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Optimization of *Arthrospira platensis* growth using organic culture medium

Aporn Bualuang[1], Bongkoj Boonburapong[2], and Surasak Laloknam[2,*]

[1]Chulabhorn International College of Medicine, Thammasat University, Pathumthani, Thailand
[2]Department of General Science, Faculty of Sciences, Srinakharinwirot University, Bangkok, Thailand
[*]Corresponding author: surasak2515@hotmail.com

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Abstract

In this study, an organic culture medium for *Arthrospira platensis* (Spirulina) was created using sugarcane molasses (SGCM), soybean meal (SBM), and small fish bone meal (SFBM) as substitutions for macronutrients and micronutrients in Zarrouk's medium. Cell growth of *A. platensis* was monitored using spectrophotometric analysis. The growth patterns of *A. platensis* under normal and lack of carbon, nitrogen, and micronutrient conditions showed that the optimal growth of a lack of carbon, nitrogen, and micronutrient was decreased by about 50% compared to normal conditions. A singular material was designed to substitute macronutrients and micronutrients in Zarrouk's medium. In the SGCM medium, the total reducing sugars were varied by concentration in the range of 0 to 3.0% (w/v), while the total protein in the SBM medium was similar. The percentage of dried SFBM in the SFBM medium was also in the concentration range of 0 to 1.0 % (w/v). The results showed that the optimum concentrations of the SGCM, SBM and SFBM media were 0.2% (w/v), 0.5% (w/v) and 0.1% (w/v), respectively. An organic combination medium was designed using optimal concentrations of SGCM, SBM, and SFBM. For a period of 30 days, cell growth of *A. platensis* was studied and compared to Zarrouk's medium. The wet weights of *A. platensis* in the combination medium and Zarrouk's medium were 31.25 g and 33.46 g, respectively. The findings indicated that the combination-medium composed of organic substance medium could be used successfully as a culture medium for *A. platensis* within organic system.

Keywords: *Arthrospira platensis*, Organic, Small fish bone meal, Soybean meal, Sugarcane molasses

1. Introduction

Cyanobacteria, also known as blue-green algae, are oxygenic photosynthetic bacteria. *Arthrospira platensis* (whose commercial name is Spirulina) is a filamentous cyanobacterium of the family Oscillatoriaceae that grows in high pH (up to pH 11) solutions that contain high levels of carbonate and bicarbonate. *Arthrospira platensis* contains a high biological-value protein content, polyunsaturated fatty acids (gamma-linolenic, linoleic), and has a low content of nucleic acids (4-6 % of its dry weight) and vitamin B12 [1,2]. Spirulina biomass has been used in many applications, including for human consumption, animal feed, feed additives, and aquaculture [3]. The US FDA (Food and Drug Administration) and EFSA (European Food Safety Authority) have reported that *A. platensis* is listed as generally recognized as safe (GRAS) [2]. This species is also used in the pharmaceutical industry, for waste treatment, and as a component of fuel production [4-7].

Organic food is concerning from the standpoints of health, ecology, fairness, and care [8], although it has caused an increase in demand from consumers [9]. In general, the Spirulina is usually cultured in Zarrouk's medium. The source of nitrogen used for culturing with Zarrouk's is either $NaNO_3$ or KNO_3. A cheaper nitrogen source, urea, has also been used [10]. Subsequently, many other media have been modified to decrease the medium cost and mass production. Molasses, the viscous concentrated liquid derived from sugarcane or sugar beets-even after removing varying amounts of sucrose-continues to contain water, sugars, other carbohydrates, ash, nitrogenous and non-nitrogenous compounds, wax, sterols, phosphatides, and vitamins [11,12]. Furthermore, soybean meal (SBM) is a by-product derived from the manufacture of soybean oil, tofu, and soy milk. The main

components of SBM are ruptured cotyledon cells and soybean seed coats, both of which are rich in cell-wall polysaccharides. The chemical characterization of these by-products reveals their protein, oil, dietary fiber, and mineral constituents, along with unspecified monosaccharides and oligosaccharides [13, 14]. Both soybean meal and full-fat soybean meal are used in the animal feed industry [15]. Small fish bone meal (SFBM) is made from small pelagic fishes, including anchovies, herring, mackerel, and sardines. These small pelagic fishes are a non-food group used in the production of fish meal, fish oil, and animal feed ingredients, which are also fed directly to animals [16]. SFBM is used to make fish sauce [17]. In addition, fish meal contains protein, lipid, antioxidants, vitamins, and minerals. Ash of fish meal has a high mineral content, especially calcium, phosphorus, and magnesium [18].

The goal of the present study was to develop a novel organic cultivation medium for *A. platensis* in a laboratory by using a combination of soybean meal, sugarcane-derived molasses, and small fish bone meal.

2. Materials and methods

2.1 Microorganism and culture conditions of the inoculum

The strain of *A. platensis* used was obtained from a collection of cyanobacteria samples currently in the possession of the Thailand Institute of Scientific and Technological Research (TISTR). The inoculum was produced by culturing *A. platensis* in Zarrouk's medium [19]. To study the effect of sugarcane molasses (SGCM), SBM, and SFBM on *A. platensis*, mid-log phase *Arthrospira* cells were transferred to a culture medium containing SGCM, SBM, and SFBM at a pH of 9.0. All constituents of the medium were then autoclaved at 121°C for 15 minutes. Hence, *Arthrospira* growth was monitored every three days, for 30 days, by measuring the optical density at 750 nm (OD_{750}) using spectrophotometric analysis (Hitachi). *Arthrospira* cells were grown under the illumination provided by fluorescent lamps at 30 ± 2 °C on a shaker rotating at 150 rpm using conical flasks. The wet weights were determined.

2.2 Pre-treatment with sugar-cane-molasses, soybean meal, and small-fish-bone meal

The sugarcane molasse was obtained from a local soy-milk shop in Ayutthaya, Thailand. The solid form of the soybean meal used was obtained from a local soy-milk shop in Bangkok, Thailand. Residual water was removed by drying the meal at 60°C, after which it was ground into a coarse powder. The small-fish-bone meal was obtained from a local shop in Songkhla, Thailand, and was also ground into a coarse powder. These three samples were prepared in a stock solution concentration of 1 % w/v. The three suspensions were individually autoclaved and then left to precipitate. The supernatants from these precipitated solutions were used to formulate the new medium.

2.3 Experimental design

The macro-and micro-nutrient components of Zarrouk's medium are shown in Table 1. For this set of experiments, sodium bicarbonate ($NaHCO_3$), the usual carbon-source, was replaced by SGCM; sodium nitrate ($NaNO_3$), the usual nitrogen-source, was replaced by SBM; and the other micronutrient-constituents (NaCl, K_2SO_4, K_2HPO_4, $MgSO_4.7H_2O$, EDTA (Na), $CaCl_2.2H_2O$ $FeSO_4.7H_2O$, A5) were replaced by SFBM.

2.4 A $NaHCO_3$-free Zarrouk's medium replaced by SGCM

$NaHCO_3$ was replaced by SGCM. The total reducing sugars concentration in SGCM was determined using the Dinitrosalicylic acid (DNS) method [20]. The total reducing sugars concentration of SGCM was 61.2 g/L, and final reducing sugars concentrations were varied between 0 % and 3% (w/v).

2.5 A $NaNO_3$-free Zarrouk's medium replacing $NaNO_3$ with SBM

In this medium, sodium nitrate ($NaNO_3$) was replaced by SBM. The total protein concentration in SBM was determined using Bradford's method [21]. The total protein concentration of SMB was 1.62 g/L. The final concentrations of total protein in SBM were varied from 0 to 3% (w/v).

2.6 A micronutrients -free Zarrouk's medium replaced by SFBM

This SFBM mediums were varied the concentration between 0 and 1% (w/v) to replace micronutrient-constituents.

2.7 The combination-medium of SGCM, SBM, and SFBM

The combination-medium mixture was comprised SGCM, which contributed 0.2 % reducing sugars, SBM, which contributed 0.5 % total protein, and SFBM at 0.1% (w/v).

2.8 Statistical analysis

All the experiments were performed in triplicate. The results were expressed as mean ± standard deviation (SD).

Table 1 Experimental design of the organic culture medium.

Chemicals	Zarrouk's medium (g/L)	Substitutions made to design the organic culture medium
$NaHCO_3$	16.8	Substituted with optimal SGCM concentrations
$NaNO_3$	2.5	Substituted with optimal SBM concentrations
NaCl	1.0	Substituted with optimal SFBM concentrations
K_2SO_4	1.0	
K_2HPO_4	0.5	
$MgSO_4.7H_2O$	0.2	
EDTA (Na)	0.08	
$CaCl_2.2H_2O$	0.04	
$FeSO_4.7H_2O$	0.01	
*A5 micronutrient	1 ml	

*A5 micronutrient: H_3BO_3, 2.86; $MnCl_2$.$4H_2O$, 1.81; $ZnSO_4$.$4H_2O$, 0.222; Na_2MoO_4, 0.0177; $CuSO_4$. $5H_2O$, 0.079 (g/L)

3. Results and discussion

3.1 Growth of A. platensis under normal, macronutrients and micronutrients deficiency

The growth pattern of *Arthrospira* cells under normal conditions in Zarrouk's medium is shown in Figure 1(A). The growth patterns with a lack of $NaHCO_3$ are shown in Figure 1(B), while growth patterns with a lack of $NaNO_3$ are shown in Figure 1(C), and growth patterns with a lack of micronutrients are shown in Figure 1(D).

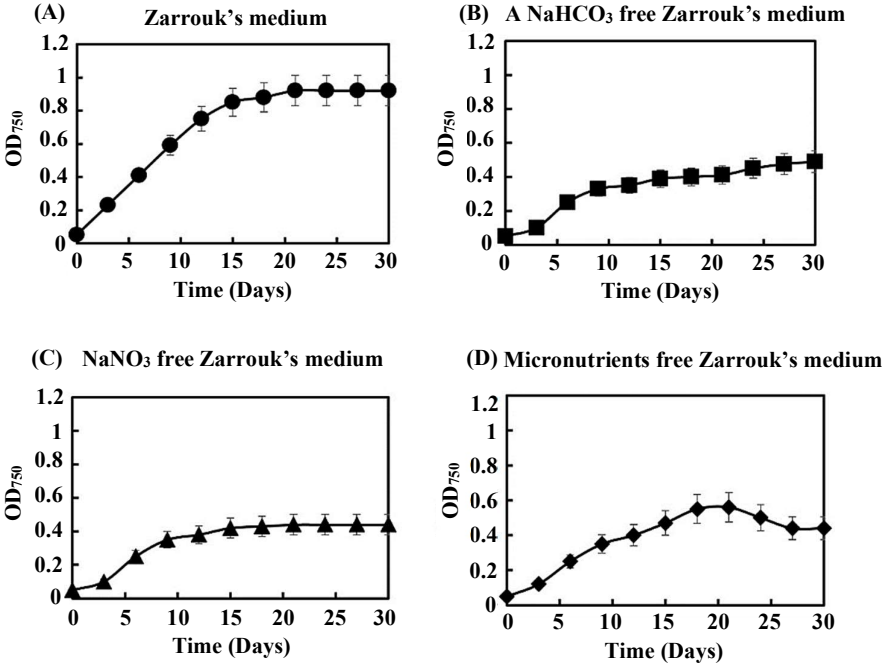

Figure 1 Optical density of *Arthrospira platensis* in (A) Zarrouk's medium (the filled circles), (B) $NaHCO_3$-free Zarrouk's medium (the filled squares), (C) $NaNO_3$-free Zarrouk's medium (the filled triangles), and (D) Micronutrients-free Zarrouk's medium (the filled diamonds). The data are representative growth curves of three separate experiments.

Figure 1 revealed the growth patterns of *A. platensis* under normal and lack of carbon, nitrogen, and micronutrient conditions. The control growth condition in Zarrouk's medium showed the OD_{750} as approximately 1.0 at day-15 (Figure 1A), and optimal growth was decreased by about 50% under the medium free $NaHCO_3$ (Figure 1B). It might be that $NaHCO_3$ is used as a carbon source to maintain alkaline conditions and osmotic pressure during the culturing of *Arthrospira*/*Spirulina*. Also, the reduction of $NaHCO_3$ concentrations in the growth medium showed a decrease in the growth rate of *Spirulina* sp. [22]. Figure 1C showed that the optimal growth rate was half that of the normal condition (Figure 1A). These results might be because sodium nitrate was necessary for growth since nitrogen is an important ingredient. El-Shouny et al reported that the growth of *A. platensis* was decreased under free sodium nitrate Zarrouk's medium after six days, and the inhibition reached 98 % after 20 days [23]. The conventional nitrogen sources were potassium or sodium nitrates, including the growth medium. In attempts to reduce the cost of the growth medium, other nitrogen sources such as ammonium sulfate, ammonium chloride, and urea have been attempted [10, 24, 25]. The growth pattern of *A. platensis* in the free micronutrient medium (Figure 1D) showed a similar reduction of 50% growth compared to the normal condition, which is similar to free carbon and free nitrogen sources. For example, phosphorus was one important ingredient in the medium to promote the growth of cyanobacteria, which is involved in many cellular processes such as energy transfer, biosynthesis of nucleic acids, and DNA [26]. A previous study reported that the biomass production of *A. platensis* was decreased by half when cultivated in batch processes to reduce phosphate (K_2HPO_4) by about 20 fold [27].

3.2 The effect of sugarcane-molasses on the growth of A. platensis

The growth of *A. platensis* was investigated in $NaHCO_3$-free Zarrouk's medium when replaced with various SGCM concentrations, as monitored by OD_{750} at day 15 of the culture, data as shown in Figure 2.

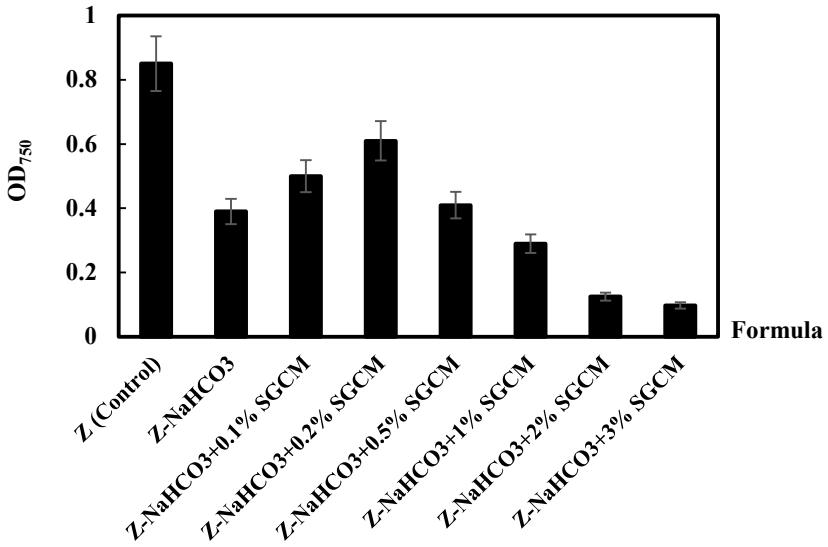

Figure 2 Optical density of *Arthrospira platensis* cells at day 15 cultivated at various concentrations of SGCM (0, 0.1, 0.2, 0.5, 1, 2 and 3% (w/v) of reducing sugar) after replacing the $NaHCO_3$ in Zarrouk's medium. The data are the average of three replicates ± S.E. (n=3)

Figure 2 demonstrates that the concentration of 0.2% SGCM for reducing sugars resulted in the highest growth rate (about 70 % compared with Zarrouk's medium control). However, an increase in the concentration of SGCM from 0.2 to 3 % reducing sugars resulted in the reduction of cell growth (from 50 – 90 %). Since molasses contains 50% of all the usual sugars, it was used as a substitute for $NaHCO_3$. The medium in which $NaHCO_3$ was substituted with SGCM at a concentration of 0.20% (w/v) for reducing sugars showed that the growth of *A. platensis* was up 70% compared to the growth in the Zarrouk's medium control unit. It appears, therefore, that it is possible to replace the carbon source with molasses, which is analogous to other reports that used supplemental molasses/sugar beet vinasse in Zarrouk's medium when cultivating *Spirulina platensis* [28-30]. However, higher concentrations of molasses (at 2 and 3% (w/v) reducing sugars) inhibited cell growth. According to Yeesang and Cheirsilp (2014) [31], the growth of *Botryococcus braunii* microalga was decreased when cultivated under a high concentration of molasses (20 g/L). Therefore, the high concentration of molasses was due to the effect of the dark color of the molasses medium, which caused less light penetration, thereby drastically reducing the growth of *B. braunii* [31].

3.3 Effect of soybean meal on the growth of A. platensis

The growth of *A. platensis* was investigated under the $NaNO_3$-free growth medium being replaced by various SBM concentrations and measuring OD_{750} at day 15 of the culture, data as shown in Figure 3.

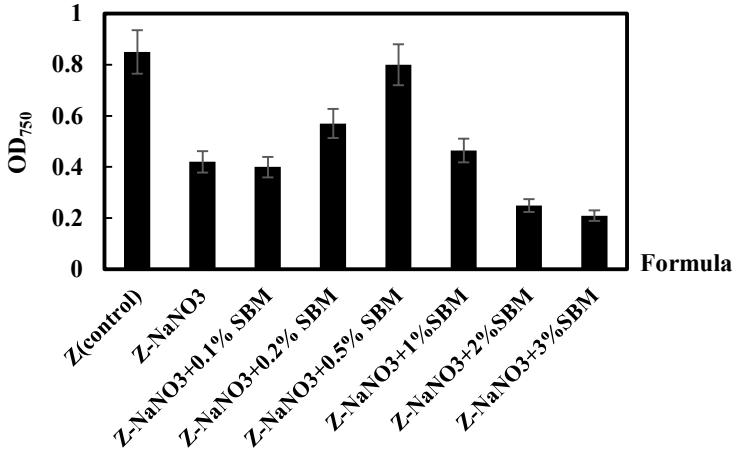

Figure 3 Optical density of *Arthrospira platensis* cells at day 15, cultivated at various concentrations of SBM (0, 0.1, 0.2, 0.5, 1, 2 and 3% (w/v) of total protein) replacing the $NaNO_3$ in Zarrouk's medium. The data are the average of three replicates ± S.E. (n=3)

Figure 3 demonstrates the results at day 15 of the culture. A concentration of 0.5 % SBM for total protein gave the OD_{750} equal to 0.8 and was similar to the control (OD_{750} = 0.85). Under other conditions, *A. platensis* grew in the 25-73% range. Lyophilized soybean meal contains 6.99% H, 46.34% C, 3.99% N, 0.25% S, and 3.59% metal oxides as ash [32]. A total protein concentration of 0.5% (w/v) via SBM promoted growth similarly to the use of Zarrouk's medium. In contrast, a higher concentration of 1-3% (w/v) SBM for total protein reduced the growth of *A. platensis*. Shanthi et al (2018) [33] reported the effect of nitrogen source replacement with 18 amino acids on the growth of *S. platensis* at a concentration of 0.05 g/L. The kind of amino acid groups affected the growth of *S. platensis* if the medium included group one (Arg, Pro, Glu, Asp, Gln, and Asn), group two (Trp, Ala, Leu, His, Ser, and MeT) and group three (Pro, Thr, Tyr, Gly, Val, and Cys), in which the growth was increased, equal and decreased when compared to the control [33]. The approximate composition of SBM contains up to 27% protein on a dry basis [32]. A higher concentration of SBM contains similar amino acids to the final group above, resulting in the reduction of *A. platensis* growth [33].

3.4 The effect of small fish bone meal medium on the growth of A. platensis

In the case of the medium in which the micronutrients were replaced by various SFBM concentrations and measured by OD_{750} at day 15 of the culture (Figure 4).

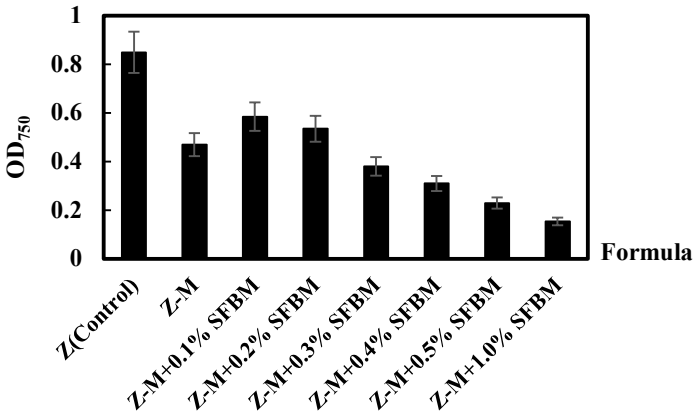

Figure 4 Optical density of *Arthrospira platensis* cells at day 15 cultivated at various concentrations of SFBM (0, 0.1, 0.2, 0.5, 1, 2 and 3% (w/v), replacing micronutrients (NaCl, K_2SO_4 K_2HPO_4, $MgSO_4.7H_2O$, EDTA (Na), CaCl2.2H2O $FeSO_4.7H_2O$ and A5) in Zarrouk's medium. The data are the average of three replicates ± S.E. (n=3)

Figure 4 shows that the normal condition had the highest growth and the micronutrient-free medium was reduced 50% compared with the normal condition. The growth of *A. platensis* under micronutrient-free medium supplementation with 0.1 and 0.2% of SFBM was increased compared with the micronutrient-free medium condition, whereas the growth of *A. platensis* was decreased under high nutrient concentration conditions (> 0.3 %). Fish meal is normally used as the main source of protein, essential amino acids, essential fatty acids, digestible energy, minerals (Calcium, Phosphorus, Sodium, Magnesium, Potassium, Iron, Zinc, and Selenium), and vitamins (Panthothenic acid, Riboflavin, Nicotinic acid, Folic acid, Choline, Vitamin B12, and Biotin) in the aquafeed industry [34, 35]. This data showed that a suitable concentration of SFBM could be used as a source of micronutrients.

3.5 The combination mixture of SGCM, SBM, and SFBM

The combination mixture of SGCM, SBM, and SFBM medium was modified to study the growth pattern of *A. platensis* compared with the control (Zarrouk's medium) and distilled water conditions (Figure 5).

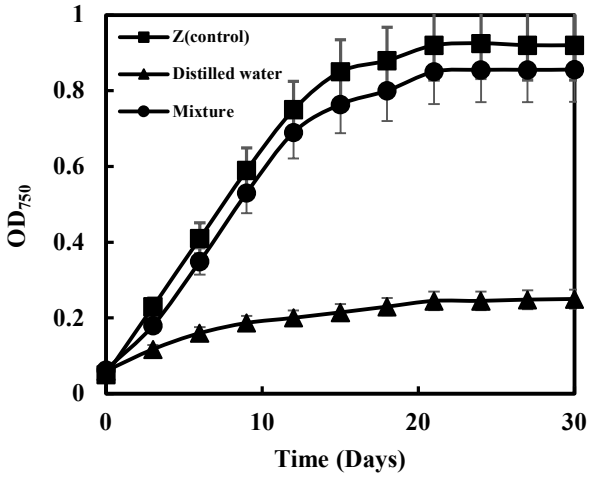

Figure 5 Optical density of *Arthrospira platensis*. The combination mixture of organic mediums. The data are the average of three replicates ± S.E. (n=3)

Figure 5 shows the growth pattern of *A. platensis* in the combination mixture of SGCM, SBM, and SFBM, which was equivalent to the control medium. After 30 days of growth culture, the wet weight of the *A. platensis* was measured under all conditions (Table 2).

Table 2 Wet weight of *A. platensis* in Zarrouk's medium, distilled water, and the combination of organic medium

Formula	Wet weight (g)*
Zarrouk's medium	33.46 ± 0.43
Distilled water	1.50 ± 0.37
Combination of organic medium	31.25 + 0.65

*The cells were grown in 1 L medium

The wet weights of *A. platensis* at day 30 of culture under Zarrouk's medium and the mixture of substances medium were 33.46 and 31.25 g, respectively (Table 2). The combination of the organic medium could promote the growth patterns (Figure 5) and wet weight similarly to the control medium (Table 2). These results might be the combination medium substituted the macronutrients and micronutrients of Zarrouk's medium with an appropriate concentration. Most published research only substituted either low-cost substances to promote the growth of *A. platensis* or selected one type of agricultural material as a replacement in Zarrouk's medium, such as molasses, soybean, cassava taro, and potato [28, 30, 36, 37]. The calculated cost of 1000 L is shown in Table 3. The cost of Zarrouk's medium and the organic medium are 297.734 US$ and 15.971 US$, respectively; the cost of Zarrouk's medium is almost 20 times more expensive than the organic medium. Table 4 shows the cost of the medium, which previous studies compare with our results. The present study showed a decrease in the cost higher than the previous study by 4-fold. This study was the first to report that *A. platensis* could grow in a novel organic medium with low-cost materials.

Table 3 Comparison of costs between Zarrouk's medium and the organic medium.

Zarrouk's medium				Organic medium			
Chemicals	Conc. (g/L)	Prices (US$ /g)	Cost (US$/1000L)	Material	Conc. (g/L)	Prices (US$ /unit)	Cost (US$/1000L)
$NaHCO_3$	16.8	12.12/1000	203.636	SGCM	0.32	0.61/2000 g	0.098
$NaNO_3$	2.5	20.91/1000	52.273	SBM	30.86	0.48/1000 g	14.964
NaCl	1	5.45/1000	5.455	SFBM	1.00	0.91/1000 g	0.909
K_2SO_4	1	14.85/1000	14.848	The SGCM we used had a total reducing sugars concentration of 61.2 g/L			
K_2HPO_4	0.5	13.64/500	13.636				
$MgSO_4.7H_2O$	0.2	10.61/500	4.242	The SBM used had a total protein concentration of 1.62 g/L			
EDTA (Na)	0.08	31.82/1000	2.545				
$CaCl_2.2H_2O$	0.04	9.09/500	0.727				
$FeSO_4.7H_2O$	0.01	10.61/500	0.212				
H_3BO_3	0.00286	10.61/500	0.061				
$MnCl_2 .4H_2O$	0.00181	23.94/500	0.087				
$ZnSO_4 .4H_2O$	0.000222	22.12/1000	0.005				
Na_2MoO_4	0.0000177	110.61/500	0.004				
$CuSO_4. 5H_2O$	0.000079	13.03/500	0.002				
		Total	297.734			Total	15.971

Cost in US$ was transformed from Thai Baht by using 33 Baht = 1 US$ Chemicals used are Kemaus and Qrec

Table 4 Cost of an *A. platensis* medium in the present study compared to previous investigations

Year	Medium	Major components in medium (g/ L)	Medium cost (US$ /1000L)	Zarrouk's Medium cost (US$ /1000L)	Fold
2006 [22]	RM_6	$NaHCO_3$ (8.00); $NaNO_3$ (2.50); SSP (1.25); MOP (0.90)	16.00	79.5	4.97
2012 [38]	Reduced cost medium	$NaHCO_3$ (16.80); NH_4NO_3 (0.35); SSP (1.25); MOP (0.90)	13.00	80	6.15
2016 [39]	CMU02	$NaHCO_3$ (8.50); $NaNO_3$ (1.50); K_2HPO_4 (0.50); NPK-16:16:16 (0.60)	13.00	74.00	5.69
In this study	Organic medium	SGCM (0.32); SBM (30.86); SFBM (1.00)	15.971	297.734	18.6

4. Conclusion

The wet weights of *A. platensis* in this combination medium (consisting of 0.2% SGCM for reducing sugar, 0.5 % SBM for total protein, 0.1% (w/v) of SFBM) and Zarrouk's medium were 31.25 g and 33.46 g, respectively. This finding indicates that a combination medium composed of these organic substances could be used as an alternative culture medium for *A. platensis* in an organic system.

5. Acknowledgements

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