Short Communication

The availability of sugar beet molasses as a substitutive medium for cultivation of *Nannochloropsis* sp. Hibberd, 1981 (Eustigmatophyta)

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An area of major concern in fish hatcheries is the provision of a dependable, nutritionally complete, economical food source for the fish larvae. In hatcheries, fish are raised from the eggs to the juvenile stages, until they are suitable for transfer to ponds or cages. Microalgae feeds are currently used in relatively small amounts in aquaculture, mainly for the production of larvae and juvenile shell- and finfish, as well as for raising the zooplankton required for the feeding of juvenile aquatic animals. Microalgae are the most important phytoplankton used as larval food (Benemann, 1992; Granvil, 1995; Hertrampf and Piedad-Pascual, 2000; INVE, 2010).

The most important parameters regulating algal growth are salinity, temperature, dissolved oxygen, pH, ammonia (NH_3), bacteria and ciliates (FAO, 2010).

Like other plants, algae require nutrients to achieve rapid growth. Concentrations of cells in phytoplankton cultures are generally higher than those found in nature. Algal cultures must therefore be enriched with nutrients to make up for the deficiencies in the seawater. Macronutrients include nitrate, phosphate (In an approximate ratio of 6:1), and silicate (Granvil, 1995; FAO, 2010).

Algal nutrient solutions are made up of a mixture of chemical salts and water. Sometimes referred to as "Growth Media", nutrient solutions (along with carbon dioxide and light), provide the materials needed for algae to grow. Nutrient solutions, as opposed

to fertilizers, are designed specifically for use in aquatic environments and their composition is much more precise (FAO, 2010).

Micronutrients consist of various trace metals and the vitamins thiamin (B_1), cyanocobalamin (B_{12}) and sometimes biotin. Two enrichment media that have been used extensively and are suitable for the growth of most algae are the Walne medium and the Guillard's $F/_2$ medium (FAO, 2010).

Water, carbon dioxide, minerals and light are all important factors in cultivation, and different algae have different requirements (FAO, 2010).

The most important parameters regulating algal growth are nutrient quantity and quality, light, pH, turbulence, salinity and temperature (FAO, 2010).

A comparative study conducted in Turkey in order to compare traditional *Nannochloropsis* bag culture and fotobioreactor culture; fotobioreactor batch culture reached 320 $\times 10^6$ cell mL^{-1} after 22 days, with a starting cell concentration at 16×10^6 cell mL^{-1} (Gökpınar *et al*., 2007).

Beet molasses is the residual syrup from the processing of sugar beet from which no more sugar can be crystallized by conventional means. The quality of molasses depends on the maturity of the sugar cane or beet, the amount of sugar extracted, and the method of extraction (Draycott, 2006).

On the basis of 73–79% dry matter and as % of dry matter, the total sugar content in sugar beet molasses which predominantly consists of sucrose is approximately 50%. The minor carbohydrates are glucose, fructose, raffinose and some other oligo- or polysaccharides. Their concentration is below 1% and depends to a significant extent on the manufacturing process. Crude protein and crude fibre contents are reported as 6.6-11.1% and 0.0-0.3% respectively. Major cations are potassium (3.2-4.7%) followed by sodium (0.6-1.9%), calcium (0.1-0.5%) and magnesium (0.01-0.3%). Their content depends mainly on soil type and water availability. Additionally, the calcium and sodium content is influenced by processing practices.

About 20% of the total mass consists of non-sucrose organic matter, in particular of non-protein nitrogen (NPN) containing substances, such as betaine (4-5%). In addition molasses contains free and bound amino acids, pryrrolidone carboxylic acid (a conversion product of glutamine), peptides and nucleic acid components (3-4%). In the manufacturing process most of the amino acids undergo changes so that less than the amounts expected from beet root are found in molasses.

Molasses contains up to 4% of organic acids predominantly lactic acid from the degradation of invert sugar (up to 1.7%) followed by malic, citric, fumaric, and oxalic acid. Molasses contains only low levels of trace elements except for iron. Sugarbeet molasses contains 9-11μgg^{-1} copper, 0.02-0.15μgg^{-1} biotin (Vit. B_7), 50-110 μgg^{-1} Pantothenic acid (Vit. B_5), 1.3 μgg^{-1} thiamine (Vit.B_1), and 5000-8000

μgg^{-1} inositol. The main inorganic anions are chloride (1.0-3.0%), sulfate (0.6-2.0%), nitrate (0.3-0.8%) and traces of phosphate (0.1-0.5%) and nitrite (3.0-170 $mgkg^{-1}$). pH of sugar beet molasses is reported as 7 to 8.76 (OECD, 2002; Stevens and Verhe, 2004; Draycott, 2006; Şeker, 2010; Türkşeker, 2010).

The purpose of this study is to determine the availability of sugar beet molasses as an additional nutritional constituent for algal cultures. Since research work on the use of sugar beet molasses in the field of algae cultivation is very limited, this study can contribute significantly to future research.

This study was conducted in the algae units of sea bream and sea bass hatchery facilities of Kılıç Group of Companies in Ören/ Milas, Muğla, Turkey.

Algal culture

Algae facilities used in this study consist of three departments, namely the Pure Culture Unit, the Pre-growing Unit and the Production Unit. The method of sharing was elaborated for the purpose of algae production. Algae, following their intensification for 10 days in the pure culture room were transferred to the pre-growing unit. In the next step, algae intensified in the pre-growing unit were transferred to the production unit. Nutrient solutions were used in each of the production stages. This study was conducted in the production stage in the Production Unit (FAO, 2010).

The water used in the Production Unit was obtained from the underground water source (Artesian well) and its salinity is 38‰. The water was treated before its use. The mechanical filtration which was the first stage of the treatment process was followed by UV sterilization. The whole treatment process allows suspended matter and pathogens to be eliminated. The dissolved oxygen concentration of water was 5 mgL^{-1} to 10 mgL^{-1}, temperature was almost constant at 27 °C and pH was 6.8 to 7.4.

Algae production was carried out in transparent LDPE (Low Density Polyethylene) bags with a 35 L capacity. 15 L min^{-1} air flow was supplied to each of the bags. Bags were illuminated by natural day light with the light intensity of more than 105.000 Lux in day time and equally by fluorescent lamps in the night. The ambient temperature of the unit was adjusted at 16°C to 25°C (FAO, 2010).

In this study, the algae, *Nannochloropsis* sp. *Hibberd, 1981* (Eustigmatophyta) obtained from Scottish Association for Marine Science(SAMS) was used. The origin of the algae was Isle of Man, UK (Algeabase, 2010; CCAP, 2010a).

The algae were counted by using Neubauer Counting Chamber (Tiefe Depth Profondeur 0.100 mm- 0.0025 mm^2) at 2 day intervals. The cilliate, *Amphorides amphora* (Claparéde and Lachmann, 1858), an undesired organism, has also been detected and counted in algae bags (Balkıs, 2004).

The study were conducted on 3 different trial groups, each consisting of three separate bags, so that triplicate determinations were obtained. Prior to the trial, 30 g molasses which was obtained from Ankara Sugar Factory, Turkey was diluted in 300 mL culture water, thus a 10% stock molasses solution was prepared. For Group I, only 150 mL molasses solution was added in each of 35 L algae bags. For Group II, 75 mL molasses solution and 20 mL f/2 were added in each of the bags and lastly for the Control Group, Group III, no molasses solution was used and only 40 mL f/2 was added in each of the bags.

Modified medium "f/2" consists of two different solutions; For the first solution of f/2; 6 kg $NaNO_3$, 600 g KH_2PO_4 and 400 g NH_4Cl were dissolved in 10 L distilled water and for the second solution of f/2; 100 g Na EDTA, 5 g $MnSO_4\,H_2O$, 5 g $CuSO_4\,5H_2O$, 5 g $Na_2MoO_4\,2H_2O$, 3 g $CoSO_4\,7H_2O$, 1 g $ZnSO_4\,7H_2O$ and 10 g $FeCl_3\,6H_2O$ are dissolved in 10 L distilled water, and then the two solutions are mixed. In order to adjust the pH of f/2 as 5.5, NaOH solution was prepared by dissolving 200 g sodium hydroxide in 1L distilled water and used in required amounts (CCAP, 2010b).

Related data obtained from the experiments were statistically evaluated by Factorial Analysis of Variance with Repeated Measurement (ANOVA) (Düzgüneş *et.al.* 1983; Zar, 1999). The results of algae trials are given in Table 1, Figs. 1 and 2. As seen in Fig. 1, the algae displayed similar growth patterns in Group II and Group III. Cell density of the algae remain unchanged for 2-4 days and then it started to increase in cell numbers and continued to increase until a peak was reached. This active multiplication phase lasted for 14 days, depending on the composition of culture medium. Differently, in Group I, cell numbers started to increase on T2 and T4 and then stayed almost constant for 10 days. From that point, the algae started to decrease in number to its starting levels and never reached the desired level of $100x10^6$.

The results of statistical analysis applied on data obtained from the study are as following:

According to the results of analysis of variance on data obtained from the algae trials there is an interaction between Time (T) and Groups (GR) in relation to number of algae ($p<0.05$). For the Group I; the mean cell number of algae at time T0 differed significantly from the means at T6, T8, T10, T12, and T14. For the Group II; the means of the number of algae at time T0, T2 and T4 differed significantly from the means at T6, T8, T10, T12, T14, T16, and T18. Mean algae numbers at T6 differed from that at T10, T12, T14, T16 and T18. Similarly mean algae number at T8 differed from that at T12, T14, T16 and T18. Finally, mean algae number at T10 differed from that at T18. In Group III; mean algae number at T0 differed from that at T2, T4, T6, T8, T10, T12, T14, T16 and T18. Also mean algae number at T2 differed from that at T6, T8, T10, T12, T14, T16, and T18. Results at T4

differ from that at T6, T8, T10, T12, T14, T16 and T18. Also results obtained at T6 were different from those recorded at T10, T12, T14, T16 and T18, and mean cell numbers of algae at T8 differed from T12, T14, T16 and T18. Results obtained at T10 differed from T14, T16 and T18, while results for T12 differed from T16 and T18. Also mean algae cell number at T14 and T16 differed from that at T18.

The means of the number of algal cells in GRI and GRII were found to be statistically significant between T8, T10, T12 and T14. Similarly, the difference between the means of the number of algal cells in GRI and GRIII were also found to be statistically significant. At T18, it was found that all groups differed from each other.

10% molasses solution, when used as a single source of nutrition showed poor performance. In Group I, the maximum number of algae was observed at time T14. A similar performance was achieved by Group II and Group III at time T4. In Group I, unlike Group II and Group III, the number of algal cells started decreasing after the climax point had been reached. The maximum number of algae in Group II was observed at T18 which was nearly the same as that in Group IIIat T10 and T12. On comparing data in Group II and Group III, it can be said that combination of molasses solution with f/2 may be considered as an efficient way. In Group I, molasses solution used as a single nutritive source had a stimulating effect on ciliate booming, while molasses solution when used in combination with f/2 was much less incentive for ciliate proliferation as seen in Group II.

Number of ciliates at T18 could not be compared statistically because of the closeness of values in Group I and Group II.

In Group I, the number of ciliates was excessively high when compared to that in the other two groups. In Group II, the algae started to approach the desired level of $100x10^6$ at time T12.

Table 1: Results of Alg trials in relation to time (here referred as T-Days) and composition of nutritive complex (here referred as GR).

	Number of microalgae (x 10^6 cells *mL^{-1}*)										
	T0 Day 0	**T2 Day 2**	**T4 Day 2**	**T6 Day 6**	**T8 Day 8**	**T10 Day 10**	**T12 Day 12**	**T14 Day 14**	**T16 Day 16**	**T18 Day 18**	***Num.*Cil. at T18 (mL^{-1})**
GR I	20	30.8	33.6	32.8	35.2	34	33.2	38.4	29.2	28.8	7
GR I	17.6	25.6	28.8	39.6	36.8	37.2	38.8	40.8	38.8	32.8	18
GR I	20.8	32.4	42	45.6	46.4	41.6	49.2	47.2	36	34	15
Mean	19.46^{aA}	29.60^{abA}	34.80^{abA}	39.33^{bA}	39.46^{bA}	37.60^{bA}	40.40^{bA}	42.13^{bA}	34.66^{abA}	31.86^{abA}	13.33
±Std. Error	0.96	2.05	3.86	3.70	3.50	2.20	4.69	2.63	2.85	1.57	3.28
GR II	18.4	28.8	37.6	60.8	81.2	95.6	101.2	106	118.4	124	4

Table 1 continued:

GR II	16.8	24.8	28	54	72.4	89.6	100.8	103.2	114.4	127	3
GR II	20	42	42	65.2	74	74	81.2	78.8	66.8	62.8	1
Mean	18.40^{fA}	31.86^{efA}	35.86^{eA}	60.00^{dA}	75.86^{cdB}	86.40^{bcB}	94.40^{abB}	96.00^{abB}	99.86^{abB}	104.60^{aB}	2.66
±Std. Error	0.92	5.20	4.13	3.26	2.71	6.44	6.60	8.64	16.60	20.90	0.88
GR III	16	26.4	31.2	68	84.8	105.2	116.8	120	134.4	160	1
GR III	22	44	50.8	68.8	82.8	96	102.4	115.2	129.6	148	1
GR III	20.8	44	53.6	69.6	82.4	92.8	105.6	124.5	133.6	149.6	0
Mean	19.60^{hA}	38.13^{gA}	45.20^{gA}	68.80^{fA}	83.33^{efB}	98.00^{deB}	108.26^{cdB}	119.90^{bcB}	132.53^{bB}	152.53^{ac}	0.66
±Std. Error	1.83	5.87	7.05	0.46	0.74	3.72	4.37	2.69	1.48	3.76	0.33

Different lowercase superscripts in the same row indicate significant differences ($p<0.05$).
Different capital superscripts in the same column indicate significant differences ($p<0.05$).
GR I: 150 mL 10% molasses solution
GR II: 75 mL 10% molasses solution+20 mL f/2
GR III: 40 mL f/2

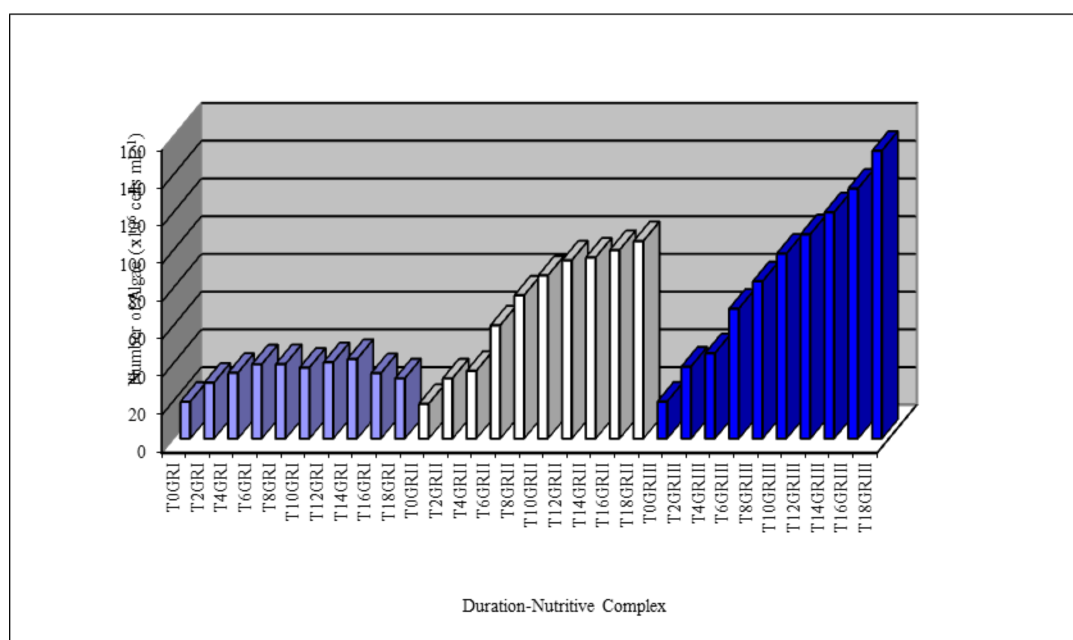

Figure 1: Number of algal cells in relation to duration and composition of nutritive complex.

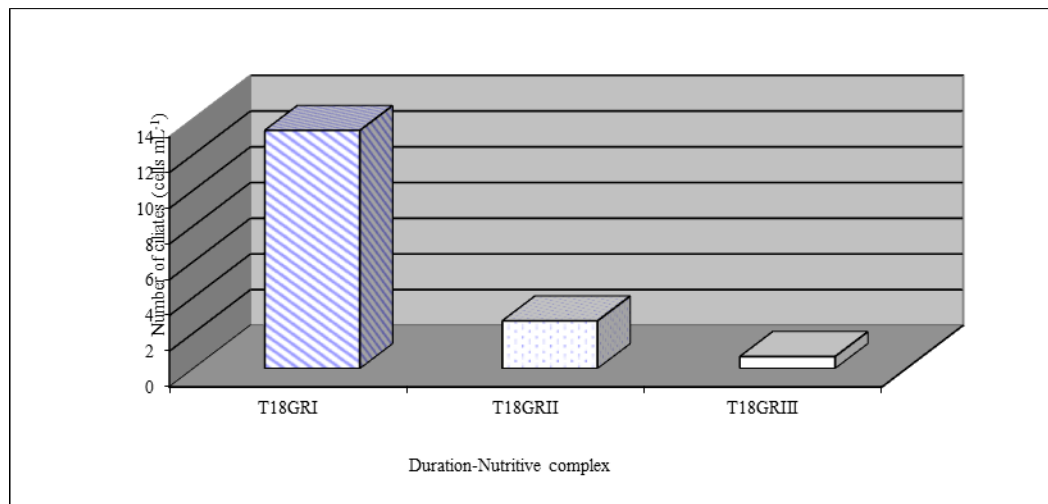

Figure 2: Number of ciliates at the time of T18 in relation to duration and nutritive complex.

Generally, it can be said that, the addition of molasses to a nutritive complex has had significant effects on the proliferation and number of algae and ciliates. From the study, it is clear that sugar beet molasses can only be used to a limited extent as a nutritional supplementary factor for algal cultures due to its stimulating effect on ciliate proliferation. For algal cultures, sugar beet molasses should not be used as a single source of nutrition, but instead they should be combined with other nutritive sources. It can be said that 75 mL molasses solution may be combined with 20 mL f/2 since it may help saving up to 50% in the amount of f/2, thus providing cost advantage in algal production processes. With its high organic acid content, sugar beet molasses can support f/2 nutritive complex. In case of using molasses for nutritional purposes, some precautions must be taken into consideration in order to control excessive ciliate proliferation. These may include washing off ciliates by using special filters, using specific chemical agents inhibiting ciliate booming and autoclaving the molasses stock to check for micro organisms including ciliates.

On comparing the contents of nutritive complex f/2 and sugar beet molasses, it is clear that; sugar beet molasses includes magnesium, calcium, inositol, vitamins (Vit B_5, Vit B_1, Vit B_7), protein and carbohydrates which were not present in the f/2 solution used in the experiments. Thus, sugar beet molasses can be assumed as a substitute for f/2 to some extent and its vitamins can support the culture for deficient components.

The feeding of ponds with wastes from sugar factories generally stimulates the growth of phyto and zooplankton. The development of the algae considerably outpaced that of zooplankton (Kyselowa, 1973). This supports the result of the present study, considering the similar chemical composition of the wastes of sugar factories and sugar beet molasses.

The cost of microalgae production is high. Algae production is also labor

intensive and the cost of chemical supply is high. Nutrients have a 4-20% share in the total cost of microalgae production (Granvil, 1995).

Sugar beet molasses with their low-cost advantage and ease of availability can draw attention of fish farmers. It is clear that sugar beet molasses can only be used to a certain extent as an additional nutritive source for algal cultures owing to its stimulating effect on the ciliate booming. Ciliate booming was a result of high content of carbohydrate in sugar beet on which bacteria feed in the medium. Bacteria can use carbohydrate and other nutrients in sugar beet, while ciliates feed on bacteria in the medium. Since algae are phototropic organisms, sugar beet can be utilized as a source of vitamins and minerals. For the purposes of algae cultivation, 75 mL molasses solution may be combined with 20 mL f/2 since this can help saving 50% on the amount of f/2, thus producers can obtain cost advantage.

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