Original Article

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Choosing the Best Microalgae and Optimising its Culture Medium to Produce More Starch for Medicinal Use

Microbiology Section

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ABSTRACT

Introduction: Some species of microalgae have the ability to produce high amount of carbohydrates, rather than lipids. The extracted starch obtained from the ultrasonic treated green microalgae has high level of antioxidant activities and in animal studies, the extracted starch from *Chlorella vulgaris* (*C. vulgaris*) exhibited anti-atherogenic, anti-cholesterolemic, anti-inflammatory and anti-tumour effects. The green microalga has been widely used as a food source containing a complete set of nutrients including carbohydrates, proteins, vitamins, and minerals.

Aim: To optimise the microalgae culture medium to increase starch production for medicinal use.

Materials and Methods: This study was conducted as a field, observational and experimental research. For this purpose, 5 mL of microalgae *C. vulgaris*, *Scenedesmus dimorphus* (*S. dimorphus)* and *Scenedesmus quadricauda* (*S. quadricauda)*, in the test tube containing Bold's Basal Medium (BBM). BBM and Z8 media were used to grow freshwater microalgae. Light absorbance, cell count and biomass of microalgae in these media were studied. The experimental design method based on Taguchi method was conducted for optimising medium for microalgae with highest starch production, with light intensity, nitrogen and phosphorus factors, in three levels (1, 0,-1).

Results: Specific growth rates of *C. vulgaris* in the media BBM and Z8 were 0.075 and 0.055 d-1, and for *S. quadricauda* and *S. dimorphus* were 0.072 and 0.008; 0.038 d-1, respectively. Due to significant difference (p=0.034) between Z8 and BBM media, BBM was selected for growing *C. vulgaris*, *S. quadricauda* (p=0.308) and *S. dimorphus* (p=0.033). In addition, growth rate and starch production of desired microalgae were achieved. Starch content in *S. dimorphus*, *C. vulgaris* and *S. quadricauda* were 19%, 29% and 20% of biomass weight, respectively.

Conclusion: It was found that starch content was increased with increasing light, reducing phosphorus and nitrogen in *Chlorella* and the results showed that *C. vulgaris* could be a good choice as a starter for producing starch for medicinal purpose.

Keywords: Biomass, *Chlorella*, Nitrogen, Optimisation, Pharmaceutical, *Scenedesmus*

INTRODUCTION

Phytoplanktons are autotrophic organisms and make their own food with the help of photosynthesis by utilsing nutrients from water in the presence of sunlight [1]. Study carried out with *Scenedesmus* spp. (*S. dimorphus* and *S. quadricauda*) and other microalgae have shown that certain polysaccharides have medical effects [2]. These polysaccharides function as protection against oxidative stress and have efficacy on gastric ulcers, wounds and constipation [3,4]. *Chlorella* is an algae belonging to the phylum Chlorophyta that is a good source of nutrients such as valuable protein, starch, calories, fat, and vitamins. These algae have the widespread use in medicine [5].

Given that different algae under varying environmental conditions would not have the same growth as well as with regard to the economic importance of algae in terms of food, medicine, health, fuel, etc., the importance of faster and cheaper growth of them felt. Therefore, by changing some of the nutrients can be achieved to maximum growth rates and cell density, especially in large quantities [6].

Juneja A et al., have shown in their study that variation in the nutrient of microalgae can change its metabolism [7]. Three factors, light intensity, the amount of nitrogen and phosphorus are effective in increasing the starch production and biomass [8].

Adding *C. vulgaris* to diet, extract significantly improves antioxidant status and attenuates lipid peroxidation in chronic cigarette smokers and might redact the mortality rate [9]. The biomass obtained from Scenedesmus spp. because of their nutraceutical properties, used as treatment for malnutrition [10].

The starch extracted from Chlorella and Spirulina are used for health care, prevention of skin dangers caused by sunlight and hair care products. Recent research by scientists shows that protein and starch in green algae can act as a HIV treatment vaccine, which means that people are immunised against these diseases by eating these algae and that there is no need for an industrial vaccine to be produced. Also, the effect of observing a three-hour starch diet on premenstrual syndrome has been proven in students [11,12].

Starch is the most abundant reserve polysaccharide in plants [13]. This material is extractable from various plant sources such as: wheat, corn, rice, potatoes and microalgae, and has been considered for its structural, physical and chemical properties. Starch is widely used in food, pharmaceutical, medical and biomedical applications, due to biocompatibility, bioassay, non-toxicity and abundant sources. The role of starch for bone tissue engineering, bone resilience, carrier for controlling the distribution of drugs and hormones in the body, is currently of particular interest [14]. Many researchers have studied the effectiveness of starch in the treatment of colon cancer in drug gelatin capsules for the better distribution of its compounds in the body, in the treatment of young women's menstrual pain in complementary medicine for the treatment of cough, which has had a significant impact on treatment of these diseases [12-15]. Microalgae are able to survive in undesirable nutritional conditions and reproduce without the need for agricultural land. Microalgae have high reproductive power. For example, *Chlorella* contains the highest levels of chlorophyll among all the plant species. *Chlorella* is a liquefied algae and is easily cultivated in large quantities and resulting starch extracted from algae being cheaper and of a higher quality than its synthetic type [16].

The aim of this study is to select the best microalgae for increasing starch production that can be used in medicine.

MATERIALS AND METHODS

This experimental study was conducted for duration of one month in May 2014 at Microalgae lab, Scientific and Industrial Research Organisation of Iran. Three freshwater microalgae *C. vulgaris*, *S. dimorphus* and *S. quadricauda*, were received from Scientific and Industrial Research Organisation of Iran. Media BBM and Z8 were selected, which are specific for the growth of freshwater phytoplankton. Composition of media used is represented in [Table/Fig-1,2] [17,18].

Medium	Components	Chemical formula	Concentration
BBM [17]	Sodium nitrate	$NaNO_3$	250 mg/L
	Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	75 mg/L
	Salt	NaCl	25 mg/L
	Dipotassium phosphate	K_2HPO_4	175 mg/L
	Calcium chloride dihydrate	$CaCl_2.2H_2O$	25 mg/L
	Zinc sulfate heptahydrate	$ZnSO_4.7H_2O$	8.82 mg/L
	Manganese chloride tetrahydrate	$MnCl_2.4H_2O$	1.44 mg/L
	Molybdenum trioxide	MoO_3	0.71 mg/L
	Copper sulfate pentahydrate	$CuSO_4.5H_2O$	1.57 mg/L
	Cobalt nitrate hexahydrate	$Co(NO_3)_2.6H_2O$	0.49 mg/L
	Ferrous sulfate heptahydrate	$FeSO_4.7H_2O$	4.98 mg/L
	Boric acid	H_3BO_3	11.4 mg/L
	Potassium hydroxide	KOH	31 mg/L
	Ethylene diamine tetraacetic acid disodium	EDTA Na_2	50 mg/L

[Table/Fig-1]: Composition of BBM medium used in present study.

Medium	Components	Chemical formula	Concentration
Z8 [18]	Dipotassium phosphate	KH_2PO_4	0.031 gL^{-1}
	Sodium nitrate	$NaNO_3$	0.467 gL^{-1}
	Calcium nitrate	$Ca(NO_3)_2$	0.041 gL^{-1}
	Magnesium sulfate heptahydrate	$MgSO_4.7H_2O$	0.025 gL^{-1}
	Sodium carbonate	Na_2CO_3	0.467 gL^{-1}
	Ferric chloride hexahydrate	$FeCl_3.6H_2O$	0.0028 gL^{-1}
	Ethylene diamine tetraacetic acid	EDTA	0.0039 gL^{-1}
	Gaffron solution	-	0.1 gL^{-1}
	Sodium tungstate dihydrate	$Na_2WO_4.2H_2O$	0.033 gL^{-1}
	Molybdic acid ammonium salt tetrahydrate	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.088 gL^{-1}
	Potassium bromide	KBr	0.119 gL^{-1}
	Potassium iodide	KI	0.083 gL^{-1}
	Zinc sulfate heptahydrate	$ZnSO_4.7H_2O$	0.287 gL^{-1}
	Cadmium nitrate tetrahydrate	$Cd(NO_3)_2.4H_2O$	0.154 gL^{-1}
	Cobalt nitrate hexahydrate	$Co(NO_3)_2.6H_2O$	0.146 gL^{-1}
	Copper sulfate pentahydrate	$CuSO_4.5H_2O$	0.125 gL^{-1}
	Ammonium nickel sulphate hexahydrate	$NiSO_4(NH_4)_2SO_4.6H_2O$	0.198 gL^{-1}
	Chromium nitrate heptahydrate	$Cr(NO_3)_3.7H_2O$	0.037 gL^{-1}
	Aluminum potassium sulfate	$Al_2(SO_4)_3$-$K_2SO_4.24H_2O$	0.474 gL^{-1}
	Vanadium sulfate hexahydrate	$V_2O_4(SO_4)_3.16H_2O$	0.035 gL^{-1}
	Boric acid	H_3BO_3	3.1 gL^{-1}
	Manganese sulfate tetrahydrate	$MnSO_4.4H_2O$	2.23 gL^{-1}

[Table/Fig-2]: Composition of Z8 medium used in present study.

Monitoring the Growth of Microalgae

Because the stock of microalgae were 10 mL, in order to increase the initial biomass, 5 mL of each microalgae was transferred to 100 mL of BBM and Z8 medium and put in shaker under 160 rpm and 50 µmoL photon m^2s^{-1} light intensity and 12L:12D photoperiod of 20 days. This test had two replicates. Under the hood, 5 mL of sample was taken from each flask every two days and after vortex with a sampler, 25 µL of sample was taken and the number of cells counted by Neubauer slide, then by using spectrophotometer, the maximum wavelength of *C. vulgaris*, *S. dimorphus* and *S. quadricauda* were obtained and optical absorbance was measured at 687 nm. The number of cells was counted using the formula; number of cells=average small cell count×4×106 [19].

Biomass Calculation

To obtain more biomass, from microalgae *C. vulgaris*, *S. dimorphus* and *S. quadricauda*, 7.5, 12, 9.4 mL were added to six PolyethyleneTerephthalate (PET) bottles of 1000 mL, with three replicates, respectively [Table/Fig-3]. For this purpose, the same bottles were selected. The caps of them were pierced for transferring the air transfer glass pipes as well as for uniform transfer of air in the bottles; Aquarium T connector tube was used. BBM culture medium was selected as the optimal medium because the growth of each alga was maximum in this culture medium. The microalgae were incubated at 25°C, to 1000 mL BBM medium and the culture continued up to 14 days. A typical pump air was used for aeration.

In order to have the same amount of microalgae inoculation, it was used from inoculation of 10%, with inverse proportions. Based on the cell count per milliliter, the microalgae with a smaller cell count per milliliter had higher inoculation and microalgae, with a larger number of cells had lower inoculation [Table/Fig-3].

Microalgae	Volume of inoculation (mL)	Inoculation (%)	Number of cell×106
S. dimorphus	12	10	8.5
S. quadricauda	9.4	7.8	10.8
C. vulgaris	7.5	6.3	13.3

[Table/Fig-3]: The amount of microalgae inoculation into the culture BBM medium.

Every two days, cell number and turbidity was measured, and biomass and the amount of starch measured at the end of the period. To obtain the biomass of microalgae, 1000 mL of medium was centrifuged at 5000 rpm for five minutes and pellet dried at 95°C for 45 minutes inside the oven dried to achieve constant weight and then it was measured. To measure the produced starch in microalgae biomass, anthrone reagent was used [20].

For making 25 mL of anthrone solution, 6 mL of distilled water was mixed with 19 mL of 98% sulfuric acid, then 38 mg of anthrone powder added to it, the solution stored in an amber container [21].

To obtain standard curve of starch, dilutions 0.001-0.5% of starch were prepared. For starch extracting, it is necessary to break the algae cell wall. To break the algae cell wall and release the starch, acid method was used. For this purpose, 0.5 gm of algae biomass was dissolved in 25 mL of 1N HCl and mixed by stirring at 70°C for 10 minutes. Then, 100 µL of this solution was mixed with 2000 µL of anthrone solution and placed at 100°C Erlen water bath for 10 minutes and after that its optical absorbance was read at 625 nm. 100 µL of distilled water was mixed with 2000 µL of anthrone solution as control and spectrophotometer zero out by it. The standard curve was obtained by using Microsoft Excel 2007. Then optical absorbance obtained from cell wall breaking of microalgae was placed into the linear equation derived from the standard curve of starch and the amount of starch produced by algae was achieved according mg/mL [22].

Preparation of Standard Curve of Cell Count by a Spectrophotometer

In this way, reading the optical absorbance of microalgae and comparing them with standard curve, the cell number of microalgae

could be determined. For standardisation, dilutions 1-50% (v/v) of media samples were prepared [23]. To calculate the specific growth rate of microalgae, following formula was used:

$$\mu=(\ln.x_m.x_0)1/t$$

(μ: the specific growth rate of microalgae, t: culture duration, xm: the final amount of cells, x0: the amount of cells at the beginning of the period) [23]. Cell doubling time was calculated from this formula: $G=\ln_2\mu-1$ (G: cell doubling time, μ: the specific growth rate of microalgae) [24].

Optimisation of Medium for *C. vulgaris*

Since, *Chlorella* algae ended up with the maximum growth in the BBM culture medium, it was selected as the optimal alga. Experimental design was took place by Minitab software using the Taguchi method, with three factors nitrogen, phosphorus and light at three levels (1,0,-1). Nitrogen source in the medium BBM was $NaNO_3$ at three levels 100, 250 and 400 mg/L. Phosphorus source was K_2HPO_4 and KH_2PO_4, from each of them, three levels 125, 250 and 500 mg/L were selected. Three light intensity levels (50.7, 41.74 and 34.66 μmoL m^{-2} s^{-1}) were considered. Light source included six pinkish and white fluorescent lamps [Table/Fig-4] [25].

Number repeat	$NaNO_3$ (mg/L)	KH_2PO_4 (mg/L) K_2HPO_4	Light intensity (μmoL $m^{-2}s^{-1}$)	Number repeat	$NaNO_3$ (mg/L)	KH_2PO_4 (mg/L) K_2HPO_4	Light intensity (μmoL. $m^{-2}.s^{-1}$)
1	400	500	46.7	16	400	125	37.66
2	250	250	46.7	17	250	500	37.66
3	100	125	46.7	18	100	250	37.66
4	400	250	41.74	19	400	500	46.7
5	250	125	41.74	20	250	250	46.7
6	100	500	41.74	21	100	125	46.7
7	400	125	37.66	22	400	250	41.74
8	250	500	37.66	23	250	125	41.74
9	100	250	37.66	24	100	500	41.74
10	400	500	46.7	25	400	125	37.66
11	250	250	46.7	26	250	500	37.66
12	100	125	46.7	27	100	250	37.66
13	400	250	41.74				
14	250	125	41.74				
15	100	500	41.74				

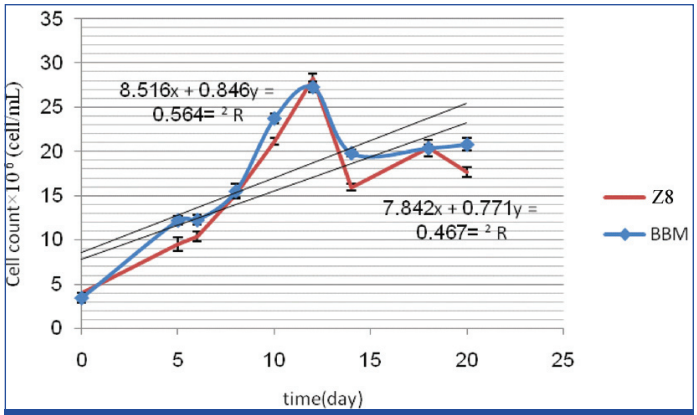
[Table/Fig-4]: Optimisation of medium for *C. vulgaris* in three level, according three factors nitrogen, phosphorus and light.

STATISTICAL ANALYSIS

The SPSS software version 16.0 was used to evaluate the growth curves; Student's t-test was used, comparing each experimental group with the control group. To evaluate the starch content, two-way ANOVA test was used. For all analysis, a 95% confidence interval was used in the statistical tests performed with graphpad prism 5. The significant level was considered at $p<0.05$.

RESULTS

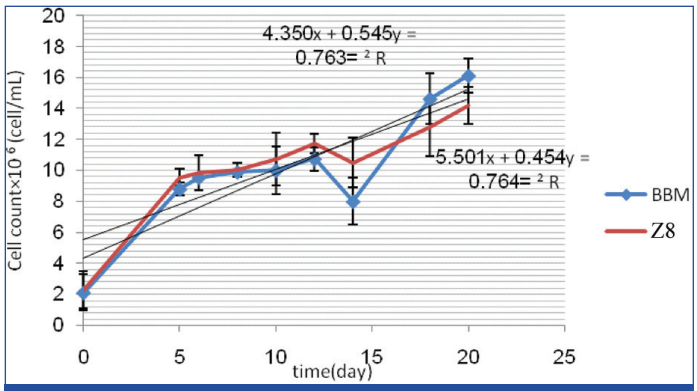
In linear equation derived from cell growth of *C. vulgaris* in media BBM and Z8; line slopes obtained from media BBM and Z8, after 20 days were 0.846 and 0.771×106 cell/mL, respectively [Table/Fig-5]. Present study showed that there is significant difference (p=0.034) between media BBM and Z8 and BBM medium was selected as a more suitable for *C. vulgaris* [Table/Fig-6]. In linear equation derived from cell growth of *S. quadricauda* in media BBM and Z8; line slopes obtained from media BBM and Z8 were 0.545 and 0.454×106 cell/mL, respectively [Table/Fig-7]. Results showed that there is no significant difference between optical absorbance of media BBM and Z8 (p=0.308) [Table/Fig-8].



[Table/Fig-5]: Cell count of *C. vulgaris* in both media BBM and Z8 after 20 days.

Culture	n	Mean±SD	p-value
BBM	9	17.19±7.02	0.034
Z8	9	15.73±7.11	
Difference	9	1.462±1.717	

[Table/Fig-6]: Comparing the value optical absorbance microalgae *C. vulgaris* between BBM and Z8.
95% CI for mean difference: (0.142; 2.782)
t-test of mean difference=0 (vs ≠ 0): t-value=2.55; p-value<0.05 was considered significant
SD. Standard deviation

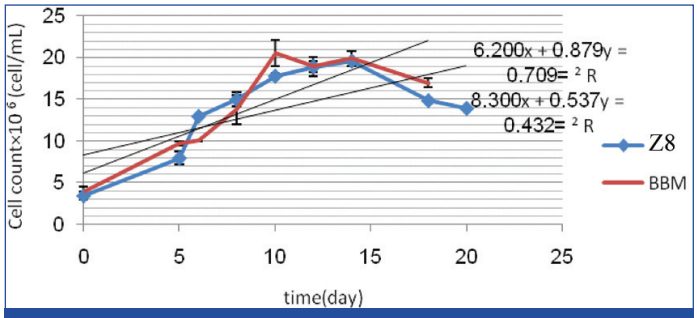


[Table/Fig-7]: Cell count of *S. quadricauda* in the media BBM and Z8 after 20 days.

Culture	n	Mean±SD	p-value
BBM	9	1.954±1.038	0.308
Z8	9	1.829±1.012	
Difference	9	0.126±0.347	

[Table/Fig-8]: Comparing the value optical absorbance microalgae *S. quadricauda* between BBM and Z8.
95% CI for mean difference: (-0.141; 0.392)
t-test of mean difference=0 (vs ≠ 0): t-value=1.09; p-value<0.05 was considered significant

According to the more line slope obtained from linear equation of BBM medium, it was considered a more appropriate medium for *S. quadricauda.* In this method, linear equation derived from cell growth of *S. dimorphus* in media BBM and Z8; line slopes obtained were 0.879 and 0.537×106 cell/mL, respectively [Table/Fig-9].



[Table/Fig-9]: Cell count of *S. dimorphus* in the media BBM and Z8 after 20 days.

The difference between optical absorbance of BBM and Z8 media was significant (p=0.033) and BBM medium was selected a more appropriate medium for *S. dimorphus* [Table/Fig-10].

Culture	n	Mean±SD	p-value
BBM	9	2.271±0.941	0.033
Z8	9	2.102±0.889	
Difference	9	0.1690±0.1965	

[Table/Fig-10]: Comparing the value optical absorbance microalgae *S. quadricauda* between BBM and Z8.
95% CI for mean difference: (0.0179; 0.3201)
t-test of mean difference=0 (vs ≠ 0): t-value=2.58; p-value<0.05 was considered significant

Present study showed that the amount of daily specific growth of *C. vulgaris* was more than *S. quadricauda* and *S. dimorphus*, 0.075 (d^{-1}) [Table/Fig-11].

Microalgae	Starch (% by weight of biomass)	Cell doubling time (d)	The amount of starch ($gL^{-1}d^{-1}$)	Z8 results as stated in results (μ) (d^{-1})	BBM results as stated in results (μ) (d^{-1})
C. vulgaris	29	2.7	6.6	0.055	0.075
S. quadricauda	20	3.21	4.14	0.008	0.072
S. dimorphus	19	2.9	3.07	0.038	0.072

[Table/Fig-11]: Starch rate in biomass of desired microalgae after 20 days and specific growth, doubling time and the amount of starch in desired microalgae.

Cell count of desired microalgae, which were cultured in 1500 mL bottles in a similar condition in terms of light intensity, culture medium, cell number of *C. vulgaris* (50×106 cell/mL) was more than *S. quadricauda* and *S. dimorphus*. Differences between microalgae was significant, in terms of the cell number (mL^{-1}) (p=0.001). According to the results of, there was significant difference between cell number of *C. vulgaris* compared to *S. quadricauda* and *S. dimorphus* and *C. vulgaris* was selected. The increase in light intensity, nitrogen and phosphorus in *C. vulgaris* led to increased cell growth. Repeat 3 showed more starch content, but according to the biomass in the repeat 2 and product of biomass multiply starch content, the amount of starch in repeat 2 was higher than others [Table/Fig-12].

Number repeat	Average cell count (× 106 cell/mL)	Average biomass (g/L)	Average produced starch (w/w %)
1	93.55	2.48	34±0.940
2	81.11	2.35	45±1.02
3	20.42	2.07	47±0.92
4	42.1	1.77	30±0.95
5	25.3	2.28	40±0.84
6	45.5	1.72	24±0.71
7	34	1.45	19±0.65
8	39.45	1.66	27±0.91
9	32.52	1.53	20±0.41

[Table/Fig-12]: Cell growth rate and the amount of starch produced by *C. vulgaris* with 9 replicates within 20 days.

Given to performed variance analysis, (p=0.001) for light intensity (lux), (p=0.001) for the amount of nitrogen and (p=0.012) for phosphorus, the effects of light, nitrogen and phosphorus on the amount of produced starch by *C. vulgaris* were significant.

By increasing the light, the amount of starch in the microalgae *Chlorella* found to be increased.

By increasing the nitrogen content from 100 mg/L to 250 mg/L, the starch content was increased but increasing from 250 mg/L to 400 mg/L, the starch content lowered very much and the amount of phosphorus in 125 mg/L led to the greatest amount of starch.

Starch production in microalgae is depends on two factors of growth rate and cell doubling time [Table/Fig-13].

Microalgae	Specific growth rate (μ)	Starch rate ($gL^{-1}d^{-1}$)	Cell doubling time (G)
Nannochloropsis oculata	0.11	3	2.9
C. vulgaris	0.13	6.6	2.7
S. quadricauda	0.08	4.14	3.21
S. dimorphus	0.11	3.07	2.9

[Table/Fig-13]: Comparison of starch production of different microalgae, based on growth rate and cell doubling time.

DISCUSSION

The most appropriate green algae for medical and pharmaceutical applications is the one that produces the largest amount of chlorophyll-containing pyruvid (starch) in appropriate conditions in terms of light and pH. In this study, the aim was to produce starch from microalgae using optimal growth conditions which are cost effective. As such, future studies may investigate the application of the extracted starch from these algae for diseases treatment and drug production.

This study is conducted for medium optimisation of microalgae *C. vulgaris* to increase usage starch production in medicine, for the first time in Iran. Other research about this microalgae in Iran, conducted on cell growth, increasing biomass and oil production. Salavatian S and Fallahi M, studied the effects of different concentrations of calcium on growth and biomass of green algae *C. vulgaris*, using pure stoke *C. vulgaris* and medium Zayndr Z8±N (with different concentrations of calcium in treatments) under laboratory conditions (at 25°C±2 and 350±3500 lux light intensity) for 96 hours [26].

The amount of nitrogen in the media BBM and Z8 was 2.5 and 0.471 g/L, respectively. The [Table/Fig-6] showed that line slope obtained from linear equation of *C. vulgaris* growth, in BBM medium was higher than Z8. Also, weight of *C. vulgaris* biomass per 100 mL of BBM and Z8 was 1.738 and 1.69 gm, respectively. These results indicate that the higher amount of nitrogen in the BBM medium compared to Z8, probably lead to further growth of microalgae in BBM medium.

The way of aeration for microalgae that used at the beginning of study was aeration by shaker. The advantage of this method over bubble technique is preventing contamination with other microorganisms.

Experiments showed that this aeration method increases the growth of microalgae; but if it is inappropriate for some of microalgae can lead to destruction of cells as a result of the introduction of high stress forces [27]. Ronda SR et al., studied the effect of aeration by air pomp on the growth and production of fatty acid gamma linolenic by Spirulina platens is in the photobioreactor. The aeration rate was considered 0.2 up to 2.5 vvm. The amounts of dissolved air of 41%, 88% and 108% was obtained 2.3, 6.5 and 7.5 ($mg.g^{-1}$ of cell dry weight), which showed that the biomass and the linoleic acid increased with increasing aeration. Special growth increased with increasing aeration [28]. Converti A et al., studied effect of nitrogen and temperature on growth and oil production of microalgae *C. vulgaris* and *Nannochloropsis oculata*, results showed that special growth of *Chlorella* was 0.14 cell in 1 mL at 25°C, but special growth decreased with increasing temperature and reached to -0.01 at 35°C. These results indicate that microalgae *C. vulgaris* had better growth at 25°C. microalgae *Nannochloropsis oculata* had better growth at 20°C [29].

Therefore, the temperature is also a factor that affects the growth of microalgae. The starch content of *Chlorella* in 50 μmoL photon $m^{-2}s^{-1}$ light intensity, before optimisation stages was 29% of biomass. The [Table/Fig-12] shows that after optimisation stages under 50.7 μmol m^{-2} s^{-1} of light intensity, it reached 47%.

In a study by Salavatian S et al., on the effect of calcium concentrations on growth and biomass of *C. vulgaris* in zinder medium it was found

that the effective concentration of calcium was in the range of 0.1-15 mg/L. The cell count at this concentration was 17575675 cells/mL. Also, the absorption rate was 0.542 and the biomass reached to 17676 cells/mL [26].

Lee J et al., suggested that *C. vulgaris* grew negligible without CO_2 and bicarbonate source. The growth rate of the algae was 2.30 and growth absorption was 0.15 [30].

In a study by Heidari SA et al., the different effects of nitrogen and ammonium on the growth of green algae *S. quadricauda* in the culture media were studied. Results showed that the highest alga cell density was 22.5×105 and 25.2×105 cells per milliliter for nitrogen and ammonium, respectively [31].

Khoeyi ZA et al., studied the effect of three light intensities of 5.5, 62.37 and 100 µmoL m^{-2} s^{-1} and different photoperiods (12L: 12D, 16L: 8D and 8L: 16D hours) on the growth rate, doubling time, and the production of *C. vulgaris* biomass. The highest growth rate of 1.13 per days, with an intensity of 100 micromoles of photon per m^2 and a photoperiod of 8:16 was observed [32].

In [Table/Fig-14] studies the researchers, show that the use of starch diet, in the treatment of some diseases, which can be produced the starch cheaper than industrial type for medical applications by cultivating green microalgae.

Study subject	Results	Year of publication	Reference
Assessment of starch dietary regimen regarding premenstrual syndrome among high school students in Sari during 2007	The results of this study revealed that starch-rich diet intake can improve premenstrual symptoms	2008	12
Effect of valerian and starch on uncomfortable breathing, coughing and snoring during usual sleep habit in 50 to 60-year-old women, Iran	The findings of this study showed that valerian and starch are effective on unpleasant breathing during women's sleep	2011	13
Nanoparticles made from novel starch derivatives for transdermal drug delivery	The potential use of these nanoparticles as transdermal drug delivery systems showed clear enhancers for flumin acid	2010	14
Resistant starch and colorectal neoplasia	As a result, the use of resistant starch significantly affects the luminal environment of the colon and causes the destruction of apoptosis of damaged genetic cells in the large intestine, some of which are known as colorectal cancer risk biomarkers	1995	15

[Table/Fig-14]: The studies done on the use of starch in medicine.

LIMITATION

There was no possibility of controlling the amount of CO_2 in the laboratory environment and by using polyethylene bottles for microalgae culture, there was a possibility of contamination of the culture medium with aerobic fungi and others microalgae.

CONCLUSION

It was found that starch content was increased with increasing light, reducing phosphorus and nitrogen in *Chlorella* and the results showed that *C. vulgaris* could be a good choice as a starter for producing starch for medicinal purpose.

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