

Screening for the Heterotrophic Cultivation of *Chlorella sorokiniana* Using an Indirect Impedance Microbiological Technique

Balázs József Nagy¹, Réka Czinkóczy¹, Áron Németh^{1*}

¹ Department of Applied Biotechnology and Food Science, Faculty of Chemical Technology and Biotechnology, Budapest University of Technology and Economics, H-1111 Budapest, Budafoki út 6-8, Hungary

* Corresponding author, e-mail: naron@f-labor.mkt.bme.hu

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Abstract

Heterotrophic culturing of microalgae is of great interest as it has the potential to produce feedstock for added-value bioproducts. The general expectation is to develop a method that can help screen for different media compositions and batch fermentation settings more easily and faster. In the current study, an indirect impedance microbiological technique was used to test the effects of various carbon and nitrogen sources on the growth of *Chlorella sorokiniana*. It was found that this technique has great potential to screen for heterotrophic cultivation conditions of microalgae as the metabolic rate of microorganisms can be determined by measuring the amount of CO₂ produced. The BacTrac 4100® was proven to be a suitable instrument to compare several different small-scale culturing settings. Considering the relative changes in impedance observed, the initial cell number exhibits an inverse linear correlation with the detection time. *Chlorella sorokiniana* exhibited an enhanced degree of growth on yeast extract and tryptone, and preferred glucose over acetate or glycerol. An optimum rate of growth at a glucose concentration of 20 gL⁻¹ was also determined. Our novel approach in the field of heterotrophic cultivation of microalgae envisages great prospects for the method in terms of the design of experiments in the field of media optimization.

Keywords

indirect impedance microbiology, high-throughput, heterotrophic, *Chlorella sorokiniana*, media optimization

1 Introduction

Chlorella species are the most studied strains of microalgae due to their high rates of growth and lipid productivity. Many studies have reported that the biomass composition of *Chlorella* strains is feasible as a resource for biorefining [1], in addition to the synthesis of biofuels [2], biopolymers [3], nutraceuticals [4] and forage [5]. Microalgae technology has also been extensively tested at wastewater treatment facilities [6, 7].

In this respect, the thermotolerance of *Chlorella sorokiniana* also attracts special attention with regard to the development of culturing technology, especially in terms of lipid production and biomass productivity. Generally, the stress factors, such as salinity [8], nutrient depletion [9] as well as osmolarity [10], affect the lipid content of *Chlorella*, however, the biomass productivity depends mainly on the culturing technique. *Chlorella sorokiniana* grows well under autotrophic conditions, but higher concentrations of biomass have been reached

under mixotrophic and heterotrophic conditions using glucose or acetate as a carbon source [11, 12]. Moreover, mixotrophic culturing can be 2.4-fold and 5.4-fold more effective in terms of biomass production than heterotrophic culturing and photoautotrophic culturing, respectively [13].

Cultivating *Chlorella* using heterotrophic culturing techniques has gained more attention in the field of algal research. Over the past five years, 148 new publication records have been listed in the Thomson Reuters' Web of Science Core Collection portal according to a search for the keywords "*Chlorella*" and "heterotrophic".

The growing interest in heterotrophic culturing of microalgae raises the need for a small-scale, high-throughput, online method suitable for selecting the right components of media and circumstances for a specific strain of microalgae. Impedance microbiological techniques may provide a solution to this general fermentation task.

Impedance microbiology is mostly implemented in the food industry to analyze food standards from the perspective of specific pathogens. Furthermore, it is used to determine the lethal dose of antibiotics on specific microorganisms, although there are new experiments where biological impedance fingerprints are used to distinguish between microorganisms based on their specific metabolic capabilities, or to measure cell lysis generated by bacteriophages [14–17].

Several instruments that measure impedance are available, one of them is the BacTrac 4100® by SY-LAB. It is designed to detect the decrease in the impedance of a medium in real time which indirectly refers to the increase in conductivity caused by charged compounds formed during the metabolism of the microorganisms. The appearance of the measurement signal results in a positive curve, even though the decrease in conductivity is indicative of a relative change in impedance when compared to the initial value. Two measuring techniques exist. In the case of the direct measurement method, electrodes are immersed directly in the media, providing the possibility to measure changes in impedance in the media as well as on the surface of the electrode. It is advised that the latter should be used for media of high salinity (i.e. conductivity), in which measurements are difficult. In the case of indirect measurements, outer vials and electrodes are immersed in 0.2% KOH to track the increase in impedance as a result of carbonate ions formed from the production of CO₂ by the culture in the inner vial. For quantitative measurements a threshold was defined, e.g. 10%, and a detection time determined until the relative change in impedance had reached the threshold. Over the past three decades both techniques have been comprehensively studied [16, 18].

The indirect method has been tested on the microalgae *Haematococcus pluvialis* to detect heterotrophic growth on different carbon and nitrogen sources. Two culturing media were tested thoroughly, the Whitley Impedance Broth (WIB) and the HKU algal selective media (Don Whitley Scientific, Shipley, England). Gong et al. observed significant differences when the experimental conditions were changed by analyzing the profile of the negative conductivity gradient. They concluded that *Haematococcus pluvialis* prefers acetate as a carbon source rather than glucose at a concentration of 1 g/L. It has also been determined that any culture media is suitable for the indirect method which facilitates the heterotrophic growth of the specific microalgae [19].

All these findings prompted us to presume that the impedance microbiological technique can provide a fast, small-scale, online method for media optimization of heterotrophic microalgae cultivation using batch fermentation. Based on these findings, the same settings, with some adjustments, were used. In this research, the indirect method of impedance microbiology was used to identify preferred carbon and nitrogen substrates for *C. sorokiniana*.

2 Materials and Methods

2.1 Microalgae cultivation

The microalgae strain *Chlorella sorokiniana* (SAG 211-32) was obtained from the Experimental Phycology and Culture Collection of Algae (EPSAG) at Goettingen University in Germany. It was maintained at 4 °C on a complex agar consisting of 5 g/L yeast extract, 10 g/L tryptone, 5 g/L peptone, 20 g/L malt extract, 5 g/L glucose, 5 g/L sodium chloride and 20 g/L bacteriological agar. The compounds were obtained from Sigma-Aldrich. The precultures were prepared heterotrophically in 250 ml Erlenmeyer flasks containing 100 ml of Whitley Impedance Broth (11.5 g/L tryptone, 10 g/L lactalbumin hydrolysate, 5 g/L meat peptone, 3 g/L yeast extract, 0.5 g/L MgSO₄·7H₂O and 0.1 g/L CaCl₂) in the absence of lactalbumin hydrolysate and supplemented with 10 g/L glucose at 25°C whilst being stirred at 250 rpm for 3 days. Each experiment was inoculated with a fresh culture.

2.2 Impedance measurements

A BacTrac 4100® instrument developed by SY-LAB (Neupurkersdorf, Austria) was used. The system is equipped with two software programs. BacMonitor® allows the parameters of the experiment to be set and the measuring points to be registered. As measurements are very sensitive, several noise factors may occur, especially as a result of changes to the ambient temperature. BacEval® provides the possibility to smooth out (by using the DropStop function) and evaluate the curves. BacEval® can export raw measured curves in the form of PDFs, thus, for further evaluation and calculations data points were readout and transferred to an Excel spreadsheet by Digitizeit (Bormissoft, Germany).

Impedance measurements were performed at 28 °C and the arrangements of the indirect measuring cells were almost the same as implemented by Gong and Chen [19] with the difference that the SY-LAB accessories were used. In our case the outer tubes were filled with 1 ml of 0.09M KOH solution supplemented with 1% agar. The inner tubes

contained 5 ml of inoculated culture media. The stock solutions for the experimental setups were prepared in disposable flasks beforehand, inoculated with 20 μl (0.1% volume ratio) of concentrated (4000 g x 5 min.) microalgae suspension, then divided into three BacTrac 4100[®] inner tubes as non-independent replicates. The initial cell count was 10^5 cells ml^{-1} in all cases. A negative, non-inoculated sample was also incubated for each experiment. All stock solutions were set to pH 6-7 and sterilized at 121 °C for 30 minutes.

The changes in impedance were recorded every 10 minutes for 60-120 hours by the BacTrac 4100[®] system. The threshold was set at -50% in M value (i.e. impedance measurement of the media in KOH solution). The comparison of the resultant curves (M% vs. time) was illustrated by SigmaPlot (v12.0). The averaged curves of the three non-independent replicates together with a fitted regression line of 5th order to all data points per experimental setting are presented in all the figures. Indirect measurements resulted in negative slopes of sigmoid growth curves, but for the sake of easier evaluation the absolute values were taken into consideration.

Except for the lag time, the same parameters were analyzed according to the BacTrac 4100[®] curves as implemented by Bancalari et al. [15], but for the determination of maximum specific growth rates a more typical approach (Eq. (1)) was used to determine this parameter [13, 20].

2.3 Experimental settings

The BacTrac 4100[®] batch cultivations were tested using 5 independent experimental settings. Different nitrogen sources were compared in media manufactured by HKU by replacing the potassium nitrate with yeast extract, urea or tryptone and applying them in a nitrogen-equivalent content of 4.4 g nitrogen per liter supplemented with 25 g/L glucose.

The carbon sources were screened in HKU and WIB media supplemented with sodium acetate at a carbon concentration of 1 g C/L, 1 g C/L glycerol and 1-2 g C/L glucose. The HKU media consisted of 0.5 g/L KNO_3 , 0.195 g/L K_2HPO_4 , 0.062 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.184 g/L $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and 1 ml/L of a trace-metal solution which consisted of 2.7 mg/L vitamin B12, 18 mg/L EDTA, 24.4 mg/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 72 mg/L ZnSO_4 , 12 mg/L H_3BO_3 , 4 mg/L $\text{CoCl}_3 \cdot 6\text{H}_2\text{O}$, 62 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 84 mg/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and 38 mg/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$.

In order to determine the glucose optimum in the case of biomass production, the changes in impedance were investigated at 8, 16, 24, 32 and 40 g C/L glucose concentrations in the WIB media at 28 °C.

2.4 Cell number determination and microscopy

At the end of the impedance measurement, the optical density of the samples was measured by a spectrophotometer (Pharmacia LKB Ultrospec) at a wavelength of 750 nm, so by estimating the final biomass concentration, each experimental setup was comparable. The possibility of the presence of infectious agents was checked by investigating the phase contrast using a Jenaval microscope. The cell numbers of some samples were also determined using a Bürker chamber under the microscope, then the final cell number was calculated using a calibration curve that plotted optical density against cell number:

$$\text{cell number (db ml}^{-1}\text{)} = 2E + 07 * OD_{750} - 7E + 06$$

$$R^2 = 0.98.$$

2.5 Calculation of the maximum specific growth rate

(μ_{max})

The registered curves of change in relative impedance were used to evaluate the growth kinetics of *Chlorella sorokiniana*. The maximum specific growth rate was specified during the exponential phase and calculated according to Eq. (1):

$$\mu_{\text{max}} = \frac{\ln N1 - \ln N0}{t1 - t0} \quad (1)$$

2.6 Statistical analysis

Statistical analyses were performed using the one-way analysis of variance (one-way ANOVA) and Tukey's multiple comparison test, a p value of less than 0.05 was considered significant. The measurements were conducted in triplicates, the mean and standard deviation (SD) were calculated using GraphPad Prism 7.

3 Results and Discussion

The metabolism of microalgae is really complex since they can grow via 3 different ways: autotrophic, heterotrophic and mixotrophic. While the biomass activity was to be measured via CO_2 formation, it needs to be taken into consideration that CO_2 is released under heterotrophic conditions (via central carbon metabolism, i.e. the tricarboxylic acid (TCA) cycle, glyoxylate cycle and pentose phosphate pathway (PPP) [21]), and is consumed under autotrophic conditions but under mixotrophic conditions its role is not fully understood. The applied BacTrac 4100[®] equipment is capable of ensuring complete darkness inside the reactor tubes, therefore, is excellent

for the study of heterotrophic growth via CO₂ formation. It is also expected that in more suitable media more cells are grown more rapidly, resulting in an increase in the rate of CO₂ formation. Thus, the profiles of CO₂ formation were used to study the growth of the alga. First the effect of the initial cell number was investigated, then optimal nitrogen and carbon sources were selected, and finally two temperatures were compared on optimized media.

3.1 Effect of different initial cell numbers on indirect impedance changes

The major outcome of impedimetric tests is the detection time, which is generally inversely correlated with the initial cell number. Our measured curves are presented in

Fig. 1. In Fig. 1A, the same colors indicate the non-independent parallel runs, which were combined and evaluated by fitting a regression line (Fig. 1B). It is clearly visible that an increase in the initial cell number results in a decrease in the detection time.

On the basis of the curves in Fig. 1C, by applying a threshold at M%=50, a strong linear inverse correlation is observed between the initial cell number and detection time.

This suggests that the consumption rate of the carbon source was higher in the case of a higher initial cell number. The relative impedance was also changed in the non-inoculated tube by 14 %. The collection of maximum specific growth rates (μ_{max}) is also presented in Fig. 1B. The results exhibit a similar trend as discussed previously.

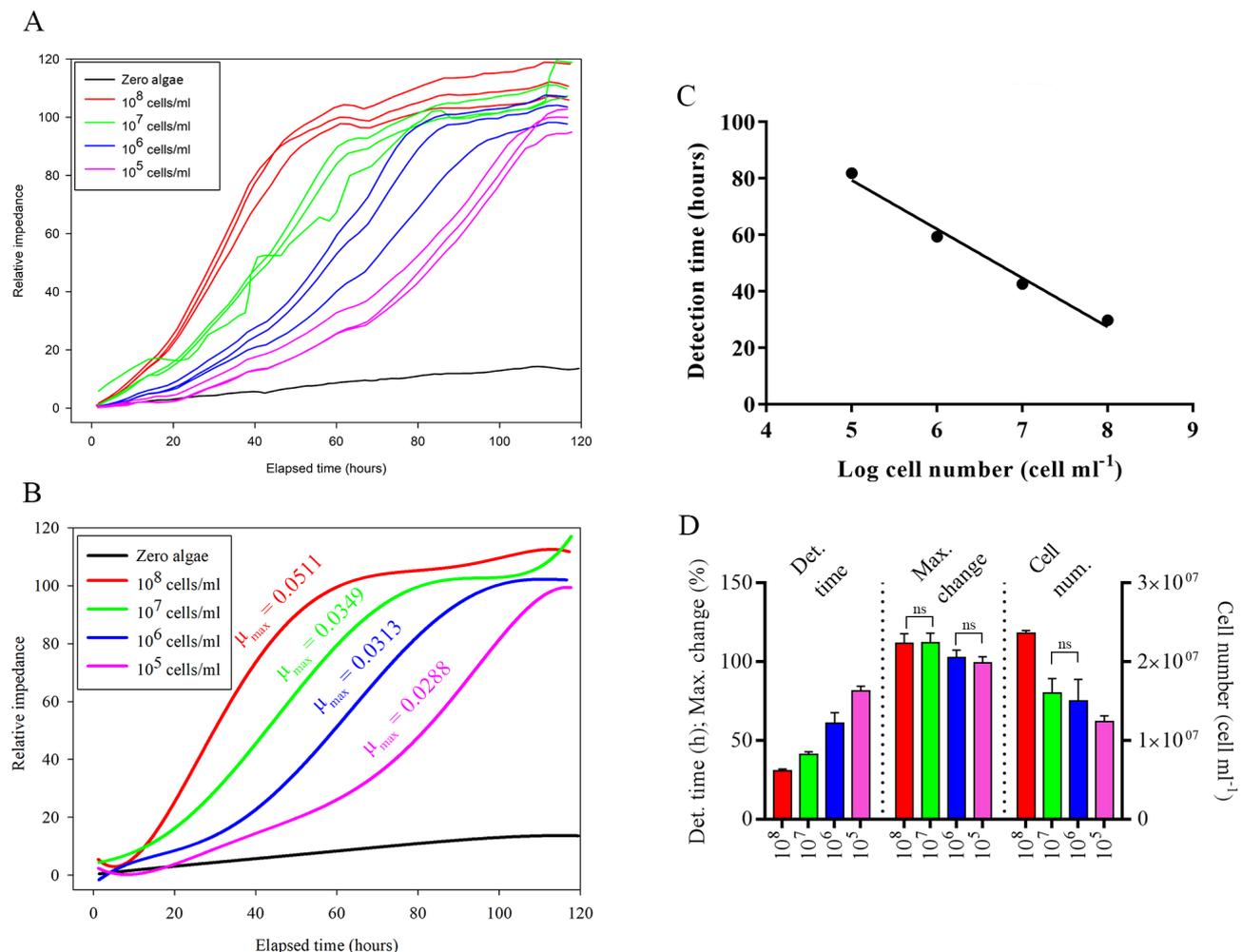


Fig. 1 Relative impedance changes of the heterotrophic culture *Chlorella sorokiniana* with different initial cell numbers at 28°C in WIB media supplemented by 16 C/g/L glucose. A) the raw data of three replications per experimental setup. B) a 5th order regression line fitted on the whole combined dataset per experimental setup. C) the observed correlations between detection time and initial cell number. D) Parameters of the measured curves (detection time, maximum change in relative impedance, final cell number). Data were expressed as the means and standard deviations of three replicates. NS – statistically not significant differences.

3.2 Effect of nitrogen sources on microalgal impedance changes

Comparative investigations have focused on the effect of different nitrogen sources on the biomass growth and lipid production of *Chlorella*. Most algal strains require a suitable type of nitrogen source, therefore, it is important to screen several nitrogen sources by culturing. Some microalgae strains are known to be more capable of using organic nitrogen sources [22, 23].

In this study, urea, yeast extract, tryptone and potassium nitrate were compared at a nitrogen concentration of 4.4 g/L. Yeast extract and tryptone are preferred by *C. sorokiniana* as can be seen according to the combined growth curves of parallel runs (Fig. 2A). This is confirmed by evaluating both detection time (the shorter the better), to achieve maximum changes in impedance and final cell number (Fig. 2B).

The impedance did not change significantly by using urea and KNO_3 compared to the control reactor which did not contain any nitrogen sources. However, they both differ from the empty cells suggesting some algal activity, which can be explained by either endogenous nitrogen utilization or according to some reports [24, 25]. According to Sharma et al., urea supports the growth of *Chlorella sp.* under near autotrophic conditions [26], but in our experiments an increase in cell numbers was not remarkable. The maximum specific growth rate was doubled when yeast extract was used instead of tryptone (Fig. 1A).

3.3 Comparison of carbon sources

The growth of *Chlorella sorokiniana* on acetate has been tested by several studies under mixotrophic conditions. *C. sorokiniana* was successfully grown on BG-11 media supplemented with 1 g C/L acetate [27] and on synthetic wastewater supplemented with up to 1.4 g C/L acetate, but growth was completely inhibited at 4.2 g C/L [28]. The acetate is assimilated through the glyoxylate cycle and can be converted into Acetyl-CoA that produces organic compounds for the metabolic pathway gluconeogenesis. Acetate is also a key molecule in lipid anabolism [29]. Therefore, the availability of acetate under mildly heterotrophic or mixotrophic conditions significantly affects the final concentration and composition of biomass. 1 and 2 g C/L were tested with a glucose origin and 1 g C/L with an acetate origin.

1 g C/L acetate resulted in a relatively low degree of growth with regard to *C. sorokiniana* as seen in Figs. 3

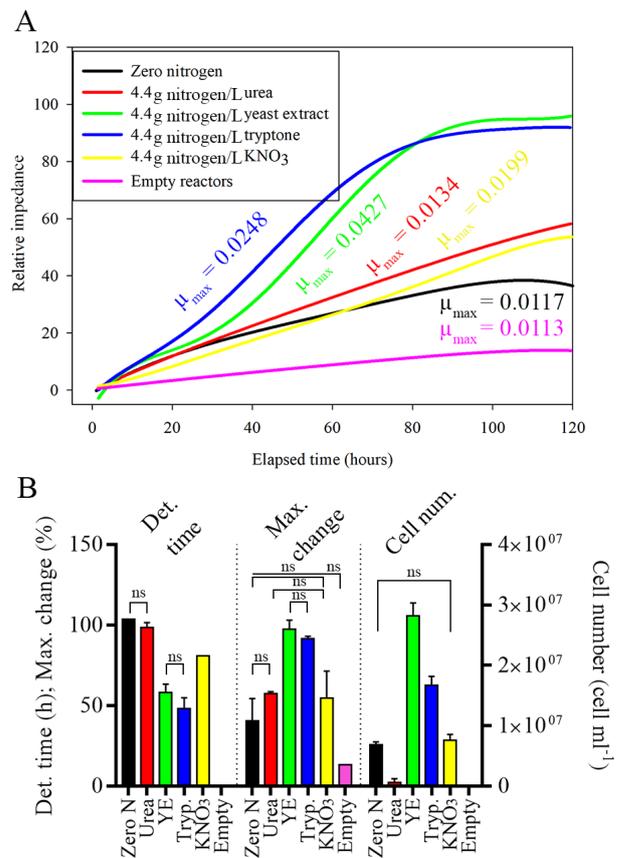


Fig. 2 Changes in relative impedance of a heterotrophic *Chlorella sorokiniana* culture with different nitrogen sources at 28°C in HKU media supplemented with 25 g/L glucose. A) 5th order regression line fitted onto the whole combined dataset per experimental setup. B) effect of the nitrogen source on the detection time, maximum changes in impedance and final cell number. Data were expressed as the means and standard deviations of three replicates. NS – statistically not significant differences.

and 4, even though the C:N:P molar ratio was sufficient (i.e. 14:2:1). The changes in impedance measured in the case of growth on acetate in the HKU medium did not differ from those controlled experiments where any carbon sources or algal cells were excluded. The preference for glucose was clear, but the difference between culturing on a glucose concentration of 1 and 2 g C/L was insignificant (Fig. 3).

These results directed our attention towards further investigations using the organic N-rich complex WIB media which can promote growth even in the absence of added carbon sources (Fig. 4). However, 1 g C/L equivalent glucose could further increase the degree of algal growth while carbon equivalent glycerol was significantly below the added-carbonless control. Acetate seemed to have a slightly positive effect in terms of the final cell

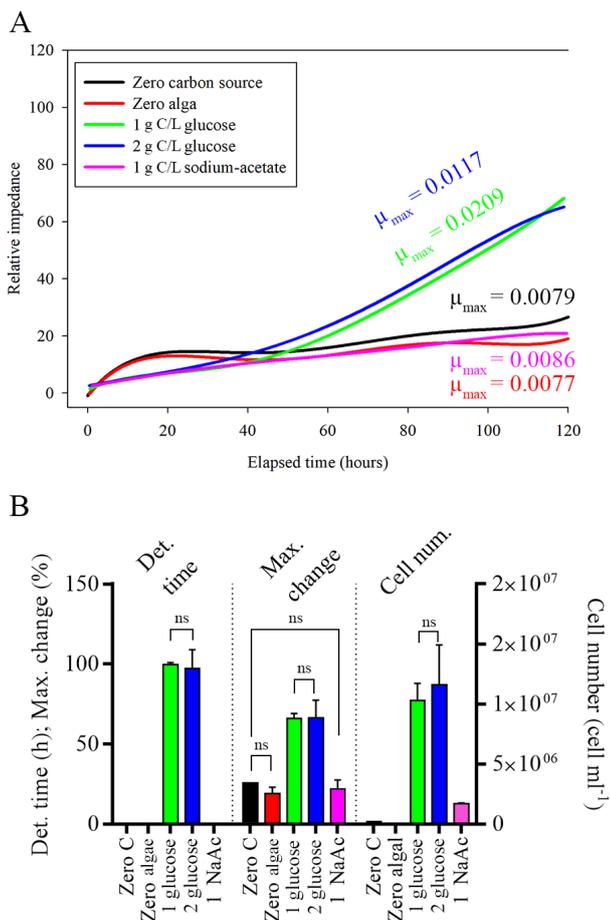


Fig. 3 Changes in relative impedance of the heterotrophic *Chlorella sorokiniana* culture with different carbon sources at 28°C in HKU media. A) 5th order regression line fitted on the whole dataset per experimental setup. B) effect of a carbon source on the detection time, maximum changes in impedance and final cell number. Data are expressed as the means and standard deviations of three replicates. NS – statistically not significant differences.

number, but no effect could be detected on the detection time, maximum changes in impedance and specific growth rate in the WIB media. It was concluded that glycerol at a concentration of 1 g C/L caused a decrease in the impedance curve compared to the controlled experiment in the absence of carbon. This effect was distinct from other research where *Chlorella* was grown efficiently on 0.9-1.2 g C/L glycerol [30, 31]. It is assumed that *Chlorella sorokiniana* can grow on glycerol when mixotrophic conditions are applied and gas exchange provided.

Glucose enhanced the degree of growth effectively according to the detection time. The preference for glucose was determined by investigating the culturing conditions of *C. sorokiniana* in several publications [32, 33].

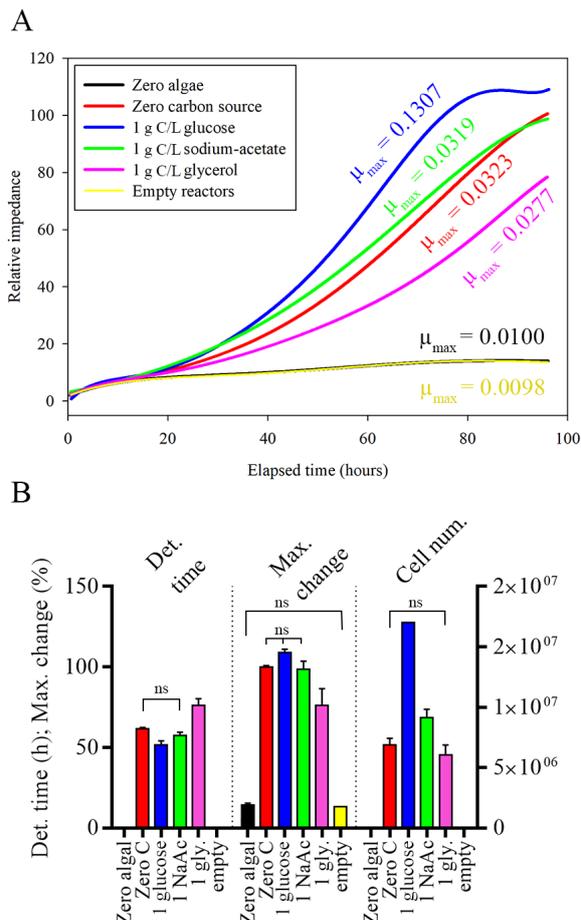


Fig. 4 Comparison of the changes in relative impedance of *C. sorokiniana* using different carbon sources at 28°C in WIB media. A) 5th order regression line fitted on the whole dataset per experimental setup. B) effect of carbon sources on detection time, maximum changes in impedance and final cell number. Data were expressed as the means and standard deviations of three replicates. NS – statistically not significant differences.

3.4 Estimating the glucose optimum

Based on our results as described above, *C. sorokiniana* exhibited a preference for glucose under the circumstances provided by the microbioreactor BacTrac 4100®, i.e. in the absence of stirring, illumination and gas exchange. The high cell density of growth in the presence of glucose has been described elsewhere [11, 34]. However, the effect of glucose concentration during indirect impedimetric measurements was of interest. Therefore, the effect on algal growth of increasing the glucose concentration from 0 to 40 g C/L was investigated at 28°C.

An optimum glucose concentration was determined. As seen in Fig. 5B, the detection time was the shortest when the concentration was 8 g C/L, whereas the maximum

change in relative impedance as well as the final cell number were the greatest. Unfortunately, it is hard to explain the shape of the curve observed in the experiment where a glucose concentration of 32 g C/L was used because based on the final cell number it correlates strongly, but in terms of the detection time and maximum change in relative impedance it does not.

Further experiments are required to understand why a lower concentration of glucose was preferred. However, several stress factors were identified in microalgae cultivations [35], but to the best of our knowledge, osmotic stress caused by glucose or the mechanism of glucose inhibition has yet to be identified in microalgae.

Similar results were observed for other *Chlorellas* such as *C. saccharophila* which exhibited an optimum glucose concentration of almost 20 g/L [36] and the growth of *C. protothecoides* which was also inhibited by a glucose concentration of 100 g/L [37]. However, a relatively high glucose concentration was used in several studies of between 20 and 35 g/L [38, 39].

4 Conclusion

In this study an indirect impedance microbiological technique was used to screen various carbon and nitrogen sources for heterotrophic microalgae cultivation. Our results showed that the technique is applicable with media of different compositions but an added carbon source was required for signal detection.

The initial cell number determines the appearance of the impedance curves, therefore, it is necessary to test all experimental setups within the same run using various controls.

It was found that heterotrophically cultured *Chlorella sorokiniana* cannot utilize sodium acetate at a concentration of 3.41 g/L (i.e. 1 g C/L), and 3.56 g/L (1 g C/L) of glycerol resulted in the inhibition of growth. Glucose is one of the most preferable organic carbon sources for microalgae and its concentration has a strong influence on specific growth rates. Further studies are required to determine the effects of other combinations of substrates and environmental parameters on growth rates. The optimum concentration of glucose was found to be 20 g/L (8 g C/L).

A comparison of microalgae growth on different nutrient compositions using a BacTrac 4100[®] microbioreactor is limited to the provision of representative results, as the indirect measuring setup is not equipped with aeration and

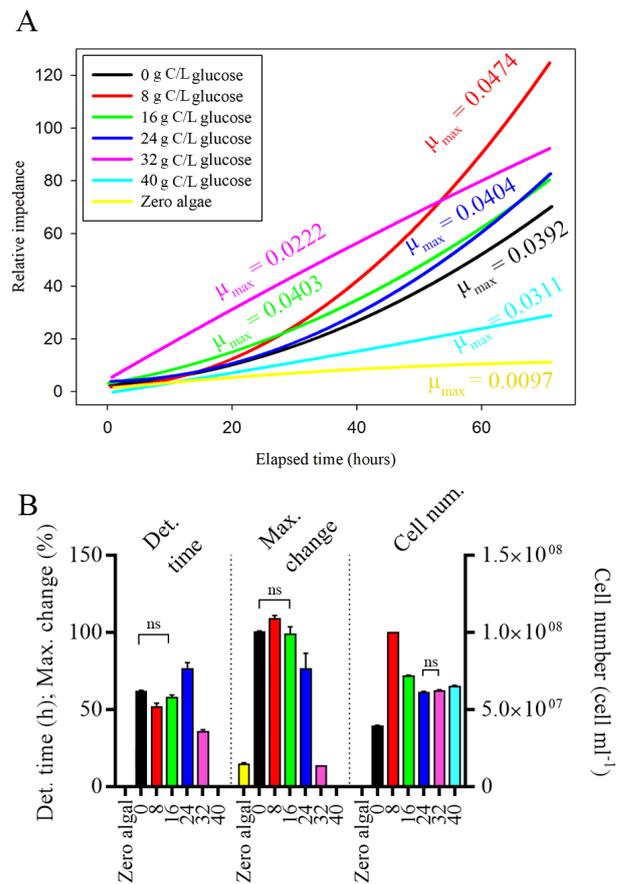


Fig. 5 Comparison of the changes in relative impedance of *C. sorokiniana* using different glucose concentrations at 28°C in WIB media. A) 5th order regression line fitted on the whole dataset per experimental setup. B) the resultant parameters of the cultures with different glucose concentrations. Data were expressed as the means and standard deviations of three replicates. NS – statistically not significant differences.

stirring. However, the concentration of oxygen is often described as a limiting factor in heterotrophic microalgae fermentation [40–42]. Therefore, an investigation into the oxygen limit in these experiments is important.

According to our results, it can be concluded that impedance microbiology could help other heterotrophic microalgae fermentation institutions to implement the factorial design of experiments for the purpose of screening for nutrients and optimizing the composition of media.

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