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# *NEK6* gene silencing using siRNA for overcome multidrug resistance in chronic myeloid leukemia cells

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**Abstract:** Tumor cells become resistant to structurally or functionally unrelated chemotherapeutics which is called multidrug resistance (MDR). There are several mechanisms including the impairment of apoptotic pathway resulting in MDR development. *NEK6* is a member of NIMA-related kinase family and it is an important mitotic kinase for proper cell cycle progression. Recent studies showed that *NEK6* gene expression, protein level, and its kinase activity are increased in variety of cancer cells. We aimed to search the involvement of *NEK6* in multidrug resistance and apoptosis in chronic myeloid leukemia. The expression levels of *NEK6* and some of the apoptotic pathway genes such as *BAX*, *BCL-2* and *SURVIVIN* were determined in sensitive and drug resistant subtypes of K652 chronic myeloid leukemia cell lines by RT-PCR method. siRNA silencing studies were performed to examine the effect of expression of *NEK6* on apoptotic behavior in parental K-562 cell line. Cell viability assay was performed by XTT method in order to investigate whether *NEK6* silencing leads to resistance in parental K-562 cells. *NEK6* expression is significantly reduced in imatinib resistant K562 cells. After *NEK6* gene is silenced by specific siRNA in parental K562 cell line, the expression levels of some apoptotic genes, such as *BAX* and *SURVIVIN* were found similar to drug resistant K562 cells. *NEK6* may have potential role in imatinib resistance which may be through apoptotic pathway in chronic myeloid leukemia.

**Keywords:** Chronic myeloid leukemia, K562 cell line, multidrug resistance, Nek6, apoptosis, siRNA



## 1 Introduction

*NEK6* is essential mitotic kinase for proper cell cycle progression. After G2-M phase transition its activity becomes important and during M phase *NEK6* activity increases. Studies showed that *NEK6* depletion results in mitotic arrest at metaphase, demonstrating that *NEK6* is essential for metaphase to anaphase transition (Fry et al. 2012; Lee et al. 2008; Moniz et al. 2011; Yin et al. 2003). After *NEK9* phosphorylates *NEK6* and *NEK7*, they phosphorylate *EG5*, microtubules, and *γ-TURC* which are important for microtubule dynamics. Depletion of either of these kinases results fragile spindles or mitotic arrest (Fry et al. 2012). Moreover, depletion of *NEK6* and *NEK7* leads to spindle assembly checkpoint (SAC) activation and inhibits metaphase to anaphase transition (Moniz et al. 2011). Overexpression of kinase dead domain *NEK6* results in M phase arrest and subsequently apoptosis (Yin et al. 2003). During cytokinesis *NEK6* localizes at midbody and *NEK6* depletion studies showed that when checkpoint is inhibited, cells delayed in late mitosis implying role of *NEK6* also in cytokinesis (O'Regan and Fry 2009).

The *NEK6* gene is situated on chromosome 9q33-34 that is a locus associated with various human cancers, such as neuroblastoma, bladder cancer, and renal cell carcinoma (Jee et al. 2010). Tissue microarray studies show that *NEK6* is overexpressed in breast, colorectal, lung, and laryngeal cancers (Capra et al. 2006). Nassirpour et al, showed that *NEK6* gene transcript, protein level and kinase activity is significantly upregulated in colon, lung, kidney and cervix cancers (Nassirpour et al. 2010).

Multidrug resistance is a complex phenomenon that cancer cells develop resistance to structurally or functionally unrelated drugs. MDR is the biggest obstacle in the treatment of cancer. There are various mechanisms behind MDR in cancer. Decreased drug-influx and increased drug efflux, impairments in apoptotic pathways, problems in drug conversion to its active form, alteration in cell cycle checkpoints and ceramide metabolism and increased drug

metabolism are the major reasons for the development of MDR (Baran et al. 2011; Kars et al. 2006).

Chronic myeloid leukemia (CML) is a form of leukemia characterized by the increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the blood (Calabretta and Perrotti 2004). The aim of current study is to investigate the role of *NEK6* in drug resistance and apoptosis in both sensitive and drug resistant variants of K562 chronic myeloid leukemia cell lines.

## 2 Materials and Method

### 2.1 Cell Culture

Ph+ K-562 (K562/S) cell line was purchased from German Collection of Microorganisms and Cell Culture, Germany. 1000 nM doxorubicin (K-562/Dox) and 1000 nM imatinib (K-562/Ima) resistant K-562 sublines were previously developed in our lab (Baran et al. 2011). The cell lines were cultured in RPMI 1640 medium (Thermo Scientific, USA) with 10% fetal bovine serum (FBS) (Biochrome, Germany) and 0.1% gentamycin (Biological Industries, Israel). Drug resistant sublines were grown with their reported drug concentrations.

### 2.2 RNA Isolation and cDNA Synthesis

RNA isolation was performed using TriPure reagent (Roche, Germany) according to the manufacturer's instructions. RNA quality and quantity was determined by NanoDrop Spectrometer (Thermo Scientific, USA). RNA integrity was checked by 1% agarose gel electrophoresis. cDNA was synthesized from 2 mg DNase I-treated RNA by using RevertAid reverse transcriptase (Thermo Scientific, USA).

### 2.3 Gene Expression Analyses

Expression analysis of *NEK6* gene was performed by TaqMan Gene Expression Assay (Roche, Germany). β-actin gene was used as internal control. Expression analyses of apoptotic genes were performed by qRT-PCR experiments by using FastStart SYBR Green (Roche, Germany). Primer sequences and amplicon sizes of apoptotic genes is shown in Table 1.

### 2.4 siRNA Transfection

Small interfering RNA was purchased from Qiagen, Germany and used to transiently silence *NEK6* gene. siRNA that did not have a target in human transcriptome was used as control (Santa Cruz, USA). siRNA delivery was performed by HiPerfect Transfection Reagent (Qiagen, Germany) according to manufactor's instructions.

### 2.5 Annexin V/PI Staining

In order to determine apoptotic status of cells, Annexin-V-FLUOS Staining Kit (Roche, Germany) was used according to manufactor's intructions. Cells were analyzed with Acuri Flow Cytometer in FL-1 and FL-3 channels.

**Table 1** Primer sequences and amplicon sizes of apoptotic genes

| Gene | Sequence 5'-3' | Amplicon size (bp) |
|---|---|---|
| *BAX* | F-TCTGACGGCAACTTCAACTG | 188 |
| | R-TTGAGGAGTCTCACCCAACC | |
| *BCL-2* | F-CCCGCGACTCCTGATTCATT | 166 |
| | R-AGTCTACTTCCTCTGTGATGTTGT | |
| *SURVIVIN* | F-AGCCAGATGACGACCCCATAGAGG | 60 |
| | R-AAAGGAAAGCGCAACCGGACGA | |
| *B-ACTIN* | F-CCAACCGCGAGAAGATGA | 97 |
| | R- CCAGAGGCGTACAGGGATAG | |

### 2.6. Tryphan Blue Exclusion Assay

Cell viability was determined by tryphan blue exclusion assay (Biological Industries, Israel). In order to analyze cell viability of siRNA treated K562 cells, $3x10^4$ cells were seeded on 6-well plates with a total volume of 1 ml. Cells were remained untreated or treated with 50 nM either mock or Nek6 siRNA. After 48 and 72 hours incubation alive cells that were not stained by tryphan blue were counted on 16 squares of hematocytometer and total cell number in 1 ml was calculated.

### 2.7 Statistical analysis

Results obtained from two experiments were analyzed by GraphPad Prism Version 5 with one-way ANOVA followed by Tukey's Test. $p<0.05$ value was considered as statistically significant.

## 3 Results

### 3.1 *NEK6* expression profiles in K-562 cell line and its drug resistant sublines

The expression profiles of *NEK6* gene in K-562 cell line and its drug resistant sublines were examined. According to the results, *NEK6* gene expression was significantly decreased in doxorubicin and imatinib resistant K-562 cell lines compared to its sensitive subline. Nek6 expression level decreased approximately 3 fold in both resistant K562 cell lines (Figure 1).

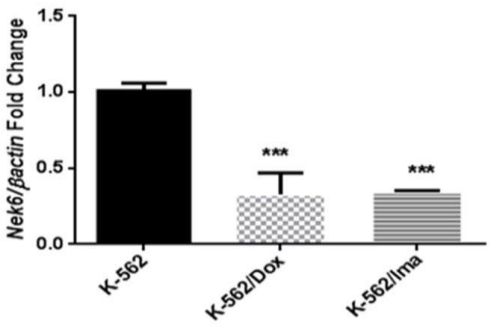


**Fig. 1** Expression levels of *NEK6* gene in drug sensitive and drug resistant K562 cell lines. ***$p<0.05$

### 3.2 Expression profile of apoptotic genes in parental K-562 cells and its resistant sublines

Screening apoptotic gene expression levels in sensitive K-562 and its resistant sublines were performed to understand the potential role of *NEK6* in MDR. According to the results, *BAX*, and *BCL-2* expression levels were significantly decreased in imatinib and doxorobucin resistant K562 cells whereas *SURVIVIN* expression was significantly downregulated and upregulated in doxorubicin and imatinib resistant K-562 cells, respectively compared to parental cells (Figure 2).

The expression level of *BAX* and *BCL-2* were significantly decreased in both doxorubicin and imatinib resistant cells. *BAX* to *BCL-2* ratio in the imatinib resistant subline was approximately 3 which may imply 3 fold increased tendency to apoptosis (Figure 2a and 2b). Interestingly, *SURVIVIN* expression was only increased in imatinib resistant subline (Figure 2c).

### 3.3 *NEK6* silencing by specific siRNA

siRNA silencing studies were performed to examine the effect of expression of *NEK6* on apoptotic behavior in parental K-562 cell line. *NEK6* expression analysis by qRT-PCR after transfection of parental K562 cells with *NEK6* specific siRNA was given in Figure 3.

The analysis shows that *NEK6* gene expression level was decreased two folds after 48 hour *NEK6* specific siRNA treatment, compared to untransfected cells (Figure 3). In siRNA transfection efficiency studies by flow cytometry, we showed that 60% of cells transfected by fluorescein conjugated siRNA (data not shown). Figure 3 shows that transfection of K562 cells with 50 nM specific siRNA leads two folds decrease in mRNA levels of *NEK6*. *NEK6* expression level did not change in cells transfected with control siRNA. Since the expression level of *NEK6* was recovered after 72 hours, further studies performed by treating cells with *NEK6* siRNA for 48 hours.

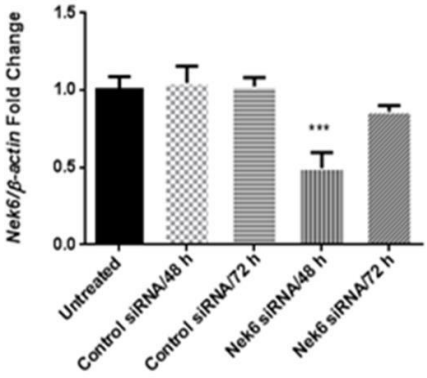


**Fig 3.** *NEK6* expression in parental K562 cell line following 48 hours and 72 hours post-transfection. *** $p$<0.00

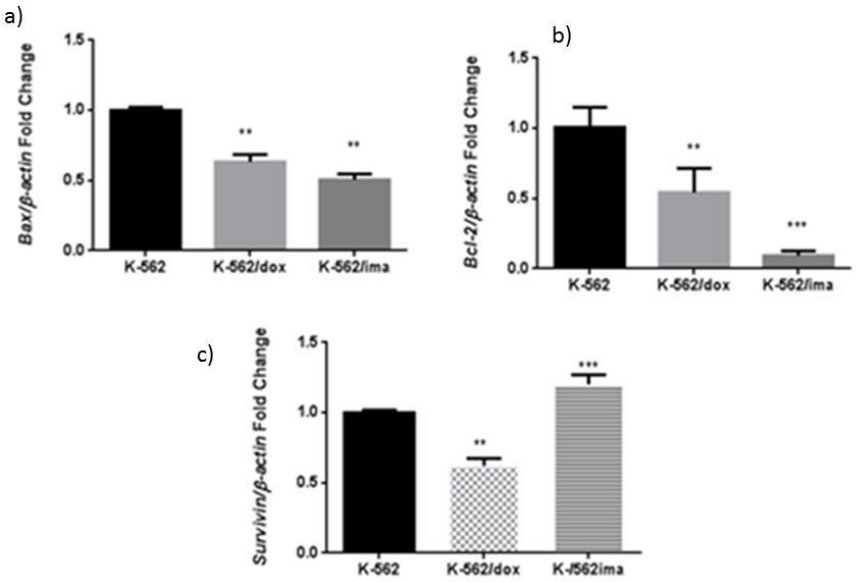


**Fig. 2** Expression levels of A. *BAX*, B. *BCL-2* and C. *SURVIVIN* genes in K562 cell lines, when $p$<0.05.

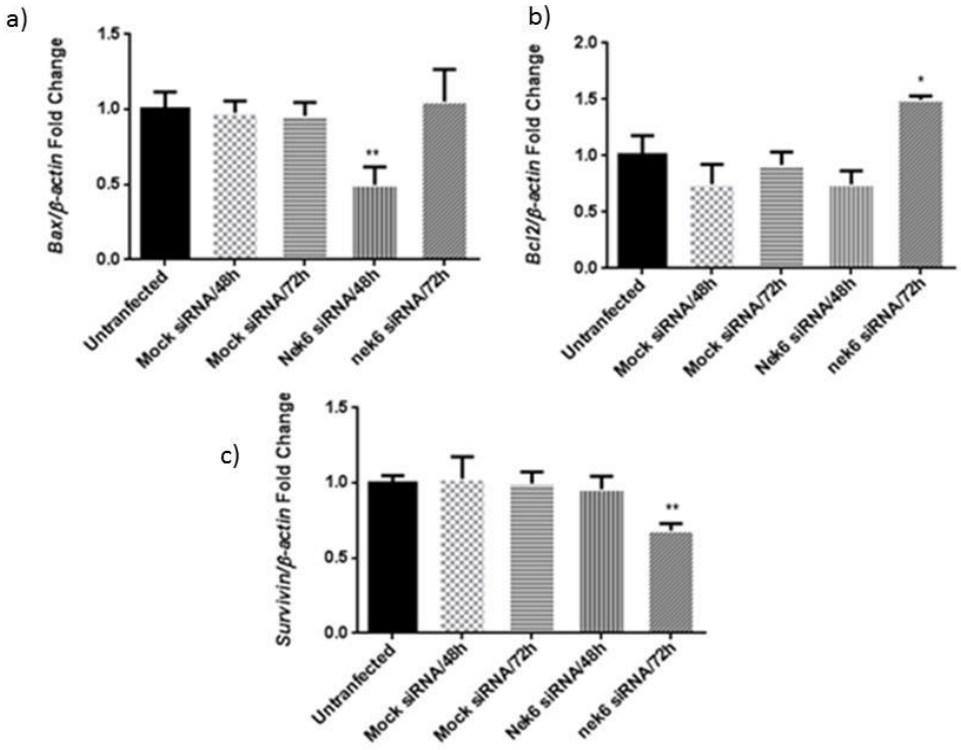


**Fig. 4** Expression levels of A. *BAX*, B. *BCL-2* and C. *SURVIVIN* genes in parental K562 cell line after silencing of *NEK6* gene, when $p<0.05$.

### 3.4 The effect of *NEK6* silencing on apoptosis in K-562 cell line

In order to show whether *NEK6* gene has a role in MDR in CML through disregulated apoptotic pathway, the expression levels of apoptotic genes after *NEK6* gene silencing in parental K-562 cell line were investigated (Figure 4). According to results shown in Figure 4, *BAX* to *BCL-2* ratio decreased to approximately 0.7 (Figure 4a and 4b). This significant decrease (approximately 4 folds) in *BAX* to *BCL-2* ratio after *NEK6* silencing indicates escape from apoptosis. This may represents parental K562 cells gain resistance to imatinib through disregulating the apoptotic pathway after *NEK6* silencing. Interestingly, when *BAX* to *BCL-2* ratio decrease, *SURVIVIN* gene expression is also decrease in this cell line (Figure 4c).

### 3.5 Apoptosis studies by Annexin V/PI staining and tryphan blue exclusion assay

Annexin V/PI staining was performed in order to show apoptotic status of transfected cells. The lower right quadrant represents early apoptotic cells and the upper right quadrant shows late apoptotic and necrotic cells (Figure 5). According to Figure 5 more than 95% of cells treated with only lipofectamine were alive. After siRNA treatment, only 7% of cells underwent apoptosis after 48 hours. Apoptotic cell amount after 72 hour of silencing declined 3%. This result is consistent with apoptotic gene expression analysis in the context of escaping from apoptosis after *NEK6* silencing. Interestingly, more than 30% of cells transfected with mock siRNA appeared as early apoptotic. The underlying reason could be off target effect of using high concentration of siRNA. The gene expression analysis did not underpin apoptotic behavior of cells transfected with mock siRNA. Figure 6 shows the cell viability of K-562 cells after variety of treatment analyzed by tryphan blue exclusion test. Results were consistent with Annexin V/PI staining. Cells transfected with *NEK6* siRNA were 90% alive and it is statistically significant with respect to etoposide treated K562 cells (Figure 6).

### 3.6 Imatinib resistance in parental K562 cells after *NEK6* silencing

K562 parental cells were subjected to different concentration of imatinib and IC50 value was determined by XTT analysis after *NEK6* silencing. Cell viability assay was performed by XTT in order to investigate whether *NEK6* silencing leads to resistance in parental K-562 cells. IC50 value for untreated K-562 cells is 150 nM whereas after *NEK6* silencing IC50 increased to 1780 nM. More than 10 fold increase in IC50 after *NEK6* silencing, indicates that K562 cells show high resistance to imatinib. When parental K-562 cells treated with imatinib only we observed an intrinsic resistance up to 200 nM. This resistance may be contributed by *BCR/ABL* dependent or independent mechanisms as mentioned earlier. In *NEK6* silenced K-562 cells treated with imatinib, cell viability did not decrease more than 50% percent. This behaviour could be explained by *NEK6* recovery after 72 hours of transfection.

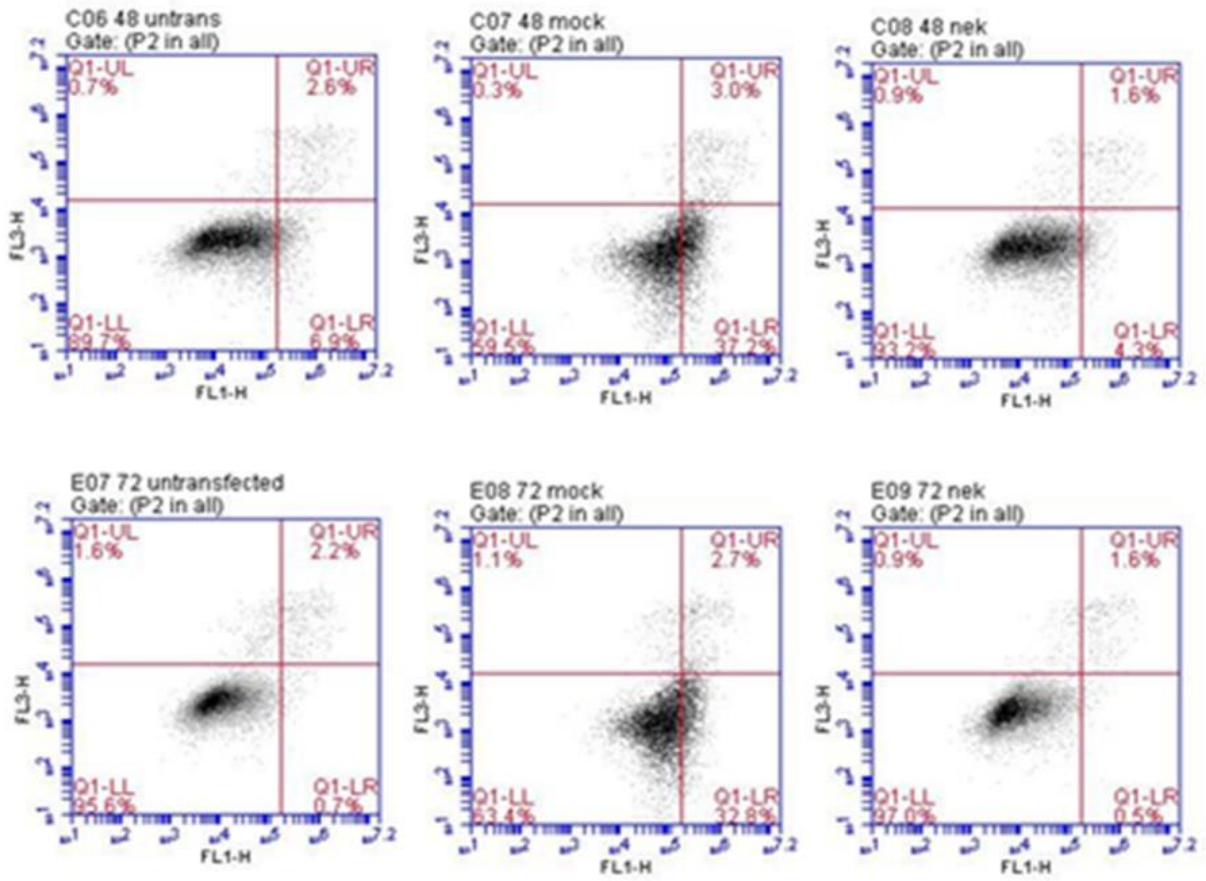


**Fig. 5** Flow cytometer results of Annexin V/PI staining. Cells treated with **A.** lipofectamine alone, **B.** mock siRNA and **C.** *NEK6* siRNA after 48 hours. Cells treated with **D.** lipofectamine alone, **E.** mock siRNA and **F.** *NEK6* siRNA after 72 hours. K-562 sensitive cells were transfected with 50 nm *NEK6* specific siRNA. After 48 and 72 hours treatment cells were stained with Annexin V/PI.

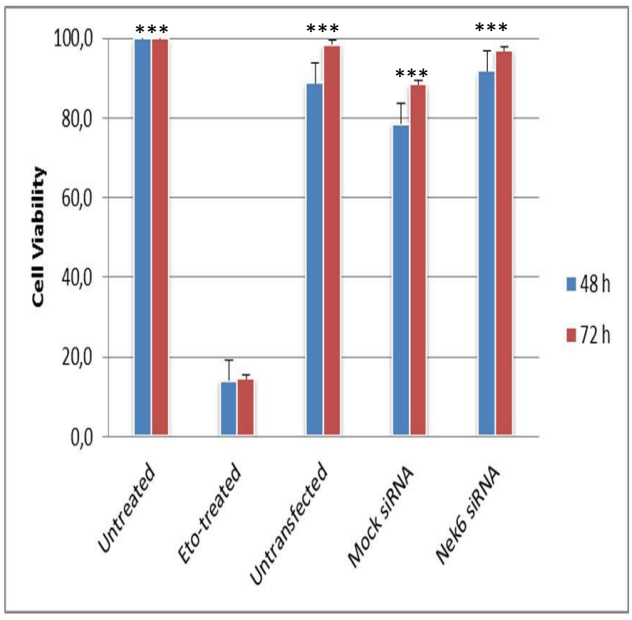


**Fig. 6** Tryphan Blue staining of K-562 cells under without treatment, with etoposide treatment, lipofectamine treatment, mock siRNA and *NEK6* siRNA treatment after 48 and 72 hours. ***$p$<0.05

## 4 Discussion

In this study, we aimed to search the possible involvement of *NEK6* in drug resistance in chronic myeloid leukemia. According to our results, *NEK6* gene expression was significantly decreased in doxorubicin and imatinib resistant K-562 cell lines compared to its sensitive subline. However, *BAX* to *BCL-2* ratio in the imatinib resistant subline was approximately 3 which may imply 3 fold increased tendency to apoptosis. This ratio is not as significant (approximately 1.4 fold) in doxorubicin resistant subline. Impairment in apoptotic pathways is one of the main reasons for MDR development. Some pro-apoptotic genes, such as *BAX* and *BAK*, are down regulated and the expression levels of some anti-apoptotic genes, such as *BCL-2* and *SURVIVIN*, are increased in various hematological malignancies (Kauffmann and Vaux 2003). In recent years, several studies have investigated the significance of *BAX/BCL-2* ratio and its correlation with several diseases including cancer. In many organisms, *BCL-2* family members modulate apoptosis via *BAX/BCL-2* ratio serving to as a rheostat to determine cell susceptibility to apoptosis. On the other hand, *SURVIVIN* expression was slightly upregulated in imatinib resistant K562 cells compared to parental cells whereas it decreased in doxorubicin resistant K-562 cell line. The different

expression patterns of *BAX* to *BCL-2* ratio and *SURVIVIN* apoptotic pathway genes in doxorubicin and imatinib resistant K562 cells may indicate that these two drugs activate different pathways during drug resistance development.

*NEK6* gene expression was transienly silenced by specific siRNA in order to show whether *NEK6* gene has a role in MDR through disregulated apoptotic pathway in CML cells. The expression levels of some apoptotic genes and *BAX/BCL-2* ratios were investigated in parental K562 cells after *NEK6* silencing. According to our results, *BAX* to *BCL-2* ratio decreased to 4 folds indicates escape from apoptosis after *NEK6* silencing. This may represent parental K562 cells gain resistance to imatinib through disregulating the apoptotic pathways. Therefore, *NEK6* gene may have a potential role in the development of drug resistance in CML.

According to literature, in HeLa and MCF-7 cells *NEK6* depletion triggers apoptosis by increasing *BAX* and *BAD* expression level. Besides, cleaved caspase-3 and PARP levels also increase after functional knockdown of *NEK6* (Nassirpour et al. 2010). *NEK6* depletion cause mitotic arrest followed by apoptosis in solid tumors. The results obtained with K562 cells seem to be contradictory to these results. This may indicate possible different roles of *NEK6* on different types of cells which either adherent or in suspension. On the other hand, in this study, *NEK6* siRNA transfection was transient and this treatment only cause two folds decrease in mRNA level of *NEK6* within 48 hours while the expression level was recovered after 72 hours. Stable transfection with siRNA or overexpression of kinase dead domain as in the case of Nassirpour study may result inducing apoptosis (Nassirpour et al. 2010). In order to validate potential role of *NEK6* in drug resistance elicited by alteration in apoptotic pathway, these studies should be repeated in stably transfected K-562 cells.

## 5 Conclusion

As a conclusion, in this study we observed that *NEK6* expression is significantly reduced in imatinib resistant K562 cells. After *NEK6* gene is silenced by specific siRNA in parental K562 cell line, the expression levels of some apoptotic genes, such as *BAX* and *SURVIVIN* were found similar to drug resistant K562 cells. These results may indicated that *NEK6* can have potential role in imatinib resistance which may be through apoptotic pathway in chronic myeloid leukemia.

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## Authors' contributions:

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Formal Analysis: Ayça Nabioğlu, Pelin Mutlu, Ufuk Gündüz

Data curation: Ayça Nabioğlu, Pelin Mutlu

Original draft preperation: Ayça Nabioğlu, Pelin Mutlu

Review and editing: Pelin Mutlu, Serap Yalçın-Azarkan, Ufuk Gündüz

## Conflict of interest disclosure:

The authors of this study declare that they have no conflict of interest.

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# Characterization of GG/GO hybrid hydrogel for strain sensor application

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**Abstract:** Hydrogel materials are 3D polymeric materials that have a wide range of applications. Strain-sensor applications, one of the application areas of hydrogels, continue to attract the attention of researchers. In this study, gellan gum-graphene oxide (GG/GO) hybrid hydrogels were synthesized for strain-sensor application. FTIR, XRD, and SEM measurements and strain sensor application analyses of the synthesized hydrogels were performed. It has been observed that the GG/GO hybrid hydrogels obtained as a result of the findings are promising for strain-sensor applications.

**Keywords:** Strain-sensor; hybrid hydrogel; 3D polymer.



## 1 Introduction

The research of flexible and wearable sensors, which fall under the class of smart devices, in the fields of electronic skin for human motion detection and health monitoring has continued exponentially in recent years (Afsarimanesh et al. 2020). Sensors with flexibility, which is one of the main features of new generation wearable electronics, can convert mechanical deformation into repeatable electrical signals due to changes in resistance under external force (Niu et al. 2020). Various attempts have been made, such as metal films, metal-polymer composite structures, metal films on flexible substrates, and nanoscale metal films, to produce wearable sensors that have this feature and can monitor user-related parameters. However, the semiconductor materials used in these devices have the disadvantage that they are brittle and allow only small strains (Liu et al. 2017; Wang et al. 2021; Wang et al. 2019).

Hydrogels are three-dimensional (3D) microstructured polymeric networks with high flexibility and hydration (Kailasa et al. 2022). Conductive hydrogels, which have inherent flexibility and biocompatibility, have received great attention in recent years as soft conductors for wearable strain sensors applications (Rahmani and Shojaei 2021). Studies of conductive hydrogels in high sensitivity, short response time, and multiple recycling applications are available in the literature (Sun et al. 2019).

Conductive hydrogels refer to a class of hydrogels that contain conductive electronic networks composed of conductive nanomaterials or conductive polymers (Zhang et al. 2019). Some issues need attention and continue to be improved in conductive hydrogel applications. E.g., in motion sensor applications, viscoelastic materials generate unwanted noise due to their electrical response to external force, usually due to dynamic changes caused by viscous deformation of conductive networks. Besides, the poor interface between hydrogel matrices and conductive fillers causes an irreversible movement or rearrangement of conductive networks, often irreversibly during deformation. This results in poor long-term reliability and a low signal-to-noise ratio as a result. For this reason, increasing the sensitivity of hydrogels used for motion sensors remains a topic of the current literature (Tang et al. 2020; Xu et al. 2019) .

In this study, gellan gum-graphene oxide (GG/GO) hydrogels with different contents were prepared as conductive hydrogels. In the analysis of strain sensor application, the sensitivities were compared after their responses to wrist joint movements, and the characterization of the hydrogel samples was carried out.

## 2 Materials and Methods

GG was dissolved in 2.5% (w/v) deionized water under constant stirring for 15 minutes at 85 °C. To prepare GG/$GO_{1.0}$, GG/$GO_{2.0}$ and GG/$GO_{4.0}$ hydrogels, it is prepared by mixing with GO at 85°C for 15 min, when used at a rate of 1.0, 2.0, and 4.0 wt. %, at different rates. The prepared solutions were was poured into molds and then cooled to room temperature to obtain three-dimensional GG/$GO_{1.0}$, GG/$GO_{2.0,}$ and GG/$GO_{4.0}$ hydrogels.

The resistance changes of the strain sensor under strain were analyzed using a multimeter (Keithley 2612A Source Meter). Produced sensors were attached to the wrist joint skin using double-sided tape to detect wrist movements. Copper wires were used to connect the sensor attached to the wrist joint. The change in relative resistance was calculated using the Eq. (1):

$$\Delta R/R_0 = (R - R_0)/R_0 \quad (1)$$

where $R_0$ and R denote the resistance of the original state and the resistance of real-time, respectively.

Hydrogel samples were analyzed using FTIR (Spectrum Two Perkin-Elmer Co.) in the wavelength range of 4000 $cm^{-1}$-400 $cm^{-1}$ by scanning at 4 $cm^{-1}$ resolution. Morphological studies were observed using scanning electron microscopy (SEM, JEOL, JMS 6060). The hydrogels, which were previously lyophilized and cut into suitable pieces, were fixed on conductive carbon bands and then covered with a thin layer of gold to ensure conductivity (Polaron CS7620). For the phase characterization of the synthesized hydrogels, analysis was carried out between 5° and 90° using Cu Ka radiation (λ= 0.15406 nm) operating at 40 kV, 30 mA, and X-ray diffractometry (XRD, D/Max 2200 LV).

## 3 Results and Discussion

After mounting a hydrogel sensor on a volunteer's wrist, the 90° flexing wrist stretched the gel and produced reproducible $\Delta R/R_0$ of 30%, 37%, and 34% for hydrogels GG/$GO_{1.0}$, GG/$GO_{2.0,}$ and GG/$GO_{4.0}$, respectively (Fig. 1). The obtained results demonstrate the great potentials of conductive hydrogels fabricated for flexible wearable devices. The sensitivity of the hydrogel sensors increased up to GG/$GO_{2.0}$ and decreased at GG/$GO_{4.0}$. The slight decrease in $\Delta R/R_0$ when the concentration of the additive material reaches 4% by weight can be attributed to the high density of the additive in the GG polymeric networks, making them difficult to replace when stretched. Therefore, GG/$GO_{2.0}$ as the optimum selected sample was used for subsequent characterizations and named GG/GO.

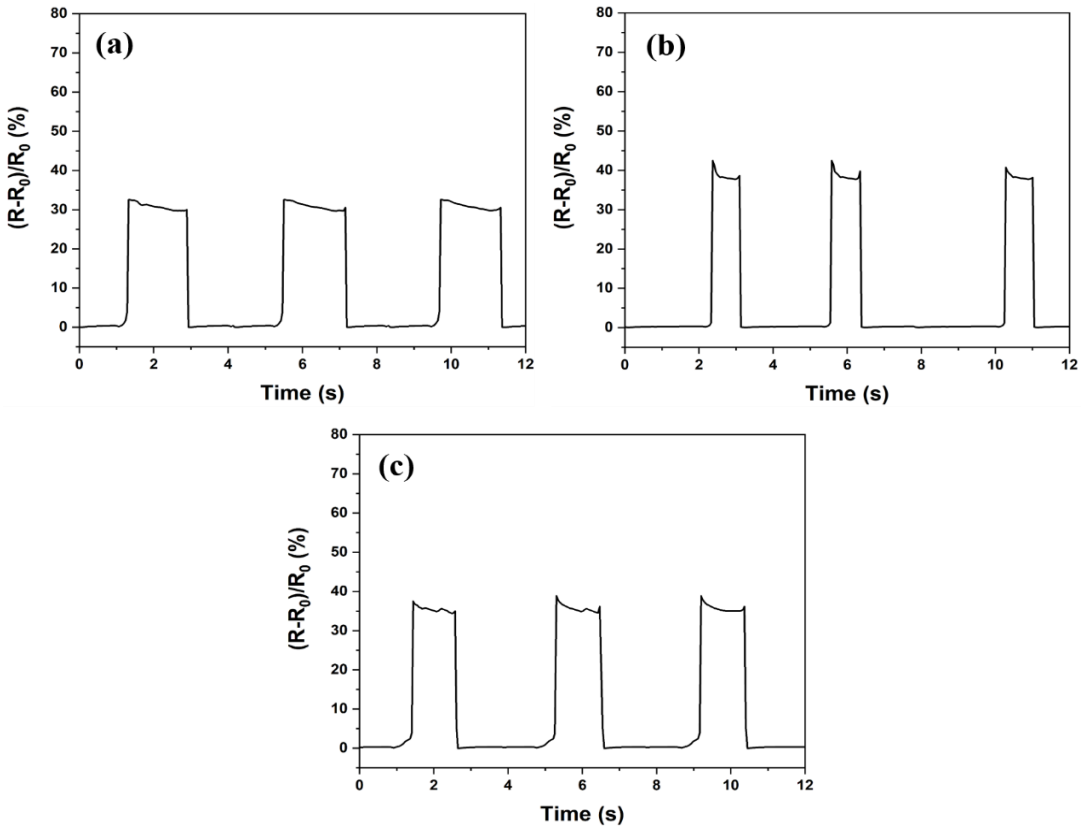


**Fig. 1** The real-time detection of human motions when the (a) GG/$GO_{1.0}$, (b) GG/$GO_{2.0}$, and (c) GG/$GO_{4.0}$ hydrogel strain sensors directly adhered to the wrist joint.

Section morphologies of the lyophilized hydrogels were examined using SEM, as shown in Fig. 2. GG-glycerol hydrogel tends to form a homogeneous structure with smaller pores and tighter networks. No obvious agglomerated structure was observed in the hydrogel sample, in which an interconnected porous network was observed.

The obtained hydrogels were also analyzed by FTIR and XRD, as shown in Fig. 3. For the FTIR spectrum of GG (Fig. 3a), the broad band at 3500−3000 $cm^{-1}$ was contributed to the stretching vibration of $OH^-$ groups, the characteristic peak at 2925 $cm^{-1}$ was attributed to the stretching vibration of C–$H_2$ groups. The characteristic peaks at 1611 and 1415 $cm^{-1}$ were ascribed to the asymmetric and symmetric stretching vibration of carboxyl groups that existed in the salt form, and 1032 $cm^{-1}$ was attributed to the $CO^-$ stretching (Lu et al. 2019; Wang et al. 2021). When looking at the FTIR spectrum of GG/GO, it is observed that there is no significant difference. Fig. 3b displays the X-ray diffraction pattern of GG and GG/GO hydrogels. GG showed a broad peak at approximately 2θ = 20°, indicating their amorphous nature with lower crystallinity. A similar amorphous structure is seen when looking at the FTIR analysis of the GG/GO hydrogel sample.

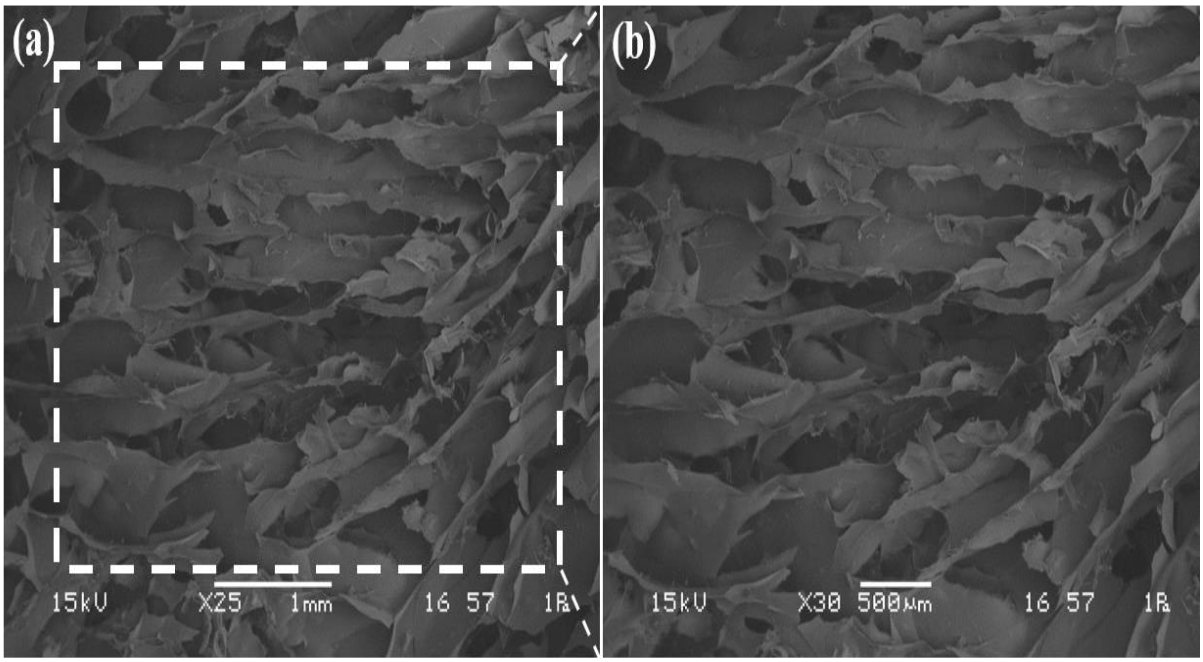


**Fig. 2** (a) Cross-section SEM image, and (b) magnified cross-section SEM image of the lyophilized GG/GO hydrogel.

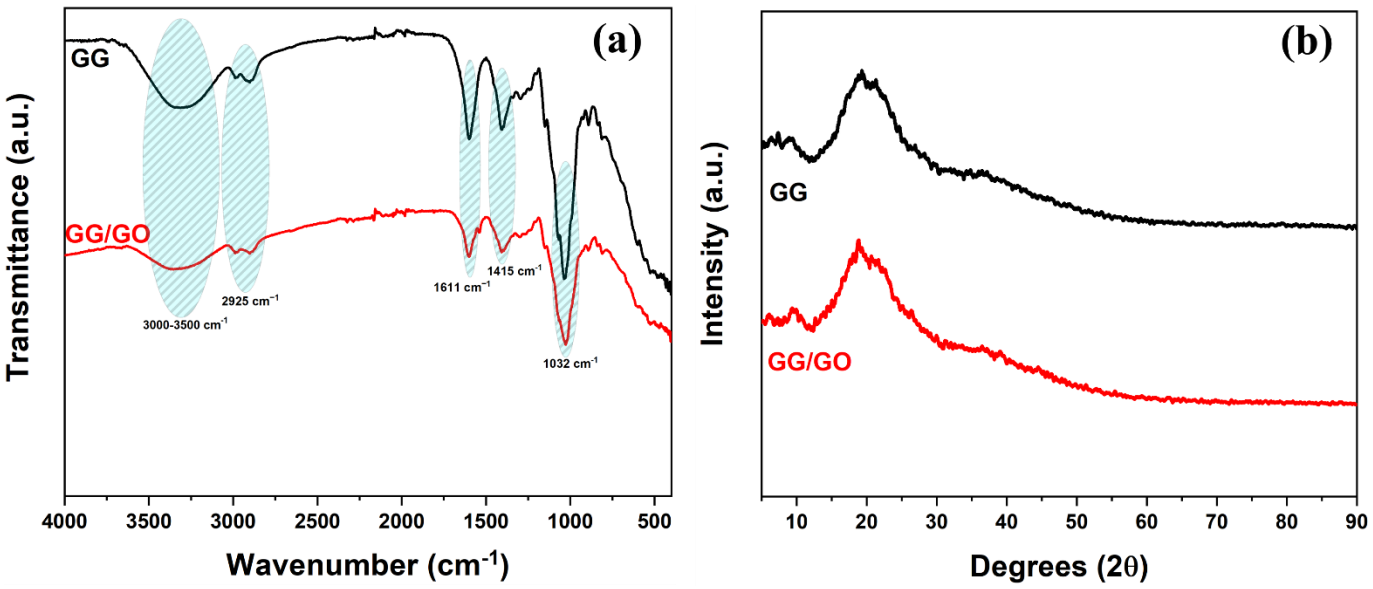


**Fig. 3** (a) FTIR spectra, and (b) XRD pattern of the GG and GG/GO hydrogels.

## 4 Conclusion

GG based hydrogels with different GO content were prepared and measured as a wrist joint movement sensor. As a result of the study, it was concluded that the sensitivity of the motion sensor feature of the GG-based hydrogel was adjusted by changing the additive ratio in its content. In addition, the promise of the prepared hydrogels as a wearable motion sensor was observed in this study. Since it is expected that hydrogel strain sensors will take place with increasing interest in future studies, the susceptibility of the obtained hydrogel to movements and vibrations such as heartbeat, speech can be examined and adhesion tests can be performed.

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**Authors' contributions:** The authors contributed equally to the article.

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# Production of Natural and Functional Pigments in *Arthrospira (Spirulina) platensis* cultivated in Laboratory Conditions

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**Abstract:** In this study, *Arthrospira (Spirulina) platensis* was cultivated under laboratory conditions at 30 ± 1 °C and 80 µmol.m$^{-2}$s$^{-1}$ illumination. Intensive cultivation of *Arthrospira (Spirulina) platensis* was carried out in 250 mL, 500 mL and 2 L flasks, and 10 L and 20 L carboys. Zarrouk Medium was used as a nutrient medium. Constant aeration also applied in the 2 L flasks and the carboys. According to the optical density results, continually increase in the biomass yields of *Arthrospira (Spirulina) platensis* observed until the 24th day of the cultivation period. During the experiment, water temperature was recorded as 29.89 ± 0.45 °C, PH 10.88 ± 0.87, oxygen level of 10.18 ± 2.67 mg/L. *Arthrospira (Spirulina) platensis* has a wide range of pigments, including chlorophyll *a*, total carotenoids and phycobiliproteins (protein-rich phycocyanin and phycoerythrin). The primary potential of these pigments seems to be their use as natural dyes, but a growing number of studies have also shown that the functional properties of these pigments related to health benefits and wide pharmaceutical applications. During the cultivation of *Arthrospira (Spirulina) platensis*, chlorophyll *a*, total carotene, phycocyanin and phycoerythrin production were determined daily by using spectrophotometric methods. The study results showed that the amount of chlorophyll *a* increased until the 29th day when the amount of β carotene increased until the 14th day. The highest chlorophyll *a* was 5.46 ± 0.57 mg/gon the 22nd day, the highest total carotene was 1.82 ± 0.25 mg/g on the 7th day. On the 14th day of the experiment, the amount of phycocyanin was 172.85 ± 7.35 mg/g and the amount of phycoerythrin was 75.54 ± 4.98 mg/g.

**Keywords:** *Arthrospira (Spirulina) platensis* culture; natural and functional pigments; chlorophyll *a*; total carotene; phycocyanin; phycoerythrin



## 1 Introduction

*Arthrospira (Spirulina) platensis* is one of the microalgae species cultivated, which is known as the superfood of the 1990s. In addition, it is a prokaryotic algae that has been living in nature for 3.6 billion years. The blue-green algae *Spirulina* contains filamentous microalgae organized microscopic cells. The optimum growth temperature for *Arthrospira (Spirulina) platensis*, which has thermophilic and alkalophilic features, is 35-37ºC. *Spirulina* is an algae species that prefers high PH (9-10) levels and it can be monocultured without contamination problems due to these particularities. The general purpose of its production is to benefit from the richness of its biochemical structure and to provide a protein source to people (Richmond 2000). All photosynthetic organisms, such as *Spirulina*, contain organic pigments to use light energy (Rizzo et al. 2015). There are three main classes of pigments in algea. These pigments are chlorophylls, carotenoids, and phycobilins. Chlorophylls (green pigments) and carotenoids (yellow and orange pigments) are lipophilic and soluble in alcohol, diethyl ether, benzene, and acetone. In addition, phycobilins are hydrophilic and soluble in water (Richmond 2000).

The only chlorophyll they contain is chlorophyll a and its amount is between 0.8-1.5% of their dry weight when the pigment formation of *Spirulina* is scrutinized. Xanthophyll content is quite high in freeze-dried *Spirulina* (6.9 g/kg). Myxoxanthophyll (37%), Beta Carotene (28%), and Zeaxanthin (17%) are the other major inside of carotenoids (Anderson et al. 1991). Consequently, its rich pigment content, *Spirulina* is used as a feed additive for ornamental fish, particularly for goldfish (Miki et al. 1986). *Spirulina* contains C-phycocyanin and allophycocyanin that are light-harvesting pigments in phycobilisomes. Furthermore, these are the pigments in protein structure with the highest economic value found in *Spirulina* (Fox 1996, Leema et al. 2010). Approximately 20% of the protein content of *Spirulina*

consists of phycocyanin, a water-soluble blue pigment. The maximum absorption of phycocyanin is 620 nm (Ciferri, 1983, Cohen, 1997). Vonshak (1997) declared that phycocyanin served as a storage nutrient in *Spirulina*. In addition, it has been reported that phycocyanin, as a natural pigment in the cosmetic, food, and pharmaceutical industries and also can replace synthetic pigments suspected of being carcinogenic (Cohen 1997, Furmaniak et al. 2017). Phycocyanin is an odorless and non-toxic powder and it is commercially known as "Lina blue" (Vonshak 1997).

The other important value of Phycocyanin is its particularity as an antioxidant pigment. And also it is known to reduce microsomal lipid oxidation and to destroy free radicals (hydroxyl and peroxyl). Aforementioned, it has been determined that phycobilin, which is found in phycocyanin, plays a role in the removal of hydroxyl radicals (Vonshak 1997). Phycobilin is utilized as a biochemical isotope in immunoassays, is used in microscopy and cytometry studies, due to its fluorescent properties (Vonshak 1997).

If improper drying methods are used during the processing of *Spirulina*, they cause about 50% of the phycocyanin to be lost. One of the extraction methods, freezing, crushing the cells with a mortar, homogenization (10000 rpm) provided 19.4 ± 0.4 mg of phycocyanin from each 100 mg of dry *Spirulina*. Extraction with water is a very slow method. In experiments with acid, it was observed that phycocyanin deteriorated. Phycocyanin remains intact at 9 ± 1 ºC and in the PH range of 5-7.5. It has been determined that phycocyanin deteriorates at temperatures above 40 ºC (Vonshak 1997).

Chlorophyll, total carotene, and phycobiliprotein (phycocyanin and phycoerithrin) amounts of *Arthrospira (Spirulina) platensis* grown under laboratory conditions were determined in this assessment.

## 2 Materials and Method

### 2.1 *Arthrospira (Spirulina) Platensis* and Culture Conditions

1 L glass flasks containing 3 Zarrouk broths were prepared for the production of *Spirulina* under laboratory conditions (Zarrouk 1966). And daylight fluorescent lamps were used to illuminate the cultures. Cultivation experiments were carried out by adjusting the laboratory temperature to 30±1 °C with an air conditioner and a light intensity of 80 µmol $m^{-2}$ $s^{-1}$. In addition, continuous lighting was applied.

The optical density of *Spirulina* was determined spectrophotometrically by taking the values of the samples taken from *Spirulina* glass flasks into test glass tubes, at a wavelength of 680 nm in a spectrophotometer calibrated with pure water. SP-300 brand spectrophotometer was used for this purpose, the temperature, oxygen, salinity, and PH values of the water were recorded during the experiment, Moreover water temperatures were determined with the help of 0.1 °C sensitive thermometer, and PH values were defined with the help of Orion PH-meter. The amount of dissolved oxygen was measured in the laboratory with the help of an Oxygen-meter.

### 2.2 Pigment Extraction and Quantification

Chlorophyll-a and total carotene content of *Arthrospira (Spirulina) platensis* were determined by the spectrophotometric method as indicated below. 5 mg of dried *Spirulina* sample was taken and treated with 5 ml of acetone, and the mixture was homogenized for 10 minutes with the help of a homogenizer for this process. It was then extracted in an ultrasound water bath at 70 °C for 10 minutes. In addition, the obtained extract was centrifuged at 3500 rpm for 10 minutes, and the liquid part containing the pigments was taken and read in the Spectrophotometer (SP-300) at 666 nm and 475 nm wavelengths. The values read were placed in the formula below and the amount of chlorophyll-a and total-carotene was determined.

Chlorophyll-a (mg/g) = 13.9 * A666

Total Carotene (mg/g) = 4.5 *A475 (Jensen 1978)

The ordered procedure was followed to determine the Phycocyanin and Phycoerithrin amount of *Spirulina*. The solutions ($K_2HPO_4$ and $KH_2PO_4$) to be used for phycocyanin were prepared, before starting the analysis. 5 mg of wet *Spirulina* sample was gathered from the cultured *Spirulina* samples by centrifugation method. $K_2HPO_4$ and $KH_2PO_4$ were added in the same proportions. Moreover, the samples were kept in the deep freezer for 10 days and filled and thawed at 12-hour intervals. The samples were kept in the Centrifuge at 3500-5000 rpm for 10 minutes after 10 days and the biliprotein pigments in the liquid part were read at A562, A615, A652 wavelengths in the Spectrophotometer and the amount of phycocyanin and phycoerythrin (mg/L) was calculated using the formulas below (Jensen 1978).

Phycocyanin (mg/L)=(A562)-0.208*(A615)*/5.09

Phycoerythrin (mg/L)=A562-2.41*Phycocyanin amount-0.849/9.62

### 2.3 Statistical Analysis

During the process, all data of the trials were analyzed using ANOVA in the SPSS program (Özdamar 2009). The whole of data was submitted as mean ± standard deviation.

## 3 Results

### 3.1 Biomass Change of *Arthrospira (Spirulina) platensis*

The growth characteristics of *Spirulina* were investigated at a constant light intensity of 80 µmol $m^{-2}$ $s^{-1}$ by keeping the temperature constant at 30 °C in this experiment. It was evaluated that there was an increase in the enema at 80 µmol $m^{-2}$ $s^{-1}$ light intensity until the 24th day when looking at the optical density changes (Figure 3.1). Average water parameters recorded throughout the experiment are presented in Table 3.1.

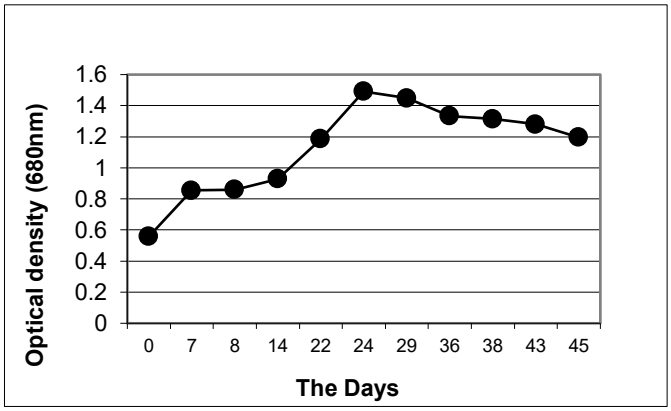



**Fig. 3.1** Biomass or optical density of *Arthrospira (Spirulina) platensis* cultivated at 30 °C at 80 µmol $m^{-2}$ $s^{-1}$ light intensity.

**Table 3.1** Water quality parameter results in *Arthrospira (Spirulina) platensis* culture units during the experiment

| | |
|---|---|
| Light intensity (µmol $m^{-2}$ $s^{-1}$) | 80 |
| Temperature (°C) | 29.89 ±0.45 |
| PH | 10.88 ±0.87 |
| Oxygen (mg/L) | 10.18 ±2.67 |

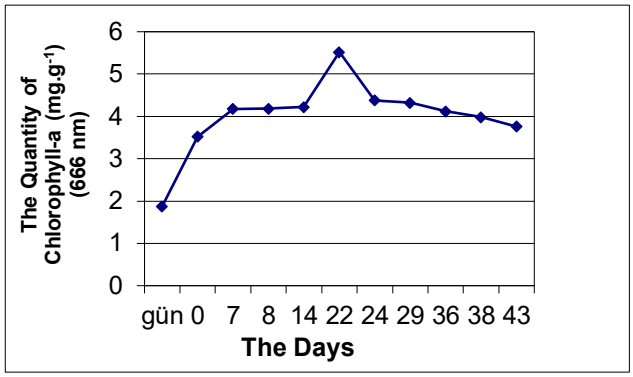
### 3.2 The Pigment Composition of *Arthrospira (Spirulina) platensis*

It is presented that Chlorophyll-a, Phycocyanin, Phycoerythrin, and total carotene amounts of *Spirulina* clearly in Table 3.2. Following the chlorophyll-a, the amount of *Spirulina* is 5.46 ± 0.57 mg/L on the 22nd day. The phycocyanin amount on the 14th day is 172.85 ± 7.35 mg/L. The amount of phycoerythrin is 75.54 ± 4.98 mg/L and the total carotene amount was found to be 1.82 ± 0.25 mg/g on the 7th day. An increase was observed until the 29th day when the changes in the Chlorophyll-a amount of *Spirulina* were examined (Figure 3.2).

**Table 3.2** The average Chlorophyll-a, Phycocyanin, Phycoerythrin, and total carotene of contents of *Arthrospira (Spirulina) platensis*.

| Pigments | N=3 |
|---|---|
| Chlorophyll a (mg/g) | 5.46 ±0.57 |
| Phycocyanin (mg/L) | 172.85 ±7.35 |
| Phycoerythrin (mg/L) | 75.54 ± 4.98 |
| Total Carotene (mg/g) | 1.82 ± 0.25 |

**Fig. 3.2** Chlorophyll-a content of *Arthrospira (Spirulina) platensis*




cultivated at 30 °C at 80 µmol $m^{-2}$ $s^{-1}$ light intensity.

There was an increase in the total carotene amount until the 14th day when the changes in the total carotene amount were examined (Figure 3.3).

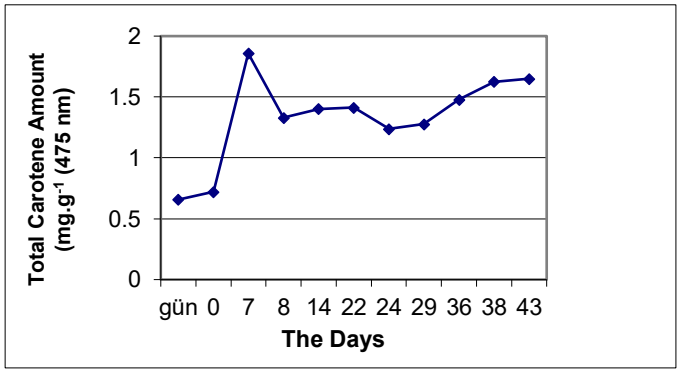



**Fig. 3.3** Total carotene content of *Arthrospira (Spirulina) platensis* cultivated at 30 °C at 80 µmol $m^{-2}$ $s^{-1}$ light intensity.

## 4 Discussion

Cultivation, extraction, and quantification of *Arthrospira (Spirulina) platensis* under laboratory conditions were defined in this study.

Some major conditional processes and steps can be ordered on growth algae. environmental conditions can cause changes in algal growth and algal biochemical structure. It affects growth, nutrient concentrations as well as the type of nutrients used in the nutrient medium (Brown et al. 1989). Hence, Zarrouk nutrient medium was preferred in this study with *Spirulina* (Zarrouk 1966).

Moreover, it is cearly observed that changes in temperature, light and salinity affect the growth and biochemical structures of algae (Vonshak 1997, Rizzo et al. 2015, Kilimtzidi et al. 2019). This examination with *S. platensis* was carried out in Zarrouk nutrient medium, illumination with 60 W fluorescent lamps at 80 µmol $m^{-2}$ $s^{-1}$ light intensity and 30 °C water temperature.

Smayda (1969) defined the photosynthetic rate as the daily carbon assimilation per unit of plant carbon. Baly equation was used as a method model. Briefly, the content of the Baly equation shows that temperature is a variable. It affects the photosynthetic rate. Smayda's work in 1969 showed that light, temperature, and salinity affect the growth of algae. It is known that algal photosynthesis is dependent on light intensity and they are integrated with different photosynthetic pigments. He gave importance to the light needs of different algae species in his studies. In addition, he ascertained that the species in the Chlorophyceae class were saturated with light between 0.032-0.048 ly.minute$^{-1}$. Emphasizing the importance of pigments at low light intensity. As a result of this, he concluded that chlorophyll and phycocyanin at high light intensity are very important for absorbing light intensity.

There are important points and steps for determining the nutritional quality, nutrient medium, and salt concentration (Kaplan et al. 1986). These are the biochemical composition of algae growth rate and growth phases (Molina-Grima et al. 1996), temperature (Kaplan et al. 1986), lighting (Brown et al. 1993; Molina-Grima et al. 1994), microelements (Akbarnezhad et al, 2020), media and nitrogen resources (El-Sheekh et al. 2021). Carotenes are of vital importance for human health because they protect cell roots and tissues from harmful factors as biological antioxidants. Moreover, many researchers have suggested carotenoids as protective against human diseases, and also carotenes such as β-carotene and lutein have been still used in cancer treatment (Richmond 2000; Ziegler et al. 1996). The main pigments in *Spirulina* are Chlorophyll-a, phycocyanin, phycoerythrin, and beta-carotene. In addition, these algal species contain secondary carotene groups such as canthaxanthin and astaxanthin. Secondary carotenoids are produced under extreme conditions such as high light intensity. Particularly, the change of color from green to red-orange as a result of nutrient restriction has been associated with an increase in secondary carotenes (Lubian et al. 2000). They also reported a decrease in chlorophyll-a and total carotene values due to the increase in light intensity in algae. In this study, the pigment composition and amounts of *Spirulina* were determined with this assessment.

Algal pigments are products presented in the beauty and health products market as various creams, milk, lotions, mud, face, and body masks. Chlorophyll is a green pigment substance that absorbs light of various wavelengths and provides photosynthesis (assimilation) to occur in the plant. Chlorophyll is responsible for absorbing the light energy used in photosynthesis, the reduction of carbon dioxide to other plant substances and sugars. In orderly, all green plants except bacteria contain Chlorophyll a, chlorophyll b is found in higher plants and green algae, red algae includes chlorophyll d, and chlorophyll c is found in diatoms, brown algae, and flagellates such as euglena. It is very important for helping to heal wounds on the skin, treat skin roughness, and accelerate tissue adaptation in skin transplantation. Chlorophyll is used comprehensively as a natural cosmetic product. It has become one of the application areas in cosmetics by being included in olive oil soaps. Furthermore, it is also used in natural cosmetic products such as face care masks and care creams besides soaps. Pigments are a rich source of vitamins, antioxidants, and minerals. These Pigments are algae such as Fucoxanthin, Phycocyanin, Phycoerythrin. It absorbs calories from the foods that people consume and prevents them from turning into fat. It helps for using in the treatment of cellulite, and also it has the impact of strengthening the immune system. Carotene has a significant role in photosynthesis as a photosynthetic pigment. Carotene can be stored in the liver and converted to vitamin A when needed, consequently, it's considered a provitamin. It s to photosynthesis by transferring the absorbed light to chlorophyll. It is responsible for providing the orange color of carrots and most other vegetables and fruits. β-Carotene is the widespread form. Leafy fruits and vegetables such as colors green, orange or yellow are contained a high level of β- carotene. In addition, brown algae contain carotene. Carotene is also effective against skin aging besides protecting the skin from the harmful rays of the sun (Cirik 1989, Cirik and Cirik 1999, Cirik and Gökpınar 1999; Cirik and Conk-Dalay, 2001;Koru and Cirik 2003).

## 5 Conclusion

In the scope of this research, aquaculture protocols and pigment compositions of *Arthrospira (Spirulina) platensis* species, which are naturally distributed in our waters, were examined in laboratory conditions. Their potential usage particularly in the field of cosmetics, neutroceuticals, and food should be studied in future research activities.

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## Conflict of interest disclosure:

No conflict of interest was declared by the authors.

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# Production of Biomass and γ-Linolenic Acid Production by *Spirulina platensis* Under Different Temperature and Nitrogen Regimes

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**Abstract:** *Spirulina* is of the worldwide cultivated and consumed microalgae. It is generally used directly or as an additive in the food industry due to its high protein content. Besides the high protein content, *Spirulina* biomass contains important fatty acids, (e.g. GLA), vitamins, minerals and other bioactive compounds. These important compounds are affected by the parameters of biomass cultivation. In the presented study, the limitation of nitrogen (25%, 50%, 75% and 100% N concentration) and temperature fluctuations (25°C and 30°C) on *Spirulina platensis* biomass yield, lipids and fatty acid profile were investigated with the comparison of Spirulina medium and Zarrouk medium. In the present investigation, the production of *Spirulina platensis* was optimized in terms of biomass and metabolites. With the increase in temperature, while the amount of biomass increased in general, dry weight decreased. The highest level of lipid accumulation was determined as 12.31 ± 1.72 % for the sample 25°C, Spirulina medium and 50% N concentration. Protein, lipid, total phenolic substance, and total carotenoid amounts were found at the highest level with the temperature increase to 30°C in all samples except the sample with the highest oil content. Consequently, the highest PUFA values were found in 30°C, Zarrouk medium and 75% N concentration as 42.610%, whereas GLA was 25°C, Zarrouk medium and 100% N concentration as 24.735%. On the other hand, GLA values were determined significantly high both during growth at 25°C and 30°C in Zarrouk medium.

**Keywords:** *Spirulina platensis*; biomass; fatty acid; GLA; nitrogen regime; temperature regime



## 1 Introduction

The use of "microorganisms" as well as plants as a source of "lipid" and "fatty acid" has been investigated for many years. Microbial oils have been researched at the industrial level in many developed countries since the 19th century, especially against famine, the decrease in traditional oil raw materials and the increase in the world population during the 1st and 2nd World Wars, and these researches have been carried out in secrecy for long periods (Ratledge 2004, 2006; Gunstone et al. 2007).

Microbial oils obtained from microorganisms and called single cell oil (THY, SCO: single cell oil) attract great attention all over the world today due to the therapeutic and nutraceutical PUFAs they contain (Ratledge 2005; Ratledge and Cohen 2008). Many groups of microorganisms (yeast, mold, bacteria and microalgae) have the ability to accumulate neutral oil under certain specific conditions. These microorganisms can accumulate 25% or more lipid in their cells and are described as “oleaginous” (Ratledge 2006; Vance and Vance 2008).

The reason for the concentration of studies on *Spirulina* is that it is a rich source of food components such as protein, vitamins (especially vitamin B12 and provitamin-A), essential amino acids, mineral substances (especially Fe) and essential fatty acids (especially GLA). 60-70% of the dry weight of Spirulina is protein and at least 20% of the total fat is composed of GLA (Ötleş and Pire 2001, Belay 2002, Colla et al. 2007). Although microalgae contain less fat than yeast and molds, the unsaturated fat profile is interesting.

The biomass yield and the change of the obtained metabolites are inevitable as a result of development in changing media compositions and temperatures, so the diagnosis and optimization of the growth conditions for the target metabolite gain importance. This study aims to determine the biomass and fatty acid composition, especially GLA, which has nutraceutical and therapeutic value, of *Spirulina platensis*, which was developed using different temperatures and nitrogen concentrations in two different growth environments.

## 2 Materials and Method

### 2.1 Material

*Spirulina platensis* (UTEX LB2356) strains were procured from UTEX, Culture Collection of Algae, Texas, Austin.

Cells were maintained with two different media: **Spirulina medium** (13.61 g $NaHCO_3$, 4.03 g $Na_2CO_3$, 0.50 g $K_2HPO_4$, 2.50 g $NaNO_3$, 1.00 g $K_2SO_4$, 1.00 g NaCl, 0.20 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, and 1 mL of micronutrient solution (50.0 mg $Na_2EDTA$, 618 mg $H_3BO_3$, 19.6 mg $CuSO_4 \cdot 5H_2O$, 44.0 mg $ZnSO_4 \cdot 7H_2O$, 20.0 mg $CoCl_2 \cdot 6 H_2O$, 12.6 mg $MnCl_2 \cdot 4H_2O$, 12.6 mg $Na_2MoO_4 \cdot 2H_2O$) and 0.15 mg of B12 vitamin), and **Zarrouk medium** (18.0 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g $K_2HPO_4$, 1.0 g $K_2SO_4$, 1.0 g NaCl, 0.04 g $CaCl_2$, 0.08 g $Na_2EDTA$, 0.2 g $MgSO_4 \cdot 7H2O$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 1.0 ml micronutrient solution (2.86 mg $H_3BO_3$; 0.02 mg $(NH_4)_6Mo_7O_{24}$; 1.8 mg $MnCl_2 \cdot 4H_2O$; 0.08 mg $Cu_2SO_4$; 0.22 mg $ZnSO_4 \cdot 7H_2O$).

### 2.2 Growth Conditions

Two different growth media (Spirulina and Zarrouk), temperatures (25±2°C and 30±2°C) and four different "$NaNO_3$" concentrations (defined as; 100%, 25%, 50% and 75%) were chosen for the evaluation of biomass production, the trial design is described in Table 1.

**Table 1** Trial groups

| | *100 % N* | *25 % N* | *50 % N* | *75 % N* |
|---|---|---|---|---|
| ***25°C - Spirulina Medium*** | A | A25 | A50 | A75 |
| ***25°C - Zarrouk Medium*** | B | B25 | B50 | B75 |
| ***30°C - Spirulina Medium*** | C | C25 | C50 | C75 |
| ***30°C - Zarrouk Medium*** | D | D25 | D50 | D75 |

The temperature was maintained by using an air conditioner and a lightening regime of 32-40 µmol photon $m^{-2}s^{-1}$ with a 14:10 hour light-dark period was applied. Cultures were aerated with 2 L $min^{-1}$ during the growth period and harvested by filtration (20 µm mesh). Obtained wet biomass was freeze-dried at -60°C overnight to obtain dry biomass, weighed and kept at -80°C for analysis.

### 2.3 Biomass and Lipid Analysis

Biomass concentration was measured daily by optical density (OD) at 680 nm (Optizen-Pop UV-Vis spectrophotometer, Korean). Lipid extraction was determined according to Folch et al. (1957) and fatty acid profiles were measured by the method described by Akpınar-Bayizit et al. (2014).

### 2.4 Statistical analysis

The descriptive statistics of the data obtained in the study and the correlations between the data were made with JMP (Version 7.0, SAS, Institute Inc. Comp., NC, USA). Results are shown as the mean ± standard deviation of 4 replicate measurements.

## 3 Results and Discussions

In this study, the changes in the GLA content of *Spirulina platensis* microalgae belonging to the *Oscillatoriaceae* family against different temperature and N source limitations were investigated. Optical density analyses were performed on *S. platensis*, which was cultured in 4 different groups until it reached the stationary phase. After the cultures that reached the stationary phase were harvested, they were dried, and their biomass amounts were calculated (Table 2).

**Table 2** Growth parameters of *Spirulina* cultures

| | $OD_{final}$ | $OD_{max}$ | *Biomass concentration* | *Lipid* |
|---|---|---|---|---|
| | | | (g $L^{-1}$) | % |
| ***A*** | 0.409 | 0.495 | $0.252^{ef}$ | $0.945^{g}$ |
| ***A25*** | 0.410 | 0.443 | $0.415^{bc}$ | $2.178^{ef}$ |
| ***A50*** | 0.380 | 0.485 | $0.284^{e}$ | $12.310^{a}$ |
| ***A75*** | 0.397 | 0.443 | $0.205^{f}$ | $2.964^{cde}$ |
| ***B*** | 0.979 | 1.043 | $0.314^{d}$ | $1.591^{fg}$ |
| ***B25*** | 0.848 | 0.988 | $0.418^{bc}$ | $3.559^{bc}$ |
| ***B50*** | 0.747 | 0.942 | $0.369^{cd}$ | $2.885^{cde}$ |
| ***B75*** | 0.936 | 1.073 | $0.218^{f}$ | $3.762^{bc}$ |
| ***C*** | 0.609 | 0.714 | $0.388^{c}$ | $3.282^{cd}$ |
| ***C25*** | 0.641 | 0.688 | $0.283^{e}$ | $3.571^{bc}$ |
| ***C50*** | 0.655 | 0.740 | $0.397^{c}$ | $2.597^{de}$ |
| ***C75*** | 0.614 | 0.717 | $0.446^{b}$ | $4.339^{b}$ |
| ***D*** | 1.365 | 1.471 | $0.511^{a}$ | $1.560^{fg}$ |
| ***D25*** | 1.033 | 1.185 | $0.428^{b}$ | $3.119^{cd}$ |
| ***D50*** | 1.036 | 1.151 | $0.332^{d}$ | $0.952^{g}$ |
| ***D75*** | 1.265 | 1.365 | $0.303^{de}$ | $1.441^{fg}$ |

**Mean values± standard deviation. Within columns, values with the different superscripts differ significantly from each other ($p < .05$).

The lipid synthesizing capacity of microorganisms is limited by some parameters. First of all, lipid production is directly related to the presence of primary nutrients and is induced by the reduction of nitrogen sources in the environment. Similarly, the increase in the amount of carbon source increases the amount by accelerating the lipid synthesis. While the decrease in nitrogen source causes an increase in lipid synthesis, it causes a slowdown in protein and nucleic acid synthesis (Ratledge and Wynn 2002; Ratledge and Cohen 2008; Ratledge 2008).

The importance of algae in human nutrition is due to the high amounts of protein, vitamins, amino acids and mineral substances in its structure. Biochemical properties in algal cultures vary depending on parameters such as species, growing region, season, water temperature, light intensity and exposure time. Compared to other seafood, the lipid content of algae is low and generally varies between 1–5% (Aguilera-Morales et al. 2005; Dawczynski et al. 2007). While the amount of lipid in green algae varies between 0.6% and 4.3%, this rate is stated as 2-12.3% for other microalgae (Chernova et al. 2001; Bigogno et al. 2002).

**Table 1** Fatty acid composition of *Spirulina platensis* biomass samples

| % | C16:0 | C18:0 | C16:1 | C18:1 | C18:2 | C18:3 (GLA) | GLA in PUFA |
|---|---|---|---|---|---|---|---|
| **A** | 27.558±0.057$^{de}$ | 3.068±0.007$^{c}$ | 4.470±0.0065$^{cd}$ | 13.966±0.0264$^{def}$ | 9.085±0.0274$^{ef}$ | 17.587±0.0412$^{de}$ | 60.43 |
| **A25** | 11.127±0.094$^{f}$ | 7.337±0.068$^{b}$ | 0.299±0.0044$^{e}$ | 35.008±0.2768$^{a}$ | 25.228±0.1134$^{a}$ | 2.970±5.1456$^{ı}$ | 1.71 |
| **A50** | 22.401±10.323$^{e}$ | 9.156±3.896$^{a}$ | 1.049±1.5065$^{e}$ | 19.763±4.7544$^{b}$ | 4.766±7.0257$^{g}$ | 0.510±0.0025$^{ı}$ | 22.19 |
| **A75** | 22.786±0.078$^{e}$ | 2.358±0.012$^{cd}$ | 3.861±0.0013$^{d}$ | 16.405±0.0622$^{c}$ | 8.637±0.0932$^{fg}$ | 14.803±0.0497$^{fg}$ | 54.76 |
| **B** | 28.578±0.480$^{cde}$ | 2.450±0.048$^{cd}$ | 6.228±0.1258$^{a}$ | 10.994±0.1535$^{ghı}$ | 9.074±0.1592$^{ef}$ | 24.735±0.4245$^{a}$ | 73.16 |
| **B25** | 38.587±0.049$^{a}$ | 2.238±0.019$^{cde}$ | 5.514±0.0817$^{abc}$ | 11.570±0.0245$^{fgh}$ | 16.919±0.0197$^{bc}$ | 11.878±0.0131$^{gh}$ | 38.97 |
| **B50** | 29.521±0.06$^{bcde}$ | 1.859±0.009$^{cde}$ | 5.674±0.0148$^{ab}$ | 12.611±0.1089$^{efg}$ | 10.666±0.0295$^{ef}$ | 22.978±0.0513$^{ab}$ | 65.27 |
| **B75** | 33.378±0.005$^{abcd}$ | 1.876±0.01$^{cde}$ | 6.113±0.0054$^{a}$ | 8.502±0.0094$^{jk}$ | 10.704±0.0073$^{ef}$ | 23.785±0.0072$^{a}$ | 68.96 |
| **C** | 33.486±0.234$^{abcd}$ | 2.272±0.125$^{cde}$ | 4.730±0.0354$^{bcd}$ | 15.000±0.1042$^{cde}$ | 16.139±0.1158$^{bcd}$ | 17.520±0.1213$^{de}$ | 50.68 |
| **C25** | 39.882±0.054$^{a}$ | 1.457±0.017$^{de}$ | 5.779±0.3540$^{ab}$ | 8.456±0.0141$^{jk}$ | 17.018±0.0190$^{bc}$ | 12.239±0.0133$^{gh}$ | 40.48 |
| **C50** | 35.844±0.081$^{abc}$ | 0.984±0.004$^{e}$ | 5.153±0.0125$^{abc}$ | 7.969±0.0222$^{jkl}$ | 17.591±0.0503$^{b}$ | 15.720±0.0472$^{ef}$ | 45.99 |
| **C75** | 36.942±0.101$^{ab}$ | 1.879±0.006$^{cde}$ | 4.743±0.0043$^{bcd}$ | 15.969±0.0534$^{cd}$ | 12.271±0.0418$^{def}$ | 9.378±0.0290$^{gh}$ | 41.07 |
| **D** | 35.561±0.103$^{abc}$ | 1.873±0.006$^{cde}$ | 4.748±0.1200$^{bcd}$ | 7.309±0.2361$^{kl}$ | 16.130±0.2223$^{bcd}$ | 22.142±0.0566$^{abc}$ | 53.53 |
| **D25** | 37.177±0.082$^{a}$ | 2.862±0.013$^{cd}$ | 5.171±0.0262$^{abc}$ | 9.062±0.0306$^{ijk}$ | 15.522±0.0244$^{bcd}$ | 20.189±0.0294$^{bcd}$ | 56.53 |
| **D50** | 35.301±0.068$^{abc}$ | 2.526±0.0172$^{cd}$ | 5.361±0.0076$^{abc}$ | 10.000±0.1279$^{hıj}$ | 17.249±0.0864$^{bc}$ | 19.104±0.0269$^{cd}$ | 52.55 |
| **D75** | 35.536±0.099$^{abc}$ | 1.986±0.007$^{cde}$ | 4.824±0.0186$^{bcd}$ | 6.817±0.0252$^{kl}$ | 17.666±0.0393$^{b}$ | 23.205±0.0623$^{ab}$ | 54.46 |

**Mean values± standard deviation. Within columns, values with the different superscripts differ significantly from each other (p< .05).

The lipid content of *S. platensis* was determined between 0.945 and 12.310 % values, and the highest value was observed in the samples of 25°C + 50% N Spirulina medium. It is reported that 6.4% to 14.3% of the dry biomass of *S. platensis* consists of lipids (Badzhanov et al. 2004; Kachroo et al. 2006). Colla et al. (2007), cultivated *S. platensis* at 4 different N concentrations at 30°C and 35°C, and determined lipid contents varied between 6.69±0.27% and 10.37±0.63. Piorreck et al. (1984) stated that the total amount of lipid in cyanobacteria does not depend on the nitrogen concentration in the medium and that nitrogen is the growth parameter limiting lipid synthesis in eukaryotic algae.

In research that optimizing the lipid ratio, values ranging from 5.21±0.1% to 18.02±0.4% were determined (Xue et al. 2010, Uslu et al. 2011, Madkour et al. 2012, Azgın et al. 2014). Griffiths et al. (2011) in their study with 10 microalgae, reported that the lipid ratio in the biomass increased with the decrease in N ratios. The data obtained in the presented study are in harmony with this study, the relationship of lipid ratio with varying N concentration, temperature and biomass amounts could not be determined clearly. The change in temperature and nutrient medium concentration applied to *S. platensis* was determined statistically, and it was determined that there was a difference in the lipid ratio ($p < 0.05$). In microbial growth, it is expected that the amount of lipid will increase as the temperature decreases and N concentration decreases in the presence of high C in the nutrient medium (Rodolphi et al. 2009, Dean et al. 2010). In this case, the best lipid synthesis occurred in 25°C + 50% N Spirulina medium (12.310). In the Zarrouk medium at the same temperature, the medium provided the best lipid value with 75% N (3.762%). This is thought to be due to the fact that the C concentration in the Spirulina medium is higher than that in the Zarrouk medium. Similarly, when the two mediums at 30°C are compared, it is seen that lipid synthesis is higher in the Spirulina medium (Table 3).

Although it varies according to the species, it is noted that the ratios of EPA, ARA, ALA and GLA in total lipid were significantly higher, especially in *S. platensis* (Habib et al. 2008). In recent years, the importance of algae has been associated with the composition of essential fatty acids, which are similar or alternative to other fatty acids of vegetable and animal origin (Vazhappily and Chen 1998; de Swaaf et al. 1999, Rosa et al. 2005; Mendes et al. 2007). 17% of the fatty acids of *S. platensis* consist of essential pigments, paraffin, sterol and terpene alcohols, which are unsaponifiable (Hoseini et al. 2013). ω-6 fatty acids in sterol structure, which make up half of the total fatty acids, are found in the galactolipid structure of algal cell walls (Certik and Shimizu 1999). However, although this content is low, it is also stated to be a good source of ω-3 and ω-6, especially GLA.

*S. platensis* contains approximately 49% GLA, making Spirulina the best source of GLA after breast milk and evening primrose and borage oils (Petkov and Furnadzieva 1988). SFA values of S. platensis grown in Spirulina broth only at 25°C showed a significant change with varying N concentrations. In other groups, changes in temperature and nitrogen concentration were not found to have a significant effect on SFA values. The SFA values obtained in the study were 70.3% for the culture developed by Durmaz and Gökpınar (2006) at 26°C, and Ambrozova et al. (2014) were found to be lower than the values of 63.18% of the culture

they developed in the photobioreactor under sunlight. Ötleş and Pire (2001) reported the SFA ratios as 55.72%, 51.64% and 51.96% in the analysis results of Spirulina powders offered for sale in the market.

It was determined that GLA accumulation was very low at high temperature and low nitrogen concentration, where MUFA synthesis accelerated in Spirulina medium (Table 3). Similar results (Piorreck et al. 1984, Griffiths et al. 2012) has also been reported. Production of PUFA and GLA is closely related to the presence of dissolved oxygen in the nutrient medium, and the absence of carbon sources. De Morais et al (2019) observed that the increase in unsaturated fatty acids, especially oleic acid, and reduction of saturated fatty acids, mainly palmitic, were proportional to the increase in the glycerol concentration in the medium.

It was reported by Knothe (2005) that the PUFA yield would increase with a decrease in temperature. However, in the presented study, it was observed that PUFA yield increased when the temperature increased from 25°C to 30°C in other algal growths, except for the samples grown at 25°C + 75% N Spirulina medium. This increase in PUFA yield was found more prominently in the 50% N sample, especially in the Spirulina medium. While Durmaz and Gökpınar (2006) reported that GLA synthesis was not affected by culture temperature, Colla et al. (2004) stated that temperature directly affects the fatty acid synthesis and that the best amount of GLA can be obtained with growth at 30°C. GLA values were detected higher in samples at 30°C than growth at 25°C. The highest GLA value was observed in the Zarrouk medium 25°C + 100% N sample group with 24.73% of total fatty acids and 73.16% of PUFA.

## 4 Conclusion

It is known that oleaginous microorganisms tend to accumulate lipids with high PUFA content inside the cell in the presence of N limitation and a high amount of C source in the nutrient medium, under optimum growth temperatures. However, unlike these microorganisms, it was not observed that the PUFA values in the biomass increased with the increase in temperature, regardless of the N limitation. Additionally, at low temperatures, algal cultivation time took longer in both media ( t25°C: 25 days, t30°C: 12 days).

It can be concluded that cultivation of *Spirulina platensis* at 25°C in Zarrouk medium and at higher than 50% concentration of $NaNO_3$ approximately 20 % of GLA could be observed. Also at these conditions, GLA concentrations were above 50% of PUFA.

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# Biodegradable Plastic and Film Production from Seaweeds

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**Abstract:** To evaluate potential bioplastic and biofilm production from seaweeds, alginate which is known as seaweed hydrocolloid and it is extracted from brown seaweeds was used as a basic material to produce bioplastic for this experiment. A colorimetric analysis of the plastic or the film indicated that the concentration of alginate directly interferes with the color difference, tending slightly yellow when alginate added. The plastic presented low opacity, below 13%, with no significant effect of the different alginate attributions on the material's transparency. The thickness of the plastic produced was directly proportional to the concentration of alginate diluted in the solution, with the addition of 0.50 g being produced in plastics with a thickness of 0.02 mm, while the addition of 5.00 g obtained 0.11 mm. The maximum elongation distance until the plastic breaks does not show differences when subjected to tension, with an average distance of 2.12 ± 1.03 mm, regardless of those analyzed. However, it was possible to observe that the tensile force for breaking the plastic with a concentration of 0.50g was 0.61 ± 0.16 kg, while at a concentration of 2.75g and 5.00g values were five times greater, 3.30 ± 1.24 kg and 3.54 ± 1.10 kg, respectively. The use of seaweed polymer has a great potential for manufacturing various types of biodegradable bioplastics or biofilms. With these properties, the concentration of 2.75g could form a very resistant film, being capable of many ecologically friendly applications in various packaging, for example for biscuits, sachets and seasonings and in developing carrier bags and plastic bottles.

**Keywords:** biodegrable; bioplastic; biofilm; seaweed hydrocolloids; algal phycocolloids; alginate



## 1 Introduction

Nowadays, people have become dependent on petroleum-plastic because it is used in many products such as pharmaceutical materials, packaging, 3D modeling, home appliances, electronics and so on. Although petroleum-based plastics are the main cause of plastic pollution, it takes thousands of years for these plastics to degrade and disappear from nature (Chae and An 2018). For this reason, it is obvious that the use of biodegradable plastics produced from natural resources is of great importance in terms of both human health and environmental sustainability. The use of bioplastics; They have many benefits such as they deteriorate more quickly in nature, are not toxic, provide energy savings during production, require less space for the wastes produced, reduce fossil fuel consumption and reduce the amount of greenhouse gases emitted. Bioplastics are now recognized as a promising approach to solving plastic pollution (Porta 2019). Bioplastics that must be produced from renewable materials; It can be produced from raw materials such as polysaccharides, proteins and lipids, but also corn, potatoes, vegetable oils, food waste, grain products, etc. They can also be produced from many biomaterials. Today, the main types are starch-based, followed by polylactic acid (PLA), poly-3-hydroxybutyrate (PHB), polyamide 11 (PA 11) and organic polyethylene (PE) (Satti and Shah 2020). Today, some bio-based plastics with short-term biodegradability are used in a variety of packaging, medical/pharmaceutical materials and agricultural products industries. The global market for bio-based plastics is estimated to be 2.11 million tons in 2018 and may increase to 2.62 million tons by 2023 (European bioplastics 2018).

Biodegradable and edible materials from plants and animals, including peptides, polysaccharides, and lipids, are profitable alternatives to synthetic packaging films (Sevindik et al. 2017; Umaraw et al. 2020). Bioplastics are derived from renewable resources. Unlike petroleum-derived plastics, which both create a high greenhouse gas effect and consume natural resources, they are sustainable materials. Many varieties have biodegradable properties. Unlike petroleum-based plastics, which continue to pollute the nature for hundreds or even thousands of years and may contain toxic substances, bioplastics prevent environmental pollution after completing their useful life, preventing environmental pollution and do not leave harmful and toxic substances

behind. The bad effects of additives such as BPA, PVC, Phthalate, Styren, which can be found in products made of petroleum-based plastics, on human health are revealed by new researches. Bioplastics obtained from natural and biological sources are safer for the environment and human health as they may not contain harmful petroleum chemicals (Umaraw et al. 2020).

Algal species are recognized as a potential raw material for bio-based plastics due to their rapid growth rate and extensive environmental tolerance (Venkatesan et al. 2016; Mathiot et al. 2019). Seaweed (or, macroalgae) (*Kappaphycus alvarezii* (Doty) Doty ex Silva) films can be used as sachets or pouches, renewable cups, plastic bags, containers and more (Siah et al. 2015).

Macroalgae derivatives called alginate, carrageenan and agar exhibit interesting film-forming properties, including plastics, and have negligible lignin content. Seaweed hydrocolloids are of great interest in the food industry due to their different functional properties (Yarnpakdee et al. 2015). Agar as a hydrophilic gel; It is the first phycocolloid used in food, pharmaceutical, cosmetic and biotechnological applications due to its ideal physical and chemical properties such as gelling, thickening, stabilizing and cryoprotective effects, high biodegradability and large water holding capacity (Guerrero et al. 2014; Sevindik et al. 2017). Red seaweed polysaccharides generally consist of agar, carrageenan, cellulose, fluoride starch, xylan, mannan and porphyra. Red algae are frequently used in film production. This is because they have film-forming polysaccharides such as carrageenan (*Kappaphycus* sp. and *Eucheuma* sp.) and agar (*Gelidium* sp. and *Gracilaria* sp.). Agar is a polysaccharide that can be found in some families of red seaweed (Rhodophyceae), usually *Gracilariaceae* and *Gelidiaceae*. Agar consists of two main components, agarose and agaropectin (Arvizu-Higuera et al. 2008). Red seaweeds are widely used in many applications such as food, agriculture, cosmetics, biomedical application. Agar was first used in the fields of food, biotechnology and pharmaceutical applications before being used as a raw material in bioplastic film production (Phan et al. 2009). Phan et al. (2009) stated that agar-based films have low moisture content, transparent, strong and flexible properties. In addition to these properties, heat seal ability makes agar-based films good practice for the food packaging industry.

**Table 1** Green extraction methods for macroalgae (Lim et al. 2021).

| **Production methods** | **Principle** | **Advantage (+)**<br>**Disadvantage (-)** |
|---|---|---|
| Enzyme-assisted extraction | Enzymes such as protease and carbohydrase break down the cell wall and release algal polymers. | +High efficiency, environmentally friendly, wide chemical selectivity<br>-Slow process, expensive enzyme |
| Microwave-assisted extraction | Microwave irradiation raises the temperature and penetrates the cell wall to release the polymers into the solvent. | +Fast process, high yield, low solvent consumption, scalable<br>-High equipment cost, explosion risk, solvent must be polar and non-volatile |
| Extraction by photo-bleaching | Sunlight is used to remove pigment through photolysis, where the sulphate content of polymers is reduced. | + Environmentally friendly, chemical-free, improves the quality of extracts, improves efficiency<br>- Affected by weather conditions, causing chemical changes in samples |
| Reactive extrusion | Mixing, shearing, heating are used to initiate chemical reactions, homogenize and melt materials to form a polymer. | + Fast and continuous processing, low solvent consumption, expandable<br>- High equipment cost can cause thermal degradation of seaweed |
| Pressure solvent extraction | The high pressure and solvent temperature below the boiling point (subcritical zone) are used to extract the polymers in a short time. | + Green solvent and low consumption, efficient, fast process, simple steps<br>-High equipment cost can cause thermal degradation of seaweed |
| Supercritical fluid extraction | The solvent (usually $CO_2$ or $H_2O$) is at the critical temperature and pressure for polymer extraction. | + Eco-friendly, fast process, high purity extracts, cheap and recyclable solvent<br>- High equipment cost, operation cost and energy consumption, big volume problem |
| Ultrasound-assisted extraction | Sound waves of more than 20 kHz penetrate the solvent within the cell wall and generate cavitation bubbles to remove polymers. | + Fast process, low solvent and energy consumption, high efficiency,<br>waste-free, simple steps, easy scale-up<br>- High equipment cost, narrow solvent selectivity |

There are mechanical, chemical and biological extraction methods for bioplastic production from macroalgae. Each extraction method has some positive and negative aspects. Answer of the question of which extraction method that should be applied is depending on the type, solvent types, environmental effects, cost, time, amount and preferred properties. Commonly used methods in the production of bioplastics from seaweeds are washing, grinding, drying, alkali, acid neutralization, formaldehyde, hot water, filtration and precipitation (Lim et al. 2021). However, these commonly used methods are not suitable in terms of time, money and efficiency compared to petroleum-based plastics. The cost is high due to the amount of water used, production method, reactants and chemicals. In addition, reactants and chemicals increase the cost as they require hazardous and appropriate waste management (Lim et al. 2021). Seaweed bioplastics with green production methods have great potential when it comes to replacing petroleum-based plastics. Besides being economical and biodegradable, materials are renewable and sustainable, and their synthesis methods can be chemical and hazardous waste-free. There are many green extraction methods for macroalgae, such as enzyme-assisted extraction, microwave-assisted extraction, photo-bleaching extraction, reactive extrusion, pressurized solvent extraction, supercritical fluid extraction, ultrasound-assisted extraction (Table 1) (Lim et al. 2021).

Today, seaweeds are of great interest in applications ranging from food to energy to products such as paper and plastic. This is due to the rich composition of seaweeds. In general, the dry weight of seaweeds contains an average of 50% total carbohydrates, 1-5% lipids, 7-73% minerals for all three groups, while proteins are lower in brown (4-24%) and higher in red and green seaweeds. (8-47%). Some types of seaweed are used to make films. To name a few, *Kappaphycus, Eucheuma, Gracilaria, Porphyra, Gelidium, Pterocladia* for red algae, *Ulva, Codium, Enteromorpha* for green algae, and *Macrocystis, Laminaria, Ascophyllum, Lessonia, Sargassum* for brown algae. Alginate is a phycocolloid and binary copolymer that can be extracted from brown algae as a raw material for bioplastic (Draget and Taylor, 2011). Alginate is a polysaccharide responsible for 22-44% of the dry weight of most marine brown algae (Li et al. 2019). Notably, alginates can cross-link with cations to form hydrogels or packaging films (Xue et al. 2019).

The general objective of this study is to produce biodegradable bioplastics and biofilms from alginate (or, alginic acid) and in order to reach the mail goal it was to analyze the mechanical, physical and barrier properties of films prepared from alginate to compare to petroleum-plastics.

## 2 Materials and Method

The preparation of biofilms was performed with three alginate concentrations: 0.50, 2.75 and 5.0 (m/v). Each of these concentrations was diluted in distilled water, with the addition of 2% Glycine dissolved in 150 ml of distilled water. The solutions were kept under stirring for 10 min at room temperature in a bioreactor. After that the temperature rise was raised to 45°C under constant stirring for 20 min. After this homogenization process, 12 g of solution was spread in ten Petri dishes and then dried in an oven at 45°C for 24 h. Dry films were removed from the Petri dish and all film samples were preconditioned for at least 48 h at constant temperature and humidity (25°C and 50% relative humidity) controlled by Sodium Bromide (NaBr).

The thickness (mm) of the films was determined using a digital micrometer at ten random points on the film body. The color and opacity of the films were determined using a colorimeter, using the CIEL*a*b system. The parameters analyzed in this color system are: L (brightness), a* (green and red) and b* (blue and yellow), using the method described by Saberi et al. (2016). Film color was expressed as total color difference (ΔE*), which is calculated according to the program.

The opacity of the films was calculated as the ratio between the opacity of the film superimposed on the black standard (Yblack) and the white standard (Ywhite) detected by the colorimeter, using the methodology described by Thakur et al. (2016). To determine the mechanical properties of the films in a texturometer, it was necessary to cut the films into strips 5 cm long and 25 mm wide. The properties studied were the tensile strength (Kg) and maximum elongation to break (mm) using the methodology described by ASTM (2002). The results of thickness, optical and mechanical properties were submitted to analysis of variance (ANOVA) and means were compared by Tukey test at a 5% significance level.

## 3 Results and Discussion

The colorimetric analysis of the film indicated that the concentration of alginate directly interferes with the color difference, tending slightly to yellow when alginate is added. The optical properties of coloring can be seen in Table 1. The film had low opacity, below 13%, and the effect of different alginate concentrations on the material's transparency was not significant. The thickness of the film produced was directly proportional to the concentration of diluted alginate in the solution, with the addition of 0.50g resulting in films with a thickness of 0.02 mm, while the addition of 5.00g obtained 0.11 mm. The maximum elongation distance until the film breaks did not show significant differences when subjected to tension, with an average distance of 2.12 ± 1.03 mm, regardless of the concentrations analyzed. However, it was possible to observe that the tensile force for breaking the film with a concentration of 0.50g was 0.61 ± 0.16 kg, while at the concentration of 2.75g and 5.00g it obtained values five times greater, 3.30 ± 1.24 kg and 3.54 ± 1.10 kg, respectively. The mechanical properties and opacity of the produced films can be seen in Figure 1 and Picture 1 shows different packages prepared by the bio-plastics/bio-films produced by macroalgal alginates.

**Table 1** The table shows the values obtained from the optical analysis, as follows: L*: luminosity; a*: green and red; b*: blue and yellow; ΔE: total color difference.

| Alginate (%) | L* | a* | b* | ΔE |
|---|---|---|---|---|
| 0.5 | 96.50 ±0.17 [a] | -0.49 ±0.08 [a] | 3.95 ±0.34 [a] | 2.45 ±0.20 [a] |
| 2.75 | 94.78 ±0.70 [b] | -0.98 ±0.13 [a] | 8.44 ±0.23 [b] | 6.89 ±0.61 [b] |
| 5 | 93.09 ±0.57 [c] | -1.05 ±0.19 [a] | 12.43 ±0.43 [c] | 11.53 ±0.52 [c] |

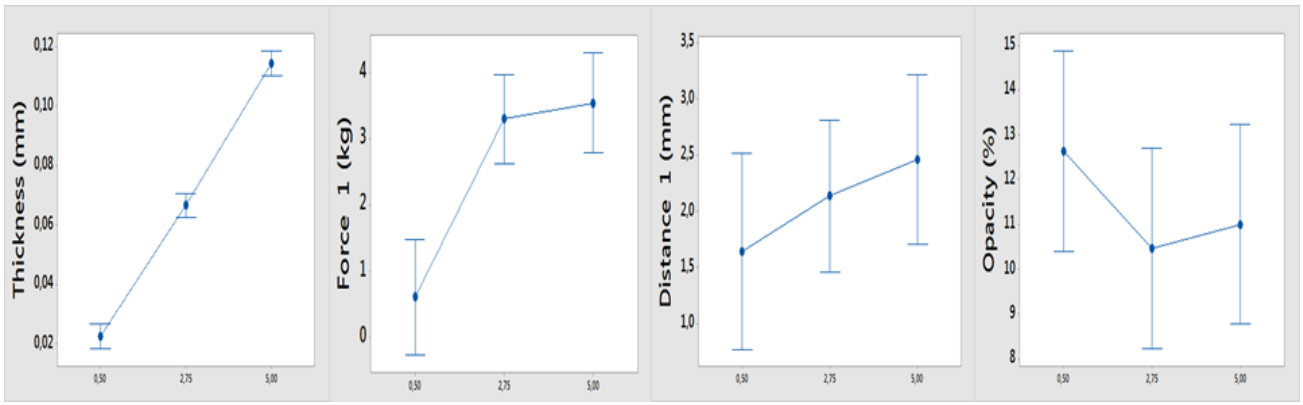


**Fig. 1** Optical and mechanical properties the bio-plastic/bio-film produced from alginate.

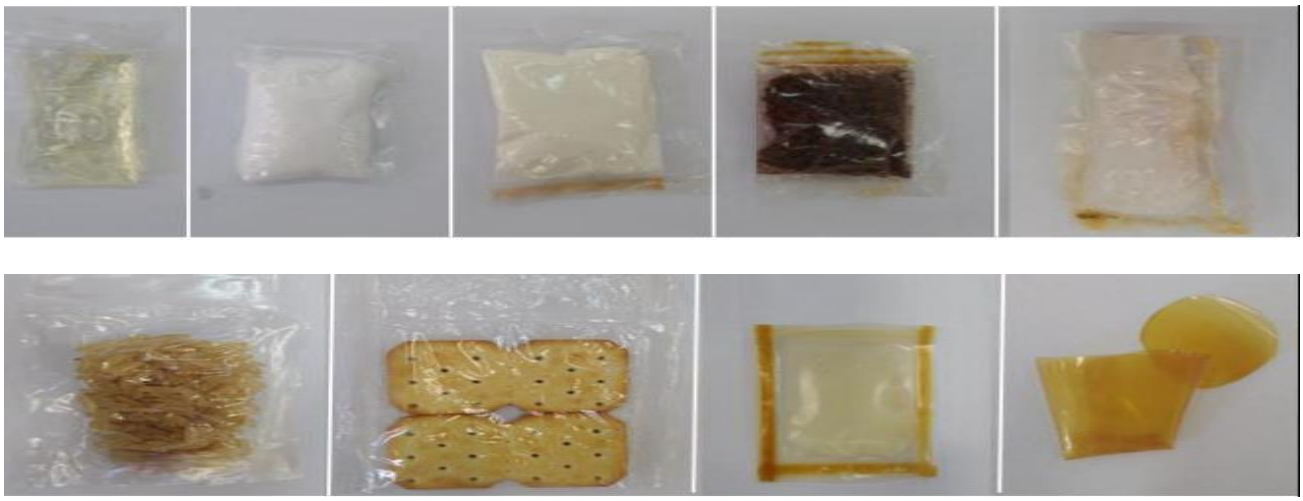

**Fig. 2** Different packages prepared by the bio-plastics/bio-films produced by the alginate.

The results of this present study shows that it is possible to produce degredable bioplastics and biofilms from seaweeds that is similar results to previous studies of Doh et al. (2020) who developed marine algal biopolymer film from 2 different brown macroalgae (*Laminaria japonica* and *Sargassum natants*).

In this present study, a film-forming solution was prepared with supernatan occuring after acid-base pre-treatment and marine algal polymer film was obtained using a bulk evaporation method. And, optical and mechanical properties were studied to determine its liability as a packaging subtrate. The result of these properties showed similarities to the findings of Sudhakar et al (2020) who developed and characterized the bioplastic film obtained from red seaweed (*K alvarezii*). In their study, the red seaweed *K. alvarezii* was used for the production of bioplastic film. The ratio of plasticizer polyethylene glycol (PEG) to seaweed biomass was optimized and a thin bioplastic film with higher tensile strength was produced. The resulting films were characterized by their thickness, tensile strength, color (L, a, b), elongation at break (EAB), water vapor transmission rate (WVTR) and oxygen transmission rate (OTR). TG-DSC, AFM, SEM and FTIR spectroscopy analysis were performed to evaluate the composition, phase transitions and chemical reaction capabilities of the film. Bioplastic film from 4% (whole seaweed) *K. alvarezii* showed better physical and mechanical properties, while TG-DSC, FTIR and AFM showed similar bioplastic properties at all concentrations. A decrease in OTR was observed against the decreasing wall thickness of the film. And, Sudhakar et al (2020) also suggested that seaweed is a potential alternative source for bioplastic production that could reduce the use of non-degradable plastics.

## 4 Conclusion

Seaweed is a leading source of polysaccharides, with properly established extraction processes allowing it to be used in a variety of fields. All types of biopolymers derived from seaweed has gained popularity in recent years. This is due to its abundance, water solubility, and outstanding film-forming abilities. And, remarkable developments in improving the properties of seaweed-based films have been described in many studies in the literature. And, there are many research opportunities to explore regarding the improvement of material properties.

Alginate (or, Alginic acid) is derived from brown algae and is a promising natural polymer. There are many studies emphasized the innovative method in the preparation, characterization and performance of seaweed-based films as food packaging by the researchers. Among the polysaccharides obtained from brown algae, Alginate has recently gained notoriety for its excellent film-forming ability. Alginate is an anionic sulfated polysaccharide classified together with carrageenan and agar for use as food additives according to European Union legislation. In light of this, numerous recent studies have demonstrated the production of sustainable, active and smart edible films based on this polysaccharide.

The use of seaweed polymer has a great potential for manufacturing several types of biodegradable films and the present study results suggest that seaweed is a potential alternative source for bioplastic production that could reduce the use of non-degradable plastics. With optical and mechanical properties obtained in this study, we suggest to use the concentration of 2.75 g could form a very resistant film, capable of being applied in different packages, for example, for biscuits, sachets and seasonings. I the further studies on bioplastics and biofilms produced from algae by using different additives are needed to obtain different mechanical and optical properties in order to reach other useful niches. The properties and characterization of seaweed bioplastic film are used to make a comparison with petroleum-based plastic. Properties and characterization data of bioplastics are generally categorized as physical, optical, mechanical, morphological, thermal, antioxidant, antibacterial and biodegradable. Physical properties include thickness, solubility, water vapor permeability (WVP), water vapor transmission rate (WVTR), and moisture content of the films. Optical properties include transparency, opacity, and light transmittance. Mechanical properties include tensile strength, elongation at break, and Young's Modulus. Morphology study Fourier transform infrared (FTIR) spectroscopy, Field Emission Scanning Electron Microscopy (FESEM or SEM), Atomic force microscopy (AFM), and X-ray diffraction analysis (XRD) can be done with Thermal properties include Thermal Gravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC).

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## Conflict of interest disclosure:

No conflict of interest was declared by the authors.

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