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Use of fermentative metabolites for heterotrophic microalgae growth: Yields and kinetics

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HIGHLIGHTS

- A diauxic effect of acetate over butyrate was observed.
- A kinetic model for biomass growth, acetate and butyrate removals was build.
- Lactate did not promote or inhibit algal growth.
- Butyrate is key for coupling dark fermentation and microalgal heterotrophy.

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ABSTRACT

The growth of two lipid-producing *Chlorella* species on fermentative end-products acetate, butyrate and lactate, was investigated using a kinetic modeling approach. *Chlorella sorokiniana* and *Auxenochlorella protothecoides* were grown on synthetic media with various (acetate:butyrate:lactate) ratios. Both species assimilated efficiently acetate and butyrate with yields between 0.4 and 0.5 g carbon of biomass/g carbon of substrate, but did not use lactate. The highest growth rate on acetate, 2.23 d^{-1}, was observed for *C. sorokiniana*, and on butyrate, 0.22 d^{-1}, for *A. protothecoides*. Butyrate removal started after complete acetate exhaustion (diauxic effect). However, butyrate consumption may be favored by the increase of biomass concentration induced by the initial use of acetate. A model combining Monod and Haldane functions was then built and fitted the experimental data well for both species. Butyrate concentration and (acetate:butyrate) ratios were identified as key parameters for heterotrophic growth of microalgae on fermentative metabolites.

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1. Introduction

Production of microalgae has gained a lot of interest in the past decades due to their ability to synthesize biomolecules having potential industrial applications such as energy generation. The use of microalgae to produce biofuels offers the main advantage of improving biomass and lipid yields and land use when compared to oil crops. Georgianna and Mayfield (2012) assessed that microalgae could produce the same amount of oil than palm oil using six times less area. Due to higher growth rates, expressed in days instead of months or years, and lipid contents up to 80% of dry biomass, microalgae are very promising candidates to produce massively and constantly biofuels (Wu et al., 2012).

Growing microalgae in darkness and on organic compounds presents several advantages compared to autotrophic cultivation systems: (1) a higher biomass density can be achieved, leading to reduce the costs of microalgae harvesting compared with low density systems operated in autotrophy (Doucha and Lívanský, 2011). (2) Higher growth rates are observed in heterotrophy, reducing the time of cultivation (Kim et al., 2013). (3) Higher lipid yields can be achieved in heterotrophic cultures, improving economic competitiveness of microalgae biofuels (Wan et al., 2012).

Industrial production of heterotrophic microalgae is hampered by the high economic and environmental costs of glucose, commonly used as main carbon source. Therefore, glycerol, acetate or wastewaters containing glucose, such as whey permeate, are considered as the most promising alternatives of low cost carbon substrates (Espinosa-Gonzalez et al., 2014). Glycerol is a by-product of biodiesel and can be used to sustain heterotrophic algal growth and reduces the overall process cost (Heredia-Arroyo et al., 2010). Acetate is a by-product of anaerobic digestion and often accumulates in dark fermentation processes. Interestingly, microalgae can easily convert acetate into acetyl-CoA which is the main

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precursor for lipid synthesis (Ramanan et al., 2013). *Auxenochlorella protothecoides* was successfully grown on glycerol and acetate, with maximal biomass concentrations of 3.97 and 3.62 g L^{-1} and maximal lipids contents of 20.33 and 52.38% of dry biomass, respectively (Heredia-Arroyo et al., 2010). These results were very similar to the values reported with glucose, i.e. 4.25 g L^{-1} of biomass and a lipid content of 25.25% (Heredia-Arroyo et al., 2010). The use of other organic substrates, such as sucrose, lactose and ethanol may not support substantial heterotrophic growth of microalgae (Perez-Garcia et al., 2011b).

Coupling dark fermentation, producing hydrogen and volatile fatty acids (VFA), such as acetate, butyrate and lactate, with microalgal mixotrophic bioprocesses has been recently investigated with the purpose of lowering the costs of the overall process by finding new sources of substrates. Hu et al. (2013) showed the feasibility of growing mixotrophically a newly isolated *Chlorella* sp. on acidogenic swine effluents containing a mixture of acetate, propionate and butyrate. The effluent had to be diluted 8 folds in order to promote the microalgae growth and it was assumed that growth inhibition was caused by high concentrations of VFAs. Consistently, Liu et al. (2012) pointed out an inhibitory effect on mixotrophic growth of *Chlorella vulgaris* when butyrate concentration was higher than 0.1 g L^{-1}. In contrast, Wen et al. (2013) found that butyrate was degraded before acetate but after valerate and ethanol by *Chlorella protothecoides* in heterotrophic conditions on anaerobically digested sugarcane wastewaters. Venkata Mohan and Prathima Devi (2012) used a mixed culture of microalgae, containing species of *Scenedesmus* and *Chlorella*, in order to convert dark fermentation effluents into microalgal biomass (1.42 g L^{-1}) and microalgal lipids (26.4% of dry weight), under mixotrophic conditions. They reported that acetate was a preferred substrate compared to butyrate and propionate. Ren et al. (2013) used sterilized dark fermentation effluents, composed of at least 95% of acetate and ethanol, to sustain heterotrophic growth of *Scenedesmus* sp. In this study, acetate was completely removed but not ethanol. The subsequent biomass production and lipids content reached 1.98 g L^{-1} and 40.9% of dry weight, respectively. Liu et al. (2013) pointed out that butyrate removal was higher under heterotrophic than mixotrophic conditions due to the competition between organic and inorganic carbon uptake. Considering all these studies, the heterotrophic growth of microalgae on a mixture of organic substrates is still difficult to estimate mainly because of the lack of a clear behavior when a mixture of substrates is used.

A. protothecoides and *Chlorella sorokiniana* are two well-known lipid-producing microalgae, their lipids content can be as high as 57 and 61.5% of dry weight, respectively (Ramanna et al., 2014; Wang et al., 2013). The aim of this study was to characterize the growth of *A. protothecoides* and *C. sorokiniana*, under strict heterotrophic conditions and in presence of three organic acids, i.e. acetate, butyrate and lactate, mainly generated in dark fermentation processes. *A. protothecoides* and *C. sorokiniana* kinetic parameters were assessed using a global kinetic model fitting biomass growth and organic carbon removal.

2. Methods

2.1. Microalgae strains and culture conditions

C. sorokiniana (CCAP 211/8K) and *A. protothecoides* (CCAP 211/7A) were obtained from the CCAP culture collection (United Kingdom). A modified BG11 medium (UTEX, http://www.utex.org/) was used to pre-cultivate the inoculum. Sodium bicarbonate (10 mM), chlorure ammonium (5 mM) and dipotassium phosphate (0.31 mM) were used as inorganic carbon (C), nitrogen (N) and phosphorus (P) sources, respectively. Since *A. protothecoides* is auxotrophic for thiamine (vitamin B1) (Huss et al., 1999), the medium was supplemented with 1 mL/L of F/2 medium's vitamins solution (CCAP, http://www.ccap.ac.uk/). The pH of the medium was set at 6.5 prior to sterilization. Ammonium and vitamins solution were sterilized using a filter with 0.2 μm pores. All other media components were sterilized by autoclaving at 121 °C for 20 min. Both species were cultivated in 500 mL Erlenmeyer flasks, containing 200 mL of modified BG11 medium. Inoculum was cultivated in autotrophic conditions to avoid a cometabolism effect, i.e. the induction of a preferred organic substrate (Narang and Pilyugin, 2005). The flasks were placed at 25 °C under a light intensity of 100 μmol photons m^{-2} s^{-1}. After 5–7 days of cultivation, the culture was used to further inoculate (10% V/V) the different culture media. Axeny was daily checked by phase contrast microscopy and DAPI coloration microscope observations as well as spreading cultures on ATCC5 solid media (ATCC, http://www.lgcstandards-atcc.org/).

2.2. Heterotrophic growth on mixtures of organic acids

In order to evaluate the cell viability of the inoculum, a positive control was cultivated under photoautotrophic conditions. A negative control containing inorganic carbon as carbon source (bicarbonate) was placed in the dark to evaluate the ability of the inoculum to grow on its own cellular reserves.

For the growth on organic carbon compounds, different initial concentrations of C sources were tested alone and in mixture (Table 1) but the C:N:P molar ratio was set at 48:16:1. Prior to sterilization with a 0.2 μm pores filter, working solutions of acetate, butyrate and lactate were neutralized at pH 6.5 with NaOH. To maintain a non-inhibitory pH throughout the experiments, pH was buffered with 100 mM of 2-(*N*-morpholino) ethanesulfonic acid (MES) (Abdelaziz et al., 2014). The initial pH was set between 6 and 6.5 (Zheng et al., 2013). Microalgae were cultivated in 125 mL black Erlenmeyer flasks with cotton plugs containing 40 mL of medium. The flasks were placed under dark conditions at 25 °C on a rotary shaker (150 rpm).

An acclimation study was also performed during 4 weeks in order to improve butyrate removal. *A. protothecoides* was successively cultivated on a medium containing 0.1 gC L^{-1} of butyrate. Medium composition was the same as described above.

All experiments and controls were done in triplicates.

Table 1
List of all the conditions tested for *Chlorella sorokiniana* and *Auxenochlorella protothecoides* to estimate and validate model parameters.

Tested experiments	Acetate (gC L^{-1})	Butyrate (gC L^{-1})	Lactate (gC L^{-1})	Estimation (E) or validation (V)[a]
Growth on acetate	**0.1**	0	0	E
	0.25	0	0	V
	0.5	0	0	V
	1	0	0	E
Growth on butyrate	0	**0.1**	0	E
	0	**0.25**	0	V
	0	**0.5**	0	V
	0	**1**	0	E
Growth on acetate and butyrate mixtures	**0.25**	**0.25**	0	E
	0.4	**0.1**	0	V
	0.9	**0.1**	0	E
Growth on lactate	0	0	**0.5**	[b]
	0.25	0	**0.25**	V
	0.25	**0.45**	**0.16**	V

[a] Data used for estimation (E) or validation (V) of model parameters.
[b] The experimental data were not used to build the model due to the absence of lactate removal during the experiments.

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