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Contributed Paper

Cultivation of *Arthrospira* (*Spirulina*) *platensis* Using Low Cost Medium Supplemented with Lac Wastewater

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ABSTRACT

Arthrospira or *Spirulina* is a well-known food supplement; however, the cost of the culture medium is pricey. Thus, the aim of this paper is to cultivate *Arthrospira (Spirulina) platensis* using a low cost medium (CMU02) supplement with lac wastewater. Four samples of lac wastewater from different lac production processes were supplemented to the CMU02 medium. The growth, biomass production, protein and pigment contents, as well as the production cost of each treatment were evaluated. It was found that the primary positive effect of the lac wastewater after water treatment was the increased biomass concentration that was recorded as high as 0.624 $g.L^{-1}$ with a protein content of 59.11 g.100 g dry $weight^{-1}$. Carotenoid and phycocyanin contents of 0.17 mg.g dry $weight^{-1}$ and 71.30 mg.g dry $weight^{-1}$, respectively, were also achieved. Lac wastewater could reduce the estimated cultivation cost by as much as 4.43 fold when compared with the Zarrouk medium. These findings indicate that lac wastewater can be used as a cost-saving supplement in large-scale cultivation systems.

Keywords: *Arthrospira,* lac wastewater, carotenoid, phycocyanin, protein content

1. INTRODUCTION

Arthrospira or *Spirulina*, is a well-known food and feed supplement. Its nutritional benefits have been acknowledged for many years [1]. In addition, this cyanobacterium also possesses high value compounds, such as pigments [2]. Although *Arthrospira* has been commercially cultivated worldwide, its high production cost remains a major challenge in terms of biomass production. With regard to the total production cost of *Arthrospira* biomass, 15-25% of the cost is accounted for by the culture medium that is needed for nutrient acquisition [3]. To address this, considerable amount of research has focused on developing a cheap and simple medium. Previous studies have successfully cultivated *Arthrospira* (*Spirulina*) *platensis* with the use of a cheap and simple medium (CMU02).

Although the productivity from this medium was lower than that of Zarrouk medium, supplements could be added to enhance *Arthrospira* production - for example, sugar cane molasses distillery slop [4] and anaerobic swine wastewater treatment effluent [5]. In addition, *A. platensis* can be cultivated in various other wastewater effluents, including the fish culture effluent [6] and sago starch factory wastewater [7].

In the lac production process (Figure 1), harvested lac sticks are crushed and washed to remove insect parts, colour and other soluble materials. After this first wash, the mixture of water, lac and debris is transferred to a sedimentation pond where a certain amount of lac is collected by sedimentation, while the remainder is collected by centrifugal extractors. Then the water acquired from the centrifuge and other soluble materials are sent to a different sedimentation pond and any leftover lac is collected by sedimentation. The final wastewater is sent to relevant water treatment units. However, if the centrifugal extractors are unavailable, the mixture from the first sedimentation step is sent to two successive sedimentation ponds, after which the final wastewater is sent to the water treatment units. This lac production wastewater is treated in three steps. Firstly, limestone is used to neutralize the pH. Secondly, the pollutants are flocculated using polymer and $FeCl_2$. Finally, the clear water is stored in a clear water pond for use in the factory. If the L pond is full, the excess water is then collected in the wastewater pond.

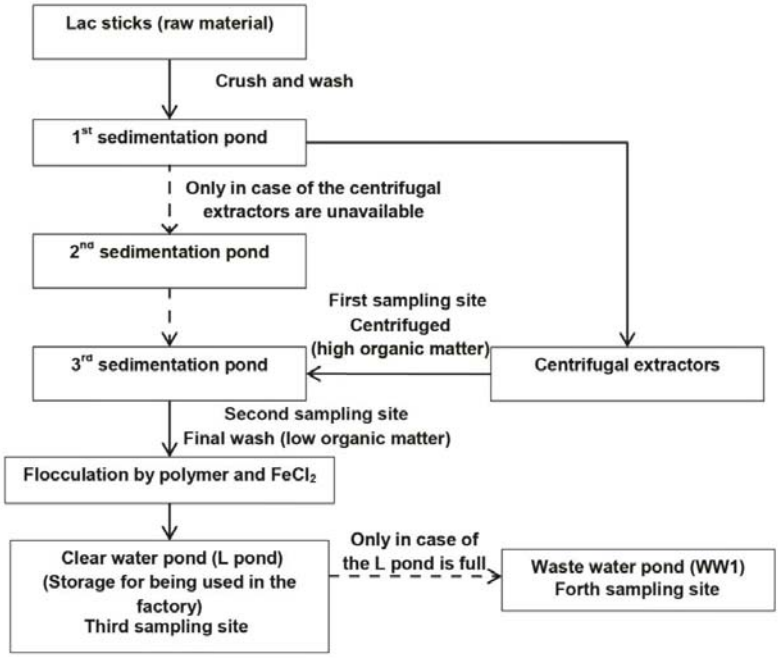

Figure 1. Schematic diagram representative of the lac production and water treatment process in the lac factory in this study.

In total, more than 10-12 L of wash water per 1 kg of seedlac is required in the making of seedlac that is free from lac dye and other organic matter. From our preliminary survey, lac wastewater is full of nutrients, especially ammonia and nitrate, which are favourable nitrogen sources for microalgae, such as *Chlorella miniata* [8] and *A. platensis* [9]. However, no one yet has investigated the cultivation of *A. platensis* supplement with lac wastewater effluent. Lac washing water may be of benefit in cultivating *A. platensis*, a cyanobacterium that can use nitrate and ammonia as a nitrogen source. In addition, the lac wastewater is also rich in organic substances that may be directly utilized as important organic nutrients or may act as a supplement growth factor [10]. Thus, this study aimed to evaluate the effect of the application of water from lac production as a nutrient supplement in *A. platensis* cultivation. The cost estimation from *A. platensis* biomass was also estimated compared with the standard medium (Zarrouk medium). In addition, the protein content and the presence of other valuable products including carotenoids and phycocyanins were also investigated.

2. MATERIALS AND METHODS

2.1 Description of Lac Production and Sampling Sites

Lac wastewater was collected from four sites (Figure 1) at the lac factory in Lampang Province, Thailand. The wastewater was collected at four specific stages of the treatment process including: (1) water after centrifugation (centrifuged), (2) water after sedimentation (final wash), (3) water after water treatment (L pond) and (4) water in the wastewater pond (WW1).

2.2 Water Quality Analysis

Some water quality parameters were measured and determined according to the Standard Methods for the Analysis of Water and Wastewater [11]. Nitrate-nitrogen was determined through cadmium reduction using NitraVer® 5 Nitrate Reagent Powder Pillows (Hatch, USA). Ammonium-nitrogen was determined through nesslerization using Nitrogen-Ammonia Reagent Set, Nessler (Hatch, USA). Soluble reactive phosphorus was determined using the ascorbic acid method by PhosVer® 3 Phosphate Reagent Powder Pillows (Hatch, USA). After the reaction was performed according to the relevant manual, the results were then read from the DR2000 Spectrophotometer (Hach, USA). Alkalinity was determined by titration with HCl and methyl orange as an indicator. Turbidity was determined spectrophotometrically at 850 nm by DR2000 Spectrophotometer (Hach, USA).

2.3 Cultivation of *Arthrospira (Spirulina) platensis* using Lac Wastewater

Arthrospira (Spirulina) platensis AARL C005 was obtained from the Applied Algal Research Laboratory, Department of Biology, Chiang Mai University, Chiang Mai, Thailand. The stock culture was maintained in Zarrouk medium pH 10, a standard medium and the conditions for *Spirulina* cultivation were as follows: [12] being kept under indoor ambient conditions (25-32°C) while being mixed by air bubbling with continuous illumination by fluorescent lamp at 27 $\mu mol_{photons}$. $m^{-2}.s^{-1}$.

In order to develop the low cost cultivation method, *Arthrospira* was cultivated in CMU02 medium, a cheap, simple *Arthrospira* medium. The pH value was adjusted to 10 using NaOH [4] and the growth was compared to that of the Zarrouk medium. The batch cultivation was conducted in 5 L medium of open glass cylindrical

chambers (radius = 15 cm) under the same culture conditions of the stock culture as has been previously described. The cyanobacterium was inoculated to obtain an initial dry weight of 0.05 $g.L^{-1}$. The growth of *A. platensis* was monitored every two days by measuring the dry weight. On the final day of cultivation, the *A. platensis* cells were harvested by 50 mesh size filtration, and analysed for protein, carotenoid and phycocyanin contents.

To improve the production from the *Arthrospira* medium with the aim of supplementing lac wastewater by substituting the trace elements in the standard medium, nine different types of media were prepared as indicated in Table 1. These media include 5 L of ten-fold diluted different lac wastewater samples in dechlorinated tap water and CMU02. The wastewater samples were filtrated through the cotton cheesecloth to remove the large debris before being diluted with the dechlorinated tap water or CMU02. The cultivation conditions were the same as those applied in the first experiment.

Table 1. Types and compositions of media used for the culturing of *Arthrospira (Spirulina) platensis*.

Types of media	Media composition
CMU02	8.5 $g.L^{-1}$ $NaHCO_3$, 1.5 $g.L^{-1}$ $NaNO_3$, 0.5 $g.L^{-1}$ K_2HPO_4 and 0.6 $g.L^{-1}$ N:P:K (16:16:16)
Centrifuged	10% (v/v) of first water sample + 90% (v/v) of dechlorinated tap water
Centrifuged+CMU02	10% (v/v) of first water sample + 90% (v/v) of CMU02
Final wash	10% (v/v) of second water sample + 90% (v/v) of dechlorinated tap water
Final wash+CMU02	10% (v/v) of second water sample + 90% (v/v) of CMU02
L	10% (v/v) of third water sample + 90% (v/v) of dechlorinated tap water
L+CMU02	10% (v/v) of third water sample + 90% (v/v) of CMU02
WW1	10% (v/v) of fourth water sample + 90% (v/v) of dechlorinated tap water
WW1+CMU02	10% (v/v) of fourth water sample + 90% (v/v) of CMU02

Biomass productivity was expressed in mg dry $weight.L^{-1}.day^{-1}$, while gross biomass productivity was expressed in terms of g dry $weight.m^{-2}.d^{-1}$ and was estimated from biomass productivity using 30 cm culture depth or 300 $L.m^{-2}$ [7].

The specific growth rate (μ, day^{-1}) was calculated following the method of Colla *et al.* [13], using the following equation:

$$\mu = (\text{maximal biomass} - \text{initial biomass})/(\text{initial biomass} \times d), \quad \text{(Eq. 1)}$$

where d is the number of days between initial and maximal biomass measurements.

Protein content was determined using semi-micro Kjeldahl in the presence of selenium as the catalyst. The liberated ammonia was distilled in a 2% boric acid solution [14].

The amount of carotenoid was measured using the method of Rodriguez-Bernaldo De Quirós and Costa [15]. Carotenoids were extracted using diethyl ether. The amount of carotenoids was spectrophotometrically measured at 450 nm. Carotenoid content (mg.g cell dry weight^{-1}) was calculated using the following equation:

$$\text{Carotenoid} = (A_{450} \times V \times 1000)/(260 \times \text{g cell dry weight}), \quad \text{(Eq. 2)}$$

where A_{450} is the absorbance at 450 nm and V is the volume of diethyl ether.

The phycocyanin (PC) content was measured by soaking dried samples in a phosphate buffer and then lyzed by freeze-thawing. The amount of PC was measured by spectrophotometer at 615 and 652 nm. The quantity of PC (mg. g cell dry weight^{-1}) was calculated using the following equation, which was provided by Bennett and Bogorad [16] with the extinction coefficients acquired from Bryant *et al.* [17]:

$$PC = ((A_{615} - 0.474(A_{652}))/5.34) \times (V / \text{g cell dry weight}), \quad \text{(Eq. 3)}$$

where A_{615} and A_{652} represent the absorbance at 615 and 652 nm, respectively and V represents the volume of the phosphate buffer.

The crude biomass cost ($.kg dry weight^{-1}) was estimated from the medium cost ($.L^{-1}) / biomass (g dry weight.L^{-1}) × 1000, (Eq. 4)

2.4 Statistical Analysis

The data were expressed as mean ± standard deviation (SD) of the three replicates. The groups were compared statistically by one-way analysis of variance (ANOVA) and post-hoc Tukey's b tests using SPSS version 14.0. *P*-values less than 0.05 were considered significant.

3. RESULTS AND DISCUSSION

3.1 Water Quality

The physical and chemical parameters of the four water samples were determined. From the overall observation (Table 2), the water sample after centrifugation (centrifuged) was the most polluted, as evidenced from its nutrient levels. As this sample was washed the least, it contained a high amount of organic matter from the lac beetle. However, after further washing, the organic matter was significantly reduced, as observed from the nutrient levels of the final wash sample. As expected, the nutrient levels from the L pond were the lowest, because the L pond served as the catchment area from the wastewater treatment system.

The major nutrient in all samples of the lac wastewater was ammonia, a common by-product of the metabolism of nitrogenous compounds [18]. The quantity of ammonia in the centrifuged sample was very high. The ammonia decreased in the samples from the final wash. However, the ammonium ion increased again in the L and WW1 pond samples, because of the degradation of the organic materials. Nitrate is another nitrogen source for *A. platensis*. In the lac wastewater, the quantity of the nitrate was very low in the centrifuged and final wash samples, as well as in the L pond sample, while its concentration was very high in the WW1 pond sample due to the bacterial activities. WW1 is a pond where the wastewater was stored. In this pond, nitrifying bacteria changed the ammonia to nitrite and then nitrate [18]. In contrast, the phosphate percentage and alkalinity were very high in the centrifuged sample, but lower in the other three samples because the phosphates and alkalized contaminants were almost completely

removed after the washing steps. This is the first report on the water quality from lac production. Compared with other types of wastewater that have been used for *A. platensis* cultivation (Table 2.), nutrients in lac wastewater are still inadequate for *Arthrospira* medium. The high turbidity (2319-6615 FAU) of the four samples is also a concern as it blocks the penetration of light needed for *A. platensis*. From our previous study, the maximum turbidity for microalgal cultivation was approximately 200 FAU (unpublished data). Thus, given the turbidity of the lac wastewater, its supplementation was limited to 10% (tenfold dilution).

Table 2. Characteristics of lac factory wastewater.

Parameter	BOD ($mg.L^{-1}$)	COD ($mg.L^{-1}$)	NH_3^+-N ($mg.L^{-1}$)	NO_3^--N ($mg.L^{-1}$)	PO_4^- ($mg.L^{-1}$)	Alkalinity ($mg.L^{-1}$ as $CaCO_3$)	Turbidity (FAU)
Centrifuged	4033.33± 694.42c	12,666.67± 377.12c	134.08± 5.44c	LD	171.47± 4.63d	7000.00± 0.00d	6615.33± 91.53c
Final wash	1666.67± 94.28b	8933.33± 1643.83b	43.33± 2.58a	0.63± 0.07a	45.40± 4.38c	2666.70± 0.00c	5534± 9.09b
L pond	380.00± 16.33a	3466.67± 377.12a	96.67± 6.25b	0.50± 0.08a	0.75± 0.21a	753.33± 12.47a	2319± 4.32a
WW1 pond	1333.33± 377.12b	4000.00± 1131.37a	130.67± 7.32c	16.80± 0.28b	10.39± 2.52b	826.67± 4.71b	2418.33± 16.68a
Digested sago effluent*	NA	1340± 520	2.87± 0.48	40.0± 1.33	21.0± 4.21	NA	NA
Digested pig waste effluents**	236.5	460	12.8	106.23	34.29	NA	NA
Fish culture effluent ***	NA	NA	5± 0.185	1.27± 0.140	0.67± 0.059	NA	NA
Pig wastewater recycling process ****	NA	2746-4157	1209-1481	NA	164-620	3099-5450	NA

All data are presented as mean ± standard deviation of triplicate, LD = Lower than detection limit (0.01 $mg.L^{-1}$ of NO_3-N), NA = data not available, the English alphabet letters stand for the statistical comparison between the groups using the ANOVA and post-hoc Tukey's b tests. Source: *[7]; **[5], *** [6]; **** [19]

3.2 Cultivation of *A. platensis* with Lac Wastewater

From the comparison of *A. platensis* growth in the cultivation with Zarrouk medium and CMU02 medium, it was found that the growth in CMU02 medium was slightly lower than that of Zarrouk medium (Figure 2). This was because this medium is lacking in trace elements [4]. However, the cost of Zarrouk medium is higher than CMU02 as the two media are priced at 0.074 US$.$L^{-1}$ and 0.013 US$.$L^{-1}$, respectively (Table S1, S2). The previous report found that the second most significant cost for *A. platensis* biomass production is the cost of the nutrients (15-25% of total operation cost)

[3]. From the estimation of *A. platensis* commercial production in Thailand, the cost of production (excluding capital costs) should be approximately 20-30 US$ per kg of dried biomass powder. However, the cost of *A. platensis* biomass in this study was comparatively high because this study was only performed on the laboratory scale. To improve the production capability of CMU02 medium, lac wastewater was supplemented in order to alternate the trace elements in the standard medium.

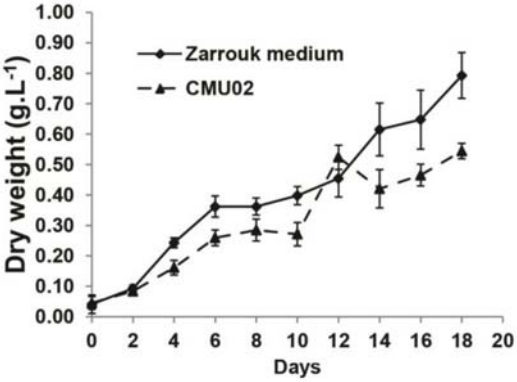

Figure 2. The growth of *A. platensis* on Zarrouk medium and CMU02 medium.

Figures 3A and 3B show the growth based on the dry weight of *A. platensis* when using various media. It was found that *A. platensis* did not survive when it was cultivated using diluted lac wastewater. However, *A. platensis* could be cultivated in CMU02 supplemented with these lac wastewater samples. However, the productivity from the treatment cultivated with CMU02 was higher than those of the treatments supplemented with centrifuged and final washed samples. This is because the organic matters in the centrifuged and final washed samples were still made up of the macro-molecules that *A. platensis* could not properly utilize. However supplying wastewater from the L pond and the WW1 pond with CMU02 medium increased the productivity, and this was higher than that which had been cultivated only with CMU02. In addition, specific growth rate (μ) was also improved (Table 3). Specific growth rates obtained from the supplementation of wastewater from L and WW1 ponds were higher than those that had been obtained from Zarrouk medium. This is because after chemical treatment, the lac wastewater was left in the L pond or passed to the WW1 pond where the remaining organic matters were degraded by microbes, resulting in it becoming nutrient rich and a growth factor for *A. platensis*. The productivity from CMU02 supplemented with WW1 is comparable with that of the previous report with regard to cultivation using anaerobic effluents from digested pig waste and digested sago effluent (Table 3), both in terms of gross biomass productivity and biomass concentration. However, the lag phase in this study was quite long, by approximately 1 week. This was perhaps because *A. platensis* needs to adapt to the media and the seed inoculation used in this study was slightly low. In further research studies, the concentrations of seed inoculation should be varied to shorten the lag phase and to increase productivity.

A crude cost estimation of *A. platensis* biomass was calculated to compare the stimulated effect of lac wastewater on cultivation. It was found that the supplementation of wastewater from the WW1 pond with CMU02 could be the most promising treatment and could reduce the biomass cost by approximately 14% from that of CMU02 only. Additionally, the cost of biomass from CMU02 plus WW1 was only one-fourth of that from the Zarrouk medium. However, the biomass cost from this study is relatively high because it was conducted in a batch culture. Generally, the culture could be harvested in 4-5 cycles

with very little nutrient addition in the semi-continuous culture [19]. The reuse of the medium after harvesting could significantly reduce the biomass costs. In addition, the crude cost was estimated only from the medium cost. The other costs, such as electricity, labour, harvesting and drying, also influence the production cost. Hence, a reduction in these production processes will also result in the cultivation process to be more economically feasible.

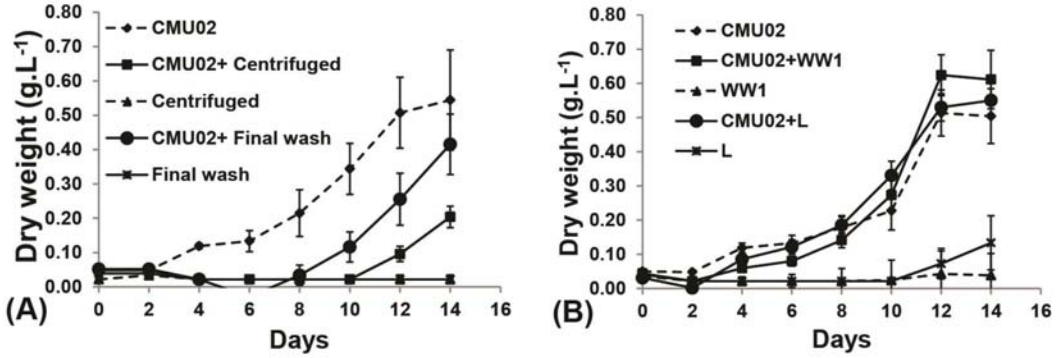

Figure 3. The growth of *A. platensis* on CMU02 medium with various lac wastewater treatments; (A) with wastewater from centrifuged and final wash treatments; (B) with L pond and WW1 pond.

Table 3. Specific growth rate, biomass productivity and estimated biomass cost of *Arthrospira (Spirulina) platensis* in various media.

	μ (day^{-1})	Biomass productivity ($mg.L^{-1}.day^{-1}$)	Gross biomass productivity ($g.m^{-2}.day^{-1}$)	Biomass concentration ($g.L^{-1}$)	Estimated cost ($US\$.kg^{-1}$)
Zarrouk medium	1.02	44.04	13.21	0.793	93.42
CMU02	0.69	37.31	11.19	0.534	24.61
Centrifuged	ND	ND	ND	0.022	603.04
CMU02+ Centrifuged	0.23	14.55	4.36	0.204	64.55
Final wash	ND	ND	ND	0.022	603.04
CMU02+ Final wash	0.50	29.66	8.90	0.415	31.66
L	0.16	9.55	2.86	0.134	98.35
CMU02+L	1.18	39.31	11.79	0.530	24.80
WW1	0.02	3.59	1.08	0.043	305.10
CMU02+WW1	1.09	52.04	15.61	0.624	21.05
Digested sagoeffluent*	0.45-0.57	NA	5.32-7.42	0.556-0.610	NA
Pig wastewater recycling process **	NA	NA	11.8-15.1	NA	NA
Fish culture effluent***	0.11	NA	NA	NA	NA

ND = not determined, NA = data not available, Source: *[7]; ** [19]; *** [6].

3.3 Nutritional and Valuable Product Determination

Protein content was measured using the *A. platensis* that had been cultivated with CMU02 supplemented with lac wastewater; the *A. platensis* cultivated with only lac wastewater produced too few cells for analysis. The protein content of the *A. platensis* cultivated with CMU02 plus lac wastewater from the L and WW1 ponds was higher than that of the other lac wastewater samples. Protein content of the CMU02 plus WW1 sample was the highest among all samples and was not significantly different from that of the Zarrouk medium. The protein content from CMU02 supplemented with WW1 was also higher than the cultivation of other wastewater samples such as the effluent from the pig wastewater recycling process [19] and fish culture effluent [6], but was at the same level of that acquired from the digested sago effluent [7]. Protein content is affected by the N:P ratio of the medium. Previous studies have reported that the recommended N:P ratio should be approximately 9:1 [7].

The *A. platensis* cultivated with CMU02 plus L and WW1 also had higher pigment content, as well as higher carotenoid and phycocyanin contents. This is because these samples contained high amounts of nutrients and growth factors for *A. platensis* from bacterial degradation, especially nitrogen sources such as ammonia. Prior studies have reported that nitrogen concentration influences the amount of protein and phycocyanins [13]. In addition, the WW1 supplement increased the turbidity, which limited light penetration. *A. platensis* must accumulate sufficient photosynthetic pigments to harvest light energy [20].

From the results in this study, it is suggested that the lac wastewater displayed advantages with regard to growth rate, cost reduction and pigment productivity, this is especially apparent with regard to lac wastewater from the WW1 pond. Even there have been a few reports about the human pathogens in the lac production. The utilization of the wastewater in food production may concern customers. Thus, CMU02 supplemented WW1 should be used for phycocyanin production instead.

In addition to increasing productivity, cultivating *A. platensis* by supplementation with lac wastewater can benefit the environment. Using lac wastewater to cultivate *A. platensis*, and the corresponding reduction in effluent discharge at the lac factory, would also improve its carbon footprint. The organic matter remaining in the ponds would then be degraded by microbes, leading to the generation of greenhouse gases such as methane (CH_4) and carbon dioxide (CO_2) during the night [21]. Thus, the supplementation of lac wastewater would reduce the volume of wastewater and organic substances, which would lead to a reduction in greenhouse gas emissions. For more efficient use and transport, the organic matter in the water could be concentrated into lac waste solids. After being digested by bacteria, these solids could be applied as organic fertilizer to cultivate either *Arthrospira* or other economically beneficial plants. The development of lac waste solids for organic fertilizer will be investigated in a future study.

4. CONCLUSIONS

Overall, the results from this study indicated that treated lac wastewater could be used to boost cultivation of *A. platensis* in low cost medium to achieve a higher yield. The supplementation of lac wastewater could reduce the estimated crude biomass cost while still maintaining

the biomass and pigments productivity comparable to those of the standard medium. CMU02 medium supplemented with treated lac wastewater may be a cheap source of nutrients for target phycocyanin production.

Table 4. Protein, carotenoids and phycocyanin of *Arthrospira (Spirulina) platensis* in various media.

	Protein content (g.100g DW^{-1})	Carotenoids (mg.g DW^{-1})	Phycocyanin (mg. g DW^{-1})
Zarrouk medium	61.02±3.12c	0.18±0.00c	114.28±10.75d
CMU02	56.85±2.17b	0.07±0.01a	38.59±3.12a
Centrifuged	ND	ND	ND
CMU02+ Centrifuged	43.81±2.66a	0.13±0.01b	56.06±2.31b
Final wash	ND	ND	ND
CMU02+ Final wash	43.65±1.52a	0.12±0.02b	50.19±4.10b
L	ND	ND	ND
CMU02+L	55.18±2.17b	0.15±0.03bc	65.95±2.78c
WW1	ND	ND	ND
CMU02+WW1	59.11±0.94bc	0.17±0.0c	71.30±2.38c
Digested sagoeffluent*	60.34±4.31	ND	ND
pig wastewater recycling process **	48.9	ND	ND
Fish cultureeffluent***	43.84	ND	ND

All data in this study are presented as mean ± standard deviation of triplicate, ND = not determined, the English alphabet letters represent the statistical comparison between the groups using the ANOVA and post-hoc Tukey's b tests. Source: *[7]; ** [19]; *** [6].

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