Bioresource Technology 184 (2015) 90–99

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Building a better mousetrap II: Using Design of Experiments with unconfounded ions to compare the growth of different microalgae

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HIGHLIGHTS

- Unconfounded media variations were used to examine microalgal growth.
- The effects of variations in NH_4^+, NO_3^-, Na^+, K^+, PO_4^-, and Cl^- were studied.
- Two strains of *Chlorella* show significant physiological and functional differences.
- Different microalgae show different effects for growth and lipid content.
- Future work could lead to the development of novel media.

ARTICLE INFO

Article history:
Received 7 September 2014
Received in revised form 2 November 2014
Accepted 3 November 2014
Available online 13 November 2014

Keywords:
Biofuels
Algae
Medium optimization unconfounded ions
Scheffe Mix Model
Response surface methodology

ABSTRACT

A large number of unconfounded media variations were used with a Scheffe Mix Model to examine in an unambiguous fashion the effects of variations in six important ions; NH_4^+, NO_3^-, Na^+, K^+, PO_4^-, and Cl^-, on the growth of *Chlorella vulgaris*. This allows several novel observations on media components, for example, the inhibitory effects of chloride, to be made. Using a side by side comparison, it is shown that two strains of *Chlorella* show significant physiological and functional differences brought out by this approach. Testing selected formulations with a diverse set of algae demonstrated different effects on both growth and cellular lipid content, in some cases driving significant lipid production. This suggests that future work using a larger portion of media composition space could lead to the development of novel media supporting maximal biomass production and lipid production.

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1. Introduction

Microalgae are currently under intense study for the production of fuels and chemicals. They are attractive in building a bioeconomy from a number of perspectives. Microalgae are a very diverse group of organisms united only by the fact that they contain a chloroplast. Therefore they represent a vast and largely untapped genetic resource, encoding a wide spectrum of potentially useful metabolic processes for coupling solar energy capture to the production of interesting compounds (Benemann, 2013; Cheung et al., 2014; Gallardo-Rodriguez et al., 2012; Hemaiswarya et al., 2011; Hosseini et al., 2013; Skjanes et al., 2013). However, the science and art of the genetic manipulation of these organisms is in its infancy. Microalgae can be readily grown on various waste streams as source of nutrients and water, thus not competing with food production for these resources (Abdelaziz et al., 2013a,b; Leite et al., 2013; Leite and Hallenbeck, 2011). As well, many species are relatively fast growers, with doubling times many fold greater than those of land plants. In addition to potentially very economically attractive compounds that might be of interest in the pharmaceutical, cosmetic and neutraceuticals industries, microalgae are well known as excellent producers of bulk commodities, such as biomass for animal feed (Benemann, 2013), or lipids for biodiesel production (Guschina and Harwood, 2006; Harwood and Guschina, 2009; Hu et al., 2008; Leite and Hallenbeck, 2011; Moody et al., 2014; Schenk et al., 2008).

In spite of these many positive attributes, until now the biotechnological potential of microalgae has been little exploited. This is due to a number of technical barriers including such things as harvesting and extraction, but perhaps of even more importance is the need to achieve high efficiency growth with maximum production of the desired compound. The environmental conditions under which algae are grown are known to play major roles in affecting the productivity and biochemical composition of the cell (Bohutskyi et al., 2014; Converti et al., 2009; Evens et al., 2008;

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http://dx.doi.org/10.1016/j.biortech.2014.11.006
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Fields et al., 2014; Gordillo et al., 1998; Griffiths et al., 2014a,b; Hallenbeck et al., 2015b; Juneja et al., 2013; Li et al., 2010; Roleda et al., 2013; Xia et al., 2013). However, in spite of this, the actual ionic composition of the basal medium used is often ignored, and many media formulations that are in common use were developed more or less haphazardly over time. Even when a systematic effort is made to ascertain the effects of one or more nutrients, say nitrogen or phosphate, they are almost invariably varied by decreasing or increasing the concentration of the salt added, such as sodium nitrate or potassium phosphate (for just two examples see Kirrolia et al., 2014 and Yang et al., 2014).

The net effect of changing nutrients in this fashion leads to confounding of the results since, at the same time, the concentration of the counter ion is changed, and the ionic strength is altered, both factors are normally unaccounted for in these types of studies. To fully understand the possible involvement of individual ions in supporting growth and controlling production of particular compounds of interest, an unconfounded approach is required where these factors are controlled. This approach has previously been used to examine the effects of five different ions on growth rates of *Chlorella vulgaris* and *Peridinium cinctum* (Even and Niedz, 2010). Very recently, an unconfounded Scheffe Mix approach was used to examine roles of six ions; NH_4^+, NO_3^-, Na^+, K^+, PO_4^- and Cl^-, on growth and lipid production of *Chlorella* sp. EN1234 (Hallenbeck et al., 2015a). Here, these results have been extended by examining the growth of the well-known *C. vulgaris* on these same mixtures and comparing this is a detailed side-by-side fashion with that of *Chlorella* sp. EN1234. Moreover, the growth and lipid production of these, and four other very diverse algae; *Chlamydomonas reidhardtii*, *Scenedesmus obliquus*, *Euglena gracilis* and *Haematococcus pluvis*, was examined and compared on eleven selected mixtures.

2. Methods

2.1. Strains and growth conditions

Chlorella sp. EN1234 was obtained from Dr. Juergen Polle, Brooklyn College, and *Chlamydomonas reidhardtii* CC124 was obtained from Dr. Maria Ghirardi, National Renewable Energy Laboratory. *Chorella vulgaris* (UTEX 259), *Haematococcus pluvis* (UTEX2505), *Scenedesmus obliquus* (UTEX 393), *Euglena gracilis* (UTEX367) were obtained from the UTEX Culture Collection of Algae. Pre-cultures were grown in the media recommended by UTEX. Six hundred microlitre of the pre-culture was subsequently inoculated into 2.4 mL of the appropriate media in a 12 well plate. Fourteen days later 2 mL of culture was removed and replaced with new media. Seven days later new 12 well plates were inoculated with 300 μL of these 12 well plate cultures and 2.7 mL of the appropriate media (final well volume of 3 mL) and used for the experiments. Cultures were grown essentially as previously described (Hallenbeck et al., 2015a) in 3 mL volumes in 12 well covered plates without agitation at 25 °C, with a light intensity of 150 μE on a 16 h light and 8 h dark cycle. Each 3 mL culture was inoculated with 3000 algae cells/μL. The indicated mixtures were used and complete details can be found elsewhere (Hallenbeck et al., 2015a).

2.2. Growth, lipid and biomass measurements

Optical density (OD) measurements were taken at 600 nm with a BioTek μQuant® microplate spectrophotometer essentially as previously described (Hallenbeck et al., 2015a). Variation between runs was ±25% and variation between biological duplicates within the same run were ±10%. Cell counts and neutral lipid productivity were determined using flow cytometry with a BD Accuri® C6 Flow Cytometer (Hallenbeck et al., 2015a). For lipid analysis flow cytometry samples were prepared in 1.5 mL conical bottom tubes with 50 μL of culture and 150 μL of deionized water (final sample volume of 200 μL). Samples were measured with (1 μL of 200X BODIPY® (200 ng/μL) in DMSO). Dyed samples were vortexed and incubated for 10 min before measuring. This dye and similar procedures have been shown to give relatively uniform staining of different algae independent of cell wall structure (Cooper et al., 2010; Govender et al., 2012).

2.3. Design of Experiments study of operational parameters

Using experimental design software (Design Expert 8) and the mixture and optimal design functions, a matrix was developed to optimize freshwater media. Such mixture designs give response surface designs that are constrained such that all the component proportions add up to 1. Here, a Scheffe Mix Model with a quadratic process order, special cubic mix order and a sixth combined order limit was used. 210 media variations were generated as previously described (Hallenbeck et al., 2015a) and consisted of a basal medium to which were added in varying amounts of H_3PO_4, HCl, HNO_3, KOH, NaOH, NH_4OH, $Mg(NO_3)_2 \times 6H_2O$, $MgCl_2 \times 6 H_2O$, and $Mg(OH)_2$ (Supplemental Table S1 in Hallenbeck et al., 2015a).

3. Results and discussion

In a previous study a set of 210 solutions was used to study in detail the response of the newly isolated *Chlorella* sp. EN1234 (Hallenbeck et al., 2015a). This involved using an unconfounded Scheffe Mix approach to examine six major cations and anions; NH_4^+, NO_3^-, Na^+, K^+, PO_4^- and Cl^- and their interactions in supporting biomass and lipid production by *Chlorella* sp. EN1234. Both Piepel (trace) plots and 3D surface analysis showed that the major media anions PO_4^- and Cl^- negatively influence final cell densities, and that maximal cell density is obtained with nitrate. In addition, mixtures were identified that supported high lipid productivity, thus defining a composition space within which further R&D might produce the best trade-off between total biomass production and high cellular lipid content. Here these important observations have been followed up upon and amplified by examining in detail the responses of a number of highly characterized algal species with particular emphasis on *C. vulgaris* and its comparison with *Chlorella* sp. EN1234.

3.1. Growth of C. vulgaris on the 210 solutions as examined by Piepel (trace) plots

It was of interest to see how another related green alga fared on the same set of 210 solutions and therefore the growth of *C. vulgaris* was examined on these solutions under the same conditions used for *Chlorella* sp. EN1234. *C. vulgaris* was chosen as it is a very well-studied *Chlorella* type strain used in numerous studies over the years. The final OD_{600}s obtained for *C. vulgaris* growing on the 210 solutions was fit to a special cubic (mix) linear (process) model. Anova (Table S1) gave a model *F*-value of 10.34. There is only a 0.01% chance that an *F*-value this large could occur due to noise. There appeared to be a good fit in terms of other parameters as well, as shown by a normal plot of the residuals and plots of residuals versus predicted and predicted versus actual (Fig. S1). The variation in response (OD_{600}) expected for changes in single variables while the proportion of the others are held constant was examined using Piepel (trace) plots (Figs. 1 and 2). Piepel plots trace out the variations in response due to changes in each

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