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Bioutilization of Cheese Whey and Corn Steep Liqour by Heterotrophic Microalgae *Crypthecodinium cohnii* for Biomass and Lipid Production

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ABSTRACT

Many different wastewater and by-products derived from industrial activities potentially support microalgal growth by providing a cost-effective and sustainable solutions. In this present study, it was aimed to biologically utilize cheese whey (CW) and corn steep liquor (CSL) for microalgal biomass and lipid production by using these wastes in culture media for heterotrophic microalga *Crypthecodinium cohnii* cultivation. To determine nutrient requirements for *C. cohnii* growing in a medium prepared with CSL and in CW, statistical screening tools were used. CSL significantly enhanced microalgal growth and it could be an alternative to yeast extract as the primary nutrient source. As for CW, it served as a good culture medium for *C. cohnii* with the supplement of some of nutrients and eliminated the need for fresh water. Thus, a new culture medium was developed by combining undiluted CW and CSL and optimized for the growth of *C. cohnii*. Lastly, in a scale-up attempt by using this new medium, microalgal production was performed in a 3 L stirred tank bioreactor. *C. cohnii* yielded relatively high biomass productivity (2.28 g/L.d) and lipid content (28.7% dry weight) in the optimized medium. Although *C. cohnii* was known for its ability to accumulate high amounts of docosahexaenoic acid (DHA), it transformed its fatty acid composition to an increased proportion of saturated and monounsaturated fatty acids (C16:0-C18:1) that comprise ~70% of total fatty acids (TFA) when it was cultivated in CW mainly supplemented with CSL. Thus, *C. cohnii* seemed to be more feasible for biodiesel production than any other purposes when it was cultivated in this new medium.

Keywords: *Crypthecodinium cohnii*, Corn steep liqour, Cheese whey, Optimization, Lipid

Peyniraltı Suyu ve Mısır Islatma Şurubunun Heterotrofik Mikroalg *Crypthecodinium cohnii* ile Biyokütle ve Yağ Üretimi Amacıyla Biyolojik Olarak Değerlendirilmesi

ÖZ

Endüstriyel faaliyetlerden elde edilen birçok farklı atık su ve yan ürünler maliyet-etkin ve sürdürülebilir mikroalg kültüvasyonu için potansiyel kaynaklardır. Mevcut çalışmada, heterotrofik mikroalg *Crypthecodinium cohnii* kültüvasyonu için kültür besiyerinde peynir altı suyu (CW) ve mısır ıslatma şurubu (CSL) kullanılarak, bu atıkların mikroalgal biyokütle ve yağ üretimi amacıyla biyolojik olarak değerlendirilmesi hedeflenmiştir. Organizmanın, her iki ortamdaki (CW ve CSL) besin ihtiyaçlarını belirlemek amacıyla istatistiksel tarama metodları kullanılmıştır. CSL'nin *C. cohnii* biyokütle gelişimini olumlu yönde desteklediği ve organizmanın kültür ortamında kullanılan maya ekstraktının alternatifi olarak kullanılabileceği belirlenmiştir. Peyniraltı suyunun ise, organizma için gerekli mineraller açısından önemli bir kaynak olduğu ve CW'in doğrudan kullanımıyla kültür ortamındaki su ihtiyacını karşılama avantajına sahip olduğu sonucuna varılmıştır. Bu şekilde *C. cohnii* kültüvasyonunda kullanılacak yeni kültür ortamı seyreltilmemiş CW ve CSL'nin birlikte kullanımı ile geliştirilmiştir ve optimizasyonu yapılmıştır. Son olarak, yapılan ölçek büyütme çalışmalarında yeni kültür ortamı kullanılarak 3 L'lik karıştırmalı tank biyoreaktörde

üretimler gerçekleştirilmiştir. Optimizasyonu yapılan kültür ortamında kısmen yüksek biyokütle verimliliği (2.28 g/L.gün) ve yağ oranı (% 28.7 kuru ağırlık) sağlanmıştır. *C. cohnii* biyokütlede son derece yüksek oranlarda dokozahekzanoik asit (DHA) üreticisi bir tür olarak bilinmesine rağmen, CSL ile zenginleştirilmiş CW ortamında biyokütlesindeki yağ asidi kompozisyonunu değiştirerek, daha çok tekli doymuş ve tekli doymamış yağ asitlerince (C16:0-C18:1) zengin bir yağ profiline (toplam yağ asidi kompozisyonunun ~70%'i) sahip olduğu saptanmıştır. Buna göre, endüstriyel atıkların değerlendirildiği bu kültür ortamında yetiştirilen *C. cohnii*'nin biyodizel üretimi için uygun bir kaynak olabileceği sonucuna varılmıştır.

Anahtar Kelimeler: *Crypthecodinium cohnii*, Mısır ıslatma şurubu, Peynir altı suyu, Optimizasyon, Yağ

INTRODUCTION

The heterotrophic marine microalga *Crypthecodinium cohnii* is particularly known for its ability to accumulate lipid (20-50% of cell dry weight) with high amounts of docosahexaenoic acid (DHA, 22:6) which is very important for nutritional and pharmaceutical applications [1]. Researchers have been trying to produce sustainable and low-cost microalgal biomass and lipid production which have been currently used as an dietary supplement in food and beverages, health foods, animal feeds and maricultural products [1-2]. In addition to these application areas for microalgal lipid, extracellular polysaccharides (EPSs) that form the basis for a wide range of applications in food, pharmaceuticals, petroleum and other industries could be produced by *C. cohnii* and might attract a commercial interest in the future for microbial EPSs production by algae [3].

A wide variety of by-products and raw materials such as cheese whey, corn steep liquor, olive-mill waste water and carob pulp syrup from the food and/or agriculture industries have been employed for microbial cultivation due to their considerable availability and low cost [4-6]. Annual world cheese-whey (CW) production is continuously increasing and new bioproducts are being sought through biotechnology in order to get full use of the produced cheese whey [7, 8]. Corn steep liqour (CSL) has also been used as an inexpensive source of essential microbial nutrients for a variety of purposes [9, 10]. Even though there exist a number of technological developments and bioproducts in the transformation of CW or CSL to other useful products [7, 10], utilization of these by-products to cultivate heterotrophic microalgae offers a new application area.

The first step in the optimization of growth medium is screening of the important factors affecting the production [11]. Taguchi orthogonal array design and Placket-Burmann (PB) are widely used statistical techniques in industrial process design, principally in the developmental trials [12, 13]. The basic principle of these methods serve as screening filters which examine the effects of many process variables and identify factors which have major effects on process parameters with a few experiments [11]. Screening experiments are followed by response surface methodology to optimize the culture medium composition factors which have a major effect on biomass productivity [11, 14]. Response surface methodology is an approach that combines various statistical and mathematical techniques, and is useful for developing, improving and optimizing a process [11, 15]. There are many applications of response surface methodology related to optimization of the composition of the growth media for microalgal growth [15-17].

In the present study, it was aimed to biologically utilize cheese whey (CW) and corn steep liquor (CSL) for microalgal biomass and lipid production by using said wastes in culture medium for heterotrophic microalga *Crypthecodinium cohnii* cultivation. Firstly, CSL and CW were separately used as cultivation medium for growing *C. cohnii*. Statistical experimental tools were used to screen the important medium components which have a significant effect on the biomass productivity by *C. cohnii* in medium containing CW and CSL, separately. Once the significant factors were revealed in screening experiments, a response surface approach had been used for optimization of predetermined medium components in the alternative cultivation medium of *C. cohnii*. Total lipid content and DHA production by *C. cohnii* in the recently formulated medium were also compared with previous results.

MATERIAL and METHODS

Microalgae and Culture Conditions

Crypthecodinium cohnii CCMP 316 obtained from the Culture Collection of Marine Phytoplankton (CCMP, USA) culture was maintained in modified ATCC 30772 cultivation medium. The modified ATCC 30772 medium contained, per liter: 1 g of yeast extract (Merck), 25 g of sea salt, 0.01 g $FeCl_3.6H_2O$, 0.15 g Na_2 β-Glycerophosphate, 0.05 g $(NH_4)_2SO_4$, 1.5 g Tris (Tris(hydroxymethyl)amino methane) buffer (Sigma), 0.01 g K_2HPO_4, 30 mg EDTA, 1.5 mg $FeCl_3.6H_2O$, 30 mg H_3BO_3, 4.5 mg $MnCl_2.4H_2O$, 0.3 mg $ZnCl_2$, 0.15 mg $CoCl_2.6H_2O$, 0.003 mg biotin, 1 mg thiamine and 9 g glucose as a carbon source. Glucose was separately sterilized in a different bottle in an autoclave at 121 ºC for 20 min and aseptically added (9 g/L) into the medium. The inoculum was prepared by growing the microalga in a 250 mL Erlenmeyer flask containing 75 mL of the modified ATCC 30772 medium incubated at 24°C for 72 h with orbital shaking at 130 rpm. The inoculum size was 10% of the total liquid volume in flasks. The inoculated flasks were then incubated at 24ºC in a rotary shaker and agitated at 130 rpm in the dark condition. During the experiments, the initial pH of the growth medium was adjusted to 6.5±0.1.

Characterization of Cheese Whey and Corn Steep Liquor (CSL)

Fresh raw cheese whey was obtained from Pınar Inc., a large dairy factory in Izmir, Turkey. It had a pH of 4.7, chemical oxygen demand (COD) of 86.3 g/L, total sugars (as lactose) of 42.6 g/L, suspended solids of 6.9 g/L, and a total nitrogen of 0.2 g/L [7]. Corn steep liqour was obtained from Cargill (Bursa, Turkey) and its pH was 4.5. CSL generally contains water (~50%), nitrogen (3-4.5%), reducing sugars (~10%), minerals and vitamins [9]. Cheese whey and CSL were both kept at -20 ºC until used.

Lipid Analysis, Methyl Ester Preparation and Analysis

The culture samples taken from the shake flasks were harvested by centrifugation at 1500 *g* for 5 min and washed at least twice with demineralised water. They were stored at –20°C until lyophilization. Lyophilization was performed in a freeze dryer (Christ, Alpha 1-2 LD plus, Germany) in accordance with the previously disclosed procedure [16]. The dried biomass obtained after freeze-drying was stored in air-tight containers at –20°C.

Oil was extracted from lyophilized algal biomass by a modified method of Bligh and Dyer [18]. Freeze-dried cells (100 mg or more) were weighed accurately into a 15 mL centrifuge tube. For extraction, 3 mL chloroform, methanol (2:1) containing 1.0 mg/mL nonadecanoic acid (19:0) and 0.5 mg/mL butylated hydroxytoluene (BHT) were used and the tube was shaken gently overnight at room temperature. After centrifugation at 1500 *g* for 5 min, the supernatant containing the extracted oil was stored at 4°C until being analysed. The extract was evaporated in a water bath (30°C) using a rotary evaporator (Stuart, RE300, UK) to remove solvents. The final lipid amount was determined gravimetrically [1].

Fatty acids were analyzed by gas chromatography (GC) after direct transmethylation with hydrochloric acid in methanol by small modifications by Christie [19]. The fatty acid methyl esters (FAMEs) were extracted with hexane and analyzed by Agilent 7890 gas chromatography equipped with a flame-ionization detector and a Supelco sp-2380 A capillary column (60m x 250µm x 0,2µm) with helium as a carrier gas according to a previously described procedure [16].

Experimental Design and Data Analysis

Fractional factorial designs like Taguchi and PB design are widely used statistical techniques for screening and optimization of medium components at shake-flask level [17, 20]. Since the present study was mainly aimed at screening of the important medium components with respect to their main effects, not the interaction effects between various medium constituents, PB and Taguchi designs were selected [12, 14]. Taguchi's method was applied to determine the medium components which significantly influence the biomass productivity of *C. cohnii* growing in medium containing CSL, while PB design was used to determine influence of medium components in CW medium. According to Taguchi's orthogonal array seven medium components in eight experiments were used to evaluate their influence on biomass productivity by *C. cohnii*. Experiments were performed according to an experimental plan given in Table 1 for medium containing CSL. A 12 run PB design was used to identify medium components having significant effects on biomass productivity in CW medium (Table 2). The low level (-1) and high level (+1) of each factor are listed in Table 2.

The data apart from optimization experiments were analyzed for one-way analysis of variance (ANOVA) and then Tukey's post hoc test was used for multiple comparisons with SPSS statistical software (SPSS for Windows ver.18.0). All statistics were based on a confidence level of 95%, so $p \leq 0.05$ was considered to denote a statistically significant difference, and $p \leq 0.01$ was also used to show the power of the significance.

Table 1. Taguchi design of variables (in actual levels) with the biomass productivity as a response in medium made with corn steep liqour (CSL, 10 g/100mL).

	Variables*							Response
Run	A (g/L)	B (g/L)	C (g/L)	D (mL/L)	E (mL/L)	F (g/L)	G (g/L)	Biomass Productivity (g/L.d)
1	0.01	1	0.01	0.1	3	0.05	0.15	0.86
2	0.001	1	0.15	0.1	0.3	0.05	1.5	0.79
3	0.001	1	0.15	1	3	0.005	0.15	1.06
4	0.01	0.1	0.15	1	0.3	0.05	0.15	0.94
5	0.001	0.1	0.01	0.1	0.3	0.005	0.15	1.25
6	0.01	1	0.01	1	0.3	0.005	1.5	0.82
7	0.01	0.1	0.15	0.1	3	0.005	1.5	0.94
8	0.001	0.1	0.01	1	3	0.05	1.5	0.80

*Seven variables to be screened in Taguchi design (A-G)-i.e., dipotassium hydrogenphosphate, glutamic acid, sodium glycerophosphate, vitamin solution, metal solution, ammonium sulphate, tris buffer.

Table 2. Plackett-Burman design of variables (in actual levels) with the biomass productivity of *C. cohnii* as a response growing in cheese whey (CW).

	Variables*											Response
Run	A	B	C	D	E	F	G	H	I	J	K	Biomass Productivity (g/L.d)
1	0.01	1	1.5	0.1	0.3	0.005	1.5	0.001	0.15	28.0	0.00	0.95
2	0.001	1	1.5	0.1	3	0.05	1.5	0.001	0.00	0.00	10.0	1.28
3	0.001	1	1.5	1	0.3	0.005	0.15	0.01	0.00	28.0	10.0	1.33
4	0.01	1	0.1	0.1	0.3	0.05	0.15	0.01	0.15	0.00	10.0	0.06
5	0.01	0.1	0.1	0.1	3	0.005	1.5	0.01	0.00	28.0	10.0	1.08
6	0.001	0.1	0.1	0.1	0.3	0.005	0.15	0.001	0.00	0.00	0.00	0.25
7	0.01	0.1	1.5	1	0.3	0.05	1.5	0.01	0.00	0.00	0.00	0.87
8	0.001	1	0.1	1	3	0.005	1.5	0.01	0.15	0.00	0.00	0.77
9	0.01	0.1	1.5	1	3	0.005	0.15	0.001	0.15	0.00	10.0	0.12
10	0.001	0.1	1.5	0.1	3	0.05	0.15	0.01	0.15	28.0	0.00	0.62
11	0.01	1	0.1	1	3	0.05	0.15	0.001	0.00	28.0	0.00	0.39
12	0.001	0.1	0.1	1	0.3	0.05	1.5	0.001	0.15	28.0	10.0	1.04

*Eleven variables to be screened in Plackett-Burman design (A-K)-i.e., dipotassium hydrogenphosphate, glutamic acid, yeast extract, vitamin solution, metal solution, ammonium sulphate, tris buffer, Iron(III) chloride hexahydride, sodium(II) β-glycerophosphate, sea salt, glucose.

Table 3. Experimental range and levels of the independent variables (sea salt, tris buffer, corn steep liquor (CSL)) and central composite design plan in actual value and observed response (biomass productivity, g/L.d).

		Actual Levels of Coded Factors				
Variables	Symbol Coded	-1.682	-1	0	+1	+1.682
Sea salt (g/L)	X1	2.52	9.0	18.50	28.0	34.48
Tris buffer (g/L)	X2	0.22	0.8	1.65	2.5	3.08
CSL (g /100 mL)	X3	0.1	3.0	7.25	11.50	14.40

Runs	X1	X2	X3	Biomass Productivity (g/L.d)
1	0	0	-1.682	1.29
2	0	0	0	1.39
3	0	0	0	1.46
4	-1	1	-1	0.75
5	0	0	1.682	1.69
6	1	-1	1	1.51
7	-1	-1	-1	0.68
8	1.682	0	0	1.45
9	0	0	0	1.39
10	1	1	1	1.97
11	0	0	0	1.59
12	0	0	0	1.54
13	0	0	0	1.41
14	0	-1.682	0	1.25
15	1	1	-1	1.77
16	-1.682	0	0	1.01
17	-1	1	1	1.86
18	1	-1	-1	1.55
19	0	1.682	0	1.50
20	-1	-1	1	1.56

RESULTS and DISCUSSION

Screening of Important Nutrient Components on Biomass Productivity of *C. cohnii* in the Medium Containing CSL

According to the preliminary tests, the concentration of 10 g CSL in 100 mL medium was chosen as the optimum CSL concentration in *C. cohnii* medium for the screening experiments with CSL. The importance of the seven components, namely, dipotassium hydrogenphoshate (K_2HPO_4), glutamic acid, sodium glycerophosphate, vitamin and metal solution, ammonium sulphate ($(NH_4)_2SO_4$) and tris buffer in a medium supplemented with sea salt (25 g/L) and glucose (9 g/L) was investigated by Taguchi design (Table 1). The biomass productivity by *C. cohnii* in the medium containing CSL (Table 1) were higher at the end of the screening experiments for all runs when compared to results obtained by using only CSL in sea water as a growth medium (data not shown). Based on the statistical analysis, dipotassium hydrogenphoshate

(K_2HPO_4), glutamic acid, vitamin and metal solution, ammonium sulphate ($(NH_4)_2SO_4$) and tris buffer were identified as the factors having significant effect on the biomass productivity by *C. cohnii* in the medium containing CSL (Table 4). All these factors caused a negative effect on biomass productivity. Thus, their lower concentrations could be used for further optimization process. Corn steep liquor was considered as a very suitable nutritious substrate in industrial fermentations since it typically contains many nitrogen compounds, vitamins, amino acids and mineral matters which are easily assimilated into normal cell metabolism [9]. In this study, the incorporation of CSL at a concentration of 10 g/100mL to the fermentation medium resulted in elimination of sodium glycerophosphate from the medium and also reduced the levels of other components (K_2HPO_4, vitamin solution, metal solution, glutamic acid, $(NH_4)_2SO_4$ and tris buffer) for biomass productivity of *C. cohnii*. Our results clearly showed that CSL could be beneficial for the cultivation of *C. cohnii*.

Table 4. Statistical analysis of medium components on the biomass productivity of *C. cohnii* growing: (A) in medium made with corn steep liqour (CSL, 10 g/100 mL) from the results of Taguchi design (B) in cheese whey (CW) from the results of Plackett–Burman design*

	Biomass Productivity (g/L.d)			
Source	MS	*F*-value		*P*>F
Model	0,029	9370.56		0.0276
K_2HPO_4	0.014	4643.45		0.0093
Glutamic acid	0.019	6222.43		0.0081
Metal solution	2.358E-003	767.15		0.0230
Vitamin solution	6.694E-003	2178.30		0.0136
$(NH_4)_2SO_4$	0.057	18550.90		0.0047
Tris buffer	0.073	23861.16		0.0041
	SS	DF		
Residual	3.073E-006	1	R^2	1.0000
Cor. Total	0.17	7	Adj. R^2	0.9999

A

	Biomass Productivity (g/L.d)			
Source	MS	*F*-value		*P*>F
Model	2.13	49.93		0.0042
K_2HPO_4	0.28	51.75		0.0055
Glutamic acid	0.057	10.62		0.0472
Yeast extract	0.21	38.91		0.0083
Tris buffer	0.88	164.09		0.0010
Na(II) B-glycerophosphate	0.23	42.25		0.0074
Sea salt	0.36	66.5		0.0039
Glucose	0.094	17.63		0.0247
	SS	DF		
Residual	0.016	3	R^2	0.9925
Cor. Total	2.15	11	Adj. R^2	0.9727

B

SS, sum of squares; DF, degrees of freedom; MS, mean square

Screening of Important Nutrient Components on Biomass Productivity by *C. cohnii* in CW Medium

According to the preliminary tests, higher biomass productivity and total lipid content by *C. cohnii* were obtained by using CW without any dilution. Plackett–Burman design was preferred as a screening experiment for growth of *C. cohnii* in CW medium. Table 4 represents the PB experimental design for 12 trials with two levels of concentrations for each components and corresponds to the biomass productivity of *C. cohnii*. Based on the statistical analysis, there were no need for using vitamin and metal solution, $(NH_4)_2SO_4$ and Iron(III)chloride hexahydride ($FeCl_3.6H_2O$) in terms of biomass productivity by *C. cohnii* in CW medium ($p>0.05$). Factors having the significant effect on the biomass productivity by *C. cohnii* growing in CW medium were identified as K_2HPO_4, glutamic acid, yeast extract, tris buffer, sodium glycerophosphate, sea salt and glucose (Table 4). Dipotassium hydrogenphosphate and sodium glycerophosphate had a negative effect on biomass productivity. On the other hand, tris buffer, sea salt, glucose, yeast extract, glutamic acid had a positive effect on biomass productivity. The results indicated that CW serves as a better culture medium with supplementation of some of ingredients found in defined medium. In addition to that, there was no need to freshwater for culturing *C. cohnii* in CW medium.

Optimization of Selected Medium Components Using Response Surface Methodology

It has been reported that *C. cohnii* requires complex nutrients for cell growth [21]. In this current study, these requirements were identified for both CW and CSL containing medium through the screening experiments (Table 4). In CSL containing medium, K_2HPO_4, glutamic acid, vitamin and metal solution, $(NH_4)_2SO_4$ and tris buffer had negative effect on the growth of *C. cohnii*. In CW medium, tris buffer, sea salt, glucose, yeast extract, glutamic acid had positive effects for the growth of *C. cohnii* (Table 4). The supplementation of nitrogen source was necessary for *C. cohnii* in CW medium. So, effects of both the yeast extract and the glutamic acid were positive for the cell growth. Glucose was used as the main carbon source by *C. cohnii* for both media. The concentration of non-significant and medium components that had negative effect for both CW and CSL containing medium were set to their lowest levels for the new medium formulation. It was concluded that, nitrogen requirement of cells could be met by using CSL in CW medium. Therefore, CSL, sea salt and tris buffer were taken as variables through the central composite design (CCD) for the investigation of their optimum levels on growth of *C. cohnii* in CW medium. In the CCD experimental model, sea salt (9-28 g/L), tris buffer (0.80-2.5 g/L) and CSL (3.0-11.5 g/100 mL) were taken as input variables. According to the implemented design, twenty combinations were performed (Table 3). The values of biomass productivity (g/L.d) which is the

response of the system obtained in different experimental conditions are also given in Table 3. The first order polynomial equation (expressed in terms of coded values) fitted to the experimental CCD data for predicting the biomass productivity was given in the following equation:

$$Y = 1.43 + 0.20X_1 + 0.11X_2 + 0.21X_3 + 0.038\ X_1X_2 - 0.23X_1X_3 + 0{,}058\ X_2X_3 \quad \text{(Eq. 3)}$$

where Y was the predicted response, i.e. the biomass productivity (g/L.d), and X_1, X_2, and X_3 were the coded values of the test variables, sea salt (g/L), tris buffer (g/L) and CSL concentrations (g/100 mL), respectively. As shown in Table 3, the biomass productivity was significantly improved with the optimized medium in comparison to that obtained with the medium containing CSL (Table 1) and CW medium (Table 2). The statistical analysis of the model was accomplished in the form of variance analysis (ANOVA) (Table 5). The model *F*-value of 13.96 indicated that the model was significant ($p<0.01$). The statistical analysis indicated that sea salt and CSL had a mutual effect on biomass productivity while tris buffer was fixed at its intermediate level ($p<0.01$). The 3-dimensional contour plot was given to illustrate the relationship between the response (biomass productivity) and the variables (sea salt and CSL) (Fig. 1). As shown in Fig. 1, there were two optimum regions for the biomass productivity by *C. cohnii* in CW medium containing CSL as the main nitrogen source. One of these optimum areas occurred in the region where the sea salt concentration was high and the concentration of CSL was low. The other one occurred in the region where the concentration of CSL was high and the sea salt concentration was low. Marine unicellular algae are generally considered to be tolerant and adaptable to a wide salinity range and several studies have been reported about the effect of salinity on cell growth of the marine dinoflagellate *C. cohnii* [21, 22]. In this study, sea salt concentrations were investigated in the range of 9-28 g/L. The biomass productivity increased with increasing salinity which had been reported before [22]. The results obtained in the current study revealed that the sea salt concentration of 9 g/L was also sufficient for *C. cohnii* CCMP316 to achieve the similar biomass productivity with the sea salt concentration of 28 g/L (Fig. 1). The sea salt concentration of 9 g/L was lower than the optimum value reported before for *C. cohnii* CCMP 316 [6]. For large scale cultivations, the sea salt concentration should preferably be as low as possible in order to prevent corrosion problems during the sterilization [23]. A decrease of the biomass productivity at extremely low concentrations of sea salt (comparing run 16 and runs at center points) could also be seen from the Table 3. The stimulation of growth with an increasing salinity to a certain degree was in agreement with the previous data [22]. In most of the cultivation media for *C. cohnii*, yeast extract is the generally used nitrogen source [6, 21, 22]. In a study where diluted carob pulp syrup was used as an alternative low-cost carbon source together with 5 g/L of yeast extract as a nitrogen source yielded lower biomass productivity values (0.096 g/L.d) when compared to the current study [6]. In this study, it has been demonstrated that CSL significantly enhances the growth of *C. cohnii* and it may be an alternative to yeast extract as the primary medium component.

It has been reported that the optimum pH for growth of *C. cohnii* is 6.5-6.6 [6,21-24]. After pH 7.0, cell growth starts to slow down [24]. So, the tris was used for buffering the pH. The statistical analysis indicated that tris buffer had a positive effect on the biomass productivity of *C. cohnii* (Table 5). Such a positive effect of buffer on cell growth could be due to the its effect by keeping cells in logarithmic phase of growth to a higher cell density [21-23].

Total lipid content of *C. cohnii* cultivated in different conditions varied between 20-33% (w/w) of cell dry weight. The lipid production of *C. cohnii* CCMP 316 in optimized CW medium containing CSL was obviously superior to that achieved with the other *C. cohnii* strains using medium containing glucose (9 g/L) and yeast extract (0-10 g/L) as principal carbon and nitrogen sources [22]. In addition to that, the lipid production by *C. cohnii* CCMP316 in the alternative medium was superior than the 4 strains of *C. cohnii* tested in pH-auxostat cultures [25]. The influence of sea salt and yeast extract concentrations on lipid accumulation by *C. cohnii* ATCC 30772 had been studied in shake-flask culture [22, 23]. In this study, it was determined that the center point of the experimental design (Table 3) in which the concentration of CSL was 7.25 g/100mL, the concentration of tris buffer was 1.65 g/L and the concentration of sea salt was 18.5 g/L was good for lipid production. The seawater salinity was slightly above the average seawater salinity (15 g/L) and it was sufficient to get an appropriate lipid content (average 27% of cell dry weight). This seawater salinity requirement for the lipid production by *C. cohnii* was in agreement with the previous data [22].

The major fatty acids found in *C. cohnii* were C12:0; C14:0, C16:0, C18:0, C18:1 and C22:6 [6, 21, 24]. In this study, the percentage of C12:0, C14:0, C16:0, C18:0 and C18:1 were within the ranges of 2.0-3.4%, 12.0-16.8%, 24-38.7%, 11.2-14.4% and 20-26% respectively. C22:6 (DHA) percentage of all 20 runs ranged from 8.5 to 17% of the total fatty acids (TFA). Regarding to fatty acid composition of *C. cohnii*, the extraordinary amount of DHA (approx. 50% of fatty acids in a vigorously-aerated culture) has been the characteristic for that microalgae [22-24]. In this work, DHA percentage of TFA was lower than the above mentioned studies. Saturated fatty acids C18:0 (11.2-14.4%), C16:0 (24-38.7%), C14:0 (12.0-16.8%) and the monounsaturated fatty acid C18:1 (20-26%) were abundant in *C. cohnii* grown in CW medium containing CSL. An increased synthesis of saturated fatty acids such as C14:0 (29.98%) and C16:0 (32.05%) and a decreased DHA proportion (16,79%) were observed previously when the cultivation temperature was higher [26]. Fatty acid composition of algal cells may also be influenced by the medium composition as the organism usually modifies its biochemical composition in response

to the compositional change [6, 25]. It has been stated that the fatty acid production by *C. cohnii* was regulated by three seperate systems, namely by, (1) the biosynthesis of saturated fatty acids, (2) the conversion of saturated fatty acids to monounsaturated fatty acids and (3) the de novo synthesis of DHA with desaturases [24]. According to the fatty acid biosynthesis pathways, the introduction of double bond is an oxidative process requiring molecular oxygen [24]. It is known that oxygen solubility is decreased by the ions and sugars normally added to the fermentation media [27]. The effect on oxygen solubility of ionic and non-ionic solutes such as molasses, corn steep liqour and protein agents has been reported in several publications [24, 27]. Also, in fermentation processes with non-filamentous microorganisms, the rises in the viscosity of the growth medium are due to the production of extracellular polysaccharides [3]. Thus, a smaller amount of intracellular molecular oxygen was available at high ionic and non-ionic solutes present in CW medium containing CSL. Therefore, it couldn't allow the oxygen-dependent enzymes to catalyze the fatty acid desaturation and made such changes in fatty acid composition.

Table 5. ANOVA for the response surface optimization of medium composition for the biomass productivity (g/L.d) of *C. cohnii* growing in cheese whey (CW) mainly supplemented with corn step liqour (CSL).

Source	SS	DF	MS	F-value	*P*>F
Model	1.74	6	0.29	13.96	<0.0001
Sea salt	0.53	1	0.53	25.66	0.0002
Tris buffer	0.16	1	0.16	7.69	0.0158
CSL	0.59	1	0.59	28.25	0.0001
Sea salt*tris buffer	0.012	1	0.012	0.56	0.4672
Sea salt*CSL	0.42	1	0.42	20.31	0.0006
Tris buffer*CSL	0.027	1	0.027	1.30	0.2741
Residual	0.27	13	0.021		
Lack of fit	0.23	8	0.029	4.11	0.0680
Pure error	0.036	5	7.107E-003		
Cor Total	2.01	19			
Std. Dev.	0.14		R-Squared	0.8657	
Mean	1.43		Adj R-Squared	0.8037	
C.V.%	10.06		Pred R-Squared	0,5938	
Press	0.81		Adeq Precision	12.033	

SS, Sum of squares; DF, degrees of freedom; MS, mean square.

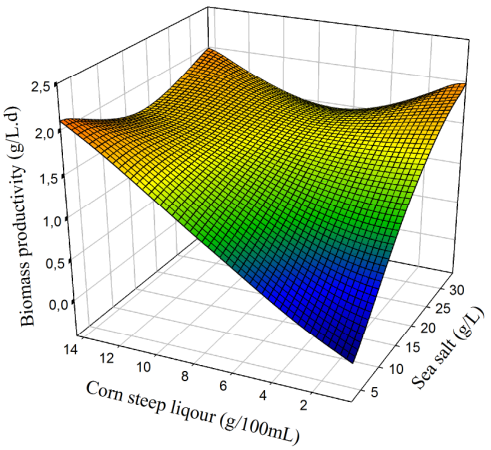

Figure 1. Contour plot of the influence of corn steep liqour (CSL) and sea salt concentrations on the biomass productivity (g/L.d) by *C. cohnii* in cheese whey (CW) medium containing CSL.

Validation of the Optimized Condition

On the basis of medium optimization, the model predicted that the maximum biomass productivity by *C. cohnii* was 1.80-1.81 g/L.d, when the CSL concentration was 11.5 g/L and that of tris buffer was 2.5 g/L when the sea salt concentration was minimized (9 g/L) and maximized (28 g/L). To verify the predicted results, validation experiments was performed in triplicate tests. Under both optimized conditions, the observed experimental biomass productivity was 1.86±0.06, suggesting that experimental and predicted values of

biomass prodcutivity by *C. cohnii* were in good agreement.

CONCLUSION

The marine heterotrophic microalga *C. cohnii* was able to grow in cheese whey medium supplemented with corn steep liqour and yielded high biomass productivity and lipid content. In order to develop algal oil production in a cost effective way, researchers should focus on the volumetric productivity. The productivity can be increased by increasing the final algal biomass concentration and the lipid content of the biomass. In the case of *C. cohnii*, heterotrophic growth on the alternative medium revealed that these two improvements were possible in CW medium containing CSL. Firstly, it was concluded that *C. cohnii* cells had high biomass productivity values (up to 1.97 g/L.d) from shake flask cultivations. Secondly, *C. cohnii* cells could contain a lipid content of at least 20% of cell dry weight. Although, it accumulated low percentages of DHA in total fatty acids composition, it still had a higher DHA percentage when compared to other ω-3 fatty acid producer microalgal strains [28, 29]. EPSs are by-products of DHA production process with *C. cohnii* which was reported by de Swaaf et al [3]. Therefore, the growing *C. cohnii* in that alternative medium might attract a commercial interest with regarding EPSs production. Also, the use of CW in the culture medium of *C. cohnii* eliminated the need for using fresh water. It could be the main motivation for production of *C. cohnii* by using CW. Moreover, the use of CW and CSL as feedstock of high value fermentation products may be a useful way of disposing of these by products, thus reducing the industrial residues and contributing to the environmental protection. To the best of our knowledge, this is the first report describing utilization of these by-products by *C. cohnii*.

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