المؤتمر الأول للابتكار وريادة الأعمال في الجامعات السعودية(فكرة واختراع وأثر) ، م ١ ع١، ص: ٢٢٣- ٢٣٣(٢٠٢٥م)
DOI:10.4197/Ieckau.1-1.10

Potentiality of huge revenue drawn through *Arthrospira* (*Spirulina* sp.) *platensis* KAU02 (Cyanophyceae) cultivation in treated wastewater rather than discharge into sea

Md. Abu Affan and Lafi Saed Al Solami*
Department of Marine Biology, College of Marine Sciences,
King AbdulAziz University, Saudi Arabia
** Major corresponding author.*
E-mail address: lalsulami@kau.edu.sa.

Abstract. Study on microalgae continues to increase due to its various usages such as live feed, feed supplements, food additives and colourization, ingredients of medicine, cosmetics and biofuels. Among microalgae, *Spirulina* (*Arthrospira*) is the most important and popular for cultivation, since it is an excellent source of high protein, polyunsaturated fatty acids (omega 3 and 6) and carotene. Its cultivation has advantages over typical traditional agriculture because of lacking of by-product and ability to grow in arid, semi-arid, and in fresh or in salt water. However, the shortage of freshwater is becoming a serious environmental problem globally, on the other hand Ca^{2+} and Mg^{2+} of natural seawater cause phosphate (PO_4^-) dilatation problem above pH of 7.10, create milky turbidity and nutrient precipitation in *Spirulina* culture medium. In the Kingdom of Saudi Arabia (KSA), the main source of freshwater is the desalination system. An alternative source for cultivation of *Spirulina* was explored by using treated waste water since there are several waste water treatment plants in KSA. Thus, the treated waste was examined for cultivation of *Spirulina platensis* KAU02 (GenBank accession number: OR723792) isolated from the Red Sea. Therefore, the objective was to use different concentration of plant's nutrients with treated wastewater to determine the profuse growth and biochemical composition of *S. platensis* KAU02 in a newly formulated medium, naming Affan-Lafi-King AbdulAziz University-Spirulina (ALKAUS), and compared the biomass production and biochemical composition with standard Zarrouk's (1966). The maximum specific growth rate ($\mu_{max.}$ day^{-1}), dry biomass production and chlorophyll *a* content of *S. platensis* KAU02 was 0.293 and 0.294 day^{-1}, 1.75 and 1.80 g L^{-1} and 15.85 and 16.18 mg. L^{-1} in culture media of ALKAUS and Zarrouk's, respectively. The biochemical composition and phycocyanin content were similar in biomass of both cultures' media. Therefore, *S. platensis* KAU02 can be produced in industrial scale by using treated waste water following our invented method.

Keywords: Treated wastewater, Spirulina platensis KAU02, culture media, biomass production and biomass quality

Introduction

Study on microalgae continues to increase due to its various usages such as live feed, feed supplements, food additives and colourization, ingredients of medicine, cosmetics and biofuels (Richmond 1986, Borowitzka and Borowitzka 1988, Cresswell et al. 1989). Among microalgae, *Spirulina* is the most important and popular for cultivation, since it is an excellent source of high protein, polyunsaturated fatty acids (omega 3 and 6) and carotene, and having advantages over any

traditional agriculture due to lack of by-product and suitability to grow in arid, semi-arid, and in fresh or in seawater (Alonso and Maroto 2000). Usages of *Spirulina* spp. are not new, Aztecs used to harvest and consume *Spirulina* spp., from Lake Texcoco, Mexico, and even today, *Spirulina* spp., grown in Lake Chad is being consumed by indigenous Africans. *Spirulina* has been commercially grown and used as a safe food supplement for several decades. Freshwater *Spirulina* is being produced in most of the farms. The strain *S. pacifica* was developed from freshwater *S. platensis* and cultivated in Cyanotech farm, Hawaii (Cyanotech, Kailua-Kona, HI). In one side, the freshwater shortage is turning out a serious environmental issue in arid and semi-arid areas of the globe (Vorosmarty et al., 2000), and on the other hand, the use of typical seawater put forward several problems such as the solubility of PO_4^{3-} is negligible and the solubility of HPO_4^{2-} decreases above a pH of 7.1 due to the presence of high concentration of Ca^{2+} and Mg^{2+}. Additionally, the culture medium becomes milky turbid with the presence of phosphate, $NaHCO_3$ and or Na_2CO_3, and in few hours turbid materials and *Spirulina* start to be sedimented which causes dies off of *Spirulina* and moreover, it makes medium nutrients deficiency (Materassi et al., 1984). Previously, human urine and pig farms treated wastewater were used for cultivation of *Spirulina* sp. in China and Korea to minimize freshwater crisis and nutrients costs (Hong and Lee, 1993; Lun and Cheng, 2006). *Spirulina* spp. were also cultured in anaerobic effluent was supplemented with natural seawater in Mexico, even though the turbidity of the culture medium was persisted (Gagneux et al., 2007).

However, the demand of freshwater is being increased due to rapid urbanization and on the other hand environmental pollution is also increasing because of wastewater releasing (WHO, 2014). Therefore, urban wastewater treatment is vital since it could be considered for renewable water resource which is proportionally increasing with the increasing of population. The usages of treated wastewater for different purposes depend on the degree of its treatment (primary, secondary and tertiary) and the tertiary level treated wastewater is free from all health hazards and can be used to irrigate all crops (Dawouda et al., 2022). Current studies of using treated wastewater as irrigation source in Saudi Arabia shown not only increasing of crops production but also act as a source of plant nutrients to minimize fertilizers utilization. Irrigation is mostly limited due to growing season of the crops though wastewater is generally produced continuously throughout the year. The kingdom of Saudi Arabia produces a total 850 million m^3 of treated wastewater annually (Dawouda et al., 2022). In Jeddah, most of the treated wastewater is being discharged to the Red Sea. The profitable and eco-friendly usages treated wastewater can bring huge revenue and created lot of job opportunity. One of the most promising way is to use of wastewater culture microalgae to minimize water crisis as well as to minimize the microalgae production cost as it contains plant's growth nutrients. In previous, municipal wastewater combined Zarrouk medium to cultivate *Spirulina* (Phang et al., 2000). Carbon and nutrients were removed with cultivation of *S. platensis* from monosodium factory wastewater by mixing with Zarrouk medium (Jiang et al., 2015; Park et al. 2013).

Zhao et al. (2017) conducted a study of *S. platensis* in treated wastewater by dilution of and they found that *S. platensis* formed the bottom. Wongsnansilp and Phinrub (2022) did culture of *Spirulina* in Thailand using different treated water by dilution of different concentration of Zarrouk's medium. They faced the precipitation of nutrients and clustering of *Spirulina* in all kinds of treated wastewater medium. Thus, a study was conducted to evaluate the growth and biomass production *Spirulina* cultured in treated wastewater using $NaHCO_3$, $NaNO_3$, K_2HPO_4, K_2SO_4, Na_2-EDTA and H_3BO_3 as additional nutrients rather than using Zarrouk's medium's (Zarrouk's, 1966) mentioned recipe. The objectives were to develop *S. platensis* culture medium and compare growth, biomass production and

biochemical composition of *S. platensis* KAU02 grown in newly formulated and traditional culture medium (Zarrouk's).

Materials and methods

The tertiary treated wastewater (TW) was collected from the Water Research Center of King AbdulAziz University, Jeddah, the Kingdom of Saudi Arabia. The TW was used to culture *S. platensis* KAU02 KAU02 (Genebank Accession number is OR723792).

Culture medium preparation

First step

Tertiary TW was used to prepare Zarrouk's medium for culture of *S. platensis* KAU02. The medium become turbid and chemical precipitated even though before inoculation of *S. platensis* KAU02. However, after inoculation of the *S. platensis* KAU02 was found to be cluster and sedimented after few hours in TW used Zarrouk's medium. Then, it was thought that high concentration of carbonic salts ($NaHCO_3$) might be related for this precipitation. The medium was again prepared with 25% concentration of $NaHCO_3$ of Zarrouk's medium recipe. Thereafter, 9 culture media *S. platensis* KAU02 were made using TW with different concentration of plant's nutrients chemicals and $NaHCO_3$ in the first step. The Zarrouk's medium was prepared using distilled water. *S. platensis* KAU02 was found to be clumping and medium was also found to be milky turbid. Therefore, culture media recipe was modified as second step (Table 1.).

Second step

Before starting second step, $NaNO_3$, K_2HPO_4 and K_2SO_4 of 2.50, 0.50, 1.00 g/L, respectively were diluted, and then three concentrations of $NaHCO_3$ were added 8.00, 9.00 and 10.00 g/L. The media were made and kept for overnight to observe whether precipitation will be occurred or not. Thereafter, 0.015 g. L^{-1} *A. platensis* was inoculated in each culture medium, and the results were not that of expecting level. Therefore, the culture media was again revised to get similar growth with standard (Zarrouk's) medium (Table 1.). In both of first and second steps, the *S. platensis* KAU02 was found to be pale in colour and died after two weeks of culture. To overcome the problem, Na_2CO_3 and KH_2PO_4 was added in newly formulated media. Thereafter, a final step culture was conducted in newly formulated media and in Zarrouk's medium (1966).

Final or third Step

In this step, culture media were prepared with dilution of $NaNO_3$, $NaHCO_3$, Na_2CO_3, K_2SO_4, KH_2PO_4, K_2HPO_4, Na_2CO_3, $FeSO_4.7H_2O$, Na$_2$-EDTA, $ZnCl_2.6H_2O$ and H_3BO_3. Then the biomass production was compared with that of the biomass production of Zarrouk's medium (Table 1).

Table 1. Selection of *S. platensis* KAU02 culture media using treated wastewater and different concentrations of chemicals in treated waste water.

	Expt. No.	$NaHCO_3$	Na_2CO_3	$NaNO_3$	KH_2PO_4	K_2HPO_4	K_2SO_4	NaCl	$MgSO_4.7H_2O$	$CaCl_2.2H_2O$	$FeSO_4.7H_2O$	Na$_2$-EDTA	H_3BO_3	Trace metal Sol.
	A1	8.00	1.00	2.50	–	0.50	1.00	–	0.120	0.041	0.010	0.090	0.005	0.5 ml
	A2	8.00	1.00	2.50	–	0.50	1.00	–	–	0.041	0.010	0.090	0.005	0.5 ml
	A3	8.00	1.00	2.50	–	0.50	1.00	–	–	–	0.010	0.090	0.005	0.5 ml
	A4	8.00	1.00	2.50	–	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml
1st step	A5	8.00	1.00	2.50	–	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml
	A6	8.00	1.00	2.50	–	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml

	B1	9.00	1.00	2.50	–	0.50	1.00	–	0.120	0.041	0.010	0.090	0.005	0.5 ml
	B2	9.00	1.00	2.50	–	0.50	1.00	–	–	0.041	0.010	0.090	0.005	0.5 ml
	B3	9.00	1.00	2.50	–	0.50	1.00	–	–	–	0.010	0.090	0.005	0.5 ml
	B4	9.00	1.00	2.50	–	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml
2nd step	B5	9.00	1.00	2.50	–	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml
	B6	9.00	1.00	2.50	–	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml
	ALKAUS-1	10.00	1.00	2.50	0.125	0.50	1.00	–	0.120	0.041	0.010	0.090	0.005	0.5 ml
	ALKAUS-1	10.00	1.00	2.50	0.130	0.50	1.00	–	–	0.041	0.010	0.090	0.005	0.5 ml
	ALKAUS-1	11.00	2.00	2.50	0.250	0.50	1.00	–	–	–	0.010	0.090	0.005	0.5 ml
	ALKAUS-1	11.00	2.00	2.50	0.125	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml
	ALKAUS-1	12.00	2.50	2.50	0.130	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml
3rd step	ALKAUS-1	12.00	2.50	2.50	0.250	0.50	1.00	–	–	–	0.005	0.090	0.005	0.5 ml
	Zarrouk's	16.80	–	2.50		0.50	1.00	1.00	0.12	0.041	0.010	0.080		1.0 ml

Growth, biomass production, chlorophyll *a* and biochemical and statistical analysis

S. platensis KAU02 biomass was determined by dry weight measurements. To estimate growth, a 20 ml of cultured samples were collected, filtered (GF/C Whatman filter paper) and dried. The samples were taken on every three days. Biomass contented filter papers were dried in an oven at temperature of 55°C, weighed, calculated (g. L^{-1}) and was used for plotting the growth curve. The specific growth rate (μ) was defined as the increase of biomass per unit time, and was calculated using the following formula (Pirt, 1975):

$\mu\ (day^{-1}) = \ln (X_1/X_2)/ (t_1-t_2)$ ……. (i)

Where, X_0 and X_1 are the biomass at the beginning (t_0) and the end (t_1), respectively of a selected time interval between inoculation and maximum biomass production.

For chlorophyll *a* estimation, culture was also collected on same day. Chlorophyll *a* content was determined following the method described by Parsons et al. (1984). Phycocyanin was determined following the methods described by Boussiba and Richmond (1994).

Biomass of *S. platensis* KAU02 was freeze dried, and the biochemical composition was determined following the Association of Official Analytical Chemist (AOAC) guidelines (AOAC,1995). The significance of differences of $\mu_{max.}\ day^{-1}$, biomass production and biochemical composition of *S. platensis* KAU02 was determined using Student's *t*-tests.

3. Results

3.1. Maximum specific growth rate ($\mu_{max}.\ d^{-1}$) of *S. platensis* KAU02

The $\mu_{max}.\ d^{-1}$varied from 0.262 to 0.277 in first step (Fig. 1A). In second step, $\mu_{max}.\ d^{-1}$was found to be varied from 0.268 to 0.280 (Fig.1B). However, the high growth rate was found in final step, in which the $\mu_{max}.\ d^{-1}$varied from 0.281 to 0.293 among the culture media (Fig.1C). In culture medium ALKAUS-6, the growth rate was 0.293 $\mu_{max}.\ d^{-1}$ which was similar with the growth rate of Zarrouk's medium (Fig.1C).

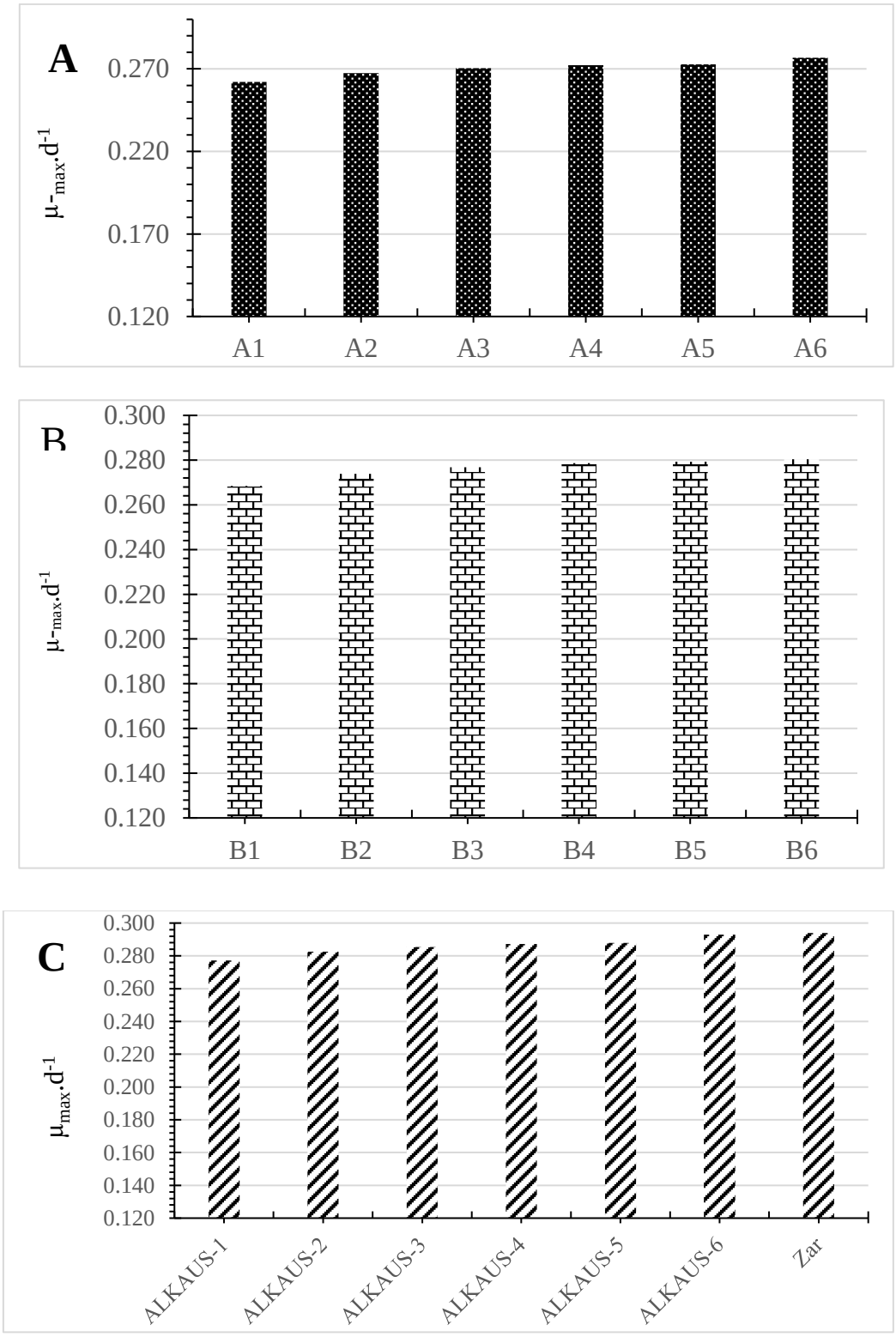
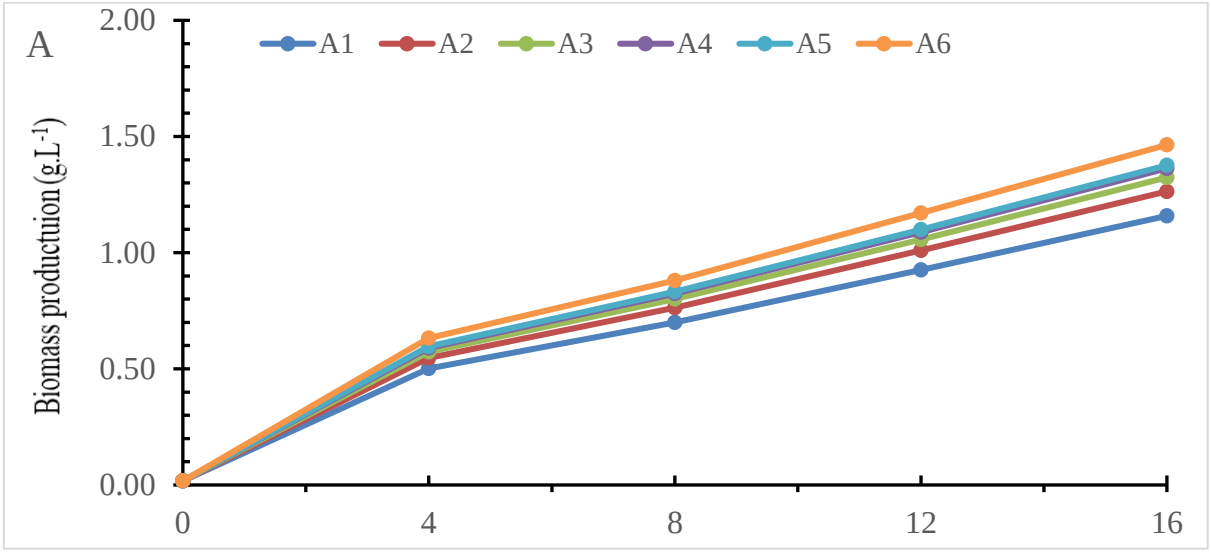
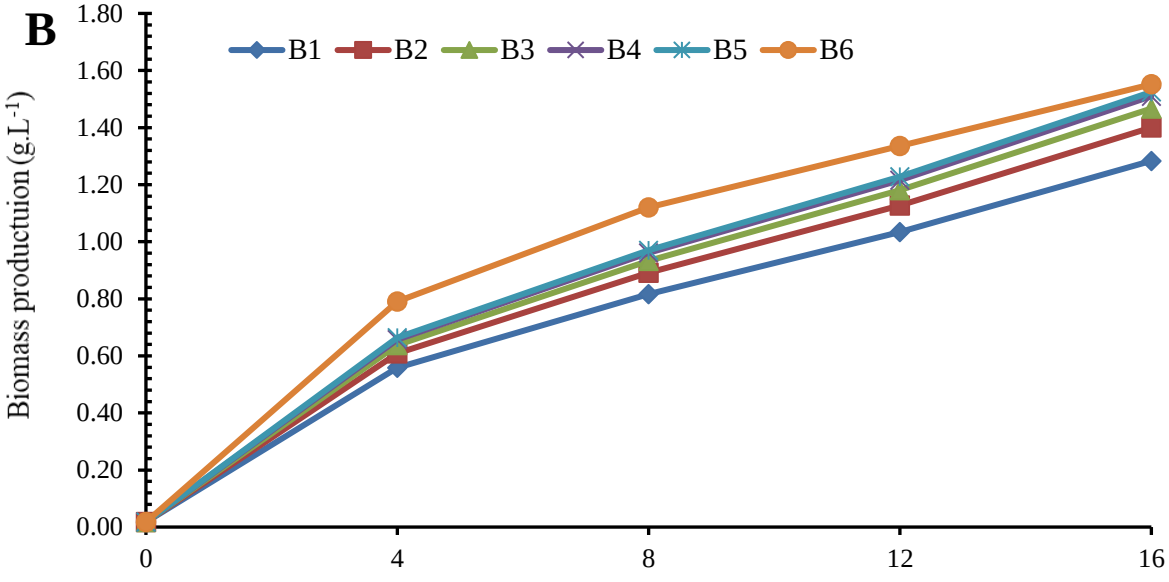


Fig. 1. Specific growth rate of *S. platensis* KAU02 grown in different steps in different culture media.

3.1.1. *S. platensis* KAU02 biomass production

Though the *S. platensis* KAU02 grew in all culture media of all steps but the best growth was found in final step or third step. The biomass production varied from 1.47 to 1.75 g. L^{-1} and the highest biomass production was found in ALKAUS-6 medium among newly formulated culture media at final step. The biomass production of ALKAUS-6 medium was similar with the biomass production of Zarrouk's medium which was 1.80 g. L^{-1} (Fig.1C).





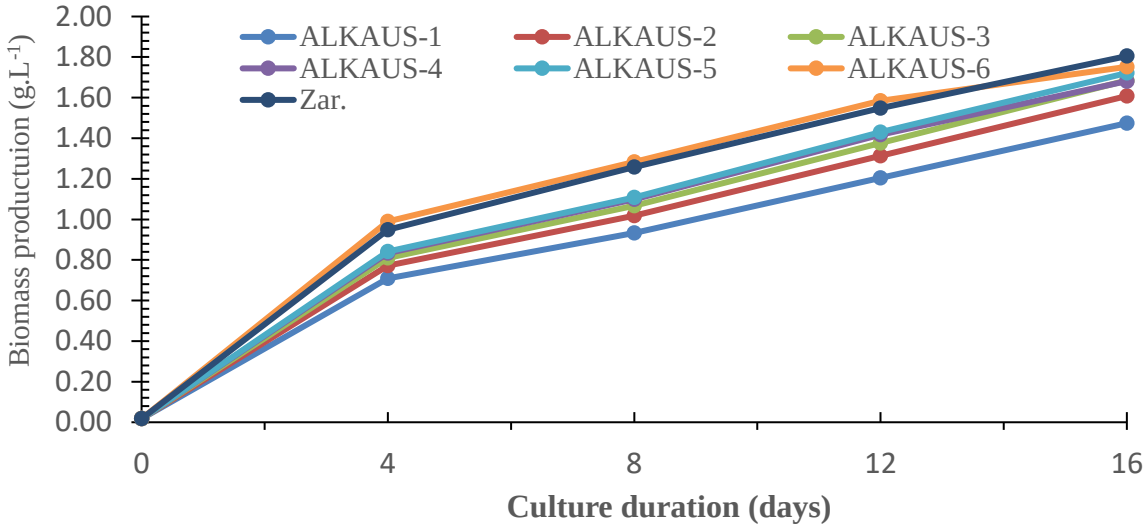


Fig. 1. Biomass production of *S. platensis* KAU02 cultured in different media prepared by using plant's nutrients in treated wastewater. First step of cultured in different media (A), Second step culture in different media (B) and Third step or final step of culture (C).

3.1.2. *S. platensis* KAU02 chlorophyll *a* content

Chlorophyl *a* content of *S. platensis* KAU02 varied from 12.75 to 16.05 mg. L^{-1} among the culture media of final step. The maximum chlorophyll *a* content was found in biomass of ALKAUS-6 culture medium. The chlorophyll *a* content of *S. platensis* KAU02 was 16.18 mg. L^{-1} in biomass grown in Zarrouk's medium (Fig. 3.).

3.1.3. Biochemical composition *S. platensis* KAU02

The Protein, lipid and carbohydrate content were 60.86, 12.65 and 13.85, and 62.23, 12.69 and 13.63% in biomass of ALKAUS-6 medium and in biomass of Zarrouk's medium, respectively. The phycocyanin content was 323.56 and 325.64 mg. g^{-1} in biomass of ALKAUS-6 medium and in biomass of Zarrouk's medium, respectively (Table 2).

Table 2. Biochemical composition in percent (%) and phycocyanin (mg. g^{-1}) of *S. platensis* KAU02 grown during the final step of culture development.

Media	Protein	Lipid	Carbohydrate	Ash	Moisture	Phycocyanin
ALKAUS-6	60.86 ± 0.12	12.55 ± 0.19	13.85 ± 0.23	7.24 ± 0.13	5.40 ± 0.23	323.56 ± 0.25
Zarrouk's	62.23 ± 0.14	12.65 ± 0.24	13.68 ± 0.17	7.15 ± 0.24	4.46 ± 0.12	325.64 ± 0.19

4. Discussion

4.1. Culture media preparation

In first step, the chemicals were found to be sedimented in white colour. In first and second step, the colour of *S. platensis* KAU02 grew very slowly, and they cells were found to be sedimented at the bottom for 4 days, then some dead yellow brown algae were found to be floated with cluster. The floating cells were pale brown colour than the mother stocks after inoculation to the end of the first and second steps study. Low concentration of phosphate and carbonic salts might be related with yellow brown colour and death of *S. platensis* KAU02. In previous, the algal colour gradually recovered from fresh green to dark green with the extension of culture time after adding different concentration of nitrogen, phosphorus and baking soda in culture media of wastewaters, fishpond

wastewater, industrial wastewater and mariculture water (Wongsnansilp and Phinrub, 2022). The culture media were again modified as third step by adding increased amount of $NaHCO_3$, $NaCO_3$ and KH_2PO_4. In this step, the cells found to be grown like mother stocks with dark blue-green colour. The algae were found to be mixed uniformly throughout the culture medium.

4.2. *S. platensis* KAU02 specific growth rate and biomass production

The μ_{max}. d^{-1} estimation is an informative way to ascertain microbial activity at their exponential rates. The growth rates and biomass production did not show any significant differences between the ALKAUS-6 and Zarrouk'culture media ($p < 0.05$). The μ_{max}. d^{-1} and biomass production of *S. platensis* KAU02 at ALKAUS-6 medium showed similarities with the *S. platensis* grown in $NaHCO_3$ pretreated seawater medium (Leema et al., 2010). Lamela and Rocha (2000) reported that the biomass production of *S. platensis* was 1.001.00 g. L^{-1} in seawater culture medium which was lower in comparison with the biomass production of *S. platensis* KAU02 grown in ALKAUS-6 medium.

The addition of $NaHCO_3$, Na_2CO_3 and KH_2PO_4 of 12.00, 2.50 and 0.250 g. L^{-1} in ALKAUS-6 medium resulted in a culture efficiency of ALKAUS-6 medium similar to that of Zarrouk's medium. In ALKAUS-6 medium was prepared with $NaHCO_3$ of 12.00 g. L^{-1} which was 4.80 g L^{-1} lower than that of $NaHCO_3$ used in Zarrouk's medium. CO_2 is important for high gowth of Spirulina (Litchfield, 1983). Traditionally used *Spirulina* culture media such as Zarrouk's (16.00 g. L^{-1} of $NaHCO_3$) or Society of toxicology (13.61 and 4.03 g. L^{-1} of $NaHCO_3$ and Na_2CO_3, respectively) (Aibaand Ogawa,1977) requires high amount of carbonic salts which is responsible for high production cost of *Spirulina*. Therefore, it could be said that ALKAUS-6 medium was better for *S. platensis* KAU02 culture using treated wastewater since it requires lesser concentration of carbonic salts in comparison with Zarrouk's medium.

4.3. Chlorophyll *a* content of *S. platensis* KAU02

The chlorophyll *a* in biomass of ALKAUS-6 medium was similar with the chlorophyll *a* content in biomass of Zarrouk's medium. Previously, $NaHCO_3$-pretreated seawater medium was used to grow *S. platensis* and the chlorophyll *a* content was 6.00 mg. L^{-1} (Leem et al., 2010) which was lower than the chlorophyll *a* content of *S. platensis* KAU02 grown in ALKAUS-6 medium. Thus, ALKAUS-6 medium may have created favourable conditions for chlorophyll *a* synthesis by *S. platensis* KAU02.

4.4. Biochemical composition of *S. platensis* KAU02

The biochemical composition of *S. platensis* KAU02 is shown in Table 2. The biochemical composition of microalgae is important for determining quality and market value (Johnston, 1970). Microalgae biochemical composition can vary across culture conditions even in the same culture medium. Oliveira et al. (1999) reported that the protein content of *S. maxima* was 70.24%, 68.01%, 68.67%, 64.58%, and 62.81% in same cultured medium with temperature of 20, 25, 30, 35, and 40 °C, respectively. Thus, it is important to determine the biochemical composition of *Spirulina* to ensure profitable production. Protein content of *S. platensis* KAU02 in biomass of ALKAUS-6 and Zarrouk's media was similar to that the biomass *Spirulina* spp., which was grown in $NaHCO_3$-pretreated seawater or an aerobic effluent-supplemented seawater (Leema et al., 2010). Affan et al. (2015) reported that protein content of *S. maxima* was 58.15% and 63.42%, of biomass grow in AKSM-2-1 and SOT media, respectively. The protein content of *Spirulina* spp., over 55% is recommended by the USA natural food industry.

The lipid content of *A. platensis* KAU02 was not significantly ($p<0.05$) different between the in biomass grown in ALKAUS-6 and Zarrouk's media. The lipid content of *A. platensis* KAU02 biomass ALKAUS-6 and Zarrouk's media was similar with the lipid content of *A. maxima* grown in pre-treated seawater and SOT media in which the lipid content was 12.20 and 12.59%, respectively

(Affan et al., 2015). Lipid content of *S. platensis* was 8.04 and 10.61% when cultured in modified Zarrouk's media prepared with undiluted seawater and Zarrouk's medium, respectively (Leema et al., 2010). Thus, it could be said that ALKAUS-6 medium may have created suitable circumstances which is similar to the Zarrouk's medium for synthesis of sufficient quantity of lipid in biomass of *S. platensis* KAU02.

The carbohydrate content of *A. platensis* KAU02 was not significantly ($p<0.05$) difference between the biomass grown in ALKAUS-6 and Zarrouk's media. The carbohydrate content of *S. platensis* KAU02 in biomass of ALKAUS-6 and Zarrouk's media was low in with carbohydrate content in biomass of *A. platensis* grown in freshwater and seawater (Villaro et al. 2023). Both soluble and insoluble carbohydrate content *S. platensis* were found to be increased with increasing of the concentrations of NaCl in the culture medium (Mutawie 2015). Previously, Affan et al. (2015) reported that the carbohydrate content of *A. maxima* was higher in biomass grown in a pre-treated sweater medium than that of the biomass grown in the SOT medium. Lamela and Rocha (2000) reported that high carbohydrate content and low protein synthesis occurred in *S. maxima* due to physiological stress in seawater. However, the culture medium of ALKAUS-6 prepared with treated wastewater did not show low protein synthesis and high content carbohydrate which indicated that the culture medium would not be stressed for *S. platensis* KAU02.

S. platensis KAU02 culture in treated wastewater medium were addressed through development of culture medium with using of lesser concentration of carbonic salts in comparison with Zarrouk's medium. The growth performance and biochemical composition of *S. platensis* KAU02 cultured in treated wastewater showed similar with Zarrouk's medium. Therefore, treated waste water could be a good source of making *S. platensis* KAU02 culture medium after enriched with $NaHCO_3$ and Na_2CO_3 and KH_2PO_4 which stimulates *S. platensis* KAU02. This study showed lower production cost of *S. platensis* compared to conventional Zarrouk's medium. Thus, treated wastewater could be used following our invented method for industrial-scale production of *S. platensis* KAU02 coastal and arid region of the world.

Author Contributions: Conceptualization— investigation of resources Affan, M. A. and Lafi, S. A. S. writing—original draft preparation, Affan, M. A. and Lafi, S. A. S,: writing — review and editing, Affan, M. A. and Lafi, S. A. S. Both authors have read and agreed to the published version of the manuscript.

Funding: This research work was supported by the Faculty of Marine Sciences, King AbdulAziz University, Jeddah, The Kingdom of Saudi Arabia.

Informed Consent Statement: "Not applicable.

Data Availability Statement: All the data are available in this manuscript.

Acknowledgments: We are particularly grateful to the staffs of the Department of Marine Biology, Faculty of Marine Sciences and Wastewater treatment centre, King AbdulAziz University for allowing us to perform this study.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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