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Evaluation of the effects of immobilization on Spirulina *platensis* cultures

Pınar Nartop*, Emine Kuşku

*Biomedical Engineering Department, Faculty of Engineering, Tekirdağ Namık Kemal University, 59860 Çorlu, Tekirdağ, Türkiye

*Corresponding author : pnartop@nku.edu.tr
Orcid No : https://orcid.org/0000-0003-2765-6133

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Abstract: Different studies are carried out to reduce the production costs of *Spirulina platensis* biomass, which is produced in liquid cultures and has a high commercial value. These studies are in the direction of determining the optimum production method by changing the nutrient medium content and culture conditions. Immobilization is a method that changes the culture conditions. In our study, *Luffa cylindrica* (luffa), a natural fiber, was used for the immobilization of *S. platensis* cells, and the growth parameters and pigment production of the cultures were investigated. In two-factor experiments, week (first and second weeks) and culture type (free and immobilized cultures), statistically significant week*culture type interactions were found in optical density, dry weight, pH, and chlorophyll-a content. Immobilization did not increase the optical density and biomass production of the cultures. The highest optical densities and biomass productions were obtained in two-week-old free cultures, where the pH value was also found to be the highest. The highest values of chlorophyll-a and total carotene content were obtained from one-week immobilized cultures (30.06 µg/ml and 48.35 µg/ml, respectively). The fact that immobilization increased pigment production in one-week-old cultures indicates that when pigment production is targeted in *S. platensis* cultures, two-stage cultures that increase pigment yield via one-week immobilization after biomass production is completed can be used.

Keywords: *Spirulina platensis*; Immobilization; Luffa, Cell culture

1 Introduction

Spirulina plantesis is a genus of *Cyanobacteria* with high nutritional values. Its most striking feature is that it lives in waters with high carbonate and bicarbonate levels and pH values up to 11. It has a high fat and high nutritional value content such as provitamins, minerals, gamma-linolenic acid, carotene and xanthophyll pigments, and beneficial amino acids. Since centuries ago, Indigenous peoples living in Africa and Mexico have utilized *S. plantesis* as a food source (Venkataraman 1997; Miranda et al. 1998).

Luffa cylindrica (luffa) is a woody plant commonly known as gourd fiber and belongs to the family *Cucurbitaceae*, which is widely used for different purposes. L. cylindrica consists of 10% wood pulp (lignin), 30% semi-cellulose and 60% cellulose. It is widely distributed in North and East Australia, Central and South America, Asia, Africa, and the Mediterranean. It is a low-cost natural fiber that is harmless to human health and recyclable. *L. cylindrica* has a round or angular stem structure in terms of vegetative characteristics (Herklots 1972; Jeffrey 1990; Ghali et al. 2009). Due to these characteristics, it is an ideal natural material that can be used in immobilization studies to be placed inside the flasks.

Immobilization is a method that allows free cells in cell cultures to adhere to surfaces to obtain more production efficiency. This method, which is frequently used in plant cell cultures, is not required in *S. platensis* cultures due to the ability of algae to multiply without the need for attachment. Immobilization of cells on natural fibers in plant cell cultures has been found to produce different results in terms of both biomass production and secondary metabolite production (Nartop et al. 2013).

When *S. platensis* immobilization studies are examined, it is seen that the cells are trapped in semi-permeable membrane or gel structures and used in the cleaning of wastewater (Purev et al. 2023; Chen et al. 2023).

Our study aimed to investigate the growth parameters and contents of *S. platensis* cultures immobilized with luffa, a natural fiber. Therefore, the growth parameters of free and immobilized cell cultures were investigated comparatively

and their contents were determined by spectrophotometric methods.

2 Materials and Method

2.1. Materials

S. platensis starter culture was obtained from Çukurova University, Faculty of Fisheries (Adana, Turkey). The nutrient medium used was modified (Zarrouk's medium) and contained (g/L);); $NaHCO_3$ - 37.2 g/L; Na_2CO_3 - 16.12 g/L; K_2HPO_4 $3H_2O$ - 2.62 g/L; $NaNO_3$ – 4 g/L; NaCl - 4 g/L; $MgSO_4$ $7H_2O$ - 0.8 g/L; $CaCl_2 2H_2O$ - 0.04 g/L; $FeSO_4 7H_2O$ - 0.04 g/L; $EDTANa_2$ - 0.32 g/L; $ZnSO_4 7H_2O$ - 0.001 g/L; $MnSO_4H_2O$ - 0.0012 g/L; H_3BO_3 - 0.01 g/L; $Na_2MoO_4 2H_2O$ - 0.001 g/L; $CoCl_2 6H_2O$ - 0.0081 g/L; $CuSO_4 5H_2O$ - 0.00005 g/L; $FeSO_4 7H_2O$ - 0.7 g/L; $EDTANa_2$ - 0.8 g/L. This nutrient medium prepared with distilled water was sterilized in an autoclave at 121ºC for 15 minutes. All chemicals used in this study were purchased from Sigma and Acros Organics and were of analytical quality (97% purity).

Fig. 1 *Luffa cylindrica* (luffa) cut for use as immobilization material

Luffa cylindrica was used as immobilization material. Luffa was cut as shown in Fig 1, washed by rinsing with detergent water, rinsed three times with tap water, washed three times with distilled water, and placed at the bottom of the flasks. The mouth of the flasks was covered with cotton wool and aluminum foil and sterilized in an oven at 170ºC for 60 minutes (Nartop et al. 2013).

2.2. Methods

S. platensis cultures were subcultured continuously at 7-day intervals by half dilution before immobilization. The volume of the immobilized cultures formed by pouring half-diluted cultures into the flasks with luffa placed on the bottom was 500 ml and the working volume was determined as 400 ml. Cultivation was carried out at 23 µmol/m^2s light intensity, 3 L/min ventilation rate, and 26±1ºC. Cultures were terminated in the first and second weeks, filtered, fresh weights were determined, and dried in an oven at 40ºC, and dry weights were recorded (Nartop and Kuşku 2023).

Before the cultures were terminated in the first and second weeks, pH measurements were performed and optical densities of 3 ml samples were measured spectrophotometrically at 665 and 680 nm (Nartop and Kuşku 2023).

For chlorophyll-a and carotenoid analyses, 5 mg samples from each experiment were mixed with 5 ml of 70% ethanol, filtered after extraction in an ultrasonic bath for 60 min, and absorbance values at 665 and 450 nm were recorded. Chlorophyll-a (Dineshkumar et al., 2015) and carotenoid (Saefurahman et al., 2021) concentrations were calculated according to the following formulae; (A_{665}: Absorbance measured at 665 nm; A_{450}: Absorbance measured at 450 nm).

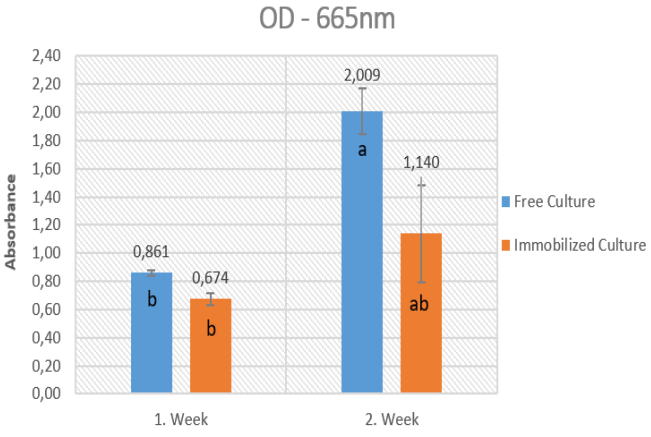
$$\text{Chlorophyll-a concentration } (\mu g/mL) = A_{665} \times 13.9 \quad (1)$$

$$\text{Carotenoid concentration } (\mu g/mL) = A_{450} \times 25.2 \quad (2)$$

In the study, each trial was carried out with three replications. Data analyses were performed by ANOVA and Tukey was used for post-hoc tests.

3 Results

In the spectrophotometric analysis at 665 nm, both weekly data and week*culture type interaction were statistically significant ($p<0.05$). The second week was in the first group with 1.574 and the first week was in the second group with 0.767 (Table 1). In the week*culture type interaction, two weeks of free cultures were in group A with 2.009, two weeks of immobilized cultures were in group AB with 1.140, and one week of free and immobilized cultures were in group B with 0.861 and 0.674, respectively (Fig 2).



(F_{week}= 17.68, p_{week}=0.014; $F_{interaction}$= 3.16, $p_{interaction}$=0.015)

Fig. 2 Optical densities of *S. platensis* free and immobilized cultures measured at 665 nm

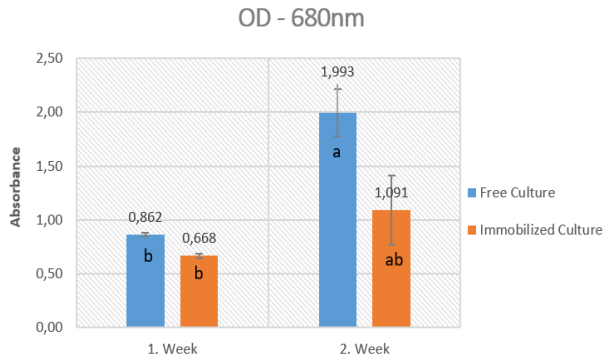
In the optical density measurement at 680 nm, both parameters and the interaction of the two parameters were statistically significant ($p<0.05$). Similar to the results obtained at 665 nm, the second week was in the first group with 1.542 and the first week was in the second group with 0.765 (Table 1). Free cultures were in group A with 1.427 and immobilized cultures were in group B with 0.879. In the week*culture type interaction, two-week free cultures were in group A with 1.992, two-week immobilized cultures were in group AB with 1.091, and one-week free and immobilized cultures were in group B with 0.862 and 0.668, respectively (Fig 3).

Table 1: Means and statistical groups of the growth parameters' results of *S. platensis* cultures depending on the duration and culture type.

Growth Parameter	Duration		Culture Type	
	1. Week	2. Week	Free Culture	Immobilized Culture
OD-665	0.767 ^{B}	1.574 ^{A}	1.435	0.907
OD-680	0.765 ^{B}	1.542 ^{A}	1.427 ^{A}	0.879 ^{B}
Fresh Weight (g)	0.659	0.873	1.025	0.507
Dry Weight (g)	0.060	0.073	0.096 ^{A}	0.035 ^{B}
pH	10.16 ^{B}	10.23 ^{A}	10.22	10.18
Chlorophyll-a (µg/ml)	28.17 ^{A}	14.30 ^{B}	21.95	20.52
Total Carotenoid (µg/ml)	47.88 ^{A}	33.57 ^{B}	44.52	36.90

1 Means marked with the same letter are statistically in the same statistical group ($p<0.05$).

The difference between the fresh weight values in the trials evaluated according to week and culture type was not statistically significant ($p>0.05$). The highest fresh weight was 1.329 g in two-week free cultures, 0.720 g in one-week free cultures, and 0.598 g and 0.416 g in immobilized cultures in the first and second weeks, respectively (Fig 4). The fresh weight results obtained were in parallel with the optical density measurements in free cultures, but the fresh weight in immobilized cultures was lower in the second week.



(F_{week}= 15.73, p_{week}= 0.017; $F_{culture\ type}$ = 7.83, $p_{culture\ type}$ = 0.049; $F_{interaction}$ = 3.26; $p_{interaction}$ = 0.015)

Fig. 3 Optical densities of *S. platensis* free and immobilized cultures measured at 680 nm

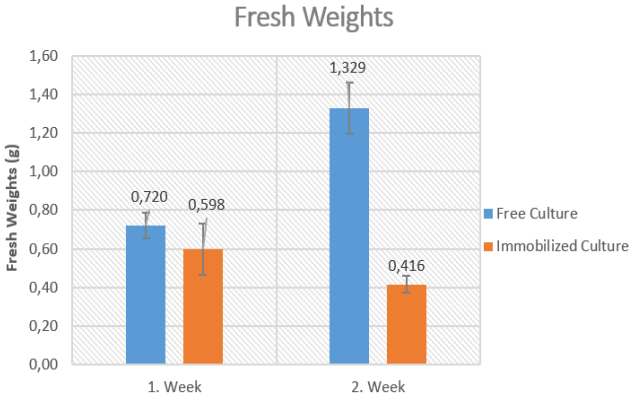
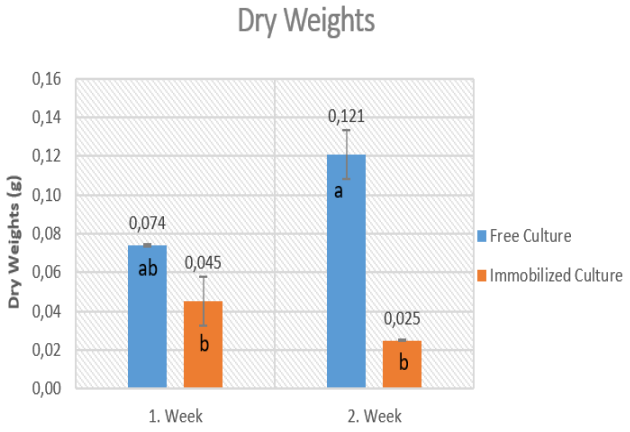


Fig. 4 Fresh weights of free and immobilized cultures of *S. platensis*

The results obtained in dry weights, culture type, and week*culture type interaction were statistically significant ($p<0.05$). Free cultures were in the first group with 0.096 g and immobilized cultures were in the second group with 0.035 g (Table 1). In the week*culture type interaction, two weeks of free cultures were in group A with 0.121 g, one week of free cultures were in group AB with 0.074 g, one and two weeks immobilized cultures were in group B with 0.045 g and 0.025 g, respectively (Fig 5).

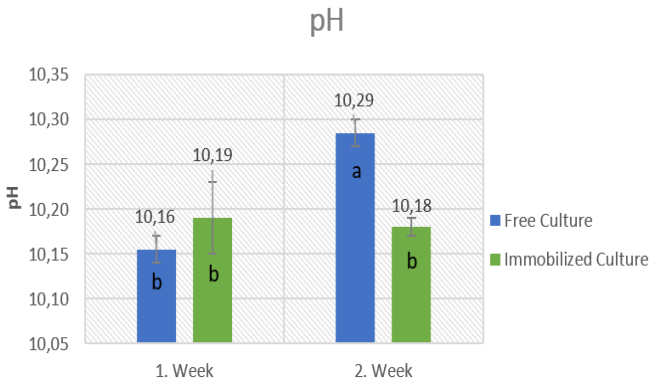
Fig 6 shows the pH values of S. *platensis* free and immobilized cultures. The week*culture type interaction was found to be statistically significant in the pH values obtained in the experiments ($p<0.05$). pH values were determined between 10.16 and 10.29 (Table 1). In free cultures, an increase was detected in the second week and the pH value of 10.29 was statistically in group A. The pH values of immobilized cultures in the first and second weeks were 10.19 and 10.18, respectively, and these values were in group B together with 10.16 obtained from free culture in the first week.



($F_{culture\ type}$= 49.99, $p_{culture\ type}$= 0,002; $F_{interaction}$ = 14.26, $p_{interaction}$ = 0.019)

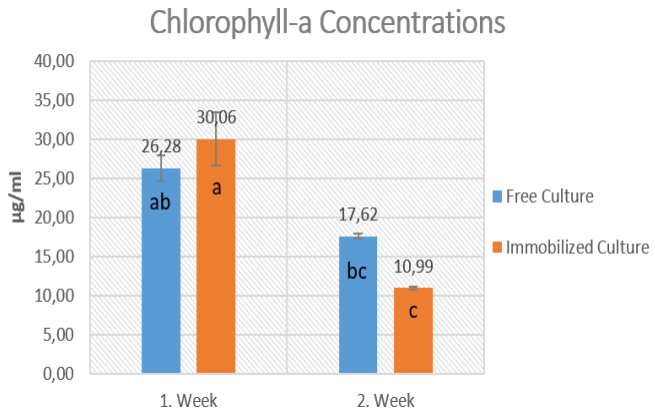
Fig. 5 Dry weights of free and immobilized cultures of *S. Platensis*

S. platensis chlorophyll-a and total carotene contents are given in Fig 7 and Fig 8. Chlorophyll-a contents were in the range of 10.99 - 30.03 µg/ml. In the results obtained in terms of chlorophyll-a concentrations, week and culture type*week interaction were found statistically significant ($p<0.05$). While the first week was in the first group with 28.17 µg/ml, the second week was in the second group with 14.30 µg/ml (Table 1). In the culture type*week interaction, one week of immobilized culture was in group A, one week of free culture was in group AB, two weeks of free culture was in group BC and two weeks of immobilized culture was in group C. One week of immobilization increased the chlorophyll-a content.



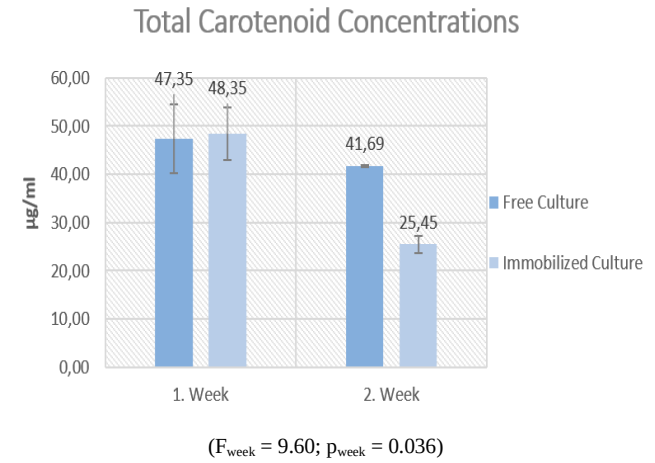
($F_{interaction}$ = 9.12; $p_{interaction}$ = 0.039)

Fig. 6 pH values of *S. platensis* free and immobilized cultures



(F_{week} = 52.83, p_{week} = 0.002; $F_{interaction}$ = 7.43, $p_{interaction}$ = 0.043)

Fig. 7 Chlorophyll-a concentrations of free and immobilised cultures of *S. platensis*



(F_{week} = 9.60; p_{week} = 0.036)

Fig. 8 Total carotene concentrations of free and immobilised cultures of *S. platensis*

Total carotene concentration values were determined in the range of 25.45-48.35 µg/ml. The difference between the data obtained according to the week was statistically significant ($p<0.05$) and the first week was in group A with 47.88 µg/ml, while the second week was in group B with 33.57 µg/ml (Table 1). The highest concentration was obtained in one week of immobilized cultures, but in the second week, the concentration decreased to a lower level than in free culture.

4 Discussion

Within the scope of our study, *S. platensis* cultures were immobilized with luffa, a natural fiber. The results of optical density measurements were parallel to each other at 665 nm and 680 nm. The highest optical density was found in two-week free cultures, while the week*culture type interaction was statistically significant in both measurements. Densities were higher in the second week as expected. Immobilized cultures were lower than free cultures. This indicates that free cells are retained in the luffa and therefore the optical density decreases. Fresh and dry weight data are in parallel with each other. Differences between fresh weights were not statistically significant, but culture type and culture type*week interaction were significant for dry weight differences. In terms of biomass accumulation, free cultures were more efficient than immobilized cultures, and the highest fresh and dry weight values were obtained in two-week free cultures. Biomass accumulation in two-week immobilized cultures was lower than in one-week immobilized cultures. This is in contrast to the situation detected in optical density measurements. This was considered as a sign that the number of cells increased over time, but the cells did not develop enough to increase the biomass.

Dry matter (biomass of liquid cultures) is an important parameter for *S. platensis* culture studies (Azgın et al., 2015). Biomass accumulation is a growth parameter that is often used in conjunction with optical density measurements for the development of cultures. Culture conditions such as temperature, light, and pH and the chemical composition of the nutrient medium (phytohormones, macro- and microelements, etc.) are known to affect the growth of *S. platensis* cultures (Abd El-Monem et al. 2018; Chen et al. 2010; Danesi et al. 2011; Romanenko et al. 2015; Gabr et al. 2020).

Immobilization is an application that changes the culture conditions and in our study, it was determined that immobilization did not increase the biomass. Lower biomass accumulation occurred in immobilized cultures compared to free cultures and biomass decreased in the second week. The pH value of the culture medium is one of the most important factors affecting the growth of S. *platensis* cultures. Thirumala (2012) reported that the optimum pH value was 10-11, but Fagiri et al. (2013) reported that the optimum pH value was 7-9. In our study, it was determined that the highest optical density and biomass accumulation were obtained at the highest pH value (10.29). The pH value was highest at two weeks of free cultures. In our study, fresh weights were determined between 0.416 - 1.329 g and dry weights between 0.025-0.121 g. In immobilized cultures, fresh weights were 0.598 g and 0.416 g and dry weights were 0.045 g and 0.025 g in the first and second weeks, respectively. Pandey et al. (2010) reported dry biomass in the range of 0.22 - 0.91 g. Ogbonda et al. (2007) determined it as 1.515 g at pH 10.

S. platensis pigment production is affected by light intensity (Chen et al. 2010; Danesi et al. 2011). However, other factors also affect pigment production. In our previous study, biosynthetic silver nanoparticles were found to affect pigment

production depending on the concentration (Nartop and Kuşku 2023). In this study, immobilization was found to affect pigment production. One-week immobilized cultures had the highest content in terms of both chlorophyll-a and total carotene. In the second week, pigment production decreased in both culture types, and the lowest value was determined in two-week immobilized cultures. In contrast to our findings, Thirumala (2012) reported that pigment accumulation increased as the culture period increased.

5 Conclusion

It is known that immobilization can cause biomass increase in plant cell cultures. In our study, it was observed that optical density and biomass did not increase with immobilization in *S. platensis* cultures, but the highest pigment production was obtained in immobilized cultures at the end of the first week. It can be recommended as a result of our study that when pigment production in *S. platensis* cultures is targeted, after obtaining high biomass, a short-term immobilization application for one week as a second step would be beneficial.

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Authors' contributions:

PN: Study conception, design, supervision, data analysis, tatistical analysis, literature review, manuscript writing, editing and laboratory experiments
EK: Laboratory experiments

Conflict of interest disclosure:

The authors declare that there are no real, potential, or perceived conflicts of interest for this article.

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