

4.3. Total Carotenoid Production (mg/g Dry Cell Weight)

Carotenoids are red pigment found in cells that work as light-absorbing and photoprotective components when coupled to protein components on the cell membrane (Saejung & Ampornpat, 2019). It is a pigment that occurs naturally in plants, algae, fungi and bacteria and is used by these organisms for photo defense, light harvesting, and species-specific coloring. In the current study, *Afifella marina* strain ME was capable to produce total carotenoid in the three light intensities despite recording significantly lower values comparatively to previous studies. As observed, the culture under 2500 lux produced the highest of 0.06 mg/g dry cell weight of total followed by 0.050 mg/g by 2000 lux and 0.0495 mg/g by 3000 lux. This indicates that a higher light intensity inhibits the total carotenoid production further. The highest total carotenoid production recorded in 112 media was 0.76 mg/g, but while *Afifella marina* was cultured in vegetable juice lower production of 0.12 mg/g dry cell weight of carotenoids was observed with 2500 lux light intensity. The increase of light intensities between the ranges of 2500 to 3000 lux proved beneficial to bacterial growth as it offered maximum efficiency of carotenoids production. Above the light intensity threshold, however, there was a significant decline in total carotenoid production. This was related to the carotenoid biosynthesis being excessively sensitive to light intensity as high light intensities may cause excessive excitation in the photosynthetic apparatus, which may lead to the creation of harmful oxygen species in the photochemical reaction (Saejung & Ampornpat, 2019; Soon et al., 2014).

In general, the low bacterial biomass output in this study may be attributed to various factors. The most important parameter is the nutrient limitation in finfish hatchery wastewater. As observed in the wastewater parameter analysis, the values of the nutrients observed were very low compared to other researchers. The amount of total nitrogen in wastewater may be the limiting factor for the growth of some species of microorganisms (Woertz et al., 2009). The low count of total nitrogen obtained may be linked to various factors. Types of culture species, sizes of species rearing technique, types of feed used in the tank, and feed management affect the nutrient dynamics and chemical nature of wastewater (Mudrak, 1981). Low nutrients improvement might be one of the options to obtain better growth profile of bacterium. These can be improved with the addition of ingredients that were used in nutrients media for the inoculum preparation. The optimum amounts of nutrients can be determined with trial-and-error basis with different proportion of ingredients. In the case of this study, the low nutrient availability may be because the wastewater collection was conducted only two days after tank-cleaning process was done therefore, it lacked in waste and uneaten feed build up. In addition, the low inoculum size used in this study may also have contributed to the low bacterial cell production. The slow growth of PNSB can be attributed to low inoculum size (Azad & Shaleh, 2015). The poor coloration of the culture stock or inoculum also indicates a possibility that there is an insufficient cell concentration within the bacterium stock samples. Furthermore, the low inoculum level results in a prolonged lag phase which in turn, results in a slower bacterial growth (Baert et al., 2008). Another factor that may have contributed to the study outcome is the agitation of culture. Agitation greatly influences the metabolite production in microorganisms. Agitation also significantly affects the bioconversion of waste into bacterial biomass production by PNSB (Chae et al., 2006; Jamrah et al., 2008). The addition of a proper agitation process would have allowed for better mixing of nutrients. This helps reduce dead zone formations in which concentrations of nutrients and oxygen are too low to support the growth of PNSB in the substrate. In this study, limited agitation was given by hand and only done occasionally during check-ups of culture every day. Further, the presence of opportunistic microbes. Collected wastewater was not sterilized to conduct the experiment. This increases the probability that other opportunistic microbes were present within the wastewater substrate and may have negatively impacted the growth characteristics of *Afifella marina*. This may result in a decrease of *Afifella marina* in the culture, hence, impacting the overall growth characteristics and bacterial biomass production. The low inoculum level of the culture may also have resulted in the inability for *Afifella marina* to suppress opportunistic microbes (Azad et al., 2019).

5. Conclusion

The optimum light intensity to produce bacterial cells and total carotenoids was 2500 lux. *Afifella marina* strain ME growth was unsatisfactory and very poor performance of the bacterial growth in finfish hatchery wastewater in terms of bacterial dry cell weight and total carotenoid production was observed. It was clear that the substrates' nutritional content was vital in determining the ability of bacterium for optimal growth development. Higher concentrations of bacterial inoculum may have resulted in higher chance of ensuring that the opportunistic bacteria which may suppress the growth of *Afifella marina* strain ME in finfish hatchery wastewater. The phototrophic bacterium *Afifella marina* strains ME is able to grow in hatchery wastewater but needs optimization of certain parameters to maximize the dry cell weight and production of total carotenoids.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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