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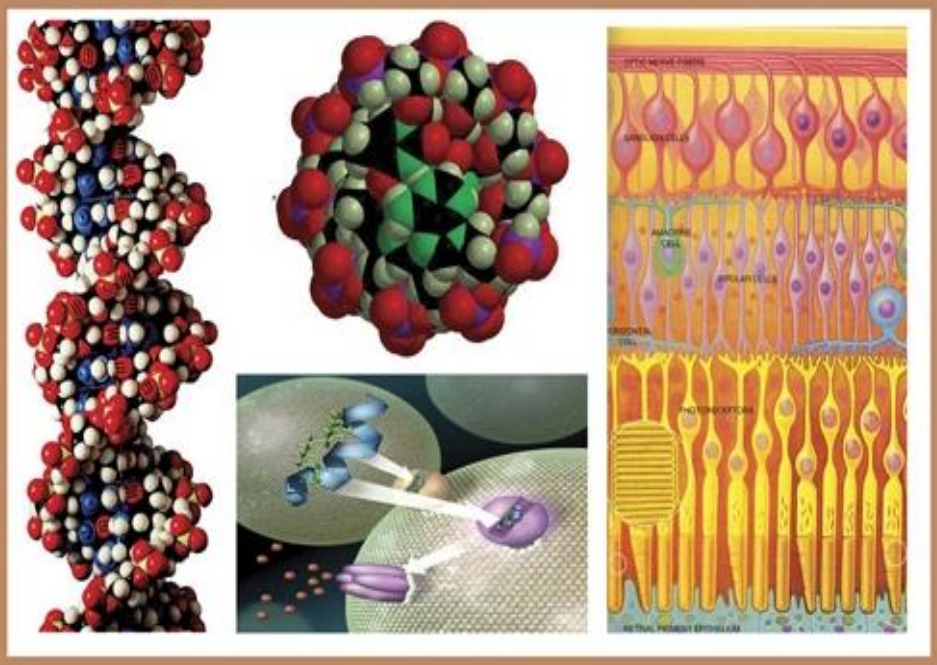

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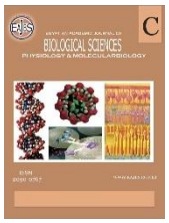

Nannochlropsis gaditana Cultivation Using Cheese Whey as Substrate for Biomass Production

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ABSTRACT

Whey represents an important disposal problem in the dairy industry. This work sought to optimize a culture medium based on sweet whey for the culture of microalgae (a *Nannochloropsis gaditana* strain was used for this study). The Deproteinized whey was then supplemented with various concentrations of heavy metals, vitamins, sodium nitrate and sodium dihydrogen phosphate, identical to that used in the F/2 Guillard medium, twenty combinations are made. *Nannochloropsis gaditana* was able to grow in all whey-based mediums at different growth rates.

Results show that deproteinized whey does not provide a good yield of cellular biomass. the high productivity in biomass (by providing the F/2 Guillard control medium) has been observed in media composed principally of deproteinized whey and heavy metals. Maximum cell number was $988.33x10^5$ from the 11^{th} day on the deproteinized whey + $NaHPO_4$ + heavy metals composed medium. However, The *Nannochloropsis gaditana* strain was faster in specific growth speed and generation time on a medium composed of deproteinised whey and heavy metals, than the other media, as well as a better biomass yield on the latter. A comparative study of growth kinetics carried out with the same strain has shown that growth takes place according to a classic pattern of discontinuous culture.

INTRODUCTION

The dairy industry is responsible for producing large quantities of whey (nine times the tonnage of cheeses manufactured). The recovery of whey or its use in various sectors offers several advantages: solving a waste treatment problem, and potentially reducing the pollution caused by whey released into waste water (Gana and Touzi, 2001; Prazeres *et al.*, 2012). Due to its high nutrient content such as lactose, soluble proteins, vitamins and minerals, whey is an excellent culture medium for microorganisms at a low cost (Boudjema *et al.*, 2009; Spalatelu, 2012; Benaissa *et al.*, 2017; Pires Arona *et al.*, 2021).

Microalgae are micro-organisms that perform a key function in ecosystems. They are close to bacteria by their ability to grow rapidly and to plants by their ability to oxygenate photosynthesis. These microorganisms are of growing interest because of their interesting biological and metabolic properties. The term “microalgae” has gradually become a term used in the scientific and industrial worlds. Indeed, in recent years, microalgal biotechnology has become a privileged research topic to explore and exploit the enormous potential of these microorganisms operating in many systems and in many industrial sectors such as pharmaceutical fields, food, agriculture, and environmental: as a depolluting of industrial waste gases (CO_2 sequestration) or liquids (fixation of nitrates, phosphates, metals...) and the production of renewable energy through the biological synthesis of hydrogen, methane and fuel.(Spolaore *et al.,* 2006; Filali, 2012; Templeton and Laurens, 2015; Han *et al.,* 2019; Remize *et al.,* 2021; Wang *et al.,* 2021).

Nannochloropsis, oleaginous microalgae, is exploited for biodiesel production (Chisti, 2007; Ilavarasi *et al.,* 2011) and is considered to be the main species of cultivated algae made up mainly of PUFA-LC that are associated with cell membranes or are found in storage compartments in the form of vesicles. These species are able to accumulate triglycerides, which represent neutral lipids. Recent studies have shown the presence of cholesterol as the main sterol. (San Pedro *et al.,* 2014; Remize *et al.,* 2021; Xiang *et al.,* 2022**)**

At the industrial scale of production, it is very important to optimize a nutrient culture medium suitable for the cultivation of this species. The nutrient environment of microalgae should be easy to prepare, economical, must achieve high growth and satisfy all the nutritional needs of microalgae. (Lu *et al.,* 2017)

The production of microalgae has become very diversified, driven by the current trend of substituting natural products for synthetic components, or even those of animal origin, and by the desire to have a renewable energy source. Microalgae, which possess exceptional qualities and important metabolic potential, can use whey to grow, multiply and give a large amount of biomass and metabolites often indispensable for humans. In order to promote these high tonnages of whey, we are interested in using sweet whey as a culture medium for Microalgae of *Nannochloropsis* genus. (Morais *et al.,* 2016; Abbasi *et al.,* 2019).

MATERIALS AND METHODS

Strain:

A *Nannochloropsis gaditana* strain used in this study belongs to the collection of the Laboratory of Aquaculture and Bioremediation, Department of Biotechnology, Faculty of Natural and Life Sciences, University Oran1, Oran, Algeria.

Media:

Sweet whey used in culture medium was kindly supplied by a local manufacturing plant for soft cheeses (camembert). Whey was deproteinized by Culture medium heating in a water bath at 100°C for 30 min, then centrifuged at 5000 rpm for 15 min. The supernatant was filtered through a standard paper filter (Durieux, ref: 66301130). (Benaissa, 2017).

In order to optimize the appropriate culture medium for *Nannochloropsis gaditana*, it was subjected to twenty different media of varying nutrient composition, listed in Table 1.

The F/2 Guillard medium (Guillard,1975) is used as a control at the other media vary by the presence of heavy metals, vitamins, sodium nitrate or sodium dihydrogen phosphate, deproteinized whey, or seawater. The final pH is adjusted to 7 with 5N NaOH and the media were autoclaved at 121°C for 15 min.

Table 1: Different media of varying nutrient composition

Medium's Code	Medium Composition
Control	f/2 Guillard (Guillard, 1975)
GM 1	f /2 Guillard (without vitamins)
GM 2	Sea water + $NaNO_3$
GM 3	Sea water + $NaHPO_4$
GM 4	Sea water + heavy metals
GM 5	Sea water + Vitamins
SWA 1	Deproteinized whey
SWA 2	Deproteinized whey+ $NaNO_3$
SWA 3	Deproteinized whey+ $NaHPO_4$
SWA 4	Deproteinized whey + heavy metals
SWA 5	Deproteinized whey + Vitamins
SWA 6	Sea water + Deproteinized whey (V/V)
SWA 7	Deproteinized whey + $NaNO_3$ + $NaHPO_4$
SWA 8	Deproteinized whey + $NaNO_3$ + heavy metals
SWA 9	Deproteinized whey + $NaNO_3$ + Vitamins
SWA 10	Deproteinized whey + $NaHPO_4$ + heavy metals
SWA 11	Deproteinized whey + $NaHPO_4$ + Vitamins
SWA 12	Deproteinized whey + heavy metals + Vitamins
SWA 13	Deproteinized whey + $NaNO_3$ + $NaHPO_4$ + heavy metals
SWA 14	Deproteinized whey + $NaNO_3$ + $NaHPO_4$ + Vitamins

Cultivation Conditions:

The cultures experiments were conducted in 100 mL of sterile medium in Erlenmeyer flasks and incubated at 23 ± 1°C under continuous illumination of 100 $\mu molm^{-1}\ s^{-1}$ intensity.

The initial cellular concentration (day 0) was $11x10^5$ cells mL^{-1}. Cultures were agitated manually three times a day for the experiment period.

Measuring Microalga Concentration:

The experiment was conducted for 18 days on the twenty media. Growth of the algal biomass was assessed at two days intervals. Biomass concentration was measured by counting cells using *Malassez* cells and results are expressed as cells/ml.

Determination of Growth Parameters:

The growth rate was calculated under Eq. 1. Where X1 and X2 are referencing the concentration of biomass measured through cell count at the beginning (t_1) and at the end (t_2) of the exponential growth phase, respectively.

Eq. 1. $$\mu = \frac{\mathrm{Ln(X2) - Ln\ (X1)}}{t2 - t1}$$

Generation time is expressed as the inverse of the growth rate.

Statistical Analysis:

The data were analysed using R software, version 3.6.3. All the analyses were carried out in triplicate, and mean values with standard deviation were reported. Effects between variables were tested using multi-way Anova. Conditions of use (normality, homoscedasticity and sample independence) have been verified. The p-value ($p< 0.05$) was considered statistically significant. Factor analysis is a statistical method used to describe variability among observed, correlated variables in terms of a potentially lower number of unobserved variables called factors.

RESULTS

Microalga Concentration:

Results of 18 days assessment of *Nannochloropsis gaditana* concentration (Fig. 1) shows that the strain seems to get a better response to media type SW4, SW8, SW10, SW12 and SW13 demonstrated by a consequent mean concentration of the microalgae under these media culture respectively 551.63, 451.65, 488.30, 477.15 and 517.67x $10^5 cellsml^{-1}$.

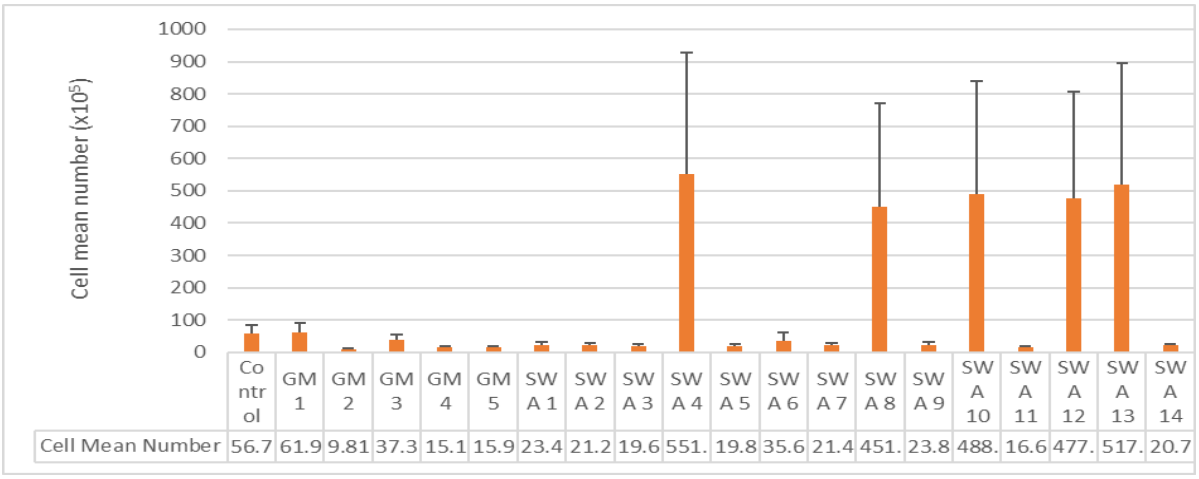

Fig. 1: Mean cell number of *Nanochloropsis gaditana* under different media cultivation.

The maximum cell'smean number of 551.63 x 10^5 cell mL^{-1} was reached using the SW4 media. While the control media cell's mean number was 56.74 x 10^5 cell mL^{-1}.

Hierarchical Clustering Analysis (HCA) (Fig. 02) obtained from mean cell number clearly shows that two categories of media can be distinguished according to the growth *N. gaditana*:

*Media SWA4, SWA8, SWA10, SWA12 and SWA13. Those media are giving consequent growth.

*Low-growth media. This is the case for control medium, GM1, GM2, GM3, GM4, GM5, SWA1, SWA2, SWA3, SWA5, SWA6, SWA7, SWA9, SWA11, SWA14 media.

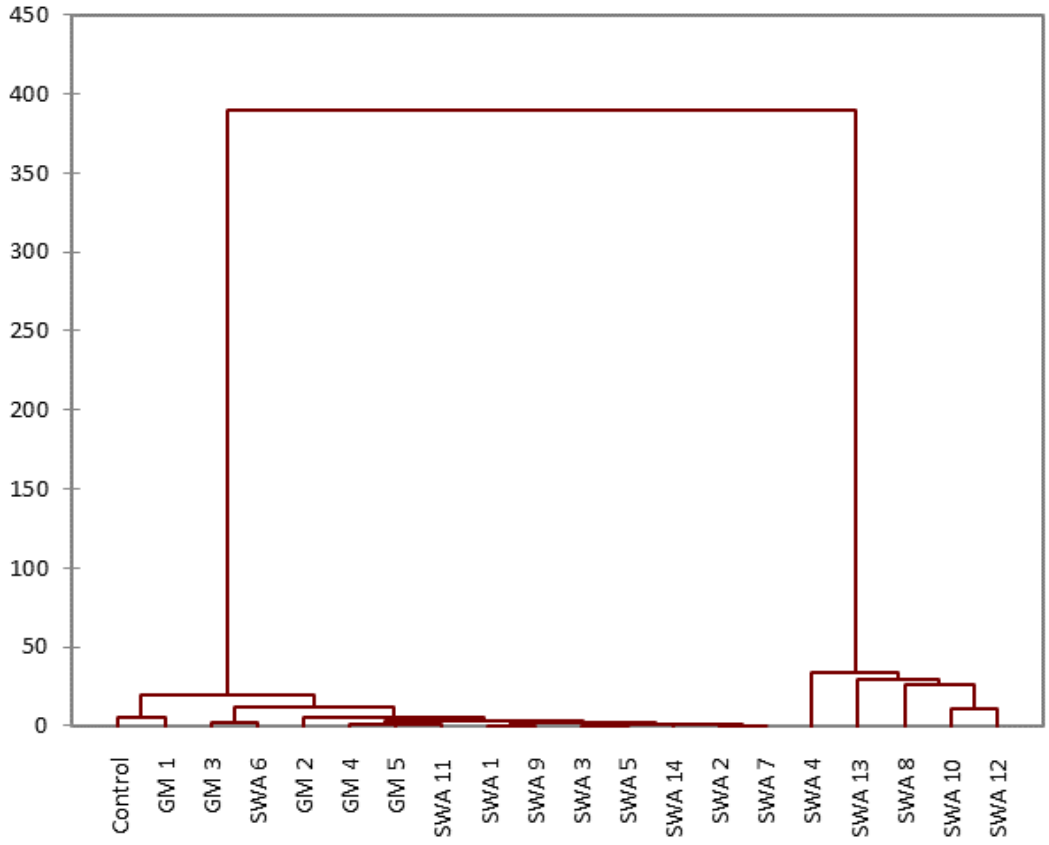

Fig. 2: Hierarchical Clustering Analysis for mean cell number on different media.

These observations indicate that the SWA4 medium appears better with a maximum growth rate found on the 7th day ($870x10^5$ cellsmL^{-1}). This may be related to its content in whey and heavy metals which allowed subsequently give a favourable combination to the growth of *Nannochloropsis gaditana*. In the same way, SWA12, SWA13, SWA8 and SWA10 also produce large biomass. These results can be attributed primarily to the presence of heavy metals and whey in combination with other elements. Despite the heavy metal content of the control medium, an average density was noted in the latter. The GM2 medium prepared from seawater and $NaHPO_4$ yielded low algal density with a maximum of 15 $x10^5$ cellsmL^{-1}. In view of all these results, heavy metals do not give satisfactory algal growth after having the associated vitamins, and seawater. Therefore, whey-based media and heavy metals can be used for seeding and obtaining significant biomass of the strain *Nannochloropsis gaditana*.

The comparison between the control medium and the vitamin-enriched media shows that they do not have a major influence on the growth of *Nannochloropsis gaditana*, these observations are consistent with the work of De La Noue (1989).

Monaco and Prouzet (2014) showed that for the growth of microalgae, the essential macronutrients are carbon, nitrogen, phosphorus. However, micronutrients include iron, cobalt, zinc, manganese and magnesium, which are essential for certain key enzymatic activities and other intracellular functions. Otherwise, according to Tebbani *et al.*, (2014) iron deficiency has also been shown to cause cellular metabolic changes through decreased microalgal cell density and size and inhibition of protein and lipid synthesis. This element also acts as a catalyst in the synthesis of chlorophyll. Also, magnesium has been shown to be essential for nitrogenase activity in cell metabolism. Indeed, a copper deficiency is likely to affect the photosynthetic mechanism of microalgae. Molybdenum deficiency can affect the metabolic process of nitrogen uptake at the cellular level (Tebbani *et al.,* 2014).

Growth Kinetics:

In light of the results of the growth of *Nannochloropsis gaditana* on different media (Fig. 3), it seems that the latency phase is common between the different media lasted a maximum of two days, depending on the composition of the medium. According to Prescott *et al.* (2010) this duration depends also on the history of the cells.

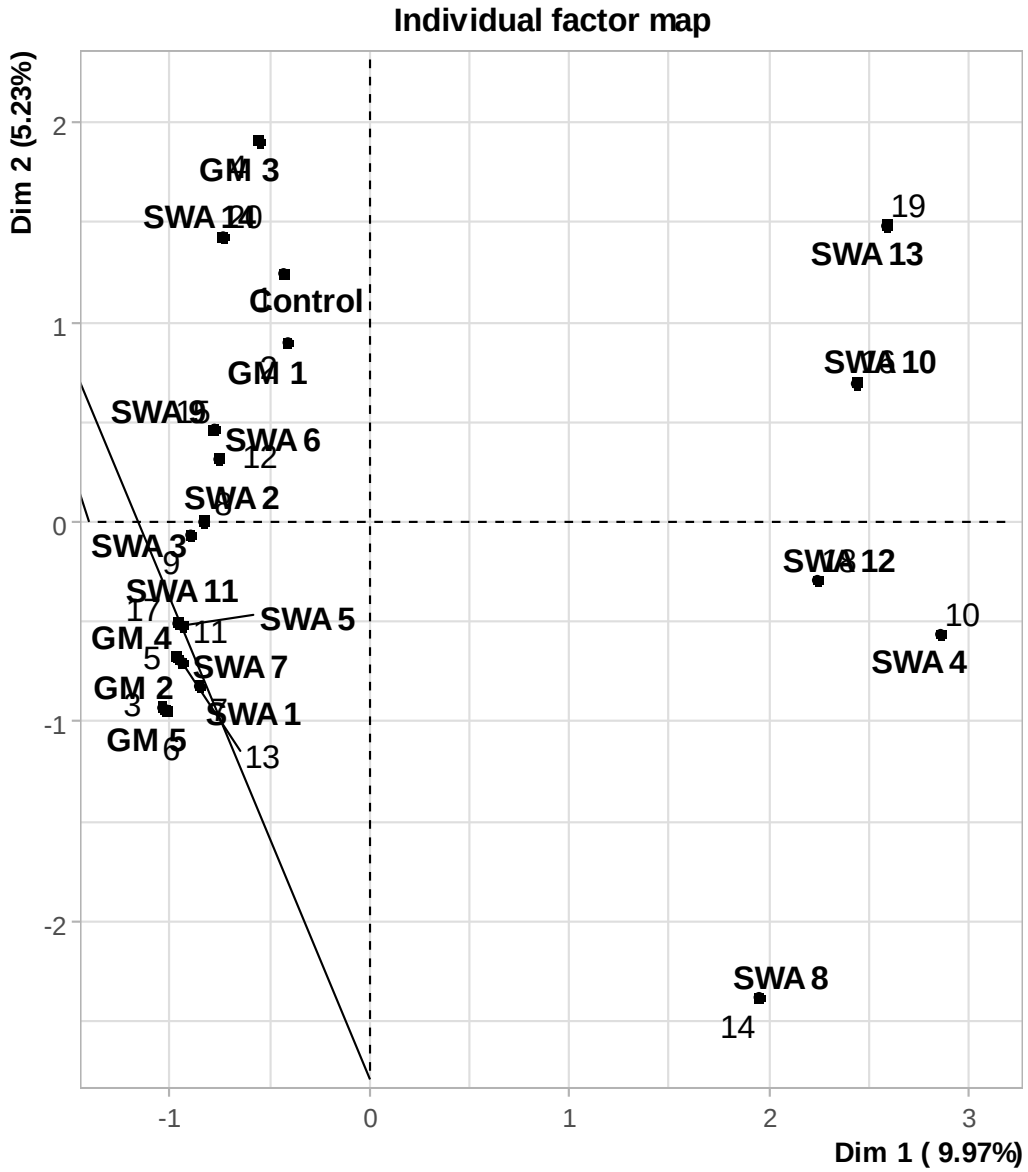

Fig. 3: growth kinetic of *Nannochloropsis gaditana* on different media

The first factorial axis groups on its positive side: SWA 4, SWA 8, SWA 10, SWA 12 and SWA 13. While it represents its negative side: GM5 and GM2 (Table 2).

The acceleration phase also took place at the same time (from the second day) in all circles.

Again, the start of the exponential phase was fast in the SWA4 medium compared to other media. In addition, high cell density was found in the SWA10 medium (987,105 cells/mL).

A remarkable growth is marked in the medium; SWA10, SWA13, SWA4, SWA8 and SWA12, However, the medium SWA2 gave the lowest algal growth during all the experiment days. It seems that the start of the exponential phase is directly related to the richness of the medium in phosphorus, and calcium provided by whey, and to the micronutrients provided by the solution of heavy metals (The SWA4 medium followed by the SWA8 medium).

The second factorial axis pits SWA 8 (-2.384) against GM3 (-1.906) (Table 2).

Table 2: Specific contribution of the first two axes

	Dim.1	**Dim.2**
Control	-0.429	1.239
GM 1	-0.411	0.893
GM 2	-1.037	-0.928
GM 3	-0.554	1.906
GM 4	-0.966	-0.679
GM 5	-1.013	-0.945
SWA 1	-0.853	-0.821
SWA 10	2.441	0.695
SWA 11	-0.957	-0.507
SWA 12	2.245	-0.294
SWA 13	2.592	1.484
SWA 14	-0.737	1.422
SWA 2	-0.827	0.004
SWA 3	-0.895	-0.07
SWA 4	2.861	-0.563
SWA 5	-0.936	-0.521
SWA 6	-0.753	0.315
SWA 7	-0.939	-0.708
SWA 8	1.949	-2.384

According to Prescott *et al*. (2018), photosynthetic microorganisms during the logarithmic phase develop and divide at the maximum possible rate based on their genetic potential, the nature of the medium and the growing conditions.

Also highlighting the addition of $NaHPO_4$ to whey and heavy metals (SWA10). Because this combination could give a remarkable biomass compared to other preparations.

Low growth averages were observed in seawater-containing media (GM2, GM3, GM4 and GM5), This can be explained by the fact that the use of NaCl at high concentrations could inhibit growth.

Growth Parameters:

According to Figure 4, the highest growth rates of *Nannochloropsis gaditana* were obtained in GM4 (1.13 d^{-1}), followed by SWA12 and SWA13 with rates of 0.9 d^{-1} and 0.45 d^{-1} respectively. However, the lowest value is found in the GM1 medium (0.19 d^{-1}). Then, a short generation time was observed in the SWA11 medium (11.62 d).

It should be noted that the values of the growth rate and the generation time vary according to the microorganisms and the conditions of the culture medium (Branger, 2007).

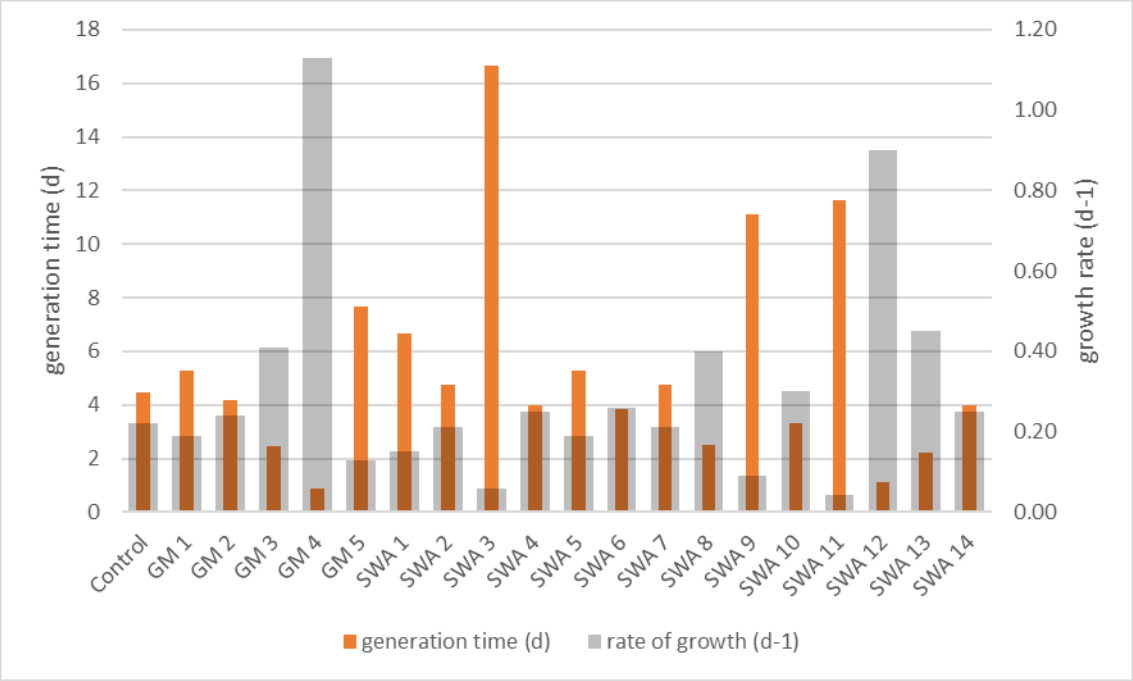

Fig. 4: Growth parameters of *Nannochloropsisgaditana* on the different media.

This can be confirmed by previous results (ref), where the presence of heavy metals in the composition of the environment influences the speed of algal growth.

It seems that whey is favourable to growth and can satisfy the nutritional requirements of *Nannochloropsis gaditana* in terms of organic elements (sugars) and mineral elements (phosphorus, calcium, copper, sodium, etc.).

After verification of normality by the Shapiro-Wilk test (p=1.239E-32) and equality of variance (p<0.00149). The Multi-Way Analysis of Variance (ANOVA) was applied to compare the number of cells in relation to two categorical explanatory variables, namely the medium and the incubation period.

Table 3: ANOVA results

	Sum Sq	Df	F value	Pr(>F)	Code
Incubation time	2893340	7	2944.68	2.20E-16	***
Environment	25506885	19	9564.01	1.87E-13	***
Incubation time: Environment	7973021	133	427.08	2.11E-15	***
Residuals	44917	320	-	-	-
Signif. codes	0 '***'	0.001 '**'	0.01 '*'	0.05 '	0.1 ' ' 1

The ANOVA reveals a highly significant difference between the number of cells in relation to the medium and the incubation period.

CONCLUSION

Cheese whey has been investigated as a potential nutrient for the green marine microalgae *Nannochloropsis gaditana* cultivation. Cheese-whey-based culture media significantly enhanced the in vitro culture biomass of *N. gaditana*. Supplementation of other nutrients and vitamins seems not necessary. Combined with heavy metals, this media was found to be the best composition for biomass growth and kinetic.

An in vitro culture duration of 11 days was found maximal for biomass

productivity, in media composed of deproteinised whey and heavy metals, in a manually agitated flask incubated at 23 ± 1°C under continuous illumination of 100 $\mu molm^{-1} s^{-1}$ intensity conditions.

Future work entails the detailed monitoring of nutrient utilisation and protein production further improving the cheese-whey-based culture medium formulation under an upscale production process.

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