

Effect of Modified Zarrouk's Medium on Growth of Different *Spirulina* Strains

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Abstract

The effect of modified Zarrouk's medium on the growth response of 6 different *Spirulina* strains was evaluated. Specific growth rate, doubling time, mean daily division rate, biomass, and chlorophyll-A contents were analyzed. Growth patterns of these strains were monitored continuously for 40 days. The results revealed significant differences in the growth parameters for different strains. *S. platensis* (SP-6) and *S. platensis* (CCMB) showed the maximum specific growth rates ($\mu = 6.1$, $\mu = 5.8$), doubling times ($T_d = 6.93$, $T_d = 6.87$), mean division rates ($k = 0.27$, $k = 0.23$) biomasses (5.1, 5.0 g/l) and chlorophyll A contents (78, 65 $\mu\text{g/ml}$) respectively, when compared with the other strains used in this study. Therefore, *S. platensis* (SP-6) and *S. platensis* (CCMB) strains can be suggested for large scale commercial cultivation with modified Zarrouk's medium. This provides the basis of a low cost medium for cultivating *Spirulina*, which is known to be a promising microalgae with several benefits.

Keywords: *Spirulina* strains, doubling time, growth response, specific growth rate, chlorophyll A

Introduction

Spirulina is a filamentous, photosynthetic, edible cyanobacterium, extensively grown for human food supplements and animal feed [1]. It has great nutritional value due to a high protein content, polysaccharides, vitamins, and minerals [2]. *Spirulina* is also rich in antioxidant enzymes, and has therapeutic potential owing to the presence of anti-viral, anti-cancerous [3], anti-hypertensive [4], immunopromoting [5], and neuroprotective [6] biomolecules. In addition, it produces various pigments, such as chlorophyll, phycocyanin etc. A major portion of commercial chlorophyll is used in the food, pharmaceutical, and cosmetic industries [7]. Moreover, it is the richest source of Gamma-linolenic acid (GLA), a precursor for biologically-active compounds such as prostaglandins (PGE1) [2], which are necessary for enhancement of the immune system [3,8]. *Spirulina* is important in bioremediation [9], due to its growth tolerance against heavy metals, and also has good nitrogen fixing capabilities [10].

Spirulina forms large colonies in tropical and subtropical surface waters containing high levels of carbonates and bicarbonates [2,11]. It requires an optimum pH of 8 - 11 and a temperature of 30 - 35 °C [12]. The cost of nutrients is considered to be a major factor that influences *Spirulina* biomass production. Cost-effective large-scale cultivation of *Spirulina* is easy to meet in terms of industrial requirements for its high protein production [13]. Zarrouk's medium has been successfully used as the standard medium for *Spirulina* culture [14]. Consequently, many modified media have been developed, using sea water, sewage water, and industrial effluents [15,16].

A study on growth kinetics is very important in understanding its ecology, physiology, genetics, and biotechnology [17]. The growth of *Spirulina* is strongly influenced by the medium components [18,19]. The aim of this study is to understand the growth response of different *Spirulina* strains under modified Zarrouk's medium and to compare the specific growth rate, doubling time, mean daily division rate, biomass, and chlorophyll-A.

Materials and methods

Strains and media composition

Six different *Spirulina* strains were obtained from different sources (**Table 1**). Modified Zarrouk media (devoid of A5 ingredients), prepared in double distilled water, was taken as the culture medium (**Table 2**). Instead of sodium nitrate (NaNO₃), potassium nitrate (KNO₃) was supplemented. Phosphate was added at the end in the preparation of the culture medium [13]. The strains were cultured in modified Zarrouk medium [16].

Table 1 Details of different strains and sources.

<i>Spirulina</i> strains	Provided by	Source
<i>S. indica</i>	CAS Botany, Chennai	CAS Botany, Chennai
<i>S. indica</i> (PCC8005)	CAS Botany, Chennai	CAS Botany, Chennai
<i>S. platensis</i> (SP-6)	CFTRI, Mysore	CFTRI, Mysore
<i>S. platensis</i> (CCMB)	CAS Botany, Chennai	CCMB, Hyderabad
<i>S. platensis</i> (PCC9438)	OrERR, Chennai	Trichy, Tamil Nadu
<i>S. maxima</i>	CAS Botany, Chennai	CAS Botany, Chennai

Central Food Technology Research Institute (CFTRI), Centre for Cellular and Molecular Biology (CCMB), Organization for Ealum Refugees Rehabilitation (PCC9438).

Table 2 Compositions of Zarrouk's media.

Ingredients	Standard Zarrouk's media (gm/l)	Modified Zarrouk's media (gm/l)
NaCl	1.0	1.0
CaCl ₂ .2H ₂ O	0.04	0.04
KNO ₃	-	2.5
NaNO ₃	2.5	-
FeSO ₄ · 7H ₂ O	0.01	0.01
EDTA (Na)	0.08	0.08
K ₂ SO ₄	1.0	1.0
MgSO ₄ · 7H ₂ O	0.2	0.2
NaHCO ₃	16.8	16.8
K ₂ HPO ₄	0.5	0.5
A ₅ micronutrient (H ₃ BO ₃ , MnCl ₂ .4H ₂ O, ZnSO ₄ .4H ₂ O, Na ₂ MoO ₄ , CuSO ₄ .5H ₂ O)	1 ml	-

Incubation and growth measurements

The temperatures for the cultures were maintained at 30 ± 2 °C. Illumination was provided with cool white fluorescent lights (2500 Lux), with a photoperiod 12/12 light and dark cycle [19]. Culturing was carried out in 250 ml conical flasks with 150 ml of media contents. The inoculum 10 % v/v [$A_{540} = 1$] was taken from a pre-culture during the log phase cultured in Zarrouk medium, pH was adjusted to 9.5, and cultures were agitated at 75 rpm in an orbital shaker. From the cultures, 5 ml samples were aseptically taken every 2 days and analyzed spectrophotometrically to determine growth measurements (Amersham 1100 Pro) at 540 nm [11]. In cell density experiments, absorbance measurements are employed, since they are rapid and relatively easier to take than cell viability methods. The absorbance was transformed into biomass by the method of Dalgaard and Koutsoumanis (2001) [15].

Estimation of specific growth rate

From the absorbance growth curve, the average specific growth rate (μ) was calculated as the logarithm of the ratio of the biomass concentration before the stationary phase to that at the start of each run (X_s/X_0) divided by the cultivation time necessary to achieve X_s .
 μ (cell day⁻¹) = $(\ln X_2 - \ln X_1)/(t_2 - t_1)$, where X_1 and X_2 represent the biomass concentrations at times t_1 and t_2 [11].

Estimation of doubling time

Doubling time (T_d) values for all the cultures were estimated $T_d = 0.693/\mu$, where T_d is doubling time and μ is the specific growth rate [15].

Estimation of mean daily division rate

The mean daily division rate, K , was determined by using the equation;
 $K = 3.3/t \times \log OD_t - \log OD_0$
where t is days since inoculation, OD_t is optical density after t days, and OD_0 is optical density when $t = 0$ [15].

Biomass estimation

Biomass or dry weight was analyzed every 5 days. From the cultures, 5 ml was taken and filtered through previously dried, pre-weighed Whatman filter paper number 1, and cell dry weights were determined after drying filters at 90 °C for 2 h [15].

Estimation of total chlorophyll-A

From the cultures, 5 ml was taken on the 15th, 25th, 35th, and 40th day, and centrifuged (Remi Cooling C-24) at 15,000 rpm for 10 min at 4 °C. Chlorophyll extraction and algorithm was done as per the Ritchie method [20].

$$\text{Chl a } (\mu\text{g/ml}) = 13.70[A]_{665} - 5.76[A]_{649}$$

Results

Specific growth rate

In the present study, 6 different strains, namely, *S. indica*, *S. indica* (PCC8005), *S. platensis* (SP-6), *S. platensis* (CCMB), *S. platensis* (PCC9438), and *S. maxima* were studied. The specific growth rate of different *Spirulina* strains (Figure 1) demonstrates that the maximum growth was observed for *S. platensis* (SP-6), followed by *S. platensis* (CCMB), with $\mu = 6.1$ and $\mu = 5.8$, respectively. While the minimum growth was observed for *S. indica* ($\mu = 2.6$) on day 40. *S. platensis* (SP-6) and *S. platensis* (CCMB) showed a gradual increase in cell density that was higher as compared to other *Spirulina* strains.

Doubling time

Doubling time is the average time required for all the components of the culture to double, and is represented by T_d . *S. platensis* (SP-6) and *S. platensis* (CCMB) showed the maximum doubling time, with $T_d = 6.93$ and 6.87 , respectively, on day 5. *S. indica* (PCC8005) and *S. maxima* had the minimum doubling time, with $T_d = 2.52$ and $T_d = 2.31$, respectively (**Figure 2**).

Mean daily division rate

A division of one mirage into 2 daughter cells is a process called mean division rate, and is represented by k . The mean daily diversion rate was higher during the 10th day for all strains except *S. indica*. The highest value was noted for *S. platensis* (SP-6), with 0.27 divisions/day, and *S. platensis* (CCMB) with 0.23 divisions/day, respectively, and the least for *S. indicia*, with 0.14 divisions/day (**Figure 3**).

Biomass

Biomasses were estimated every 5th day during the 40 days cultivation period. Comparing all the strains, it was found that the maximum biomass was obtained by *S. platensis* (SP 6) and *S. platensis* (CCMB), with nearly 5.1 and 5.0 gms/l, respectively, whereas the least biomass of 2.6 gms/l was achieved by *S. indica* (PCC8005) on day 40 (**Figure 4**).

Total chlorophyll A amount

Chlorophyll estimation was performed on the 5th, 15th, 25th, 35th, and 40th days. Maximum chlorophyll content was found in *S. platensis* (SP-6) and *S. platensis* (CCMB). The present results revealed that *S. platensis* (SP 6) and *S. platensis* (CCMB) contained 78 and 65 $\mu\text{g/ml}$, respectively, while *S. indica* contained the minimum level of chlorophyll, 23 $\mu\text{g/ml}$, during the 40th day (**Figure 5**).

Discussion

In mass cultures of *Spirulina* strains, nutritional conditions are the key factor that control their growth and productivity [19]. The present investigation was conducted with the basic aim of providing a simple modified medium and choosing the correct *Spirulina* strain for large scale production.

The present results revealed that *S. platensis* (SP-6) and *S. platensis* (CCMB) showed higher maximum specific growth rates, doubling times, and mean daily division rates when compared with other strains (**Figures 1 - 3**). The growth of the strains and the doubling times are recognized as some of the most important factors that play major roles in determining the biochemical composition of microalgae [19]. In the microalgae, declining growth normally occurs in cultures where there is a specific requirement for mean division rate. In this phase, growth and biomass is often very high.

This biomass accumulation and chlorophyll-A production is higher in *S. platensis* (SP-6), *S. platensis* (CCMB) when compared with other strains (**Figures 4 and 5**). On the basis of these studies, biomass was found to be a reliable indicator of cell growth [23], and maximum growth rate was observed for *S. platensis* (SP-6) and *S. platensis* (CCMB). Chlorophyll-A is a very important pigment, extensively used in the cosmetic, pharmaceutical, and food industries [24]. Selecting and culturing of the right strain will be a good alternative choice for industrial application. Cultivation of photosynthetic blue green microalgae like *Spirulina* is an attractive process for obtaining the value added biochemical components [25]. It was also reported that *S. platensis* has high levels of phycocyanin, chlorophyll-A, β -carotene, polysaccharides, and γ -linolenic acid [24,26]. Similarly, our investigation also reveals that *S. platensis* (SP-6) and *S. platensis* (CCMB) strains have the maximum chlorophyll-A content (**Figure 5**).

India, China, Israel, Thailand, Taiwan, Japan, the US, etc. are important producers of *Spirulina* on a large scale. Many of the *Spirulina* farms are located in Asia-Pacific region [27], and productivity is low and unstable. For instance, China has a productivity of $7 \text{ g m}^{-2} \text{ d}^{-1}$ against the world average of $14 \text{ g m}^{-2} \text{ d}^{-1}$ [28]. Even though many of the *Spirulina* cultivating Asian countries have ideal and optimal environmental culture conditions, poor strain selection is considered to be one of the main reasons for low productivity. So, this would be helpful in choosing the right strain for higher biomass production at the commercial level.

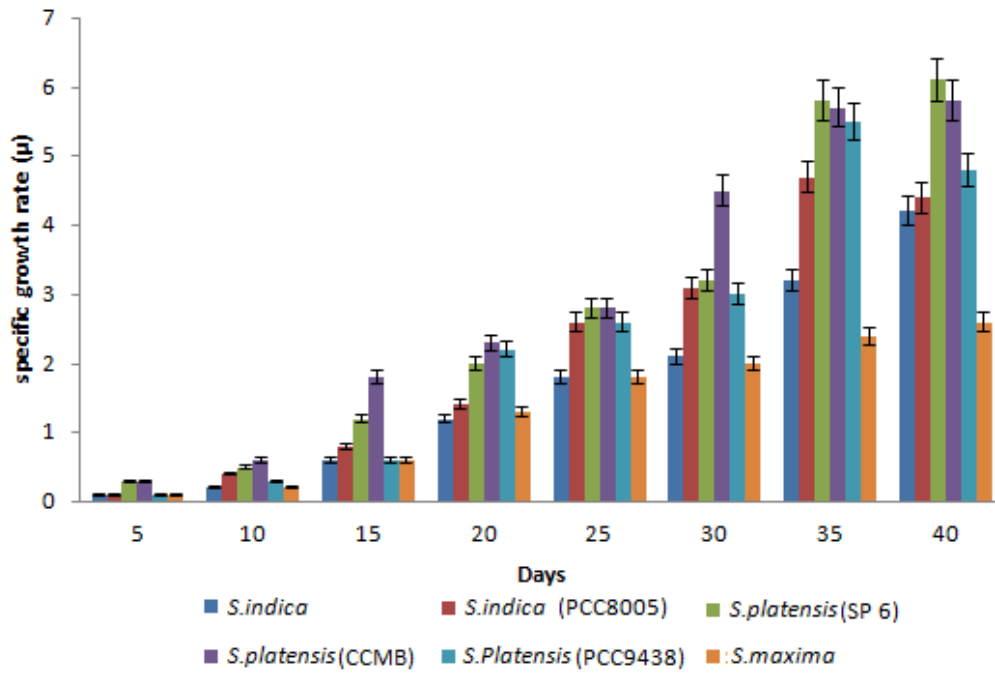


Figure 1 Specific growth rate of different *Spirulina* strains grown in modified Zarrouk media.

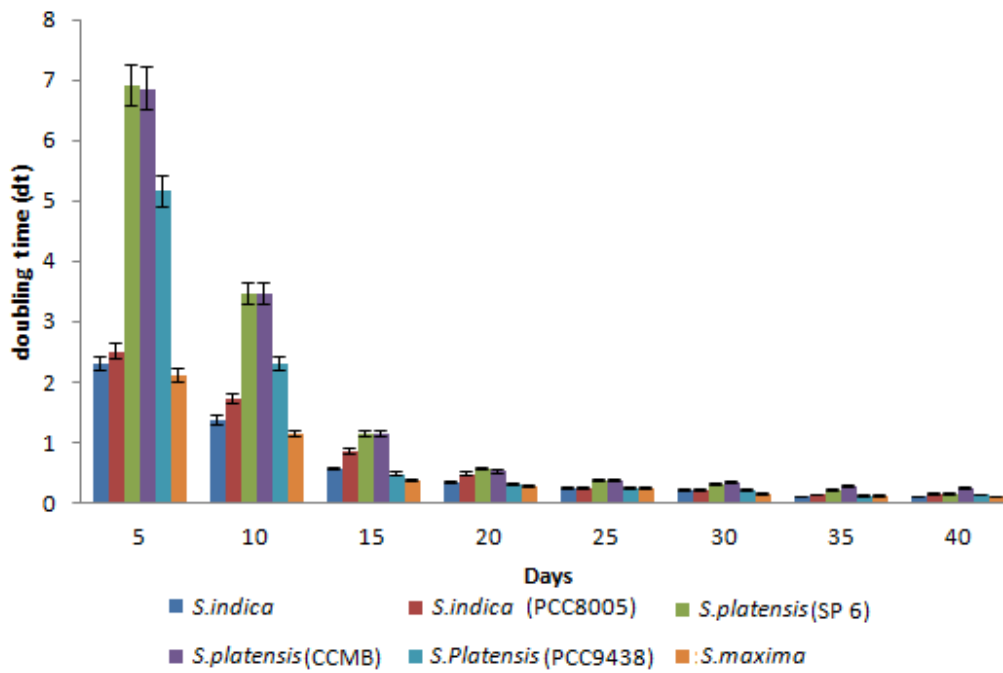


Figure 2 Doubling times of different *Spirulina* strains grown in modified Zarrouk media.

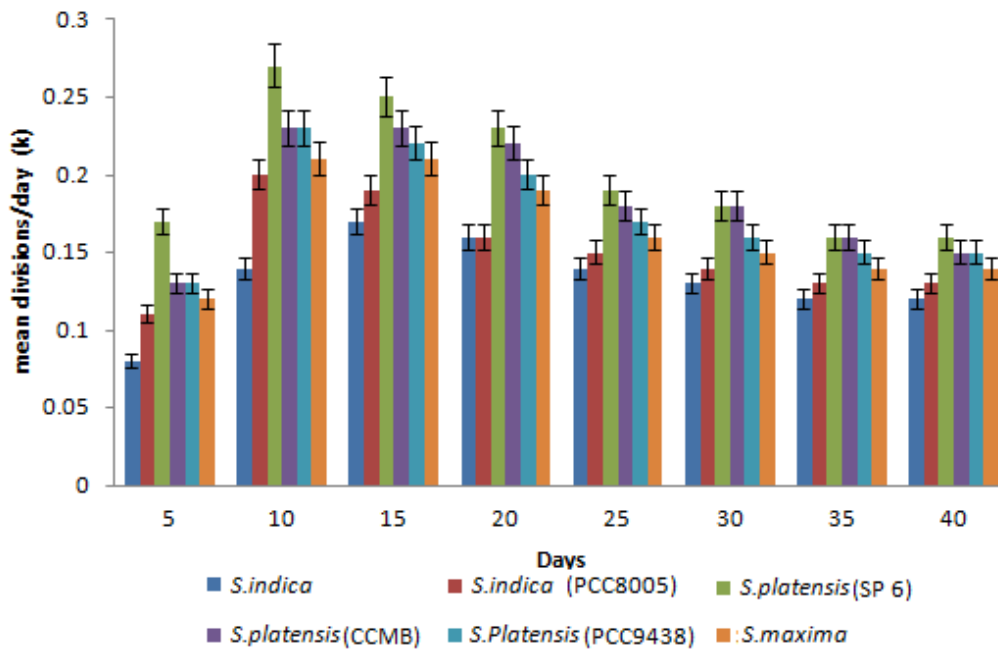


Figure 3 Mean daily division rate of different *Spirulina* strains grown in modified Zarrouk media.

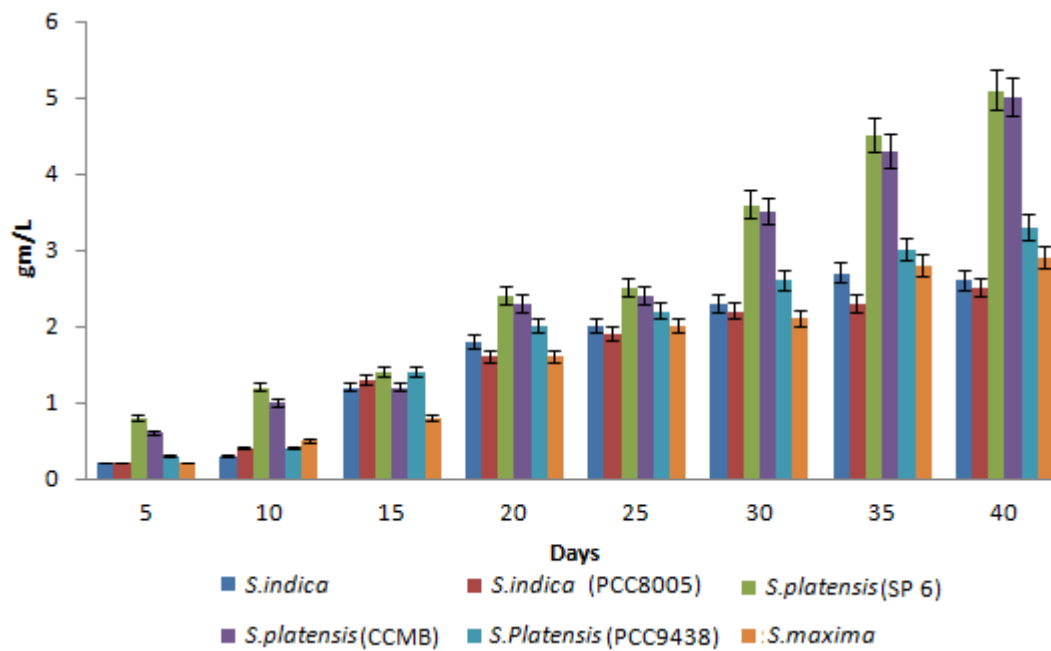


Figure 4 Biomass of different *Spirulina* strains grown in modified Zarrouk media.

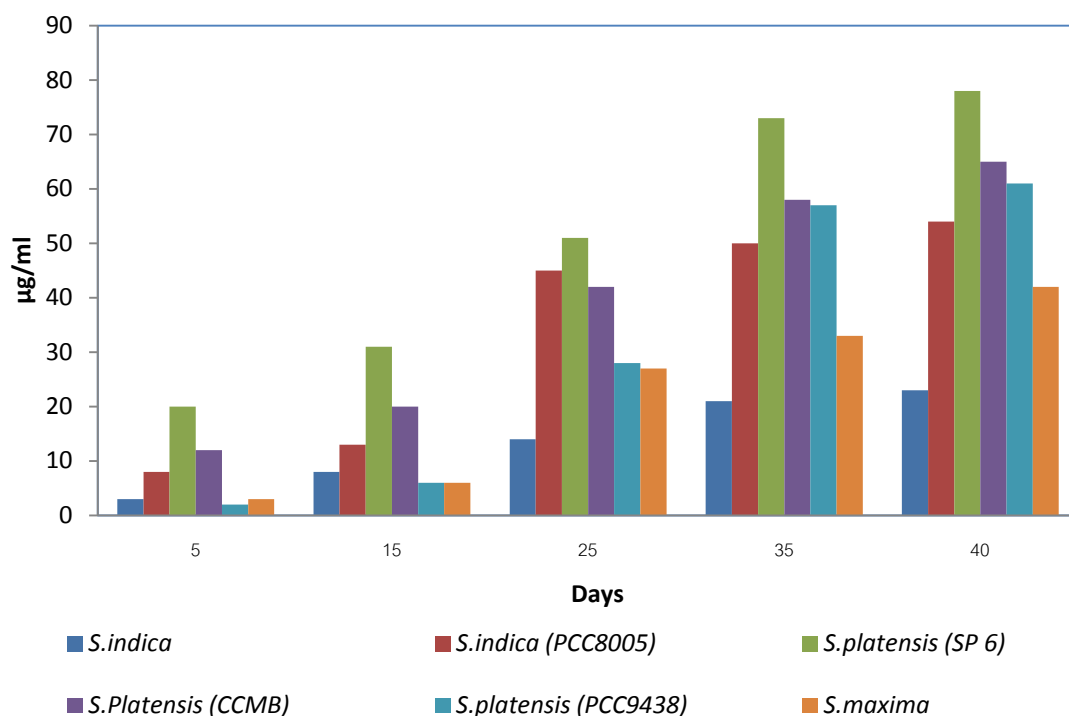


Figure 5 Chlorophyll a content of different *Spirulina* strains grown in modified Zarrouk media.

Among the nutrients required for the growth of *Spirulina*, potassium is considered to be an important component which plays an essential role in maintaining high growth rate of microalgae like *Spirulina*. Replacement of potassium nitrate instead of sodium nitrate and changes in the source of culture media limits the intensive growth of microalgae. Zarrouk's media substituted with commercial single super phosphate (SSP) instead of di-potassium hydrogen phosphate, EDTA, and A5 micronutrients have enhanced the growth, biomass, and chlorophyll content in *S. platensis* [18]. Similarly, in the present study, sodium nitrate was replaced by potassium nitrate and the modified Zarrouk's media were tested to determine their effectiveness in different strains, and the results revealed that *S. platensis* (SP-6) and (CCMB) strains were found to be more efficient. Potassium plays a major role to provide the appropriate ionic environment for metabolic processes in the cytosol, and is a regulator of various processes, including growth regulation, photosynthesis, protein synthesis, and nitrate source in the medium, which increases the biomass concentration [29]. Sodium ions are found mostly outside the cells, whereas potassium ions are found inside the cell fluids [30]. A5 micronutrients (Boric acid, manganese chloride, zinc sulphate, sodium molybdate, copper sulphate) are not considered to be used to check the growth of the strains because of their cost.

The present study was taken up with the aim of providing a simple media for large scale production of *Spirulina* strains. The results indicated that modified Zarrouk's medium is suitable for *Spirulina* cultivation, when evaluated in terms of growth rate, biomass, and chlorophyll-A content. *S. platensis* (SP-6) and *S. platensis* (CCMB) are found to be better when compared with other strains (**Figures 1 - 6**). Therefore, the merits of the modified Zarrouk's medium are clearly emphasized as a low cost alternative medium, and also as a capacity of highly productive input, which can be used profitably for large scale biomass production of *Spirulina* for commercial purpose.

Conclusions

The present investigation was chosen with the basic aim of selecting correct strains for mass cultivation of *Spirulina* for high biomass production. The present study concluded that modified Zarrouk's medium, devoid of A5 micronutrients and with replacement of potassium nitrate in place of sodium nitrate, is better for the growth performance of *Spirulina*, in terms of specific growth rate, mean daily division rate, biomass, and chlorophyll-A. Among the 6 *Spirulina* strains, it was found that *S. platensis* (SP-6) and *S. platensis* (CCMB) revealed the highest growth, biomass, and chlorophyll-A synthesis. This modified Zarrouk medium can be used as a low cost medium variant for cultivation of the *S. platensis* (SP-6) and *S. platensis* (CCMB) strains.

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