Couplage de la fermentation sombre et de l'hétérotrophie microalgale: influence du mélange de métabolites fermentaires, de la lumière, de la température et des bactéries fermentaires sur la croissance algale

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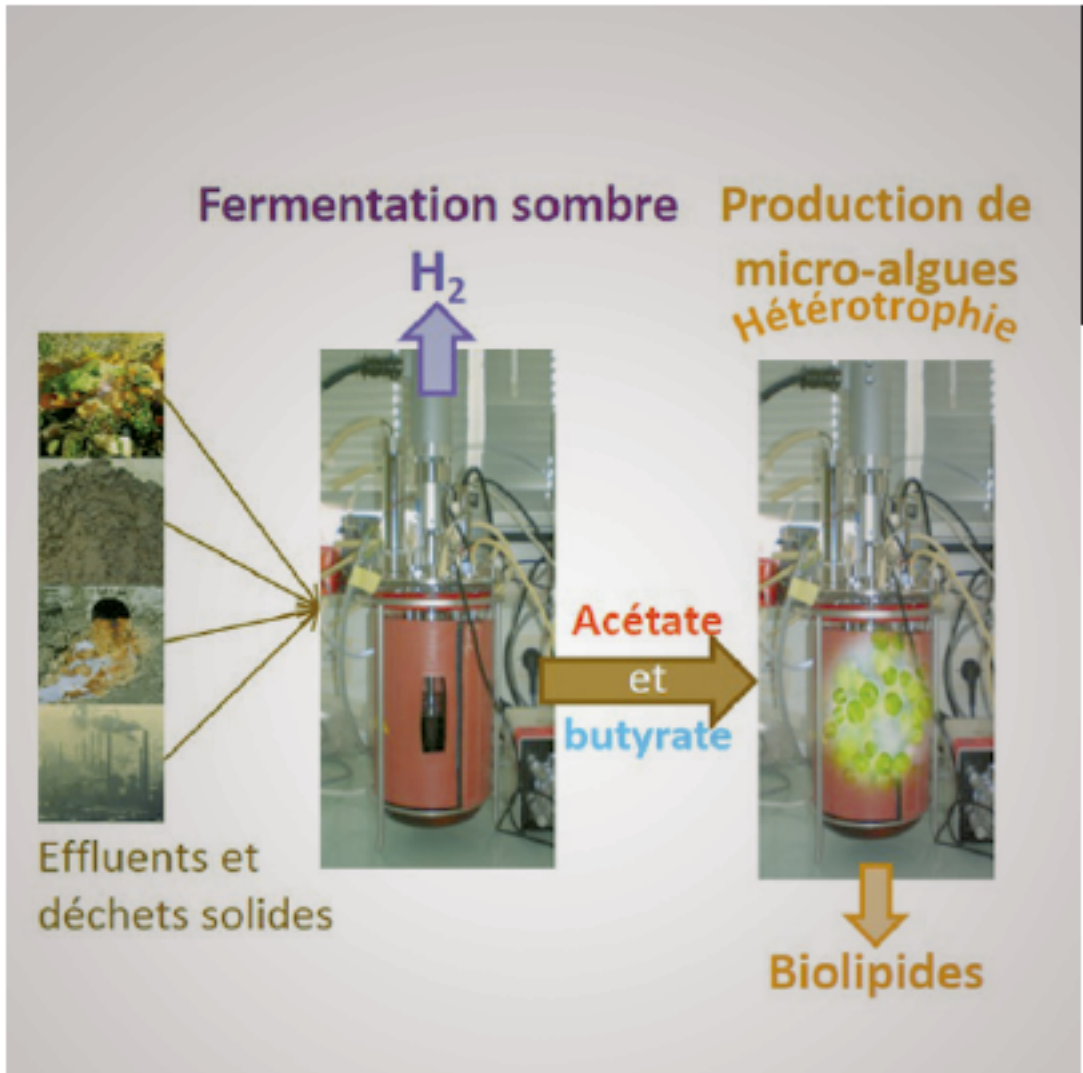

COUPLAGE DE LA FERMENTATION SOMBRE ET DE L'HÉTÉROTROPHIE MICROALGALE

INFLUENCE DU MÉLANGE DE MÉTABOLITES FERMENTAIRES, DE LA LUMIÈRE, DE LA TEMPÉRATURE ET DES BACTÉRIES FERMENTAIRES SUR LA CROISSANCE ALGALE

COUPLING DARK FERMENTATION WITH MICROALGAL HETEROTROPHY: INFLUENCE OF FERMENTATION METABOLITES MIXTURES, LIGHT, TEMPERATURE AND FERMENTATION BACTERIA ON MICROALGAE GROWTH

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Coupling dark fermentation with microalgal heterotrophy: influence of fermentation metabolites mixtures, light, temperature and fermentation bacteria on microalgae growth

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Coupling dark fermentation with microalgal heterotrophy: influence of fermentation metabolites mixtures, light, temperature and fermentation bacteria on microalgae growth

Growing microalgae in heterotrophic mode present several advantages over autotrophic mode such as a higher productivity in terms of biomass and lipids for biofuels production. Nevertheless, this process is limited by the production cost associated with the organic substrate (i.e. glucose) and fermenters sterilization costs. Dark fermentation effluents, mainly composed of acetate and butyrate, could be used as a low-cost medium to grow microalgae heterotrophically or mixotrophically. The aims of this PhD were i) to optimize microalgae growth on various mixtures of fermentations metabolites using the presence or absence of light and different cultivation temperatures and ii) to assess the feasibility of using unsterilized fermentation effluents.

First, a model based on mass balance was built to characterize heterotrophic growth rates and yields when *Chlorella sorokiniana* and *Auxenochlorella protothecoides* were supplemented with different mixtures of acetate and butyrate. Results showed that the acetate:butyrate ratio and the butyrate concentration *per se* were two key parameters for promoting heterotrophic growth. Then, further studies showed that the presence of light and the use of suboptimal temperature (30 °C) could reduce the butyrate inhibition on growth by either triggering autotrophic production of biomass or enhancing growth on acetate. Finally, it was shown that microalgae could outcompete fermentation bacteria for acetate when growing on raw dark fermentation effluents, thanks to a fast algal growth on acetate ($1.75\ d^{-1}$) and a drastic change of culture conditions to the detriment of bacterial growth.

KEYWORDS: Heterotrophy; Mixotrophy; Dark fermentation; Volatile fatty acids; *Chlorella*; microalgae-bacteria interactions

Couplage de la fermentation sombre et de l'hétérotrophie microalgale: influence du mélange de métabolites fermentaires, de la lumière, de la température et des bactéries fermentaires sur la croissance algale

La production de microalgues en hétérotrophie présente plusieurs avantages pour la production de biocarburants par rapport à la production autotrophe, comme une productivité plus importante en termes de biomasse et de lipides. Cependant, le développement industriel de ce procédé est limité par les coûts de productions associés au substrat organique (i.e. glucose) et à ceux liés à la stérilisation des fermenteurs. Les effluents de fermentation sombre, composés principalement d'acétate et de butyrate, pourraient être utilisés comme milieux de culture peu onéreux pour la culture hétérotrophe ou mixotrophe de microalgues. Les objectifs de cette thèse étaient i) de mieux appréhender la croissance algale sur des mélanges variés d'acétate et de butyrate en fonction de la présence ou l'absence de lumière et de la température de croissance et ii) d'évaluer la faisabilité d'utiliser des effluents de fermentation non stérilisés pour soutenir la croissance de microalgues oléagineuses.

Tout d'abord, un modèle basé sur des bilans de masse a été construit afin de caractériser (taux de croissance et rendements) la croissance hétérotrophe de *Chlorella sorokiniana* et *Auxenochlorella protothecoides* sur des mélanges d'acétate et de butyrate. Les résultats ont montré que le rapport acétate:butyrate et la concentration en butyrate étaient deux paramètres clés pour soutenir la croissance hétérotrophe. Puis, il a été démontré que la présence de lumière et l'utilisation d'une température suboptimale (30 °C) pour la croissance algale permettaient de réduire l'inhibition du butyrate en permettant une production de biomasse autotrophe ou en améliorant la croissance sur acétate. Enfin, il a été montré que les microalgues peuvent être compétitives sur l'acétate lors de la croissance sur des effluents bruts de fermentation sombre en présence de bactéries fermentaires, grâce à la croissance rapide des microalgues sur acétate ($1.75\ j^{-1}$) et à un changement drastique des conditions de culture peu favorables à la croissance des bactéries d'origine fermentaire.

MOTS-CLES: Hétérotrophie; Mixotrophie; Fermentation sombre; Acides gras volatils; Chlorella; interactions microalgues-bactéries

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A:B	Acetate:butyrate ratio	**MES**	2-(N-morpholino)ethanesulfonic acid
Ac-CoA	Acetyl-Coenzyme A	**MFC**	Microbial Fuel Cell
AD	Anaerobic Digestion	**MS**	Malate Synthase
AF	Acidogenic Fermentation	**N**	Nitrogen
ANOVA	Analysis of Variance	**NAD**	Nicotinamide adenine dinucleotide
ATP	Adenosine Tri-Phosphate	**NADPH**	Nicotinamide adenine dinucleotide phosphate
C	Carbon	**OD**	Optical Density
COD	Chemical Oxygen Demand	**ODE**	Ordinary Differential Equation
DAPI	4',6-diamidino-2-phenylindole	**OP**	Oxidative Phosphorylation
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea	**PHA**	Polyhydroxyalkanoates
DF	Dark Fermentation	**P**	Phosphorus
DHA	Docosahexaenoic acid	**PS**	Photosystem
DW	Dry Weight	**PUFA**	Poly-Unsaturated Fatty Acids
EPA	Eicosapentaenoic acid	**qPCR**	Quantitative Polymerase Chain Reaction
FAD	Flavin adenine dinucleotide	**rDNA**	Ribosomal Deoxyribonucleic acid
G3P	Glyceraldehyde 3-Phosphate	**S/X**	Substrate :Microorganism ratio
H	Heterotrophy	**SUC**	Succinate
HRT	Hydraulic Retention Time	**TAG**	Triacylglycerol
ICL	IsoCitrate Lyase	**TS**	Total Solids
M	Mixotrophy	**VFA**	Volatile Fatty Acids
MEC	Microbial Electrolysis Cell	**VS**	Volatile Solids

Résumé

Introduction

Au cours des dernières décennies, une grande attention a été portée sur la production de microalgues pour synthétiser un large panel de molécules d'intérêt industriel. En effet, les microalgues sont capables de produire, entre autres, des lipides et des pigments pouvant être utilisés dans les industries agroalimentaires, cosmétiques, pharmaceutiques ou encore pour la production de biocarburants (Raja et al., 2014; Wu et al., 2014). L'intérêt d'utiliser les microalgues pour la production de biodiesel à partir de lipides algaux repose sur des rendements élevés en termes de biomasse et de lipides ainsi que sur une plus faible utilisation de terres arables par rapport aux plantes oléagineuses (Wijffels and Barbosa, 2010). Cependant, pour subvenir aux besoins en biodiesel de l'Union Européenne (0,4 milliard de m^3 par an), la surface nécessaire pour la culture de microalgues serait équivalente à celle du Portugal (Wijffels and Barbosa, 2010). Ainsi, malgré les avantages d'une telle production, une augmentation de la productivité et une baisse du coût total de production sont nécessaires.

Les microalgues peuvent être cultivées en autotrophie (la lumière étant source d'énergie et le CO_2 source de carbone), en mixotrophie (la lumière étant source principale d'énergie et à la fois le CO_2 et des composés organiques comme sources de carbone) et en hétérotrophie (des composés organiques étant sources d'énergie et de carbone en absence totale de lumière). Les avantages majeurs des systèmes de culture en hétérotrophie par rapport à ceux en autotrophie sont : (1) des densités de biomasse plus importantes (Doucha and Lívanský, 2011) ; (2) des taux de croissance plus élevés (Kim et al., 2013b) ; (3) de plus forts rendements en lipides (Wan et al., 2012) ; (4) une production volumétrique plutôt que surfacique (Venkata Mohan et al., 2015) ; (5) la possibilité d'utiliser des fermenteurs (comme pour la croissance bactérienne ou fongique) et non pas des photo-bioréacteurs dont l'optimisation technologique est toujours un sujet de recherche actuel. Ces avantages font de l'hétérotrophie une source prometteuse de biodiesel.

Cependant, à cause du fort coût du glucose, le principal substrat utilisé en hétérotrophie, seules les productions de molécules à haute valeur ajoutée, comme le DHA, sont à ce jour économiquement viables (Fei et al., 2014). Afin de produire des molécules à faible valeur ajoutée, dont le biodiesel, il est donc nécessaire de trouver une source de carbone peu onéreuse, alternative au glucose (Liu and Chen, 2014). L'acétate, un acide organique

s'accumulant pendant les procédés de fermentation sombre, est l'une des alternatives au glucose comme substrat pour l'hétérotrophie. De plus, l'acétate peut être facilement converti en acétyl-CoA qui est le précurseur principal de la synthèse des lipides chez les microalgues (Ramanan et al., 2013).

Très récemment, le couplage entre la fermentation sombre (FS), productrice de biohydrogène, et de la culture hétérotrophe de microalgues, productrice de lipides, a été proposé afin de produire de manière durable des biocarburants à partir de déchets et d'effluents (Park et al., 2014). La FS est un procédé simple permettant à des bactéries anaérobies de convertir une large gamme de déchets (provenant de l'agriculture ou de l'industrie agro-alimentaires) et d'effluents (provenant de l'agriculture ou des industries papetière et sucrière) en biohydrogène, un gaz à haut rendement énergétique, et en acides organiques, majoritairement l'acétate et le butyrate (Sambusiti et al., 2015). L'avantage majeur de la FS est de rendre des composés organiques complexes, issus d'effluents et non assimilables tels quels par les microalgues, en acides gras volatils (AGVs) simples pouvant servir de source de carbone pour la croissance algale (Singhania et al., 2013). De plus, grâce à la minéralisation de l'azote (N) et du phosphore (P) en ammonium et en orthophosphate au cours de la FS (Cai et al., 2013), les effluents de FS contiennent également des quantités substantielles de N et P, nécessaires à la croissance des microalgues.

Au cours des dernières années, quelques études ont montré des productions prometteuses de biomasse microalgale et de lipides à partir d'effluents de FS, composés majoritairement d'acétate et de butyrate (Cho et al., 2015; Fei et al., 2014; Hongyang et al., 2011; Hu et al., 2013, 2012a; Liu et al., 2013, 2012; Moon et al., 2013; Ren et al., 2014b; Venkata Mohan and Prathima Devi, 2012). Cependant, les performances des cultures hétérotrophes et mixotrophes sur des effluents de FS sont toujours difficiles à estimer, principalement à cause d'une absence de comportement clair des microalgues (surtout en termes d'inhibition par les substrats et de cinétique de croissance) lorsqu'un mélange de substrats est utilisé.

Des connaissances plus approfondies sur la croissance des microalgues sur acétate et butyrate, utilisés en mélange, sont nécessaires afin de mieux comprendre et prédire le comportement des microalgues en croissance sur des effluents de fermentation sombre et ainsi étayer la faisabilité du couplage. En effet, certains **prérequis cruciaux** n'ont toujours pas été établis, tels que :

- *la connaissance des rendements et cinétiques associés aux assimilations de l'acétate, du butyrate ou encore du CO_2 (en mixotrophie)*
- *la compréhension de l'inhibition par le butyrate*
- *la caractérisation des interactions entre microalgues et bactéries fermentaires.*

L'**objectif principal** de cette thèse était d'**apporter des éléments de réponse** à ces questions afin de **caractériser** aussi finement que possible **la croissance de microalgues hétérotrophes sur des mélanges d'AGVs**. Ainsi, la croissance de microalgues a été étudiée dans un premier temps sur **effluents synthétiques**, en fonction de la variation des mélanges d'acétate et de butyrate ainsi que de certains paramètres abiotiques (lumière et température), et dans un second temps, sur **effluents bruts de fermentation sombre**, contenant en particulier des bactéries fermentaires potentiellement compétitrices des microalgues.

Cette thèse se divise en cinq chapitres. Le premier introduit brièvement les procédés de fermentation sombre (FS) et présente un état de l'art du couplage entre FS et croissance des microalgues. D'après cet état de l'art, une inhibition de la croissance des microalgues sur des mélanges d'AGVs a été suggérée pour des ratios acétate:butyrate faibles. En effet le butyrate serait assimilé lentement et en fonction de sa concentration, inhiberait la croissance algale et l'assimilation de l'acétate. Ces hypothèses ont été testées expérimentalement dans le chapitre 2. Ensuite, l'influence de la lumière et celle de la température, combinées ou non, ont été étudiées dans le chapitre 3. D'une part, la lumière pourrait déclencher la production d'ATP et de biomasse, via la fixation du CO_2, ce qui permettrait d'accélérer la dégradation du butyrate. D'autre part, à la température optimale, la croissance des microalgues pourrait bénéficier d'une amélioration de l'activité enzymatique et d'une réduction de la perte d'énergie cellulaire via la thermorégulation. La compétition possible entre les microalgues et les bactéries fermentaires, issues des effluents de FS, pour les AGVs a été étudiée dans le chapitre 4. Finalement, les conclusions et perspectives de ces travaux de thèse sont discutées, d'un point de vue fondamental puis appliqué, dans le chapitre 5. Dans ce résumé, seuls les résultats de recherche sont présentés. La bibliographie n'est pas détaillée.

Cinétiques et rendements de croissance de microalgues hétérotrophes associés à l'assimilation diauxique d'AGVs

Les premiers objectifs de cette thèse étaient (i) d'étudier les interactions entre les assimilations de l'acétate et du butyrate et de déterminer si ces assimilations sont diauxiques, c'est-à-dire toujours séquentielles, et (ii) d'évaluer le seuil (concentration) d'inhibition du

butyrate sur la croissance hétérotrophe des microalgues. Deux espèces de microalgues, connues pour produire des lipides (avec un taux de lipides pouvant dépasser les 50% du poids sec) et capables d'hétérotrophie et de mixotrophie, *Chlorella sorokiniana* (CCAP 211/8K) (Ramanna et al., 2014) et *Auxenochlorella protothecoides* (CCAP 211/7A) (Wang et al., 2013), ont été choisies pour cette étude. Ces deux espèces ont ainsi été cultivées sur acétate et butyrate, utilisés comme seule source de carbone, et sur des mélanges d'acétate, de butyrate et de lactate, les trois métabolites principaux des effluents de fermentation sombre. Pour chaque expérience, les paramètres cinétiques (taux de croissance et taux d'assimilation des substrats) et les rendements de biomasse ont été déterminés en développant un modèle basé sur un bilan de masse. Seuls les résultats obtenus avec *C. sorokiniana* sont présentés dans ce résumé.

Les différents matériels et méthodes utilisés sont résumés dans la Figure 1.

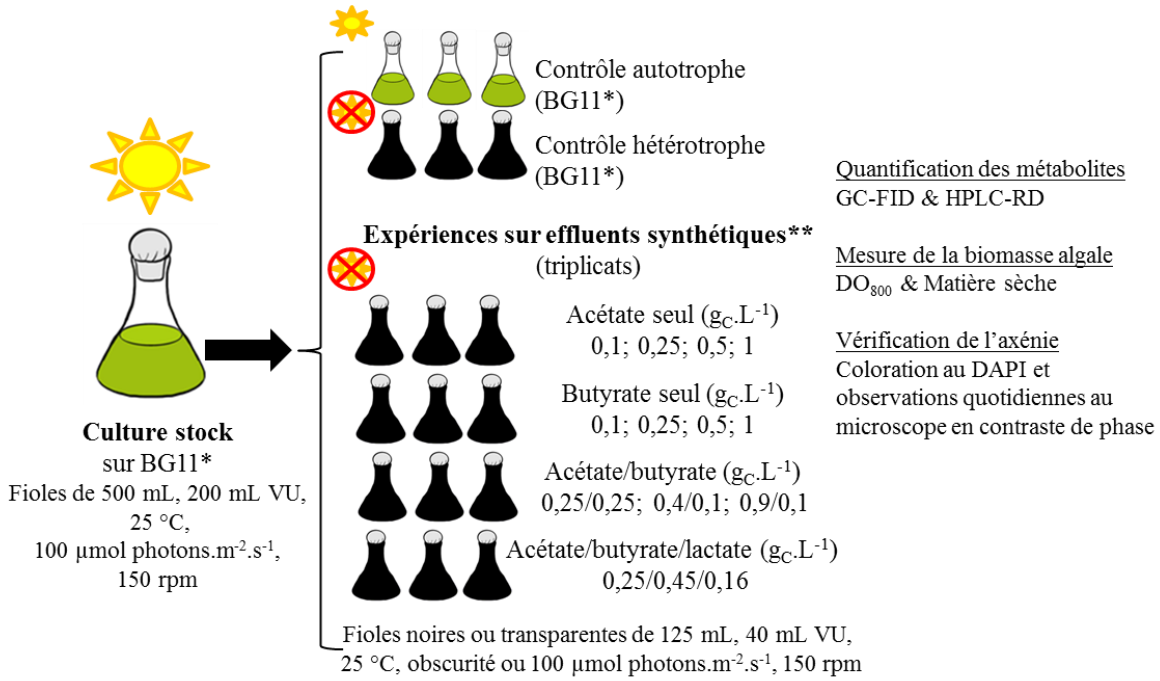

Figure 1. Représentation schématique des expériences.

BG11*: milieu BG11 modifié (NH_4Cl à 5 mM, KH_2PO_4 à 0,31 mM, $NaHCO_3$ à 10 mM et solution de vitamines, solution tampon MES à 100 mM (pH 6-6,5), **effluent synthétique: mélanges d'acétate, butyrate et lactate, BG11 modifié sans bicarbonate avec un ratio C:N:P de 48:16:1 (carbone limitant); VU: Volume Utile. L'ensemble des milieux et de la verrerie a été stérilisé soit par filtration (0,2 µm) soit par autoclave (121 °C, 20 min). La densité optique mesurée à 800 nm (DO_{800}) a été corrélée à la matière sèche: MS (g_{MS}.L-1) = 1.24*OD_{800} (R^2 = 0,95) pour *C. sorokiniana*).

Un modèle mathématique a été développé afin de déterminer les paramètres cinétiques associés à la culture en batch sur des métabolites de fermentation. Le modèle est composé de trois équations différentielles, la première se référant à l'assimilation de l'acétate (équation de

Monod, Equation 1), la deuxième à celle du butyrate (équation d'Haldane modifiée pour prendre en compte la diauxie, Equation 2) et la dernière à la production de biomasse à partir de ces substrats (Equation 3).

$\frac{dS_a}{dt} = \underbrace{\mu_{a\_max} * \left(\frac{S_a}{S_a + K_{S_a}}\right)}_{\mu_a\,(S_a)} * \frac{1}{Y_a} * X$	Equation 1
$\frac{dS_b}{dt} = \underbrace{\mu_{b\_max} * \frac{K_D}{K_D + S_a} * \frac{Sb}{S_b + \frac{\mu_{b\_max}}{\alpha} * \left(\frac{Sb}{S_{b\_opt}} - 1\right)^2}}_{\mu_b(S_b)} * \frac{1}{Y_b} * X$	Equation 2
$\frac{dX}{dt} = \mu_a\,(S_a) * X + \mu_b(S_b) * X$	Equation 3

avec, S_a la concentration en acétate ($g_C.L^{-1}$), K_{Sa} la constante de demi-saturation pour l'acétate ($g_C.L^{-1}$) , μ_{a_max} le taux de croissance maximal associé à l'assimilation de l'acétate (j^{-1}), Y_a le rendement de biomasse sur acétate ($g_{MS}.g_C^{-1}$), X la biomasse microalgale ($g_{MS}.L^{-1}$), S_b la concentration en butyrate ($g_C.L^{-1}$), S_{b_opt} la concentration en butyrate pour laquelle μ_b (S_b) (le taux de croissance sur butyrate) est maximal ($g_C.L^{-1}$), α la pente initiale ($L.j.g_C^{-1}$), μ_{b_max} le taux de croissance maximal associé à l'assimilation du butyrate (j^{-1}), K_D la constante de demi-inhibition associée avec la diauxie ($g_C.L^{-1}$), Y_b le rendement de biomasse sur butyrate ($g_{MS}.g_C^{-1}$).

Les paramètres cinétiques ont été estimés en utilisant la moitié des jeux de données expérimentales puis validés avec l'autre moitié en utilisant l'algorithme de Nelder-Mead.

Aucune inhibition de la croissance de *C. sorokiniana* sur acétate seul (0,1; 0,25; 0,5 et 1 $g_C.L^{-1}$) en hétérotrophie n'a été observée. Les dynamiques de croissance (Figure 2-A) et de dégradation de l'acétate ont été prédites avec précisions par le modèle. Le taux de croissance (2,23 d^{-1}) et le rendement de biomasse (0,42 $g_C.g_C^{-1}$) sur acétate ont pu être estimés grâce au modèle. Ces valeurs sont en accord avec celles de la littérature pour la même espèce sur acétate (Ogbonna et al., 2000). Pour les conditions sur butyrate seul (0,1; 0,25; 0,5 et 1 $g_C.L^{-1}$), une croissance de microalgues n'a été observée que pour la concentration la plus faible en butyrate (Figure 2-B). Ces résultats ont confirmé que l'inhibition du butyrate sur la croissance des microalgues était liée à sa concentration. Cette inhibition a été représentée en utilisant l'équation d'Haldane et les dynamiques de croissance et d'assimilation du butyrate ont pu être

prédites (Figure 2-B). Un taux de croissance (0,16 j^{-1}) très faible par rapport à celui sur acétate et un rendement de carbone (0,56 $g_C.g_C^{-1}$) supérieur à celui sur acétate ont été estimés. Ainsi, la faible croissance sur butyrate est due à une croissance très lente et non pas à un faible rendement de biomasse. Le seuil d'inhibition du butyrate, au-delà duquel le taux de croissance décroît avec des concentrations croissantes, a été estimé à 0,05 $g_C.L^{-1}$ (correspondant au paramètre S_{b_opt}) via le modèle mathématique. Cette inhibition par le butyrate pourrait être due à une acidification du pH cytosolique (Lin et al., 2015).

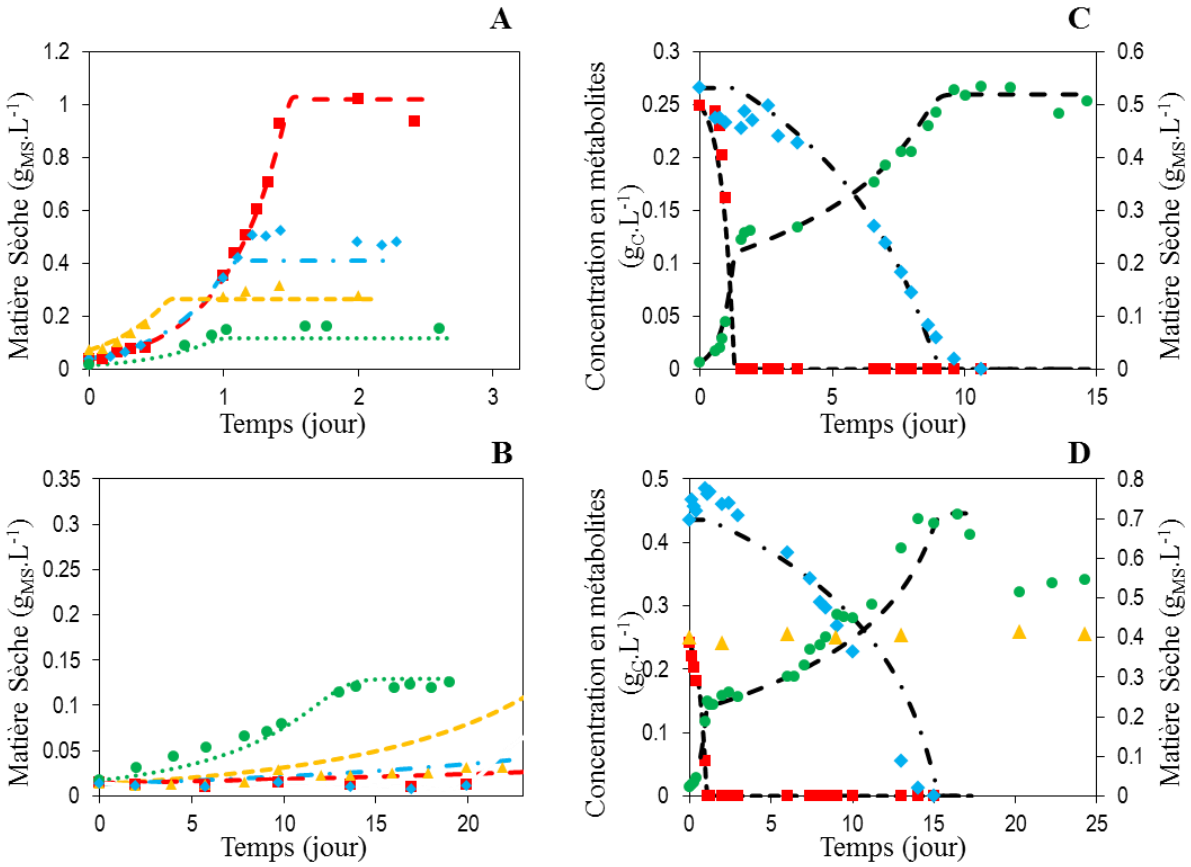

Figure 2. Croissance hétérotrophe de *Chlorella sorokiniana* sur acétate, butyrate et lactate, seuls ou en mélange.

Croissance de *C. sorokiniana* sur acétate seul (A) et butyrate seul (B) à 0,1 $g_C.L^{-1}$ (●), 0,25 $g_C.L^{-1}$ (▲), 0,5 $g_C.L^{-1}$ (◆) et 1 $g_C.L^{-1}$ (■). Evolution de la biomasse algale ($g.L^{-1}$,●), de l'acétate ($g_C.L^{-1}$,◆), du butyrate ($g_C.L^{-1}$, ■) et du lactate ($g_C.L^{-1}$,▲) lors de la croissance sur un mélange acétate et butyrate (C) et acétate, butyrate et lactate (D). Les lignes en pointillés représentent les simulations du modèle.

Plusieurs mélanges d'acétate et de butyrate ont ensuite été utilisés (0,25/0,25 ; 0,4/0,1 et 0,9/0,1 en $g_C.L^{-1}$) pour soutenir la croissance hétérotrophe. Au cours de toutes ces expériences, un phénomène de diauxie a été observé. En effet, l'assimilation du butyrate commençait seulement après la dégradation totale de l'acétate par les microalgues (Figure 2-C). Ce phénomène peut être expliqué par la préférence pour l'acétate par rapport au butyrate

chez les microalgues. Ce phénomène de diauxie a été inclus dans le modèle via une constante d'inhibition (K_D, $2{,}10.10^{-10}$ $g_C.L^{-1}$) portant sur l'assimilation du butyrate. De plus, alors que le butyrate n'était pas consommé par les microalgues lorsqu'utilisé comme seul substrat, à 0,25 $g_C.L^{-1}$, (Figure 2-B), le butyrate a été consommé par les microalgues en croissance sur les mélanges d'acétate et de butyrate (Figure 2-C). Grâce à l'assimilation de l'acétate dans un premier temps, une certaine biomasse algale a pu être générée et ainsi permettre l'assimilation du butyrate dans un second temps.

La croissance de *C. sorokiniana* sur un mélange d'acétate (0,25 $g_C.L^{-1}$), de butyrate (0,45 $g_C.L^{-1}$) et de lactate (0,16 $g_C.L^{-1}$), tel que retrouvé dans un effluent de fermentation sombre (Rafrafi et al., 2013), a également été étudiée (Figure 2-D). Le lactate n'a pas été consommé par les microalgues. Sa présence n'a eu aucune influence sur la croissance sur acétate et butyrate. Le modèle a pu être utilisé sans modifications pour décrire cette croissance. La présence de lactate dans des effluents brut de fermentation sombre ne semble pas interférer avec la croissance algale.

Cette première partie de la thèse a permis d'évaluer et de modéliser la croissance de microalgues sur des effluents synthétiques de fermentation sombre. Un phénomène de diauxie a été mis en évidence ce qui a confirmé la préférence des microalgues pour l'acétate. De plus, le butyrate s'est avéré inhibiteur à de très faibles concentrations. Réduire l'inhibition du butyrate au sein d'un mélange d'AGVs a donc été identifié comme une étape clé pour permettre la faisabilité du couplage entre la fermentation sombre et l'hétérotrophie des microalgues.

Effets contrastés de la lumière et de la température sur la croissance de microalgues sur des mélanges d'AGVs

Afin de réduire l'inhibition de la croissance algale par le butyrate, l'objectif de la deuxième partie des résultats était d'étudier les interactions entre l'assimilation de l'acétate et celle du butyrate en fonction de la présence de lumière et de la température de culture. *C. sorokiniana* étant thermotolérante, avec une température optimale de croissance en autotrophie et en mixotrophie sur glucose ou acétate généralement obtenue pour des températures entre 35 et 37 °C (Janssen et al., 1999; Li et al., 2014; Van Wagenen et al., 2014b), il est suggéré qu'une croissance optimale sur AGVs serait observée à 35 °C en hétérotrophie. Par ailleurs, cette croissance à 35 °C pourrait en plus bénéficier de l'apport d'énergie supplémentaire par la lumière en culture mixotrophe.

Ainsi, les effets de (i) la lumière (avec ou sans lumière), (ii) de la température (25, 30 et 35 °C) et (iii) la combinaison entre présence de lumière et forte température (35 °C) sur les taux de croissance et rendements ont été testés lors de la croissance de *C. sorokiniana* sur un mélange d'acétate et de butyrate (à 0,3 $g_C.L^{-1}$ chacun). Afin de mieux comprendre les interactions entre les mécanismes d'assimilation de l'acétate et du butyrate, des contrôles avec un seul des deux substrats, acétate ou butyrate (à 0,3 $g_C.L^{-1}$) ont également été réalisés. De plus, des contrôles autotrophes, dans lesquels le bicarbonate était la seule source de carbone, ont été effectués permettant d'estimer la part de croissance en autotrophie (fixation du CO_2) de celle en hétérotrophie (assimilation des AGVs), lorsque la microalgue est cultivée en mixotrophie.

La préparation des cultures stock de microalgues, les mesures en métabolites, en biomasse algale et la vérification de l'axénie ont été réalisées comme présentée dans la Figure 1. De même, les milieux de culture pour les expérimentations étaient composés du même milieu BG11 modifié avec soit du $NaHCO_3$ (à 0,3 $g_C.L^{-1}$) pour l'autotrophie, soit de l'acétate et/ou du butyrate (à 0,3 $g_C.L^{-1}$ chacun) pour la mixotrophie et/ou hétérotrophie. Pour les conditions d'autotrophie et de mixotrophie, les fioles Erlenmeyer (transparentes) ont été placées en triplicata, à la lumière (123 ± 10 µmol photons.$m^{-2}.s^{-1}$) et celles pour l'hétérotrophie (noires) à l'obscurité, avec la même agitation (150 rpm).

C. sorokiniana a été cultivée sur acétate seul, sur butyrate seul et sur un mélange d'acétate et de butyrate en hétérotrophie (30 °C et 35 °C) et en mixotrophie (en présence de lumière à 25 °C et 35 °C). Ces résultats ont été comparés à l'hétérotrophie à 25 °C en réalisant des simulations du modèle décrit dans la première partie. Quelles que soient les conditions de croissance, une croissance diauxique sur acétate et butyrate a été observée (Figure 3). La croissance sur acétate puis celle sur butyrate ont pu être analysées indépendamment. Les paramètres utilisés pour la comparaison entre les différentes conditions sont les taux de croissances apparents (Tableau 1) et les rendements carbones apparents (Tableau 2).

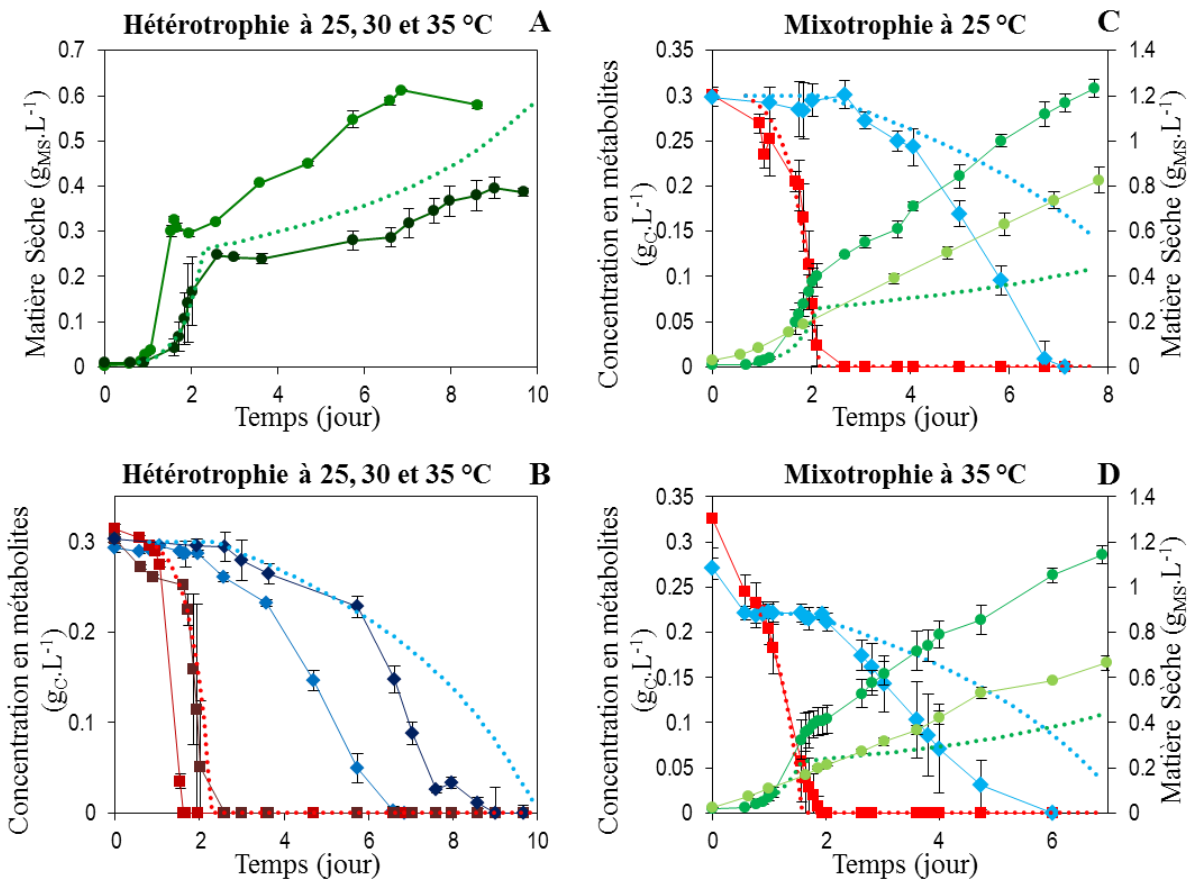

Figure 3. Influences de la température et de la lumière sur la croissance de *C. sorokiniana* sur un mélange d'acétate et de butyrate.

(A) Biomasse microalgale ($g.L^{-1}$) pendant la croissance hétérotrophe à 30 °C (●) et 35 °C (●). (B) Concentration en acétate et butyrate ($g_C.L^{-1}$) pendant la croissance hétérotrophe à 30 °C (■ ,◆) et à 35 °C (■ , ◆). (C) et (D) Biomasse mixotrophe ($g.L^{-1}$, ●), concentrations ($g_C.L^{-1}$) en acétate (■), en butyrate (◆) et biomasse autotrophe (contrôle) ($g.L^{-1}$, ●) durant la croissance en présence de lumière à 25 °C et 35 °C. Les simulations de la croissance hétérotrophe à 25 °C (ligne pointillée verte), de la concentration en acétate (ligne pointillée rouge) et en butyrate (ligne pointillée bleue) sont représentées.

La température et la présence de lumière ont eu un effet positif sur le taux de croissance sur acétate, utilisé comme seule source de carbone (Tableau 1). Les maxima ayant été atteints à 35 °C que ce soit en hétérotrophie (5,88 j^{-1}) ou en mixotrophie (5,65 j^{-1}). Ces résultats ont confirmé qu'une température élevée, proche de son optimum théorique, pouvait favoriser la croissance de *C. sorokiniana*.

Tableau 1. Taux de croissance sur acétate et butyrate en fonction de la température et de la présence de lumière.

C. sorokiniana a été cultivée sur acétate seul (A seul), butyrate seul (B seul) et sur un mélange d'acétate et de butyrate (A+B) en hétérotrophie (absence totale de lumière) et en mixotrophie (en présence de lumière) à différentes températures. Les taux de croissance apparent (μ_{app}) et les taux de productivité (r_{lin}**) atteints lors de la dégradation de l'acétate et du butyrate sont présentés.

		Hétérotrophie			Mixotrophie	
	Conditions de croissance	25 °C (modèle)	30 °C	35 °C	25 °C	35 °C
μ_{app} (j^{-1}) sur acétate	A seul	2,23*	4,65 ± 0,16	5,88 ± 0,39 b	4,14 ± 0,35	5,65 ± 0,55
	A + B		4,12 ± 0,19	3,17 ± 0,45	2,68 ± 0,12	2,53 ± 0,16
μ_{app} (j^{-1}) et r_{lin}** ($g.L^{-1}.j^{-1}$) sur butyrate	B seul	0,16*	0,13 ± 0,01	Pas de croissance	0,14 ± 0,00**	0,18 ± 0,01**
	A + B		0,16 ± 0,01	0,11 ± 0,02	0,16 ± 0,01**	0,16 ± 0,00**

* : Les taux de croissance obtenus à 25 °C en hétérotrophie sont ceux estimés via l'utilisation du modèle présenté dans la partie précédente. Il s'agit de taux de croissance maximum.

** : Pendant la dégradation du butyrate en mixotrophie, la croissance algale était linéaire. Ainsi, les valeurs représentent les taux de productivité (r_{lin}) de biomasse et sont exprimées en $g.L^{-1}.j^{-1}$.

Tableau 2. Rendement de carbone sur acétate (A) et butyrate (B) en fonction de la température et de la présence de lumière.

Les rendements apparents (Y_{app} en g_C de biomasse par g_C d'AGVs dégradés) ont été calculés en estimant que 50% de la biomasse algale était constituée de carbone (Chen and Johns, 1996).

		Hétérotrophie			Mixotrophie	
	Conditions de croissance	25 °C (modèle)	30 °C	35 °C	25 °C	35 °C
Y_{app} sur acétate ($g_C.g_C^{-1}$)	A seul	0,42	0,58 ± 0,04	0,64 ± 0,06	0,8 ± 0,05 ($X_{hét}$: 70%)	0,71 ± 0,03 ($X_{hét}$: 85%)
	A + B		0,51 ± 0,01	0,41 ± 0,02	0,79 ± 0,04 ($X_{hét}$: 61%)	0,6 ± 0,06 ($X_{hét}$: 60%)
Y_{app} sur butyrate ($g_C.g_C^{-1}$)	B seul	0,56	0,42 ± 0,03	Pas de croissance	1,69 ± 0,02 ($X_{hét}$: 26%)	1,61 ± 0,03 ($X_{hét}$: 45%)
	A + B		0,56 ± 0,01	0,28 ± 0,03	1,19 ± 0,11 ($X_{hét}$: 38%)	1,48 ± 0,02 ($X_{hét}$: 38%)

$X_{hét}$: En condition de mixotrophie (présence de lumière), le pourcentage du rendement dû uniquement à l'assimilation des AGVs ($X_{hét}$) a été estimé en retranchant la fraction autotrophe (fixation du CO_2), estimée via le contrôle en autotrophie stricte, au rendement apparent (Y_{app}).

Cependant, la présence de butyrate dans le mélange d'AGVs à 35 °C a réduit ces taux de croissance sur acétate, environ d'un facteur 2, en hétérotrophie et en mixotrophie (Tableau 1). Ainsi, dans le cas du mélange d'AGVs, les taux de croissance sur acétate étaient supérieurs en hétérotrophie à 30 °C (4,65 j^{-1}) qu'à 35 °C et en mixotrophie à 25 °C (2,68 j^{-1}) qu'à 35 °C. De la même manière, alors que la plus haute température testée (35 °C) a entrainé des rendements élevés en carbone sur acétate seul, la présence de butyrate a réduit ces rendements lors de la croissance sur des mélanges d'AGVs (Tableau 2). Il est suggéré que pour les microalgues en croissance très rapide, une perturbation telle que la présence de butyrate ait des conséquences dramatiques sur cette croissance. En effet, dans des conditions de croissance moins rapides

(hétérotrophie à 30 °C et mixotrophie à 25 °C), l'effet négatif lié à la présence de butyrate semblait moins prononcé. Il est à noter que la plus forte biomasse obtenue, ainsi que les rendements en biomasse, en mixotrophie par rapport à l'hétérotrophie (Figure 3 et Tableau 2) est probablement liée à la co-assimilation de carbone organique et de carbone inorganique (CO_2). En soustrayant la fraction autotrophe, estimée via le contrôle en autotrophie stricte sur bicarbonate, à la biomasse totale mixotrophe, il a ainsi été estimé que 15 à 30% des rendements sur acétate en mixotrophie étaient effectivement dus à l'assimilation du CO_2.

Lors de la croissance en hétérotrophie sur butyrate seul, seule la température à 30 °C a eu un effet positif sur la croissance comparée à celle à 25 °C (où aucune croissance n'aurait été observée d'après une simulation du modèle) (Figure 3). A 35 °C, aucune croissance n'a été observée (Figure 3). A 25 °C, le taux de croissance maximal sur butyrate, μ_{b_max}, 0,16 j^{-1}, (Equation 2), ne peut être atteint que pour une concentration en butyrate de 0,05 $g_C.L^{-1}$ (S_{b_opt}) (voir première partie des résultats). D'après la simulation du modèle (Figure 3-A et B), cette concentration n'a été obtenue qu'après 9,5 jours de croissance. A 30 °C, le taux de croissance apparent (0,16 j^{-1}) a été calculé pour une concentration en butyrate de 0,29 $g_C.L^{-1}$ atteinte après seulement 2 jours de culture. La dégradation complète du butyrate a ainsi été réduite de 3 jours en hétérotrophie à 30 °C par rapport à 25 °C (Figure 3). Contrairement à ce qui était attendu, la croissance à haute température, 35 °C, n'a pas permis de réduire l'inhibition du butyrate sur la croissance algale.

En présence de lumière, la croissance de *C. sorokiniana* sur butyrate seul et sur un mélange acétate et butyrate à 25 °C a bénéficié de la croissance autotrophe sur CO_2 (Figure 3). En effet, comme indiqué dans le Tableau 2, plus de 60% de la biomasse formée lors de la dégradation du butyrate a été induite par la fixation du CO_2. Grâce à cette co-assimilation de carbone organique et inorganique, l'assimilation du butyrate a été raccourcie de 3 jours comparée à l'hétérotrophie à 25 °C (Figure 3-C). Par contre, contrairement à ce qui était attendu, l'augmentation de la température en mixotrophie de 25 à 35 °C n'a pas apporté d'avantages majeurs pour la croissance sur butyrate (Figure 3, Tableau 1, Tableau 2).

Cette deuxième partie de la thèse a permis de mettre en évidence que l'utilisation de la température optimale théorique (35 °C) pour la croissance de *C. sorokiniana* a eu un impact négatif sur la croissance hétérotrophe et mixotrophe lorsqu'un mélange d'acétate et de butyrate est utilisé. Cependant, la croissance de *C. sorokiniana* a été améliorée (par rapport à 25 °C) avec succès en hétérotrophie à une température sub-optimale (30 °C) via

l'augmentation du taux de croissance sur acétate (4,1 j^{-1}) et la réduction de l'inhibition du butyrate sur la croissance. Un effet positif de la lumière sur l'assimilation du butyrate a également été observé à 25 °C. Cet effet de la lumière est lié à une augmentation de la biomasse algale (atteignant 1,14 $g.L^{-1}$) à travers la combinaison de la croissance par hétérotrophie et autotrophie. En conclusion, *C. sorokiniana* pourrait croître avec succès sur des effluents de fermentation sombre contenant acétate et butyrate à une température sup-optimale (30 °C) et en présence de lumière.

Compétition pour les AGVs entre microalgues et bactéries fermentaires lors de la croissance sur un effluent brut de fermentation sombre

A ce jour, très peu d'études se sont focalisées sur l'utilisation d'effluents de fermentation sombre non stérilisés, contenant des bactéries fermentaires, pour la croissance de microalgues (Hu et al., 2013, 2012a; Venkata Mohan and Prathima Devi, 2012). La croissance bactérienne et son éventuel impact sur celle des microalgues, via notamment une compétition pour les métabolites fermentaires, n'ont jamais été étudiés alors que les conséquences peuvent s'avérer majeures sur le succès du couplage. Ainsi, dans la troisième partie de la thèse, l'impact sur la croissance microalgale de la présence de bactéries fermentaires provenant d'une culture batch de fermentation sombre a été étudié. Etant donné que les microalgues ont une croissance rapide sur acétate (partie 1) et que la majorité des bactéries fermentaires devraient être anaérobies strictes (*Clostridium* sp.), il est supposé que les microalgues pouvaient s'avérer être compétitives pour les AGVs. Un effluent de fermentation non stérilisé (obtenu après fermentation sombre du glucose), composé d'acétate (0,29 $g_C.L^{-1}$) et de butyrate (0,69 $g_C.L^{-1}$), a été utilisé pour évaluer les interactions entre microalgues et bactéries et ces résultats ont été comparés à la croissance algale en condition axénique sur le même effluent stérilisé.

La mise en place de l'expérimentation est résumée succinctement dans la Figure 4.

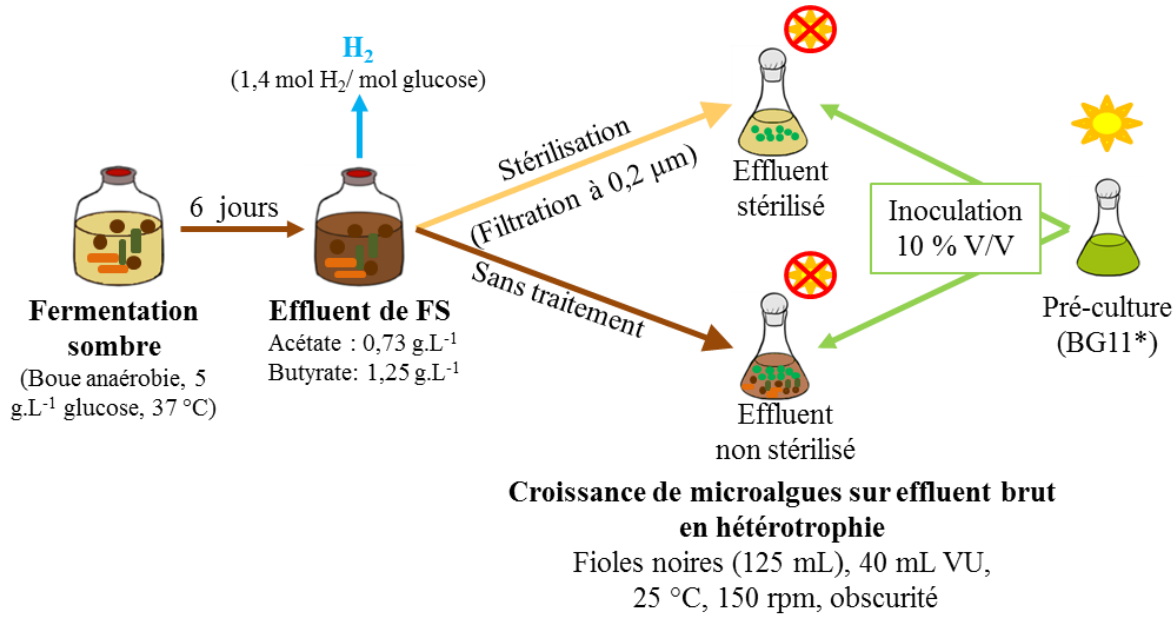

Figure 4. Représentation schématique de l'expérience.

BG11*: milieu BG11 modifié, VU: Volume utile; FS: Fermentation sombre. L'expérience a été réalisée en triplicata (3 fioles par condition).

Dans un premier temps, la croissance de *C. sorokiniana* a été suivie par PCR quantitative en utilisant des amorces spécifiques au phylum *Chlorophyta* dans l'effluent stérilisé et non stérilisé (Figure 5-A). Une augmentation similaire de la biomasse algale, exprimée sur la Figure 5-A en log(copies d'ADNr 18S.mL^{-1}), a été observée au cours des 2,7 premiers jours sur milieu stérilisé et non stérilisé. La biomasse maximale mesurée était de 0,33 g.L^{-1}. Le rendement carbone associé était de 0,55 g_C de biomasse par g_C d'acétate. Ce rendement est supérieur à celui trouvé sur milieu synthétique (0,42 $g_C.g_C^{-1}$, Tableau 2). Cette différence pourrait être expliquée par la présence d'autres composés organiques provenant de l'effluent, par exemple des acides aminés ou des vitamines (Singhania et al., 2013), qui pourraient être également assimilés par les microalgues. Au cours de ces 2,7 jours, l'acétate a été entièrement dégradé en milieu stérilisé tout comme en milieu non stérilisé. Ces résultats ont suggéré que (i) la croissance de *C. sorokiniana* sur effluent non stérilisé est probablement liée à la dégradation de l'acétate, comme sur effluent stérilisé, et que (ii) la présence de bactéries fermentaires n'a pas impacté la croissance des microalgues. Dans les deux conditions, aucune croissance n'a été observée après dégradation de l'acétate. Sur milieu stérilisé, le butyrate n'a pas été consommé. Sa concentration initiale étant élevée, il est possible qu'elle ait empêché la croissance de *C. sorokiniana*. En milieu non stérilisé, le butyrate a été entièrement dégradé en 9 jours, sans croissance algale. Les bactéries fermentaires étaient très probablement responsables de cette dégradation du butyrate.

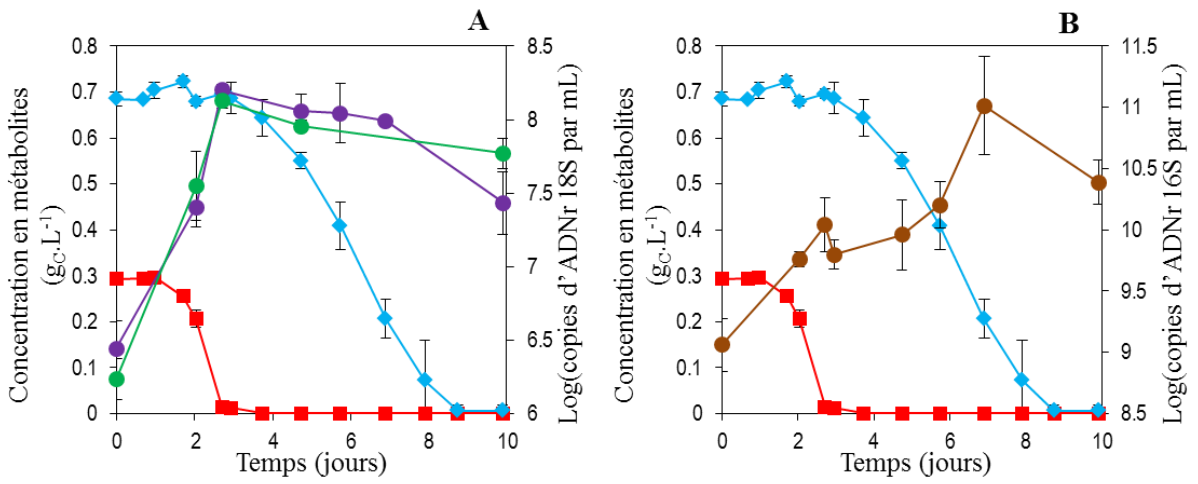

Figure 5. Croissance des microalgues et des bactéries fermentaires sur effluent de fermentation.

(A) Logarithme du nombre de copies d'ADNr 18S (microalgues) pendant la croissance hétérotrophe sur milieu stérilisé (●) et non stérilisé (●) avec les concentrations, en $g_C.L^{-1}$, d'acétate (■) et de butyrate (◆) pendant la croissance sur effluent non stérilisé. (B) Logarithme du nombre de copies d'ADNr 16S (bactéries) (●), concentration en acétate (■) et en butyrate (◆) pendant la croissance sur effluent non stérilisé.

La croissance bactérienne a également été suivie par qPCR en utilisant des amorces génériques pour les bactéries et deux phases de croissance ont été observées (Figure 5-B). La première phase a eu lieu pendant la dégradation de l'acétate (2,7 jours) et la seconde pendant la dégradation du butyrate. Etant donné que les microalgues étaient très probablement responsables de la dégradation de l'acétate, les bactéries présentes dans l'effluent ont pu se développer sur d'autres composés organiques de fermentation ou des exsudats de microalgues (type exopolysaccharides). La croissance bactérienne observée pendant la dégradation du butyrate (sans croissance de *C. sorokiniana*) a confirmé que le butyrate a été totalement consommé par les bactéries fermentaires. Aucune compétition pour le butyrate, entre microalgues et bactéries, n'a été observée.

Un séquençage des échantillons a été réalisé afin d'identifier les espèces bactériennes présentes au cours de l'expérience (début de l'expérience, fin de dégradation de l'acétate et fin de la culture après dégradation du butyrate) (Figure 6).

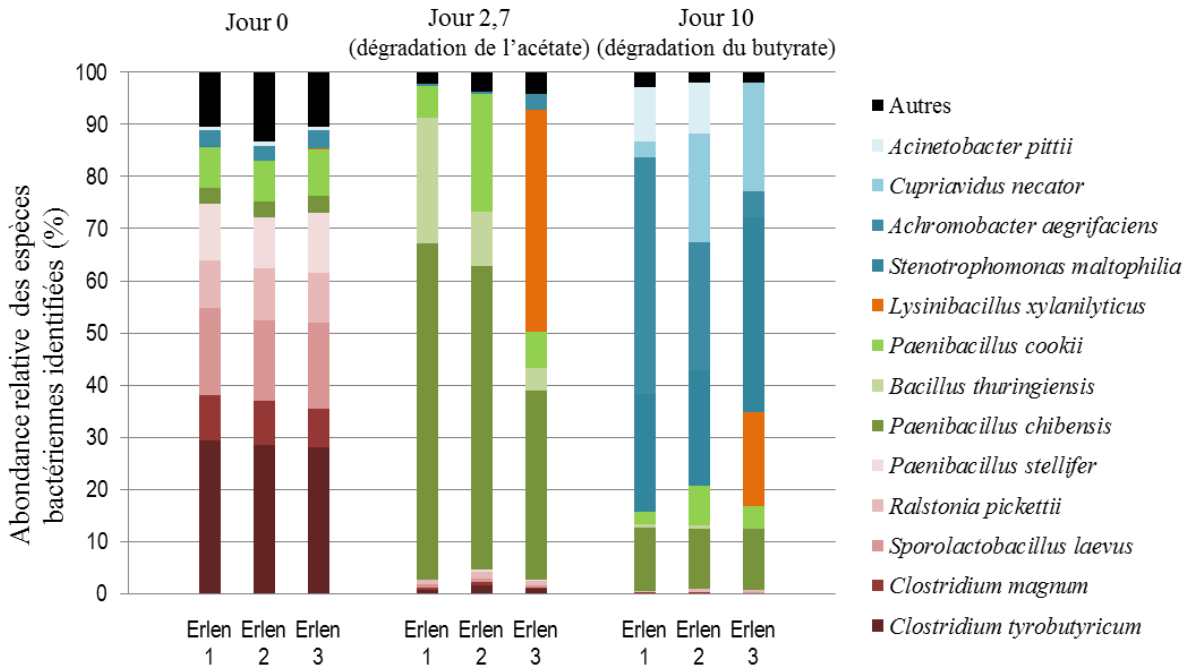

Figure 6. Diversité de la communauté bactérienne pendant la croissance aérobie sur effluent de fermentation non stérilisé.

Les taxons avec moins de 2% d'abondance relative individuelle ont été regroupés dans le groupe « Autres ».

Les espèces bactériennes majoritaires étaient anaérobies strictes en début de culture (*Clostridium* sp. et *Sporolactobacillus* sp.), puis des espèces anaérobies facultatives ont émergé à la fin de la dégradation de l'acétate (*Paenibacillus* sp.) et, à la fin de la culture, des espèces aérobies strictes sont devenues majoritaires (*Stenotrophomonas maltophilia*). Dans deux des fioles, une espèce très proche de *Paenibacillus chibensis* (99% d'identité) était majoritaire à la fin de la dégradation de l'acétate. D'après Shida et al. (1997), *Paenibacillus chibensis* est incapable de croître sur acétate. Dans la troisième fiole, une espèce proche de *Lynsinibacillus xylaniticus* était majoritaire à ce moment de la culture. Etant donné que la dégradation de l'acétate était la même dans les trois fioles (Figure 6), il est probable que cette espèce n'était pas non plus impliquée dans la dégradation de l'acétate. Ces observations confirment la suggestion selon laquelle *C. sorokiniana* était très compétitive pour l'acétate et entièrement responsable de sa dégradation. A la fin de la culture, après dégradation complète du butyrate, des espèces proches de *Stenotrophomonas maltophilia* et *Cupriavidus necator* étaient majoritaires (Figure 6). *Cupriavidus necator* est connue pour assimiler le butyrate (Grousseau et al., 2013). L'émergence de ces espèces pendant la dégradation du butyrate, durant laquelle aucune croissance de *C. sorokiniana* n'a été observée, a confirmé que ces

espèces aérobies strictes (Assih et al., 2002), initialement présentes dans l'effluent, étaient responsables de l'assimilation du butyrate.

Cette troisième partie de thèse a permis de montrer que les deux obstacles majeurs à l'industrialisation de la culture hétérotrophe, les coûts associés à l'utilisation du glucose et à la stérilisation du milieu de culture, pouvaient être contournés en utilisant un effluent brut de fermentation sombre. D'une part, l'assimilation de l'acétate par *C. sorokiniana* a été suffisamment rapide pour permettre aux microalgues de gagner la compétition par rapport aux bactéries. D'autre part, le changement drastique des conditions de culture entre la fermentation sombre et l'hétérotrophie, en passant de conditions anaérobies à 37 °C à aérobies à 25 °C, a défavorisé les bactéries fermentaires en faveur de la croissance microalgale et est une solution prometteuse afin d'éviter la stérilisation du milieu de culture.

Conclusions et perspectives

Le couplage entre la fermentation sombre, productrice d'**hydrogène** et **d'acides organiques**, et **l'hétérotrophie des microalgues**, productrices de **lipides**, est une nouvelle approche permettant une double valorisation de déchets et d'effluents. Au cours de cette thèse, plusieurs aspects de la faisabilité du couplage ont été étudiés :

- la capacité des microalgues (*Chlorella sorokiniana* et *Auxenochlorella protothecoides*) à croître sur divers mélanges synthétiques d'acétate, de butyrate et de lactate,
- les effets de la présence de la lumière et de fortes températures de croissance sur l'inhibition du butyrate,
- et la compétition potentielle pour les acides organiques entre les microalgues et bactéries fermentaires en croissance sur des effluents bruts de fermentation.

Ces travaux ont permis de mettre en évidence que :

- la concentration en butyrate et le rapport acétate:butyrate étaient des paramètres clés pour favoriser et maitriser la croissance hétérotrophe sur AGVs, grâce au développement d'un modèle permettant de caractériser et de prédire la croissance algale sur mélanges d'AGVs,
- la présence de lumière (mixotrophie) et une température à une valeur sub-optimale (30 °C) étaient des paramètres abiotiques permettant de réduire significativement l'inhibition du butyrate,

- les microalgues étaient capables de croître sur l'acétate provenant d'effluents de fermentation non stérilisés malgré la présence de bactéries fermentaires.

Afin de limiter l'inhibition du butyrate sur la croissance des microalgues, des recherches futures sur la compréhension de l'impact du butyrate sur les mécanismes cellulaires, comme l'acidification du pH cytosolique, sont nécessaires. De plus, la prise en compte de la présence de la lumière et de la température dans le modèle établi dans cette thèse pour la croissance hétérotrophe est une étape importante afin de maîtriser au mieux la culture des microalgues en fermenteurs. En outre, ce travail de thèse s'est focalisé sur l'étude de la croissance de microalgues, riches en lipides, utilisant les effluents de fermentation mais sans avoir évalué l'impact sur la production de lipides. Ainsi, l'étude de la capacité des microalgues à produire des lipides de réserve en fonction de la composition des effluents de fermentation sombre (compositions en métabolites et leur ratio, présence de bactéries fermentaires) est également une étape cruciale dans le développement du couplage de ces deux procédés.

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Introduction

Production of microalgae has gained a lot of interest in the past decades due to their ability to synthesize a wide range of molecules with many industrial applications. Indeed, microalgae can produce polysaccharides, lipids, unsaturated long-chain fatty acids and pigments. These products can be used industrially as food supplements (DHA), cosmetics (antioxidant such as astaxanthin), drugs (antibiotics), animal feeding for aquaculture, food colorants (β-carotene), for wastewater treatment (nutrient removal and CO_2 mitigation) or energy (biodiesel from lipids or bioethanol from starch) (Raja et al., 2014; Wu et al., 2014). They are particularly interesting for biofuel production since they offer higher biomass and lipid yields and use less land when compared to oil crops. As an illustration, Georgianna and Mayfield (2012) assessed that microalgae could produce the same amount of oil than palm oil using six times less area. Nevertheless, suppling biodiesel from microalgae for the European Union (0.4 billion m^3 per year), the land required to grow microalgae would be similar to the surface area of Portugal (Wijffels and Barbosa, 2010). Therefore, an increase in productivity, so far estimated at 40 000 liters per hectare per year, and a decrease in general production cost, by a factor of 10, are still required (Wijffels and Barbosa, 2010).

Different cultivation modes exist for growing microalgae. Microalgae can be cultivated autotrophically (with light as the energy source and CO_2 as the carbon source), mixotrophically (with light as the main energy source and both CO_2 and organic compounds as carbon sources) and heterotrophically (in the dark with only organic compounds as sources of both energy and carbon). Growing microalgae in darkness and on organic compounds presents several advantages compared to autotrophic cultivation systems: (1) higher biomass density can be achieved 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., 2013b); (3) higher lipid yields can be achieved in heterotrophic cultures, improving economic competitiveness of microalgae biofuels (Wan et al., 2012); (4) higher volumetric production (Venkata Mohan et al., 2015). Furthermore, industrial fermenters, designed for bacteria and yeasts, are readily available to grow microalgae heterotrophically whereas photobioreactors design is still a challenge (Venkata Mohan et al., 2015). Overall, the absence of light significantly reduces both the cost and the cultivation area required for the overall process (Perez-Garcia et al., 2011b).

Nevertheless, industrial production of heterotrophic microalgae is still far from being competitive with traditional agriculture or fossil fuels, because of the high cost of glucose, used usually as the carbon source. Heterotrophically grown microalgae are currently only economically viable for high-value molecules such as DHA production from glucose (Fei et al., 2014). Roquette (France), Fermentalg (France) and Solazyme (USA) are the world leaders in heterotrophic production of such high-value molecules. To be able to produce low-value molecules, such as biofuel, it is mandatory to use low-cost carbon substrate (Liu and Chen, 2014).

Wastewaters containing glucose, such as whey permeate or cane molasses, glycerol (from biodiesel production effluents) or acetate (from dark fermentation effluents) are considered as the most promising sources of low cost carbon substrates (Espinosa-Gonzalez et al., 2014). However, growing microalgae on agricultural waste such as cane molasses implies high cost because of the necessary pretreatments to release sugars (Yan et al., 2011). 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 accumulates in dark fermentation processes. Interestingly, microalgae can easily convert acetate into acetyl-CoA which is the main precursor for lipid synthesis in microalgae (Ramanan et al., 2013).

In recent years, coupling bacterial dark fermentation (DF), which produces hydrogen, and heterotrophic cultivation of microalgae, which produces lipids, has been suggested as being a very promising sustainable approach for producing gaseous and liquid biofuels (Park et al., 2014). DF is a simple process that can convert a wide range of solid waste and effluents into hydrogen, a high-energy gas (Sambusiti et al., 2015). During DF, anaerobic bacteria break down complex carbon compounds from the organic matter contained in waste (e.g., food waste or agricultural waste) and wastewater (e.g., wastewater from agriculture, paper or sugar industries) into simple organic acids (Lee et al., 2014). Acetic and butyric acids are the two main end-products of DF and can be further used as low cost carbon sources to sustain the growth of heterotrophic microalgae (Ren et al., 2014b). The main advantage of DF is that organic carbon compounds from complex waste that are not directly available to microalgae degradation are simplified into low molecular weight volatile fatty acids (VFAs) (Singhania et al., 2013). Moreover, thanks to nitrogen and phosphorus mineralization into ammonium (N) and orthophosphate during DF (Cai et al., 2013), effluents also contain substantial amounts of N and P that are required to sustain the heterotrophic growth of microalgae.

Very recently, few studies investigated the feasibility of using dark fermentation effluents, composed of acetate and butyrate, to sustain microalgae growth and showed promising perspectives in terms of production of microalgae biomass and lipids (Cho et al., 2015; Fei et al., 2014; Hongyang et al., 2011; Hu et al., 2013, 2012a; Liu et al., 2013, 2012; Moon et al., 2013; Ren et al., 2014b; Venkata Mohan and Prathima Devi, 2012). However, considering all these studies, the performances of heterotrophic and mixotrophic growth of microalgae on a mixture of organic substrates are still difficult to estimate because of the lack of a clear growth behavior (substrate inhibition and growth kinetics) when a mixture of VFAs is used.

Deeper knowledge on microalgae growth on VFAs, used in mixtures, is required to better understand and predict microalgae growth on dark fermentation effluents and show the feasibility of the coupling. Indeed, crucial knowledge such as yields and kinetics associated with acetate and butyrate or CO_2 uptakes (under mixotrophic conditions), butyrate inhibition, interactions between fermentative bacteria and microalgae are still lacking. The main objective of this PhD was to characterize microalgae heterotrophic growth response to different mixtures of VFAs by first studying microalgae growth response to various acetate and butyrate mixtures using synthetic effluent with changing abiotic parameters (light and temperature) and then using raw DF effluent.

The thesis is composed of five chapters. The first chapter introduces DF processes and reviews current knowledge on their coupling with microalgae growth. According to this literature review, it was first hypothesized that microalgae growth on mixtures on VFAs was inhibited at low acetate:butyrate ratio due to both a slow uptake of butyrate and a concentration-related inhibition of butyrate on microalgae growth and acetate uptake. These hypotheses have been experimentally tested in Chapter 2. In this chapter, the interactions between acetate and butyrate uptakes during heterotrophic growth of microalgae have been characterized using a modelling approach. Then, the influence of light and temperature, two abiotic parameters identified as key parameters to promote microalgae growth on DF effluent and more particularly on butyrate, was studied in Chapter 3. On the one hand, light could trigger ATP synthesis and biomass synthesis, through CO_2 fixation, which could accelerate butyrate exhaustion. On the other hand, under optimal temperature, microalgae growth, and subsequently VFAs uptake rates and yields, could benefit from the enzymatic activity enhancement and the reduction of the requirement for thermoregulation. Possible competition for organic substrates, VFAs, between microalgae and fermentation bacteria originated from

DF was investigated in Chapter 4. Finally, conclusions and perspectives are discussed, from both fundamental and applied point of views, in Chapter 5.

The results of this PhD were valorized in the following publications:

- Turon V, Baroukh C, Trably E, Fouilland E, Steyer J-P, *Use of fermentative metabolites for heterotrophic microalgae growth: Yields and kinetics*, **published in** Bioresource Technology (2015), 175: 342-344.

- Turon V, Trably E, Fayet A, Fouilland E, Steyer J-P, *Raw dark fermentation effluent to support heterotrophic microalgae growth: microalgae successfully outcompete bacteria for acetate*, **published in** Algal Research (2015), 12:119-125.

- Turon V, Trably E, Fouilland E, Steyer J-P, *Growth of Chlorella sorokiniana on a mixture of volatile fatty acids: contrasted effect of light and temperature*, **published in** Bioresource Technology (2015), 198: 852-860.

- Turon V, Trably E, Fouilland E, Steyer J-P, *Potentialities of dark fermentation effluent as substrates for microalgae growth: A review*, **submitted to** Process biochemistry in October 2015.

- Baroukh C, Turon V, Trably E, Fouilland E, Steyer J-P, Bernard O, *Towards metabolic modeling of heterotrophic and mixotrophic microalgal growth on wastes*, **to be submitted to** PNAS

The results obtained in this PhD have also been presented in international conferences as listed below:

- Turon V, Fayet A, Trably E, Fouilland, E, Steyer, J-P, *Successful heterotrophic microalgae growth on raw fermentation digestates: influence of bacteria*, International Conference on Algal Biorefinery, 2014 – Copenhagen (Danemark) (**oral presentation**)

- Turon V, Trably E, Fouilland, E, Steyer, J-P, *New insights on heterotrophic microalgae growth on dark fermentation effluents*. Alg'n'Chem, 2014 – Montpellier (France) (**oral presentation**)

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Chapter 1

Literature review

1.1 Converting waste into H_2 and/or VFAs by dark fermentation

Nowadays, both volatile fatty acids (VFAs) and hydrogen productions are based on fossil fuels which precludes their usages for low costs applications as biofuel or electricity generation (Ghimire et al., 2015; Lee et al., 2014). On the other hand, energy and chemicals production not relying on traditional oil refinery that emits high amounts of greenhouse gases is one of the most crucial challenges of the 21th century. Another main challenge is to reduce the pollution unleashed by the constant increase of human waste. Environmental biorefineries, as sustainable platforms "producing bio-based products (food, feed and chemicals) and energy (fuels, heat and electricity) from biomass" (Chang et al., 2010), have been identified as a solution for both these issues. Combined VFAs and hydrogen production from waste by biological processes is a perfect example of an environmental biorefinery. Indeed, VFAs can be further used as substrates for the production of chemicals (bioplastics) and energy (biodiesel and electricity) by various microorganisms (Lee et al., 2014). Moreover, hydrogen is considered as one of the most promising solution of replacement of fossils fuels since it is a very high energy carrier (122 kJ.g^{-1}) and its oxidative combustion produces only water vapor

as end-product (Guo et al., 2010). Last but not least, hydrogen could be used as a sustainable source of electricity for wide usages such as transportation through fuel cells technologies (Ghimire et al., 2015).

Hydrogen production by biological processes can be classified into two categories: the light-dependent and the light-independent technologies (Ghimire et al., 2015). The first group is carried out by photosynthetic organisms such as microalgae, cyanobacteria and purple non-sulfuring bacteria. Light-independent processes known as dark fermentation (DF) processes are carried out by fermentative organisms that convert anaerobically organic carbon compounds into hydrogen and small organic acids (VFAs) and alcohols. VFAs can also be produced through acidogenic fermentation (AF), a special type of dark fermentation where operating conditions are slightly different, with no hydrogen production. The principles of DF and AF as well as the downstream uses of their effluents are presented in this section.

1.1.1 Principles of DF and AF fermentations in mixed cultures

1.1.1.1 From waste to hydrogen and VFAs: DF and AF steps

Anaerobic digestion (AD) is a biological process carried out by a complex microbial community that converts complex organic matter into a simple biogas composed of CH_4 and CO_2, in four steps (Figure 1-1). (1) During **hydrolysis**, the complex organic matter is degraded into simple monomers (monosaccharides, amino acids and fatty acids) by extracellular enzymes secreted by a broad range of strict anaerobic species, such as *Clostridium* sp., and facultative anaerobic species, such as *Acetobacter* sp. or *Streptococcus* sp. (2) During **acidogenesis**, fermentative bacteria, such as *Bacillus* sp., *Enterobacter* sp. and *Clostridium* sp., convert organic monomers into VFAs (acetate, propionate, butyrate), organic acids (lactate), alcohols (ethanol, butanol), hydrogen and CO_2. (3) In the following step, **acetogenesis** all organic metabolites (butyrate, ethanol, etc.) are transformed into acetate and hydrogen by acetogenic syntrophic bacteria such as *Synthrophomonas* sp. H_2 and CO_2 are also transformed into acetate by homoacetogenic bacteria such as *Acetobacterium* sp. and *Clostridium* sp. (4) Finally, **methanogenesis** is carried out by methanogenic archaea that consume acetate, CO_2 and H_2 to produce CH_4.

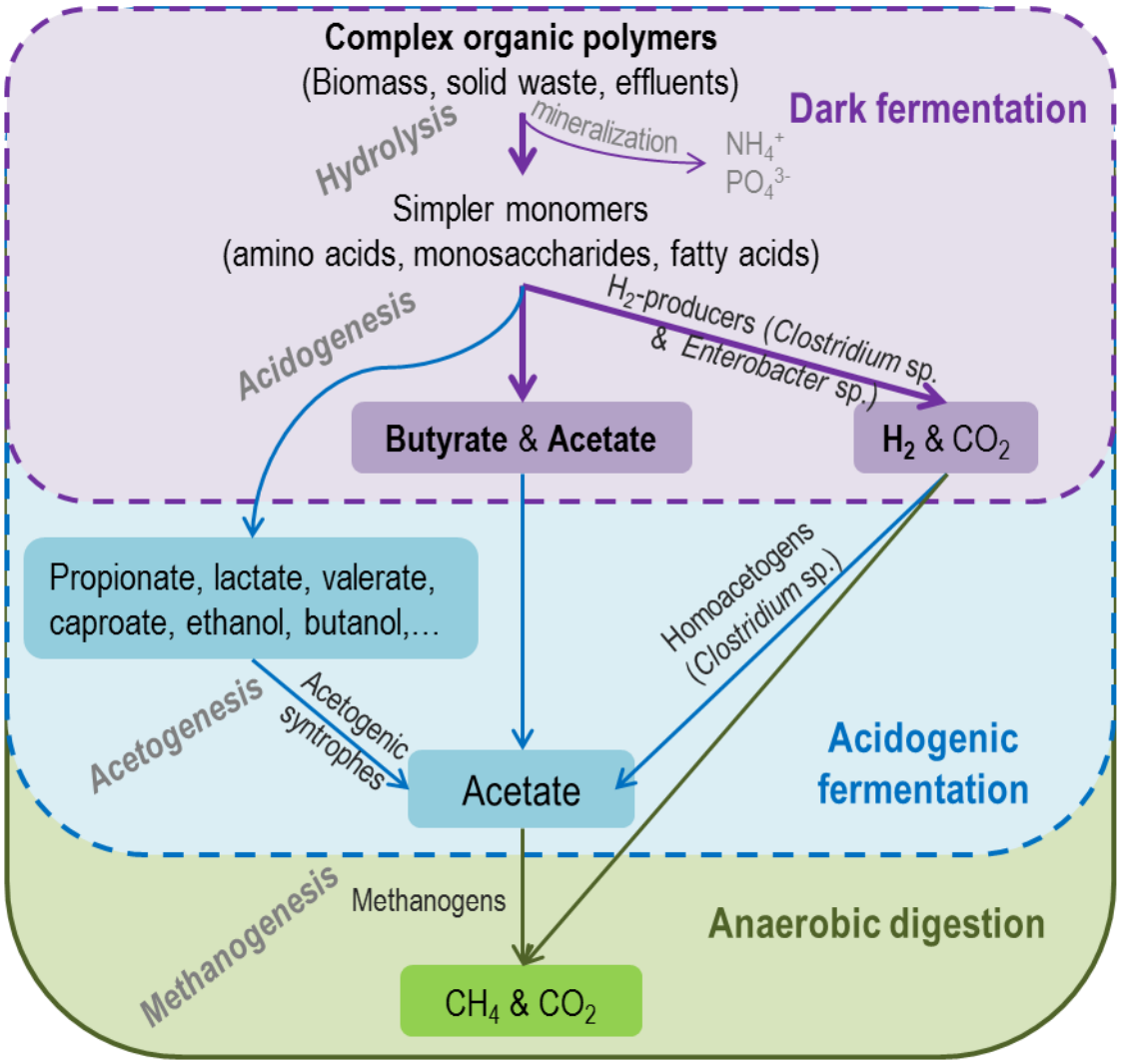

Figure 1-1. Links between anaerobic digestion, acidogenic fermentation and dark fermentation.

DF is part of the full anaerobic digestion process, ending with hydrogen production after acidogenesis (Figure 1-1). AF targets VFAs production instead of H_2 production (Syngiridis et al., 2013). In that particular process, H_2 is exhausted by favoring acetogenesis to maximize the acetate production (Saady, 2013).

1.1.1.2 Microbiology of hydrogen and VFAs production in mixed cultures

Hydrogen and VFAs productions by DF and AF have been intensively studied using either mixed cultures from soils or anaerobic digesters or co-cultures (two selected species) or pure cultures of hydrogen-producing species (Lee et al., 2014; Monlau et al., 2013). Mixed cultures allow the use of a broad range of unsterilized waste and lower the cost of the overall process due to the lack of aseptic conditions (Wong et al., 2014). Moreover, the diversity of the microbial community can stabilize the degradation of waste (several species can perform the same task according to their affinity with the substrate) (Jobard et al., 2014). The main

disadvantage of using mixed culture is the presence of non-H_2-producing species which use the substrate or waste for other pathways (Ntaikou et al., 2010). The main advantages of co-cultures and single-cultures are the lack of undesired by-products and, consequently, higher hydrogen yield are achieved when compared to mixed cultures (Ntaikou et al., 2010). Unfortunately, these cultures require aseptic conditions which drastically increase the cost of the process and also preclude the use of a wide range of wastes as feedstocks. In addition, if complex waste is used as feedstock, one species might lack the entire set of enzymes required to appropriately degrade the organic matter (Jobard et al., 2014). Single and co-cultures have been intensively used to study dark fermentation of specific waste such as energy crops or lignocellulosic residues but mixed cultures are preferred when more complex waste, such as food waste or sugar factory wastewaters, are used (Ntaikou et al., 2010). Among the H_2-producing species, *Clostridium* sp. (strict anaerobes) and *Enterobacter* sp. (facultative anaerobes) have been the most studied species in single and co-cultures and are also the dominant H_2-producing species in mixed cultures (Monlau et al., 2013).

1.1.1.2.1 Hydrogen metabolism, associated theoretical yields and involved species

Hydrogenase enzymes are the key enzymes involved in the biological production of hydrogen. Their cellular role is to evacuate the excess of electrons issued from substrate oxidation, with protons, H^+, to generate H_2. Hydrogen production in mixed cultures is produced through two main pathways: the acetate and butyrate pathways (Hawkes et al., 2007). The main hydrogen producing fermentative pathways during DF are illustrated in Figure 1-2.

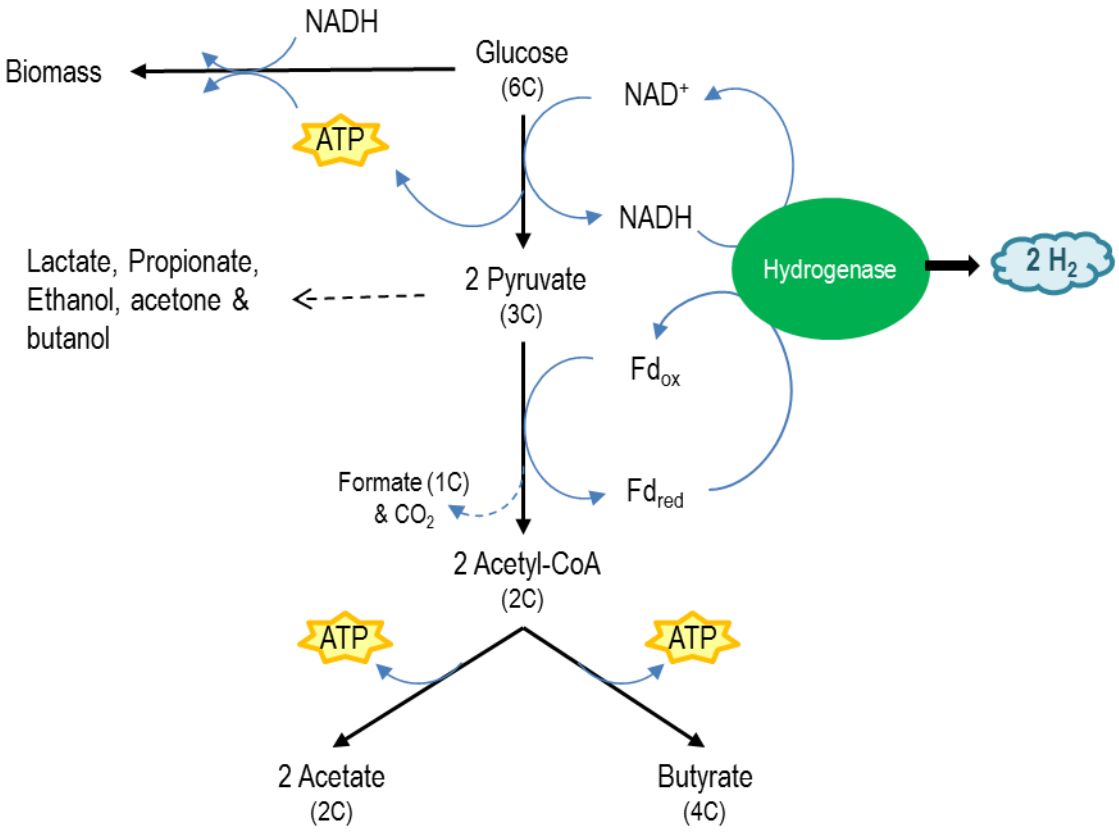

Figure 1-2. Production of H_2 through the acetate and the butyrate pathways.

The theoretical hydrogen yields of the acetate and butyrate pathways are 4 and 2 mol of hydrogen per mol of glucose consumed, respectively (Equation 1-1 and Equation 1-2).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \text{Equation 1-1}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad \text{Equation 1-2}$$

In mixed cultures, hydrogen is produced by both pathways and the molar ratio of acetate:butyrate depends on many parameters (bacterial species involved, pH, hydraulic retention time (HRT), etc.) (Hawkes et al., 2007; Lin et al., 2007). Few studies have shown that a high butyrate content, as opposed to a high acetate content, could be linked with a high hydrogen yield (Hawkes et al., 2007; Lin et al., 2007). Indeed, production of acetate through acetogenesis and/or homoacetogenesis is not correlated or negatively correlated with hydrogen production (Guo et al., 2013). Even though hydrogen production through the acetate pathway should theoretically lead to the highest hydrogen yield, low H_2 yield can be observed with high acetate production. Hawkes et al. (2007) suggested the following equation (Equation 1-3) to describe hydrogen production in mixed cultures with an average acetate:butyrate molar ratio of 0.66:

$$4C_6H_{12}O_6 + 2H_2O \rightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 2CO_2 + 10H_2$$ **Equation 1-3**

According to Equation 1-3 , the theoretical hydrogen yield in mixed cultures should be 2.5 mol of hydrogen per mol of glucose consumed. Practically, H_2 yield in mixed culture ranges broadly between 0.4 to 3 mol H_2/mol of glucose consumed (Ntaikou et al., 2010; Wong et al., 2014). In mixed cultures, *Clostridium sp*, a sporulating strict anaerobic bacteria, are usually the main H_2-producing bacteria (Wong et al., 2014). Among the *Clostridium* genus, *C. butyricum*, *C. acetobutylicum*, *C. tyrobutyricum* and *C. saccharolyticum* are the most representative members found in dark fermentation reactors operated under mesophilic conditions (20 - 45 °C) and with mixed cultures (Wong et al., 2014). Even though both acetate and butyrate pathways are used in mixed cultures, according to the dominant *Clostridium* species, one of these pathways may be favored (Lin et al., 2007). As an illustration, *C. butyricum* (Rafrafi et al., 2013) and *C. tyrobutyricum* (Zhu and Yang, 2004) are known to use the butyrate pathway preferentially. In thermophilic (50 – 70 °C) and hyperthemophilic (above 80 °C) conditions, hydrogen producers mostly belong to the genus *Thermoanaerobacterium* and *Thermotoga*, respectively. They produce high amounts of hydrogen with acetate as main end-product (Verhaart et al., 2010).

H_2 can also be produced in two steps through the acetate-formate pathway with a yield of 4 mol of H_2 per mol of glucose consumed (Equation Equation **1-4**). This pathway is typical to *Enterobacteria*, such as *Escherichia coli* under anaerobic conditions (Ntaikou et al., 2010).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2\,CH_3COOH + 2HCOOH + 2H_2$$
$$2HCOOH \rightarrow 2CO_2 + 2H_2$$ **Equation 1-4**

Another H_2-producing pathway is the acetate-ethanol pathway, with a yield of 2 mol of H_2 per mol of glucose consumed (Equation Equation 1-5). *Ethanoligenens sp*, strict anaerobic species, are known to produce hydrogen through this pathway.

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2$$ **Equation 1-5**

1.1.1.2.2 Hydrogen consumption or non-H_2 producing fermentative pathways, and related species

In mixed cultures, H_2 experimental yields are always lower than theoretical yields because of the presence of pathways consuming directly hydrogen or competitive non H_2-generating pathways (Ghimire et al., 2015; Guo et al., 2010).

The main hydrogen consumers are methanogenic archaea. Sulfate and nitrate reducers also directly consume H_2 to produce H_2S and NH_3, respectively. Hydrogen can also be used as an electron donor for propionate, caproate, succinate or acetate synthesis. For instance, homoacetogenic bacteria, such as *Clostridium aceticum*, can convert H_2 and CO_2 into acetate. This step is favorable to VFAs production and is therefore very important in AF processes. Competition for the substrate arises, for example, because of environmental changes, such as a pH change. *Clostridium* sp. can use different substrate to generate propionate, ethanol, lactate, valerate, formate, acetone or butanol when pH is low or at high concentrations of VFAs (Figure 1-2). In addition, some other species, such as lactic bacteria, *Lactobacillus* sp. or *Sporolactobacillus* sp., directly outcompete hydrogen producers for the substrate.

Nevertheless, at low concentration, facultative anaerobes can play a beneficial role in removing residual-oxygen in DF processes which subsequently favors the growth of *Clostridium* strict anaerobic bacteria and H_2 production (Hung et al., 2011). Non-H_2-producers can also degrade complex substrates, such as cellulolytic organic matter, into smaller molecules during the hydrolysis step providing readily available substrate for *Clostridium* species (Figure 1-1) (Hung et al., 2011).

1.1.2 Operating parameters influencing H_2 and VFAs production

1.1.2.1 Feedstocks for dark or acidogenic fermentation

As a definition, a sustainable feedstock for dark or acidogenic fermentation (DF and AF) should be abundant, readily available, cheap and highly biodegradable (Guo et al., 2010). Theoretically, any waste containing high amounts of carbohydrates, proteins and fats could be used as feedstocks (Ntaikou et al., 2010). Crop residues, animal manure, food waste, sludge from anaerobic treatment plants, effluents from sugar or paper industries are among the most studied feedstocks for sustainable hydrogen production (Guo et al., 2010; Lee et al., 2014). According to the waste and its content in carbohydrates, protein and fats, a broad range of H_2 and VFAs production can be achieved (Table 1-1). Indeed, carbohydrates-rich waste achieve higher H_2 production since carbohydrates have been identified as the main source of hydrogen production from waste (Guo et al., 2013; Ntaikou et al., 2010). For thermophilic and hyperthermophilic fermentation, the range of useable waste is narrow since only waste discharged at high temperature are sustainable from an economic and environmental point of view, such as waste from food processing or beverage production industries (Cheong and Hansen, 2007; Wong et al., 2014). Simple sugars such as glucose and sucrose (glucose +

fructose) are routinely used as model substrates to study and characterize hydrogen production with mixed cultures (Davila-Vazquez et al., 2008; Rafrafi et al., 2013).

1.1.2.2 Inoculum pretreatments

Physical pretreatments (heat, ultraviolet irradiation, freeze) and chemical pretreatments (incubation at very acidic or alkali pH) have been used to induce cell lysis of non-sporulating and non-H_2-producing bacteria. *Clostridium* species survive the pretreatment thanks to their sporulation capacity. In the DF reactor, favorable growth conditions allow germination of spores of *Clostridium* species. Usually, heat shock treatment gives the best results (Wong et al., 2014).

1.1.2.3 Substrate concentration and organic loading rate

Under batch, semi-continuous and continuous modes, hydrogen production can be modulated by the substrate concentration (Moletta, 2008). The optimal glucose concentration is around 10 $g.L^{-1}$. This concentration does not inhibit H_2-producers but inhibits methanogens. Low initial glucose concentration also prevents the accumulation of end-products, acetate and butyrate, which can lead to sporulation of *Clostridium* sp. or a shift to ethanol, propanediol or butanol production and a reduced H_2 yield (Guo et al., 2010; Hawkes et al., 2007). Similarly, when using waste, high organic loading (or organic loading rate under continuous mode) can lead to VFAs accumulation and thus decrease hydrogen production (Gómez et al., 2011). Under thermophilic conditions (55 °C), increasing the total solids (TS) contents of the substrate, i.e. wheat straw, (> 19 % TS) can lead to a shift from hydrogen, acetate and butyrate production by *Clostridium* sp. to lactate production and reduce hydrogen production by bacteria belonging to *Bacilli* and *Clostridia* classes and *Bacteroidetes* phylum (Motte et al., 2014). Under mesophilic conditions, H_2 production was reduced for TS contents higher than 28 % due to partial hydrolysis of wheat straw induced by a reduction in free water (Motte et al., 2013). A shift from butyrate:acetate molar ratio lower than 1 to higher than 2 was observed for TS contents higher than 28 %.

Organic loading rate (OLR) and related hydraulic retention time are two key parameters influencing the bacterial community structure, and thus performances of fermentation when operated in continuous mode (Hawkes et al., 2007). A short hydraulic retention time (HRT ≤ 6 h) favors the fast growing H_2-procing *Clostridium sp* and tends to wash out slow growing methanogenic bacteria (Ghimire et al., 2015).

Table 1-1. H_2 and metabolites production according to the feedstock and operating conditions.

Feedstock (organic load)	Inoculum	Culture mode	pH	T °C	H_2	Acetate ($g.L^{-1}$)	Butyrate ($g.L^{-1}$)	Ethanol ($g.L^{-1}$)	Lactate ($g.L^{-1}$)	Propionate ($g.L^{-1}$)	Reference
Food waste (60 g $VS.L^{-1}$)	Anaerobic sludge (HT)	B	8	35	289 [a]	12.2	6.7	-	-	0.7	(Chi et al., 2011)
Hydrolyzate of corn stover (5.5 $g.L^{-1}$ of mixed sugars)	Anaerobic sludge (HT)	B	5.5	35	2.84 [b]	0.57	0.88	-	-	-	(Datar et al., 2007)
Buffalo slurry and cheese whey (20.6 $gVS.L^{-1}$)	Lagoon sediments	B	6.5	37	117 [a]	0.85	2.04	0.51	0.1	2.8	(Marone et al., 2015)
Food waste (13 g $COD.L^{-1}$)	Anaerobic sludge (HT)	C*	6.5	37	310 [a]	0.5 [e]	0.8 [e]	0.9 [e]	0.9 [e]	0.2 [e]	(Han and Shin, 2004)
Rice slurry (5.5 $g.L^{-1}$ of carbohydrates)	Anaerobic sludge (HT)	B	4.5	37	346 [c]	0.9	2.3	-	-	-	(Fang et al., 2006)
Wheat straw (25 $g.L^{-1}$)	Cow dung compost (UV-treated)	B	7	36	68.1[a]	1.6	1.6	0.48	-	< 0.4	(Fan et al., 2006)
Cornstalk waste (15 $g.L^{-1}$)	Cow dung compost	B	7	36	150[a]	0.9	1.1	0.25	-	0.7	(Zhang et al., 2007)
Hydrolyzed bagasse (10 $g.L^{-1}$)	Anaerobic sludge (HT)	B	5.4	50	13.39[d]	-	0.8	-	0.3	-	(Chairattanamanokorn et al., 2009)
Food waste (15 g $COD.L^{-1}$)	Anaerobic sludge	B	10	28	0 (AF)[d]	4	0.6-0.7	-	-	1	(Dahiya et al., 2015)

[a]: mL H_2 gVS^{-1}; [b]: mol H_2 per mol of sugars consumed; [c]: mL $H_2.g^{-1}$ of carbohydrates; [d]: mL $H_2.gTS_{added}^{-1}$; [f]: Acidogenic fermentation; [e]: maximal metabolites concentration during fermentation. VS: Volatile Solids, TS: Total Solids; *: HRT: 2.3 - 4.5 d^{-1}; T: Temperature; HT: Heat-treated; B: Batch; C: Continuous

1.1.2.4 pH and temperature

pH is a critical operating parameter in DF since its variation can affect the hydrogenase activity and metabolic pathways up to the microbial community structure (Moon et al., 2014). Due to the production of organic acids during fermentation, pH has to be buffered (usually around 6 to favor *Clostridium* sp (Figure 1-3) or regulated through alkaline solution addition in continuous cultures. A drop of pH due to the production of VFAs is known to lead to a shift in metabolism, from H_2 production to solventogenesis (Hawkes et al., 2007). As an illustration, *Clostridium tyrobutyricum* is known to use mainly the butyrate pathway at pH 6 and at pH 5, a metabolic shift from butyrate fermentation to lactate and acetate fermentation occurs (Zhu and Yang, 2004).

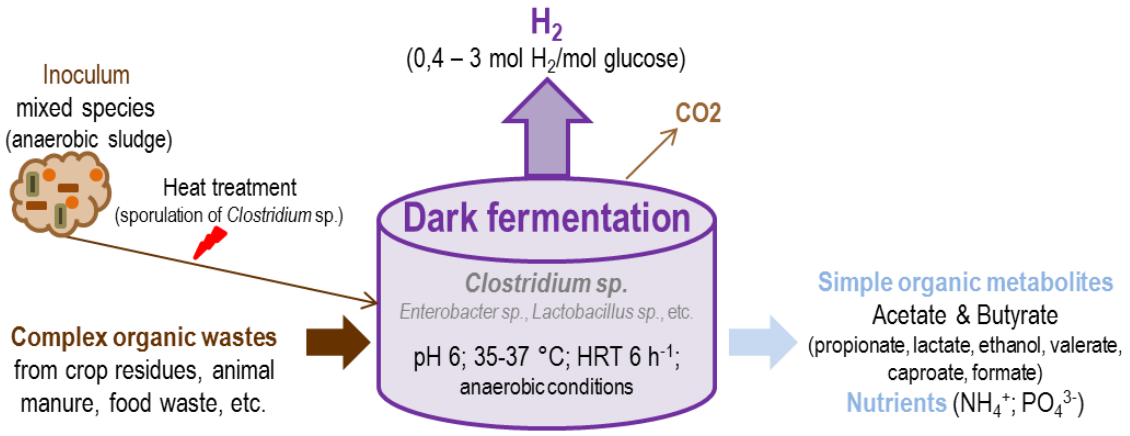

Figure 1-3. Schematic representation of dark fermentation with mixed cultures under mesophilic conditions.

A broad range of temperatures, i.e. mesophilic (35 – 37 °C), thermophilic (50 – 70°C) and hyperthermophilic (> 80 °C), has been used to carry out the dark fermentation process (Ghimire et al., 2015). Temperature can cause a shift in bacterial community thus leading in variations in the distribution of metabolic end-products. Generally, acetate is the main metabolite present when processes are performed at thermophilic or hyperthermophilic temperatures whereas butyrate is dominant in processes performed at mesophilic temperature. Nevertheless, counterexamples exist since hydrogen metabolic pathways depend also on the type of microbial community, pH, the type of feedstock and the organic loading rate (Ghimire et al., 2015). In addition, non-readily degradable substrates, such as lignocellulosic compounds, prefer thermophilic or hyperthermophilic temperatures which improve the substrate hydrolysis.

1.1.2.5 Specificities of AF operating conditions

VFAs production through AF is linked to DF but the optimized operating conditions differ (Lee et al., 2014). Operating conditions are set so as to improve waste degradation through the presence of additional non H_2-producing species (Lee et al., 2014). pH is for example adjusted to target specific VFAs production. For instance, pH 6 – 6.5 will promote *Clostridium sp* and acetate, butyrate production whereas propionate production can be enhanced by the presence of *Propiobacterium sp* which the growth is promoted at pH 8 (Lee et al., 2014).

Temperature is not as critical as pH for robust AF and is usually set around 35 – 37 °C, as in DF, to avoid cost related issues due to higher temperatures.

HRT in AF are longer than for DF, between 2 to 4 days, since H_2 has to be converted into VFAs. Shorter HRT tend to favor butyrate production over propionate production (Lee et al., 2014). Organic loading rate, usually expressed as the Chemical Oxygen Demand (COD), also affect VFAs distributions. COD higher than 20 $g.L^{-1}$ can lead to a shift from acetate to propionate (Lee et al., 2014). According to Lee et al. (2014), COD should be greater than 4 $g.L^{-1}$ to avoid inhibition of AF.

Hydrogen and VFAs productions vary greatly according to the substrate (type and organic matter contents), pH, HRT, H_2 partial pressure, concentration of fermentation products, presence of methanogens, presence of non-fermentative bacteria and composition of the microbial community of hydrogen-producing bacteria. To improve conversion of waste into VFAs, optimization of these parameters and control of bacterial community are still under investigation.

1.1.3 Valorization of AF and DF effluents

Between 50 and 80% of the COD from the feedstock is transformed into soluble metabolites during DF (and 100% during AF) (Sarma et al., 2015). Theoretically, 66.7% and 83% of the COD from glucose is converted into acetate through the acetate-pathway (Equation 1-1) and into butyrate through the butyrate-pathway (Equation 1-2), respectively. Fermentation metabolites such as propanediol, succinic acid and ethanol are considered as high-added value molecules for chemical applications (textile, detergent synthesis and fuel) (Sarma et al., 2015). The two critical points are the high cost of extraction and purification steps and the production of these products are negatively correlated with H_2 production. AF and DF effluents can also be used directly as low cost substrates for microbial growth for various biotechnological applications (Figure 1-4) (Sarma et al., 2015).

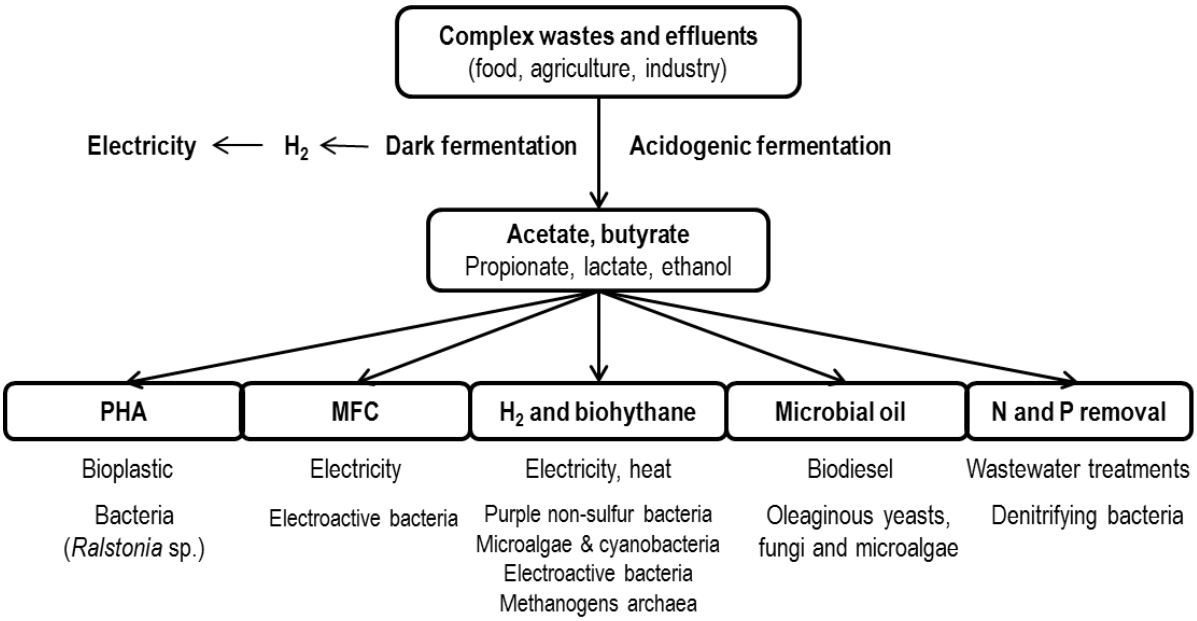

Figure 1-4. Valorization of effluents from dark fermentation and acidogenic fermentation.
MFC: Microbial fuel cell; PHA: polyhydroxyalkanoates

1.1.3.1 Characteristics of DF and AF effluents

1.1.3.1.1 Carbon compounds composition and concentration

Due to a great variation in substrates and operating parameters used for DF and AF, one cannot define a general composition of metabolites in fermentation effluents (Table 1-1). Acetate and butyrate concentrations, along with the acetate:butyrate ratio (between 0.4 and 6.7), can vary greatly. According to the acetate, butyrate, acetate-butyrate or acetate-ethanol pathways (Equations 1-1 to 1-4, respectively), theoretical VFAs production from 10 $g.L^{-1}$ of glucose can reach 6.7 $g.L^{-1}$ of acetate, 4.9 $g.L^{-1}$ of butyrate, 3.3 $g.L^{-1}$ of acetate and 3.7 $g.L^{-1}$ of butyrate, and 3.3 $g.L^{-1}$ of acetate and ethanol, respectively.

1.1.3.1.2 Nutrients content

Nutrient composition in anaerobic digestion (AD) effluents has, so far, received more attention than DF effluents. Since nutrient assimilation by methanogenic bacteria is relatively low, nutrient contents of both AD and DF effluents should be similar. Ammonium (NH_4^+) and orthophosphate (PO_4^{3-}) are the main forms of nitrogen and phosphorus in AD effluents (Cai et al., 2013). NH_4^+ and PO_4^{3-} are released during the hydrolysis step of both DF and AD through mineralization of complex organic compounds and proteins (Figure 1-1). NH_4^+ concentrations , ranging from 1.5 to 6.8 g per kg of fresh matter, and PO_4^{3-}, 1.5 to 6.8 gP per kg of fresh matter, in the effluent mainly depend on the substrate used for AD (Möller and Müller, 2012). Protein-rich feedstocks such as pig manure or food waste lead to ammonium and

orthophosphate-rich effluents (Cai et al., 2013). On the opposite, effluents from crop residues digestion are poor in nutrients (Möller and Müller, 2012). Whatever the feedstock comes from, some chemical reactions such as magnesium (Mg^{2+}), PO_4^{3-} and NH_4^+ precipitation to form struvite ($MgNH_4PO_4 \cdot 6H_2O$) or magnesium-phosphate ($Mg_3(PO_4)_2$) or aggregation with suspended solids can reduce the nutrients availability in the effluent. Similarly, precipitation and aggregation involving Ca^{2+} and Mg^{2+} may also reduce the nutrients availability in the effluent. Sulfur, such as sulfate (SO_4^{2-}), is also a crucial component to sustain the microbial growth. In DF, sulfate is volatized into H_2S by sulfate reducing bacteria which use hydrogen as second substrate (sub-section 1.1.2.2).

As main advantage of using AD or DF effluents to sustain the microbial growth, it has been suggested that vitamins could be produced by the microbial community of digestion processes (Möller and Müller, 2012).

To use fermentation effluents as media to grow microorganisms, detailed analysis of the nutrients content of the effluents is crucial since some microorganisms have special nutrient requirements, such as Mg^{2+} (part of chlorophyll) and high nitrogen content for microalgae. For example, DF effluents lacking iron and molybdenum are not suitable effluents to grow *Rhodobacter* species since these nutrients are part of hydrogen producing enzymes involved in H_2 production through photofermentation (sub-section 1.1.3.4) (Özgür et al., 2010).

1.1.3.2 Coupling DF and Bioplastics (polyhydroxyalkanoates) production

Polyhydroxyalkanoates (PHA) are biodegradable polymers that could replace petrochemical-based plastics. PHA can be synthesized by microorganism such as *Ralstonia sp* and *Cupriavidus sp* (Grousseau et al., 2013; Lee et al., 2014). For efficient PHA production, DF or AF effluents should be filtered and excessive N and P removed (using struvite precipitation for example). PHA production by mixed cultures from activated sludge, fed with AF effluents, have already shown high production of PHA with contents as high as 77% of cellular dry weight (CDW).

1.1.3.3 Coupling DF and electricity generation: Microbial Fuel Cell

Basically, the principle of microbial fuel cell (MFC) is to use microorganisms able to use organic substrates as electrons and protons donors at the anode and create an electric current between the anode and the cathode. DF and AF effluents rich in VFAs have been used as substrates for electricity production in MFC (Lee et al., 2014). As summarized by Lee et al. (2014), MFC fed with acetate exhibit higher yields than with butyrate.

1.1.3.4 Biogas: hydrogen and biohythane

Hydrogen yield from DF could be further improved by coupling DF with other biological H_2-producing processes such as photofermentation or microbial electrolysis cells (MEC). Coupling DF with photo-dark fermentation could critically enhance the H_2 yield from 4 to 12 mol of H_2 per mol of glucose consumed (Azwar et al., 2014). Photo-dark fermentation is usually performed by purple non-sulfur bacteria such as *Rhodobacter sphaeroides* under anaerobic conditions. Acetate is preferred for photo-dark fermentation compared to butyrate by *Rhodobacter* species (Ren et al., 2008; Uyar et al., 2009). Some microalgae species have also been studied for H_2 production through photofermentation. Recently, *Micractinium reisseri* has been grown on mixtures of acetate and butyrate and as for *Rhodobacter,* acetate was a preferred substrate compared to butyrate (Hwang et al., 2014). The filtration of DF or AF effluent is required in order to reduce the concentrations of suspended solids and to allow a better light exposure (Gómez et al., 2011). Separating solid residues from the waste of DF or AF and further treatments, through anaerobic digestion, could enhance light availability for photo-dark fermentation and improve the yield of the process (Xia et al., 2014, 2013).

In MEC, the protons released from the oxidation of organic substrates at the anode are transferred to the cathode where they are converted into H_2 thanks to an electrical input. H_2 yields as high as 9.95 mol H_2 per mol of glucose have already been achieved by coupling DF with MEC (Sambusiti et al., 2015). To avoid the cost of the electrical input required for MEC, a system combining DF, MFC and MEC have shown promising results with a 41% H_2 yield increased when compared with DF alone (Sambusiti et al., 2015). As for photo-dark fermentation, acetate is also the preferred substrate (Lee et al., 2014).

DF and AF effluents could also be used to sustain subsequent anaerobic digestion to produce methane. In the case of coupling DF with AD, both H_2 and CH_4 could be recovered and produce a biogas called biohythane (Sambusiti et al., 2015). As biohythane, composed generally of 5- 20 % H_2, the main advantages over methane only are an increase in the combustion efficiency and a cleaner combustion (decrease in hydrocarbons, CO and NOx releases) (Luo et al., 2011). This coupling is so far the most probable and feasible technology since AD processes are readily available at industrial scale.

1.1.3.5 Coupling DF and nitrogen and phosphorus removal processes

VFAs from DF and AF could also be used as low cost carbon sources to support growth of phosphorus-removing and denitrifying bacteria in wastewater treatment plants (Singhania et

al., 2013). Carbon content in municipal wastewaters is generally too low to efficiently support the microbial growth (Perez-Garcia et al., 2011a). VFAs from acidogenic fermentation have been successfully used for nitrogen (N) removal, up to 92% removal efficiency, and phosphorus (P) removal, up to 99% removal efficiency (Lee et al., 2014). N removal rate is generally higher with acetate, followed by propionate and butyrate (Singhania et al., 2013). For P removal, propionate seems the preferred substrate, followed by acetate and then butyrate (Lee et al., 2014).

1.1.3.6 Biodiesel from microbial oil

Biodiesel production from oleaginous microorganisms is based on a chemical transesterification reaction between triacylglycerides (TAGs) from microbial oil and short chain alcohol, methanol or ethanol (Lam and Lee, 2012). The products are Fatty acids methyl esters (FAME), the main components of biodiesel (Lam and Lee, 2012), and glycerol. Since biomass and lipids production are not concomitant biological processes, two-stage cultures are usually used to produce lipids from oleaginous microorganisms (Bumbak et al., 2011; Xiong et al., 2015). In the first stage, biomass is produced. In the second stage, carbon is provided but one of the nutrients, usually nitrogen, is limiting so that carbon is used by micro-organisms to produce lipids.

DF and AF effluents have been suggested as a good alternative to glucose to produce microbial oil from yeasts, fungi or microalgae (section 1.3) (Park et al., 2014). Examples of biomass yields and lipid contents achieved recently using oleaginous yeasts and fungi are presented in Table 1-2. As for the previous mentioned applications, acetate is a preferred substrate compared to butyrate to sustain microbial growth for oil production. Acetate uptake is much faster, up to 6 to 10 fold, than butyrate uptake for most microorganisms including *Yarrowia lipolytica* and *Cryptococcus* sp. (Fontanille et al., 2012; Vajpeyi and Chandran, 2015). In addition, as illustrated in Table 1-2, lipids production by oleaginous yeast was favored for high acetate:butyrate ratios (> 2.1). Butyrate uptake by microorganisms under aerobic conditions and the influence of VFAs ratios and concentrations on microbial growth are further discussed in sections 1.2 and 1.3.

Table 1-2. Biomass yields and lipid content achieved during microbial growth on DF and AF effluent.

Effluent	Organic acids load ($g.L^{-1}$)	A:B ratio ($g\ g^{-1}$)	Species	Biomass yield ($g.g^{-1}$)	Lipid content (%)	Reference
Synthetic	1.5[a]	3	*Yarrowia lipolytica*	0.47	40	(Fontanille et al., 2012)
Synthetic	2	8	*Cryptococcus albidus*	0.6	27.8	(Fei et al., 2011)
AF	> 9	> 8	*Cryptococcus curvatus*	0.28	50 - 60	(Xu et al., 2015)
AF	> 5	2.1	*Cryptococcus albidus*	0.2	14.9	(Vajpeyi and Chandran, 2015)
DF	> 19 [b]	1.82	*Cryptococcus curvatus*	0.5	13.8	(Chi et al., 2011)

[a]: in $g.L^{-1}.h^{-1}$

[b]: the final organic acids concentration after effluent dilution was 0.8 $g.L^{-1}$.

Waste and effluents valorization into H_2 and VFAs through dark fermentation and acidogenic fermentation is based on the environmental biorefinery concept. H_2 can be used to produce energy, such as electricity, and VFAs can be used by a broad range of microorganisms to produce bioplastic, biogas, electricity and biodiesel. DF and AF effluent valorization is crucial for the industrial development of both processes (Ghimire et al., 2015). The composition and quantity of VFAs produced, as long as other end-products such as lactate and ethanol, will greatly impact the process following the fermentation step.

1.2 Growth of microalgae on acetate and butyrate as single substrates

Acetate and butyrate are the main end-products of dark fermentation (DF) (section 1.1). The use of these VFAs, as single substrates, to sustain microalgae growth is discussed in this section while the use of DF effluents, composed of mixtures of acetate and butyrate, will be discussed in the next section (1.3).

Acetate has been successfully used to promote microalgae growth and lipid production by microalgae (Table 1-4). It has been described as one of the best alternatives to glucose, if not the best (Lowrey et al., 2015; Perez-Garcia et al., 2011b). In contrast, little is known on microalgae growth on butyrate due to an inhibition of the growth at relatively low concentration (section 1.2.2). It is well known that short chain fatty acids, such as butyrate, inhibit microbial growth, for concentration as low as 10 $g.L^{-1}$, and are therefore extensively used as preservative agents in the food industry (Ricke, 2003; Rodrigues and Pais, 2000).

Acetate assimilation by microalgae has been studied under two aerobic conditions: in heterotrophy and in mixotrophy. Indeed, many microalgae are known to be able to grow under heterotrophy, autotrophy and mixotrophy, also referred to as photoheterotrophy (Table 1-3) (Chen and Chen, 2006). Heterotrophy is defined as the use of preformed organic source

from the environment to sustain growth, without light (Perry et al., 2004). In contrast, autotrophy is defined as the use of only inorganic CO_2 and light to sustain the growth (Perry et al., 2004). Some microalgae species are able to use both organic and inorganic C compounds in the presence of light (i.e. mixotrophy). Understanding mixotrophic metabolism (a combination of autotrophic inorganic C fixation and heterotrophic organic C assimilation) is still a challenge and is further discussed in the next sub-section. These metabolisms can be differentiated by (i) the source of energy, i.e. the way to synthesize adenosine triphosphate (ATP) which allows endergonic cellular reactions such as active transport and anabolic reactions to occur; (ii) the source of carbon, one of the major chemical components of biomass which is first transformed into precursors and then into macromolecules composing the biomass and (iii) the source of electrons, i.e. electron donors to provide reducing power, NADH, $FADH_2$ and NADPH, necessary for reducing anabolic reactions.

Table 1-3. Nutritional types of microalgae.

Nutritional type	Carbon source	Energy source	Electron source (donor)
Chemoorganoheterotrophy "**hetrotrophy**"	Organic source (sugars, organic acids, etc.)		
Photolithoautotrophy "**autotrophy**"	Inorganic (CO_2)	Light	H_2O
"**mixotrophy**"	Organic (and inorganic)	Light (and organic)	H_2O (and organic)

Heterotrophy and mixotrophy share most of their metabolic pathways (break-down of organic molecules) but differ greatly by the use of light and CO_2 for cell growth in mixotrophy. To better understand the differences observed in terms of growth on acetate between these two metabolisms (sub-section 1.2.1.4), similarities and major differences of these metabolisms are presented in sub-sections 1.2.1.1 and 1.2.1.2. Productions of lipids and other molecules of industrial interest from heterotrophic microalgae grown on acetate are also presented (sub-section 1.2.1.4).

1.2.1 Acetate, an ideal carbon source?

1.2.1.1 Assimilation of acetate in heterotrophy: the glyoxylate cycle

Acetate is actively assimilated by eukaryotic microorganisms by a monocarboxylic/proton transport protein (Perez-Garcia et al., 2011b). Then, acetate is carried into the glyoxysome

where it is transformed into acetyl-CoA, a central precursor metabolite, by a acetyl-CoA synthetase. Acetyl-CoA participates to the glyoxylate cycle, a variant of the Krebs cycle allowing the synthesis of precursor metabolites from two-carbon substrates (Figure 1-5). Indeed, the major difference between glucose and acetate metabolisms is the replenishment of oxaloacetate (OA) pool, necessary to run the Krebs cycle, which is depleted for amino acids synthesis. For glucose metabolism, OA pool is replenished by the anaplerotic reactions. For acetate and other two-carbon substrates, two enzymes specific of the glyoxylate cycle, the isocitrate lyase (ICL) and malate synthetase (MS), allow the formation of four-carbon metabolites from acetyl-CoA. If glucose or intermediary metabolites of the Krebs cycle, such as succinic acid, are present in the medium, ICL synthesis is blocked in microalgae (John and Syrett, 1968). To subsequently produce energy, an intermediate metabolite of the glyoxylate cycle, succinate, is then transported into the mitochondrion for the synthesis of reducing power, through the Krebs cycle. Reducing power is then used for ATP synthesis using oxidative phosphorylation of O_2. As for many heterotrophic microorganisms, roughly 50% of the assimilated carbon is lost through the Krebs cycle in microalgae (Chen and Johns, 1996; Goulding and Merrett, 1966). Thanks to the glyoxylate cycle and Krebs cycle, major biomass precursors are synthesized and ensured the production of all necessary molecules for cell functioning (proteins, DNA, RNA, carbohydrates).

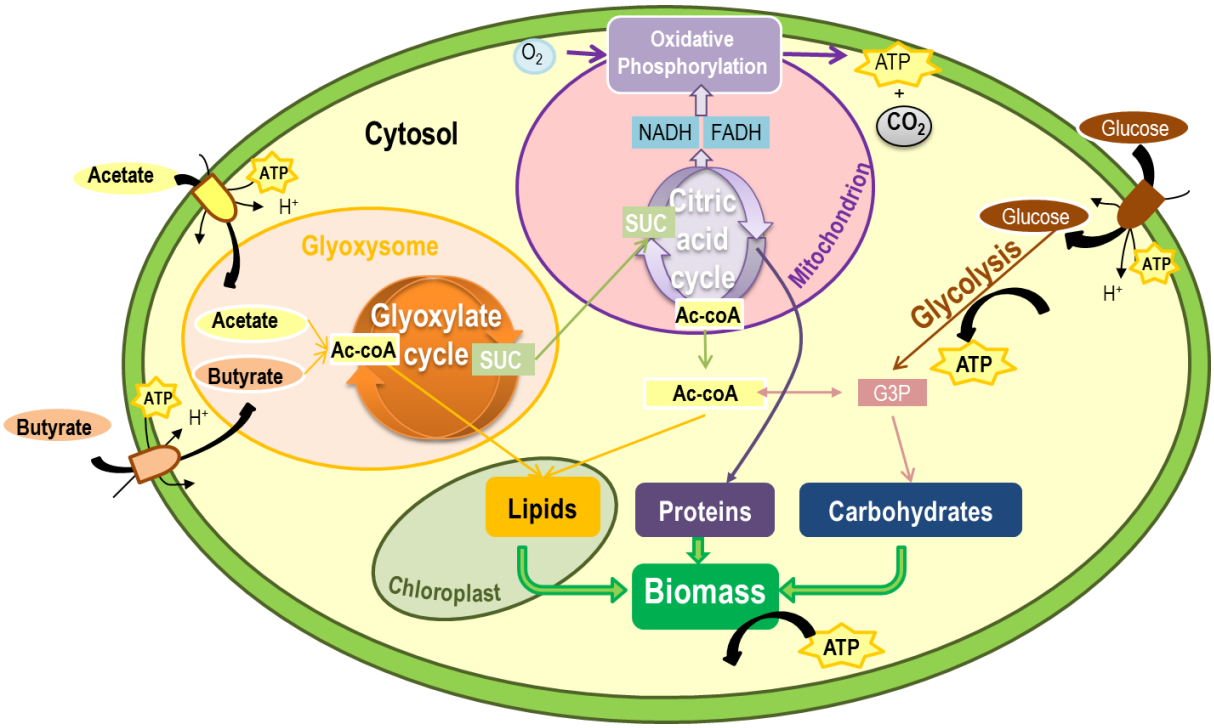

Figure 1-5. Schematic representation of heterotrophic metabolism of acetate, butyrate and glucose in microalgae.

During heterotrophic growth, acetate and butyrate are actively transported into the cell and then metabolized in the glyoxysome. Both VFAs are break down into Precursor Metabolites (PM) for biomass synthesis, and succinate (SUC) which is reintegrated in the citric acid cycle (TCA) in the mitochondrion. Reduced power (NADH and FADH) is then regenerated during the TCA cycle and enables the oxidative phosphorylation (OP) involving O_2, which is the source of ATP in heterotrophy. The release of CO_2 during the TCA cycle is the main source of carbon loss in heterotrophy.

1.2.1.2 Assimilation of acetate in the presence of light: interactions between heterotrophic and autotrophic metabolisms

1.2.1.2.1 Brief overview of the autotrophic metabolism

The presence of light induces assimilation of inorganic carbon, i.e. autotrophic metabolism, even in the presence of organic carbon. Ability of microalgae to grow on CO_2 relies on photosynthesis in the chloroplast. Very briefly, photosynthesis can be divided in two parts: the light-dependent reactions, photophosphorylation, and the light-independent reactions, so called dark reactions (Figure 1-6).

During photophosphorylation, thanks to the light energy, channeled via pigments and photosystem (PS), water is split into O_2 and electrons which are subsequently transferred to successive thylakoid membrane proteins until they reduce $NADP^+$, the final electron acceptor, into NADPH. During electrons transfer, a charge separation is created via the influx of protons in the thylakoid lumen. Thanks to this charge separation, ATP is produced in the

chloroplast stroma by an ATP synthetase. Alternative electron flow pathways, such as the cyclic electron flow in PSI, exist and allow a surplus of ATP production.

During the dark reactions of photosynthesis, ATP and NADPH are mostly reinvested into the Calvin-Benson Cycle to produce a three-carbon metabolite, glyceraldehyde-3-phosphate (G3P) by fixing 3 molecules of CO_2 thanks to the ribulose-1,5-biphosphate carboxylase/oxygenase, also known as the RuBisCO enzyme. CO_2 fixation has a high ATP demand, since 3 mol of ATP are required to fix 1 mol of CO_2. G3P is subsequently used as a precursor metabolite for cell functioning.

1.2.1.2.2 Possible interactions between heterotrophic and autotrophic metabolisms in mixotrophy

Understanding the interactions between heterotrophic and autotrophic metabolisms, i.e. the proportion of biomass produced from CO_2 or from organic carbon, has been the aim of many studies over the past 50 years and no consensus has been reached on the underlying pathways. The difficulty to unravel mixotrophic metabolism relies in the possible interactions between autotrophic and heterotrophic metabolisms. Indeed, precursor metabolites, such as acetyl-CoA and glyceraldehyde-3-phosphate (G3P), energy (ATP) and reducing power (NADH or NADPH) are produced by both metabolisms. In heterotrophic metabolism, O_2 is consumed and CO_2 is produced whereas it is the opposite in autotrophy.

Positive interactions between heterotrophic and autotrophic metabolisms could theoretically enhance the acetate uptake and assimilation by microalgae during mixotrophic metabolism: (i) cellular energy, ATP, produced through photophosphorylation in the chloroplast could be used to boost organic carbon uptake, (ii) O_2 released during photooxidation of water in the chloroplast could increase respiration rate in the mitochondrion and (iii) CO_2 released during the Krebs cycle initiated by organic carbon assimilation could be recycled through the Calvin cycle and increase biomass yield (Wan et al., 2011; Yang et al., 2000). Aside from interactions between acetate metabolism and autotrophic metabolisms, acetate has recently been shown to protect *Chlamydomonas reinhardtii* from photodamage, induced under high light and subsequent production of toxic singlet oxygen ($^1O_2$) (Roach et al., 2013). According to Roach et al., (2013), acetate can directly interact with PSII and reduce the charge recombination events leading to $^1O_2$.

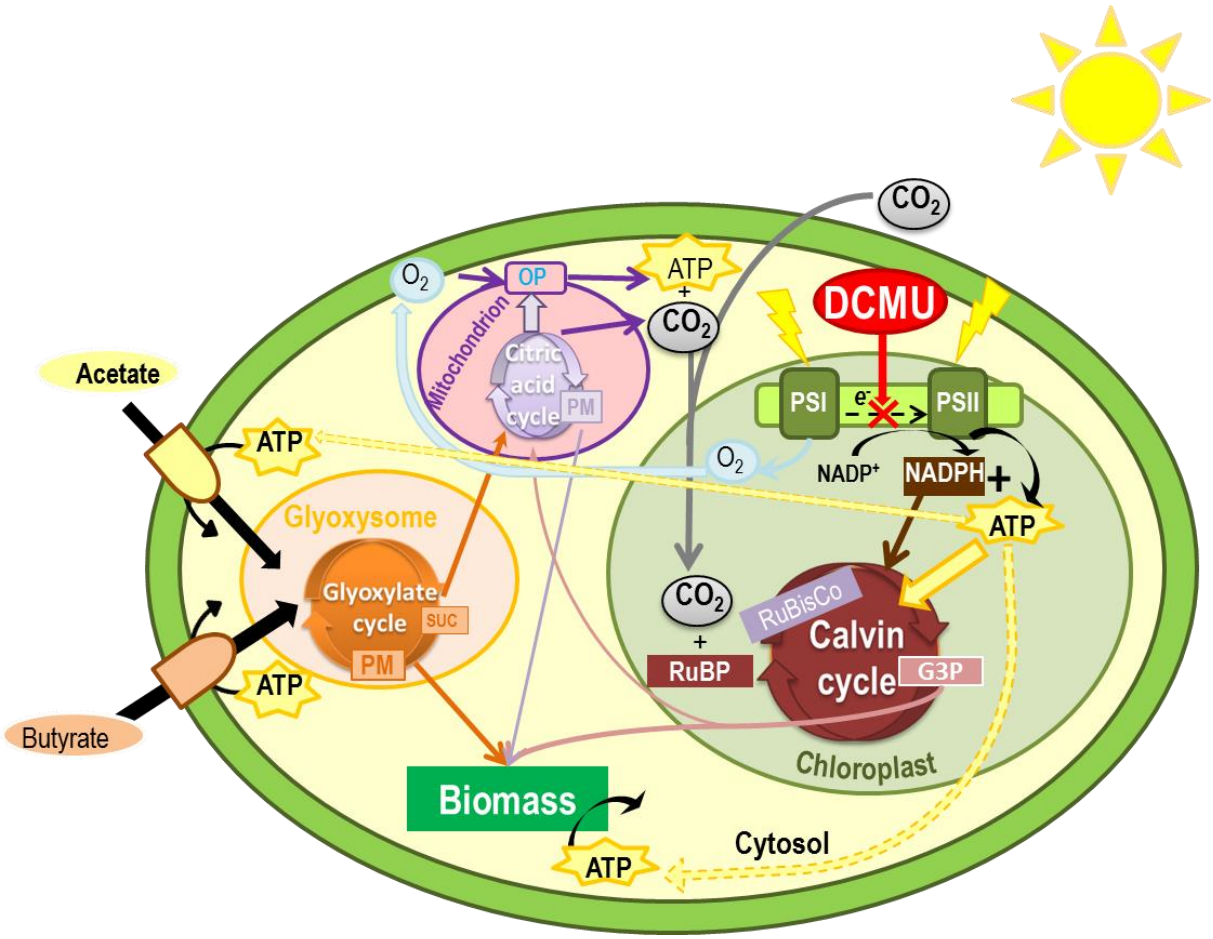

Figure 1-6. Possible positive interactions between heterotrophic and autotrophic metabolism.

Under mixotrophic conditions, the ATP produced through photophosphorylation could be used not only for the fixation of CO_2 through the Calvin cycle but also to support heterotrophic metabolism. Moreover, O_2 released during the oxygenation of water, the first step of photophosphorylation, could be used to increase mitochondrial respiration which is often limiting in heterotrophy. CO_2 released through OP could be recycled into the Calvin cycle and thus increase biomass. Biomass growth through CO_2 fixation is very ATP demanding (3 mol of ATP per mol of CO_2 fixed) and usually slower than through organic carbon fixation.

Since Acetyl-CoA is a central metabolite for cell biology and is produced by both heterotrophic and autotrophic metabolisms, it is very unlikely that both metabolisms do not interact via a feedback control (negative interactions) (Sheen, 1994). For instance, in some microalgae such as *Chlamydomonas*, some genes related to the photosynthetic activity (proteins involved in light harvesting, the RuBisCO enzyme and carbonic anhydrase) are down-regulated in presence of acetate (Fett and Coleman, 1994; Kovács et al., 2000; Sheen, 1994). At saturating light and CO_2 levels, the proportion of carbon biomass from photosynthesis declined by 50% in the presence of acetate in *Chlamydomonas* sp. (Heifetz et al., 2000). Even under non-saturating light and CO_2 levels, acetate induced a metabolic shift from autotrophy to heterotrophy in *Chlamydomonas* sp. and also in *Chlorella sorokiniana*

(Kovács et al., 2000; Qiao et al., 2012). As a consequence, photosynthesis was shortly impaired because of NADPH accumulation, causing $NADP^+$ shortage, due to ATP use for acetate uptake instead of CO_2 fixation (Kovács et al., 2000; Qiao et al., 2012). According to a flux balance analysis based on *Chlamydomonas reinhardtii* genome, the accumulation of NADPH leads to a reduce electron flow from PSII to PSI and ATP generation in the chloroplast relies mainly on cyclic electron flow at PSI during mixotrophic growth on acetate (Chapman et al., 2015). As a consequence, photosynthesis, i.e. CO_2 fixation, was predicted to be downregulated.

Kinetics and control of acetate assimilation in the presence of light has been extensively studied in *Chlorella* species, particularly in *C. vulgaris* and *C. sorokiniana*, previously known as *Chlorella pyrenoidosa* (Goulding and Merrett, 1966; Merrett and Goulding, 1968, 1967a, 1967b; Syrett, 1966; Syrett et al., 1964; Wan et al., 2011). All results showed that the presence of light enhanced acetate uptake rate and biomass yield on acetate. It was generally agreed that ATP production through photosynthesis provides energy to enhance the isocitrate lyase production and acetate uptake thus increasing the acetate assimilation. Nevertheless, a consensus was not reached on the origin of ATP, from non-cyclic photophosphorylation (Goulding and Merrett, 1966), or from cyclic electron flow a PSI at low light intensity (Kovács et al., 2000; Syrett, 1966).

Obviously, these complex interactions are controlled by the source (acetate or another compound) and concentration of organic carbon, the light intensity and CO_2 availability which further complicates a deep understanding of the mixotrophic metabolism (Markou and Georgakakis, 2011). As a consequence, microalgae growth response to glucose under mixotrophic conditions cannot be generalized to all carbon substrates. As an illustration, chlorophyll content of *Micractinium inermum* decreased by almost 90% and 25% during mixotrophic growth on glucose and acetate, respectively.

1.2.1.2.3 Control of acetate uptake by the acetate:CO_2 ratio and light intensity

The balance between carbon from acetate, or any other organic carbon source, and carbon from CO_2 fixed seems to depend greatly on their concentrations. CO_2 fixation is favored at high CO_2 levels and organic carbon uptake is favored at low CO_2 levels as shown with *Euglena gracilis*, *Chlorella protothecoides* and *Nannochloropsis salina* (Ogbonna et al., 2002; Sforza et al., 2012). This might be due to a slow fixation rate of CO_2 by the RuBisCO enzyme at low CO_2 level. Interestingly, under high CO_2 availability and light intensities, the

production of isocitrate lyase (the central enzyme of the glyoxylate cycle) has been shown to be reduced in *Chlorella sorokiniana* (Syrett, 1966).

Under a constant acetate supply, *Chlamydomonas reinhardtii* metabolism shifted from heterotrophy-like to autotrophy-like metabolism with increasing light intensities (Boyle and Morgan, 2009). Similar results were obtained during the mixotrophic growth of *Euglena gracilis* on glucose (Ogbonna et al., 2002).

Several authors showed that when light and/or CO_2 are low, mixotrophy is simply the sum of heterotrophy and autotrophy based on biomass yield and/or growth rate (Kobayashi et al., 1992; Markou and Georgakakis, 2011; Martinez and Orus, 1991; Ogbonna et al., 2002; Smith et al., 2015; Zhao et al., 2012). However, this statement does not reach a consensus (Markou and Georgakakis, 2011; Wang et al., 2014). Under light intensities between 100 and 200 µmol photons.m^{-2}.s^{-1}, *C. sorokiniana* growth rate has been shown to be higher than the combination of heterotrophic and autotrophic growth rates (Li et al., 2014). Recently, Smith et al. (2015) observed a synergetic effect between the two metabolisms when CO_2 and O_2 were limiting. Indeed, mixotrophic growth rate of *Micractinium inermum* obtained under CO_2 and O_2 limiting conditions with acetate was 1.74 times higher than the sum of autotrophic and heterotrophic growth rates. On one hand, autotrophic metabolism provided dissolved oxygen for heterotrophic Krebs cycle and respiration despite the O_2 limitation of the culture. On the other hand, dissolved inorganic carbon, produced through the Krebs cycle, was available for the Calvin cycle despite the lack of CO_2 in the culture medium.

Despite the current trend to favor mixotrophic over heterotrophic cultivation for microalgae, based on very promising biomass results (see sub-section 1.2.1.4), mixotrophic metabolism is still poorly understood and no consensus has been reached on the possible interactions between autotrophic and heterotrophic pathways in mixotrophy in the scientific community. In addition, the regulation of operating conditions, i.e. light, CO_2 and organic carbon loads, appears to be more complicated than for heterotrophic cultivation.

1.2.1.3 Acetate assimilation and lipids synthesis

Acetyl-CoA is the main precursor metabolite for the synthesis of lipids, pigments-like lipids and neutral lipids (Figure 1-7). The latter includes triacylglycerols (TAGs) used for biofuel production (De Swaaf et al., 2003; Perez-Garcia et al., 2011b). Therefore, lipid synthesis from acetate is theoretically much more straightforward than from glucose or CO_2 (De Swaaf et al., 2003). As an illustration, lipids content in *Chlorella sorokiniana* was found to be always

higher during growth on acetate than during growth on glucose in either heterotrophy or mixotrophy (Christophe et al., 2012). In *Chlorella vulgaris*, the presence of light stimulates lipids, proteins and also carbohydrates synthesis from acetate (Syrett et al., 1964). Nevertheless, as for autotrophic and heterotrophic growth on glucose, high lipids synthesis is mostly achieved thanks to an environmental stress, the most studied being nitrogen starvation or limitation, which shifts metabolism from a biomass growth strategy to a carbon storage strategy.

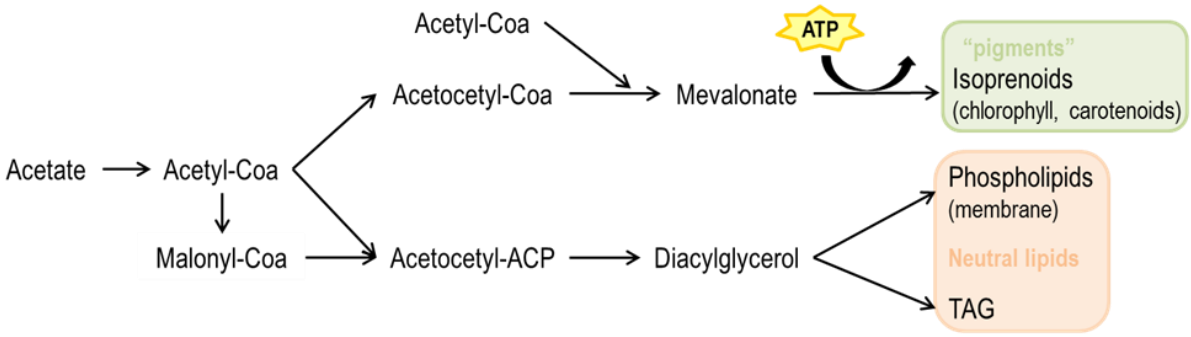

Figure 1-7. Lipids synthesis from acetate.

Abbreviations: Coenzyme-A (CoA), Acyl carrier protein (ACP).

1.2.1.4 An overview of products obtained with growth on acetate

Acetate has been used to sustain successfully heterotrophic or mixotrophic growth of microalgae in the contexts of (i) wastewater treatment and NH_4^+ removal, (ii) low-added value molecules production, i.e. lipids for biofuel, and (iii) high-added value molecules such as pigments (Table 1-4).

Table 1-4. Overview of products and yields obtained with acetate.

Microalgae species	Cultivation mode[a]	Acetate concentration ($g.L^{-1}$)	Growth rate (d^{-1})	Final biomass ($g.L^{-1}$)	Aim (and yield)	References
Chlorella sorokiniana	H	<10	2		Ammonium removal	(Ogbonna et al., 2000)
Chlorella sorokiniana	M	2.4	2.9	8.8	Lutein (3.4 ‰ of biomass)	(Cordero et al., 2011)
Chlorella sorokiniana	M	2		5	Biomass (chlorophyll and lutein)	(Van Wagenen et al., 2014a)
Chlorella protothecoides	H	20.5		3.24	Lipids (21.8% of biomass)	(Heredia-Arroyo et al., 2010)
Chlorella protothecoides	M	8	1.45	3.29	Biomass	(Sforza et al., 2012)
Chlorella vulgaris	H	7.2			Ammonium removal in municipal wastewater	(Perez-Garcia et al., 2011a)
Chlorella vulgaris	M	20.5		3	Lipids (14% of biomass)	(Heredia-Arroyo et al., 2011)
Crythecodinium cohnii	H	< 8		109	Lipids (56% of biomass) DHA (17%)	(de Swaaf et al., 2003)
Haematoccocus pluvialis	H	2.7			Astaxhantin (9 $mg.L^{-1}$)	(Kobayashi et al., 1997)
Scenedesmus sp	H	10	0.67 d-1	1.86	Lipids (34.4% of biomass)	(Ren et al., 2013)

[a]: H (heterotrophy), M (mixotrophy)

Removal of ammonium and phosphate from municipal wastewaters by microalgae under autotrophic conditions, known as tertiary wastewater treatment, can be relatively slow. Because of a poor light access, low microalgae densities and consequently slow nutrients removal rates are achieved (Perez-Garcia et al., 2011a). Under heterotrophic conditions with acetate supplementation, *Chlorella sorokiniana* and *Chlorella vulgaris* removed rapidly nutrients from the wastewater thanks to high growth rate, as high as 2 d^{-1} (Table 1-4) (Ogbonna et al., 2000; Perez-Garcia et al., 2011a). Supplementing acetate to wastewaters containing low organic carbon concentration has been identified as an efficient way to enhance nutrient removal rates by microalgae.

Production of microalgae biomass rich in lipids, mainly TAGs, to further produce biofuels has also been achieved with acetate as substrate for heterotrophy and mixotrophy (Table 1-4).

Nitrogen stress, i.e. nitrogen limitation, could be further used to enhance these yields. Some microalgae, such as *Chlamydomonas reinhardtii* and *Chlorella sorokiniana*, are also able to produce H_2 during anaerobic growth on acetate in presence of light (Driver et al., 2014). Nevertheless, due to O_2 production and H_2 production inhibition by O_2, H_2 yields by microalgae are still relatively low. Most microalgae store carbon as starch in the chloroplast. High content of starch and cellulose (from cell wall), i.e. carbohydrates, can be used to produce bioethanol through microbial fermentation (Ho et al., 2013).

Eicosapentanoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), essential polyunsaturated long chain fatty acids (PUFAs), more commonly known as omega-3, are industrially produced from microalgae to make up for the shortening supply of these PUFAs from fish-oil (Parsaeimehr et al., 2015). PUFAs are very important for good visual and neurological developments in young children (De Swaaf et al., 2003). They are currently used as food supplements (De Swaaf et al., 2003). EPA production by the diatom *Navicula saprophila* was enhanced by almost 30% in presence of acetate under mixotrophic conditions. Even more strikingly, acetate was used as sole carbon source resulting to the highest DHA reported production by the apochlorotic *Crypthecodiniium cohnii* (15.3 $g.L^{-1}$, De Swaaf et al., 2003).

β-carotene, astaxanthin and lutein are the main carotenoids produced industrially for food, cosmetic and pharmaceutical applications (Mojaat et al., 2008). They act as natural colorants and also as anti-oxidant agents due to their capacity to protect against oxygen free radicals (Mojaat et al., 2008). In microalgae, carotenoid pigments are produced from acetyl-CoA (Figure 1-7). The combination of acetate addition (4 $g.L^{-1}$) with oxidative induced stress by $FeSO_4$ successfully enhanced the production of β-carotene in *Dunaliella salina* under mixotrophic conditions (Mojaat et al., 2008). Similarly, relatively high astaxantin production, 9 $mg.L^{-1}$, were achieved during heterotrophic growth of *Haematococcus pluvialis* on acetate under salt-induced stress conditions (Kobayashi et al., 1997). Under optimal conditions for growth, i.e. without stress conditions, lutein production from mixotrophic *Chlorella sorokiniana* in the presence of acetate reached 30 $mg.L^{-1}$(Cordero et al., 2011).

By combining multiple products, i.e. low-added value and high-added value molecules production, heterotrophic and mixotrophic cultivations of microalgae on acetate could become more economically viable (da Silva et al., 2014). Indeed, an attractive prospect for microalgae cultivation relies on their ability to produce simultaneously lipids, carbohydrates, pigments

and proteins (Liang et al., 2009). As an illustration, it was shown that *C. vulgaris*, grown heterotrophically on acetate, was composed of 23% carbohydrates, 31% of lipids and 42% of proteins which could be further used in the bioenergy field and food or feed industry (Liang et al., 2009).

1.2.1.5 Main challenge: concentration-related toxicity

High concentration of non-diffusing substrate, such as acetate and glucose, are known to inhibit microalgae growth mainly due to a high osmotic pressure of the medium (Chen and Johns, 1995). Only few microalgae strains, such as *Galdieria sulphuraria*, are able to support concentration of glucose higher than 30 $g.L^{-1}$ (Bumbak et al., 2011). Even the intensively studied and heterotrophic *Chlorella* species usually do not tolerate glucose concentrations higher than 30 $g.L^{-1}$ (Bumbak et al., 2011). As a consequence, in batch cultivation, biomass concentration of more than 100 $g.L^{-1}$ cannot be achieved. High concentration of acetate may change pH gradient across the microorganism membrane leading to a change in the osmotic pressure and intracellular pH which alters the entire cellular activity (Yan et al., 2013). Maintenance energy required to overcome this osmotic pressure by import or export ions and osmotic work leads to a decrease in cell growth, i.e. growth rate and biomass yield (Chen and Johns, 1996). Acetic acid, undissociated form of acetate, is also particularly inhibitory to microorganisms and its concentration increases when pH decreases (Béligon et al., 2015; Chen and Johns, 1994). If salt of acetate are used, Na-acetate or Ca-acetate, high amounts of ions will be released in the medium, leading to an increase in the ionic strength of the medium. As a consequence, the microbial growth can be inhibited due to water losses to equilibrate ions concentrations between the medium and the cell. Another issue related to acetate concentration is the change of pH during cultivation. Indeed pH rises because of acetate depletion in the medium (Bouarab et al., 2004).

To avoid growth inhibition by high acetate concentration (or glucose) and to reach high-density cultures, operating conditions such as fed-batch cultivation, pulse addition of substrate and perfusion techniques have been proposed (Bumbak et al., 2011; de Swaaf et al., 2003; Ratledge et al., 2001). Fed-batch cultures were successfully employed to avoid acetate concentration-related inhibition, which was maintained under non-inhibitory concentration, below 8 $g.L^{-1}$, for growth of *Crypthecodinium cohnii* which reached 109 $g.L^{-1}$ of biomass in 16 days (de Swaaf et al., 2003; Ratledge et al., 2001). Similarly, fed-batch addition of acetate through pH control was used to reach 80 $g.L^{-1}$ of DW with the oleaginous yeast *Cryptococcus curvatus* in only 2.5 days (Béligon et al., 2015). Pulsed addition of highly concentrated

substrate prevent the dilution of biomass and has successfully been used as well to achieve high microalgae cell concentration during heterotrophic cultivation on glucose (Bumbak et al., 2011). Nevertheless, if acetate is provided under a salt form, ions will again accumulate (Chen and Johns, 1995). Fed-batch strategy can be successfully used but the potential of microalgae growth will be limited at some point. In perfusion culture, also known as cell-recycle, the culture is continuously fed with the medium but the cells are physically retained in the culture, usually by the use of a selective membrane. This way, biomass is not diluted and at the same time the inhibitory ions do not accumulate (Chen and Chen, 2006). A perfusion system was used to grow heterotrophically *Chlamydomonas reinhardtii* on acetate and showed the best performances in terms of accumulation of biomass compared with batch, fed-batch and chemostat systems (Chen and Johns, 1995).

1.2.2 Butyrate, a possible inhibitory substrate?

1.2.2.1 Evidences of butyrate uptake by microalgae and related issues

Butyrate assimilation by microalgae, *Chlorella* sp., was first evidenced in the late 40's under mixotrophic conditions by Myers (1947) and under heterotrophic conditions by Eny (1950, 1949).

During mixotrophic growth of *Chlorella pyrenoidosa*, the O_2 respiration rate decreased with an increase in the supply of butyrate, whereas such effect was not observed when acetate was used (Myers, 1947). Under heterotrophic growth of *Chlorella* sp. on 50 mM of butyrate, Eny (1950) observed that even though O_2 was taken up, growth was inhibited by increasing the butyrate concentration. No physiological hypothesis to explain this phenomenon was suggested (Eny, 1950, 1949). More recently, growth of *Chlorella protothecoides* was completely inhibited for butyrate concentration as low as 0.5 $g.L^{-1}$ under heterotrophic conditions (Chang et al., 2012). During heterotrophic cultivation on glucose (5 $g.L^{-1}$), the addition of 1 $g.L^{-1}$ of butyrate completely inhibited growth of *Chlorella pyrenoidosa* and *Chlorella ellipsoidea* (Samejina and Myers, 1958). The same pattern of butyrate inhibition has also been evidenced in the fast growing yeast *Yarrowia lipolytica* for butyrate concentration as low as 0.09 $g.L^{-1}$ (Rodrigues and Pais, 2000). Recently, *Scenedesmus sp* was grown heterotrophically on 7.3 $g.L^{-1}$ butyrate. Even though microalgae growth was slow (0.46 d^{-1}) and the biomass yield was low (0.09-0.11 $g.g^{-1}$), this species seems more resistant to butyrate than the aforementioned *Chlorella* species (Ren et al., 2013). There was no suggestion explaining the resistance of this species to butyrate. When pH is controlled, the

non-photosynthetic *Crypthecodinium cohnii* can grow on butyrate for concentration as high as 1.2 $g.L^{-1}$ in mixture with yeast extract but a concentration-related butyrate toxicity was still observed (Zhang, 2012).

1.2.2.2 Metabolism of butyrate assimilation by microalgae

Unlike acetate assimilation, butyrate assimilation by microalgae has not been extensively studied. As for acetate, butyrate is probably actively transported into the cell via a monocarboxylic / proton transporter and further metabolized in the glyoxysome (Kurihara et al., 1992; Perez-Garcia et al., 2011b). In the glyoxysome, butyrate might be activated into acetyl-CoA through β-oxidation and then enter the glyoxylate cycle (Figure 1-5) (Kurihara et al., 1992).

1.2.2.3 Hypothesis explaining butyrate inhibition on cell growth

Despite their use as preservative agents for more than 70 years, the exact mechanism explaining the inhibition of bacterial growth by short chain fatty acids, such as butyrate, is not yet well understood (Defoirdt et al., 2009; Ricke, 2003). Recently, Hu et al (2012) pointed out that the effect of butyrate on algae metabolism still has to be deciphered (Hu et al., 2012). Two aspects of butyrate uptake have to be considered: butyrate inhibition on cell growth and, when growth occurs, a butyrate uptake much slower than acetate uptake.

1.2.2.3.1 pH related toxicity ?

Inhibition of microorganisms' growth by weak acids (both undissociated and dissociated forms of the acid are present in water), such as acetate (pKa 4.8 at 25 °C) and butyrate (pKa 4.8 at 25 °C) was previously suggested as a result of an acidification of the cytosolic pH (Lin et al., 2015). Indeed, weak acids have been extensively used to study cytosolic pH variations (Frachisse et al., 1988). At low pH values, acids are assimilated under their undissociated form (Figure 1-8). Due to neutral pH of cytosol, acids are dissociated in the cytosol, which leads to a decrease in pH value. Since regulation of cytosolic pH is very sensitive, most enzymes are effective within a pH range of ± 0.4 pH unit, protons are actively pumped out the cell to maintain a neutral pH in the cytosol (Palmqvist and Hahn-Hägerdal, 2000). This process is ATP-demanding and can thus lead to low microorganisms' growth (da Silva et al., 2013). In *Chlamydomonas reinhardtii*, with an external pH of 7.4 and 4.4 $g.L^{-1}$ of sodium butyrate, butyric acid (undissociated form) caused a cytosolic pH decrease of 0.5 unit which had to be compensate by H^+ expulsion (Nagel et al., 2002). In addition, protons pumps are also linked with Ca^{2+}, K^+ and Na^+ flux which are important cell messengers and a change in

their concentration might be very inhibitory to cell growth (Plieth et al., 1997). Even at low concentration ($\leq$ 1 g.L^{-1}), inhibition of growth by butyrate might be due to the undissociated form of butyric acid rather than its concentration per se (Rao and Mikkelsen, 1977). Obviously, these two parameters are linked since the concentration of undissociated form of an acid depends on concentration and pH.

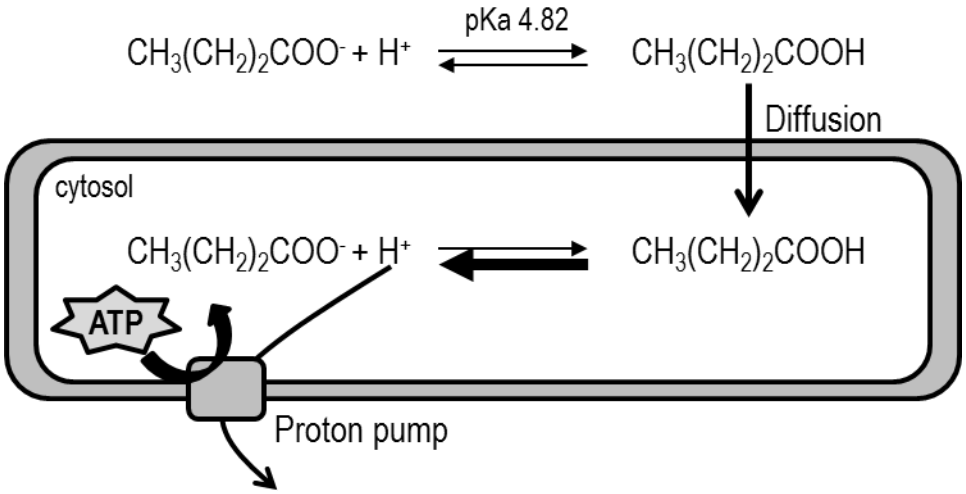

Figure 1-8. Effect undissociated acid on cytosolic pH regulation for butyric acid.

Membrane polarization is affected by the concentrations of acids outside and inside the cells. A remaining presence of butyrate for a long period might be detrimental to the cell (Frachisse et al., 1988). In contrast, since acetate uptake is fast, membrane depolarization due to acetate might be shorter. Thus, high concentration of acetate, as high as 8 g.L^{-1}, can be used to sustain microalgae growth without inhibition (Ogbonna et al., 2000).

1.2.2.3.2 Modification of DNA expression?

In mammalian cells, butyrate has been known to influence the gene expression, i.e. modification of transcription and replication of DNA, through the inhibition of histone deacetylase (Gupta et al., 2006). Nevertheless, inhibition of DNA replication in plant cells and microalgae by butyrate might not be due to hyperacetylation of histones (Pedersen and Minocha, 1988; Waterborg, 1998). The specific effect of butyrate on DNA replication in microalgae has never been studied.

1.2.2.3.3 Metabolic repression of butyrate assimilation?

It is possible that butyrate inhibition and slow uptake is caused by a negative feedback control by an intermediary metabolite of butyrate assimilation, or a slow affinity for its transporter or

slow enzymes reactions of β-oxidation. There is still no study on the cause of metabolic inhibition by butyrate in microalgae. On the opposite, only an non-inhibitory effect of butyrate on the isocitrate lyase activity, the main enzyme of the glyoxylate cycle, was shown (Ramananda and Mcfadden, 1965).

The two main end-products of DF are not similarly assimilated by microalgae. Indeed, on one hand, acetate seems to be a very suitable substrate to sustain both heterotrophic and mixotrophic microalgae growth. On the other hand, butyrate might not be a suitable carbon source to sustain microalgae growth, particularly if supplemented alone. Nevertheless, in DF effluents, acetate and butyrate are both present. Therefore, microalgae growth on VFAs might change due to interactions between the two potential substrates from DF effluents.

1.3 Coupling DF or AF fermentation with microalgae growth

The feasibility of growing microalgae on raw DF and AF effluents has been recently evidenced under heterotrophic and mixotrophic conditions (Table 1-7, references therein). Based on the results obtained under both synthetic and raw fermentation effluents, abiotic and biotic parameters pointed out or appearing to be influencing the microalgae growth are discussed. Since there were only few studies carried out under strict heterotrophic conditions, results obtained under both heterotrophic and mixotrophic conditions are discussed from the beginning of the section but the effect of light on microalgae growth on VFAs mixtures is discussed in sub-section 1.3.1.3. Comparisons with the results obtained during the growth of oleaginous yeasts and fungi are also discussed.

1.3.1 Influence of abiotic parameters on microalgae growth on synthetic fermentation effluents

Until recently, organic carbon substrates were only studied as single substrate to sustain microalgae growth in heterotrophic conditions (section 1.2). Nevertheless, in carbon-rich wastewaters or industrial effluents, several carbon sources are usually available for microalgae (Lowrey et al., 2015). DF and AF effluents are composed of various proportions of VFAs and other organic metabolites such as lactate and ethanol (section 1.1). During microbial growth on multiple carbon substrates, either simultaneous uptake of different substrates or sequential uptakes, also called diauxic effect, are possible (Kovárová-kovar and Egli, 1998). Diauxic phenomena are usual when one of the substrate present in the medium is preferred over another, for example when the growth rate during the uptake of one of the substrate is higher than with the other substrate (Narang and Pilyugin, 2005). Enzymatic

repression is often the cause of a diauxic effect, e.g., repression of the synthesis of the transporter protein (Kovárová-kovar and Egli, 1998).

Microbial growth on multiple organic substrates is driven by the composition and proportion of each substrates, the total organic substrate concentration and the substrate concentration (S):initial biomass concentration (X) ratio (S/X) (Egli et al., 1993; Kovárová-kovar and Egli, 1998). These parameters are all linked together. Nevertheless, understanding their individual influence on microalgae growth and then the interactions between them is crucial to further promote microalgae growth on fermentation effluent. In addition, the culture conditions, heterotrophic or mixotrophic, pH and temperature control, also have tremendous effects on growth as previously discussed (section 1.2). Since all these parameters were almost never studied individually, dissecting the results to highlight the influence of each parameter on microalgae growth on raw effluents is difficult. In this section, unravelling the effect of the proportion of each substrate, the total organic substrate concentration, the S/X ratio, light, pH and temperature on microalgae growth on synthetic effluent was attempted in order to understand the results observed during growth on raw effluents (section 1.3.2).

1.3.1.1 Influence of VFAs content on microalgae growth

Only few studies clearly investigated the impact of VFAs ratio on heterotrophic microalgae growth at a constant concentration of total VFAs (Fei et al., 2014; Zhang, 2012). According to Fei et al. (2014), a high acetate concentration compared to butyrate and propionate was favorable for *Chlorella protothecoides* growth (Table 1-5). In contrast, Zhang (2012) showed that whatever the acetate:butyrate ratio, between 4 and 0.25 in $g.g^{-1}$, similar biomass yields were achieved by *Crypthecodinium cohnii*. Nevertheless, the media used to sustain *C. cohnii* growth were supplemented with yeast extract, 2 $g.L^{-1}$, which contains organic carbon and therefore might have biased the results.

By decreasing the acetate concentration, biomass yields, g biomass per g total VFAs (Y_{X/S_total}), were lowered due to an incomplete exhaustion of butyrate and/or propionate (Table 1-5) (Fei et al., 2014). Slow butyrate and propionate uptakes (not investigated) could explain this finding. To speed up butyrate and propionate uptakes, a low S/X ratio, could be used (sub-section 1.3.1.2). The S/X in the study of Fei et al. (2014) could not be calculated since the initial biomass was too low to be measured (authors' personal communication) indicating nonetheless that it must have been high, very probably higher than 20. Given the biomass yields based on only VFAs consumed by the microalgae ($Y_{X/S\_consumed}$), it could be concluded

that microalgae growth was inhibited during cultivation with acetate:butyrate:propionate (A:B:P) ratio of 4:3:3 (Table 1-5) (Fei et al., 2014). Similar conclusions were reached for growth of the oleaginous yeast *Cryptococcus albidus* on VFAs (Chang et al., 2012; Fei et al., 2011). Microalgae biomass yields were calculated without differentiating acetate, butyrate or propionate assimilation (Fei et al., 2014). Because the biomass yield may differ relatively to the type of substrate assimilated, it would be important to know the fraction of biomass due to acetate or butyrate or propionate uptakes. In addition, Fei et al. (2014) mentioned that butyrate and propionate uptakes were accelerated after acetate exhaustion. This observation suggests that the presence of acetate inhibited butyrate and propionate uptakes. A diauxic phenomenon might have happened. Therefore the direct interaction between the assimilation of the different substrates required further investigations in order to characterize the microalgae growth on VFAs in mixture.

Table 1-5. Effect of VFAs ratio on heterotrophic growth of *Chlorella protothecoides* (Fei et al., 2014).

A:B:P ratio [a]	Acetate removal (%) [b]	Butyrate removal (%) [b]	Propionate removal (%) [b]	$Y_{X/S\_consumed}$ ($g.g^{-1}$) [c]	Y_{X/S_total} ($g.g^{-1}$) [d]	Lipid content (%)
4:3:3	100	50	20	0.25	0.16	35
6:3:1	100	75	90	0.31	0.29	46.5
7:1:2	100	90	50	0.33	0.3	47.4
8:1:1	100	100	95	0.33	0.33	48.7

[a]: Acetate:butyrate:propionate ratios in grams tested with a total VFAs concentration of 2 $g.L^{-1}$.
[b]: The percentages of VFAs consumed, at the end of cultivation, were obtained from the authors (personal communication).
[c]: biomass yield, g biomass per g VFAs consumed by the microalgae.
[d]: biomass yield, g biomass per g of total VFAs.

So far, understanding microalgae growth on different VFAs ratio is based only on hypothesis, such as slow butyrate uptake and possible interactions between the assimilations of the substrates. The effect of the presence of butyrate on acetate uptake by microalgae, in terms of both yields and kinetics, still remains unknown.

1.3.1.2 Influence of total VFAs concentration and S/X ratio on the microalgae growth

As previously pointed out, high acetate and butyrate concentrations, when used as single substrate, could be inhibitory to the microalgae growth (section 1.2). Under both heterotrophic and mixotrophic conditions, increasing the total VFAs concentration above 2 $g.L^{-1}$ inhibited biomass yields of *Chlorella protothecoides* and *Chlamydomonas reinhardtii* (Table 1-6) (Fei et al., 2014; Moon et al., 2013). It is important to note that the pH of the media was not

buffered during the experiments. Moon et al. (2013) reported an increase in pH from 7.5 to 9.5 during microalgae cultivation. Inhibition of microalgae growth at high VFAs concentration might have been caused by an increase in pH due to VFAs exhaustion. Whereas the oleaginous yeast *Yarrowia lipolytica* can grow efficiently with 80 $g.L^{-1}$ of glucose, growth was inhibited from 5 $g.L^{-1}$ of VFAs even with pH control (Fontanille et al., 2012). Biomass yield achieved during C*ryptococcus albidus* growth on VFAs decreased with increasing VFAs concentration from 2 to 10 $g.L^{-1}$, due to an incomplete VFAs exhaustion for initial concentrations higher than 5 $g.L^{-1}$ (pH control was not mentioned) (Fei et al., 2011).

Table 1-6. Effect of total VFAs concentration on biomass yield and lipid content.

Total VFAs concentration ($g.L^{-1}$)	*Microalgae*				*Yeast*	
	C. protothecoides [a]		*C. reinhardtii* [b]		*C. albidus* [c]	
	$Y_{X/S}$ ($g.g^{-1}$) [d]	Lipid content (%)	$Y_{X/S}$ ($g.g^{-1}$)	Lipid content (%)	$Y_{X/S}$ ($g.g^{-1}$)	Lipid content (%)
1	0.39	48				
2	0.29	48	0.85	14	0.58	27
4	0.08	41				
5			0.38	19	0.51	25
8	N.G.	N.G.			0.26	12
10			0.17	17	N.G.	N.G
Reference	(Fei et al., 2014)		(Moon et al., 2013)		(Chang et al., 2012; Fei et al., 2011)	

[a]: A:B:P ratio was set at 6:3:1 and the experiment was carried out under heterotrophic conditions.
[b]: A:B:P ratio was set at 8:1:1 and the experiment was carried out under mixotrophic conditions.
[c]: A:B:P ratio was set at 6:3:1.
[d]: biomass yield, g biomass per g VFAs.
[e]: N.G.: no growth was detected.

At constant initial microalgae concentration, increasing the concentration of VFAs results in a higher S/X ratio. To shorten VFAs exhaustion and reduce VFAs inhibition, the initial S/X could be lowered, by either diluting the medium or increasing the initial microalgae load (Liu et al., 2012). Under mixotrophic conditions on butyrate, lowering the S/X from 4.8 to 1.1, resulted in an increase in final biomass production by 50% and residual butyrate decreased from 50% to 0% during growth of *Chlorella vulgaris* (Liu et al., 2012). Butyrate uptake rate was slightly enhanced by the surplus of initial biomass, from 0.17 $g.L^{-1}.d^{-1}$ to 0.23 $g.L^{-1}.d^{-1}$. Nevertheless, the content of VFAs still has to be taken into account to discuss the results. It could be expected that the S/X ratio has to be set according to the acetate:butyrate ratio. For

instance, with a high A:B:P ratio of 8:1:1 and a high S/X of 20, *Chlamydomonas reinhardtii* could grow without inhibition on 2 $g.L^{-1}$ of VFAs (Moon et al., 2013) (Table 1-6).

As for microalgae growth on VFAs, lowering the initial S/X ratio to enhance yeast growth on VFAs has been successfully used for both *Cryptococcus albidus* and *Yarrowia lypolitica* (Chang et al., 2012; Fontanille et al., 2012). In both cases, the S/X ratio was lowered thanks to a first growth on glucose which provided high microbial biomass. A low S/X of 1.6 enabled the use of a high concentrated mixture of VFAs, 9 $g.L^{-1}$, with A:B:P ratio of 6:3:1 to grow *Cryptococcus albidus*. Similarly, *Yarrowia lypolitica* was grown on VFAs, A:B:P ratio of 3:1:1, at a feeding rate of 1.5 $g.L^{-1}.h^{-1}$ with an intial S/X lower than 0.1, after glucose or glycerol exhaustion (Fontanille et al., 2012). Glycerol being a co-product of biodiesel production from oleaginous micro-organisms, the use of both effluents containing glycerol and VFAs could be used to reduce the cost of carbon source and lower VFAs inhibition on growth.

1.3.1.3 Mixotrophic cultivation: effects light and CO_2

During mixotrophic growth on VFAs, microalgae are expected to assimilate CO_2 thus increasing biomass and speeding up the apparent uptake of VFAs (sub-section 1.2.1.2). Autotrophic growth could be considered as another mean to lower the S/X ratio. The results obtained by Liu et al. (2012) evidenced that both butyrate and inorganic carbon uptakes occurred simultaneously during mixotrophic growth of *Chlorella vulgaris*. Indeed, only a simultaneous assimilation of substrates can explain the biomass yield observed on butyrate, 2.1 $g.g^{-1}$ (Liu et al., 2012). This observation highlights once more the necessity to discuss with caution the biomass yields obtained at the end of mixotrophic growth. The authors pointed out that inorganic carbon was a preferred substrate compared to butyrate. Indeed, *C. vulgaris* growth rate on a mixture of butyrate and bicarbonate (HCO_3^-), ranging from 0.52 to 0.63 d^{-1}, was significantly lower than the one observed during autotrophic growth on HCO_3^- as single substrate, 0.97 d^{-1} (Liu et al., 2012). In addition, the butyrate:HCO_3^- ratio also appeared to be important in order to avoid inducing completely autotrophic metabolism (Liu et al., 2012). Similarly, light intensities also need to be carefully managed to be not inhibitory (Liu et al., 2013). These parameters are probably species specific and need to be further investigated. The interactions between heterotrophic and autotrophic metabolisms during growth on mixtures of VFAs are still not completely described. As for heterotrophic conditions, the interactions between the assimilations of acetate and butyrate have not been investigated yet. For instance,

the effect of the different butyrate concentrations combined with CO_2 fixation on acetate uptake under mixotrophic conditions has not been investigated yet.

1.3.1.4 Influence of temperature and pH

Despite the beneficial effects caused by an optimal temperature on microbial growth, such as enzymatic activity enhancement and reduction of the requirement for thermoregulation, temperature could have adverse effects on microalgae growth on VFAs. Indeed, the RuBisCO enzyme, which catalyzes CO_2 fixation in the Calvin cycle (section 1.2.1.2.1), has a stronger oxidase activity at high temperature (depending on the microalgae species), meaning that CO_2 fixation is reduced due to a higher photorespiration rate (Bernacchi et al., 2001). In addition, the pKa of acids is linked to temperature and temperature variation may change the concentration of the undissociated toxic form of VFAs (sub-section 1.2.2.3.1). Effect of temperature on microalgae growth on VFAs has been scarcely studied but the first results highlighted the importance of studying this parameter. *Chlorella protothecoides* was grown heterotrophically on a mixture of VFAs, A:B:P ratio of 6:3:1, at 25 °C and 30 °C. 30 °C is close to the optimal temperature for this species which ranges, under heterotrophic and autotrophic conditions, between 28 and 30 °C (Fei et al., 2014; Sforza et al., 2014; Shi et al., 2006). Nevertheless, when supplemented with a mixture of VFAs, microalgae growth was higher at 25 °C than at 30 °C (Fei et al., 2014). Optimal conditions for growth on favorable substrates might not be the same as for growth on inhibitory substrates. Therefore, the influence of abiotic parameters such as temperature must be looked into to better understand microalgae growth on mixtures of VFAs.

As pointed out in section 1.2, the control of pH is required to reduce the pH-related inhibition caused by VFAs. In addition, without pH control, pH rises due to organic acids exhaustion leading thus to an increase in toxic ammoniac (NH_3) if ammonium (NH_4^+) is present in the medium (pKa NH_4^+/NH_3 = 9.5). pH control, via automatic titration of either base or acid, was successfully used to increase biomass production by 38% and reduce butyrate removal by 19% during mixotrophic growth of *Chlorella vulgaris* on butyrate (Liu et al., 2012). As pointed out previously, when analyzing microalgae growth on VFAs, the control of pH has to be carefully considered to better understand the yields observed.

1.3.2 Growth of microalgae on raw effluents: successes and challenges

1.3.2.1 Successful microalgae growth on raw effluents thanks to controlled abiotic parameters

Despite the very different raw effluents tested, with varying compositions and concentrations of fermentation metabolites, *Chlorella* sp. and *Scenedesmus* sp. grew on DF and AF effluents thanks to various operating conditions strategies (Table 1-7).

1.3.2.1.1 High acetate concentration lead to higher microalgae production

High acetate concentration ($\geq$ 3 $g.L^{-1}$) combined with high A:B ratio and pH control have been identified as key parameters to reduce the inhibitory effects caused by high initial concentration of VFAs and thus promoting the microalgae growth on raw effluents (Table 1-7) (Cho et al., 2015; Hongyang et al., 2011; Hu et al., 2013, 2012; Ren et al., 2014a). Indeed, effluents with high total metabolites concentrations (> 5 $g.L^{-1}$), and with A:B ratio ranging from 4.5 to 20, have been successfully used to reach high microalgae concentration ($\geq$ 2 $g.L^{-1}$) even without low S/X (no dilution) under both heterotrophic and mixotrophic conditions (Cho et al., 2015; Ren et al., 2014a). Since ethanol was not assimilated by *Scenedesmus* sp. (Ren et al., 2014b), biomass yield could be further enhanced by using ethanol-consumer species such as *Crypthecodinium cohnii*, *Chlorella protothecoides* and *Chlorella sorokiniana*, (Lowrey et al., 2015; Ogbonna and Tanaka, 1998; Sforza et al., 2012). As suggested by several authors, including Fei et al., (2014), microalgae grew well in fed-batch cultivation on raw effluents thanks to the control of VFAs concentration remaining lower than inhibitory concentration ($\leq$ 2 $g.L^{-1}$) (Hu et al., 2013). As previously pointed out (sub-section 1.3.1.3), microalgae concentration and yields achieved under mixotrophic conditions have to be analyzed cautiously. For example, the high biomass yield (> 1 $g.g^{-1}$) and microalgae concentration reached by *Chlorella pyrenoidosa* were probably due to both inorganic carbon uptake and also unidentified organic compounds from the effluents (Table 1-7) (Hongyang et al., 2011). As could be expected, when acetate concentration was low (0.3 $g.L^{-1}$) because of dilution of the effluent, final microalgae concentration was low (0.35 $g.L^{-1}$) despite mixotrophic conditions and an A:B ratio of 10 (Table 1-7) (Hu et al., 2012). Since pH was neither controlled nor buffered, the AF effluent used in the study of Hu et al. (2012) had to be diluted 8 to 20 fold to avoid growth inhibition by an increase in NH_3 concentration (Hu et al., 2013, 2012a).

Table 1-7. Overview of studies carried out with raw fermentation effluents.

Fermentation process		Effluent treatments	Microalgae growth							Studies/ Comments	Reference
	Mixed or pure culture Substrate		Metabolites concentration ($g.L^{-1}$)	A:B [a]	H/M	pH control	species	S/X	$Y_{X/S}$ [b]	X_{prod} ($g.L^{-1}$) [c]	
Dark fermentation	Pure (*Clostridium*) Glucose, xylose	Dilution: ¼ Sterilized	Acetate : 0.3 Butyrate : 0.8 Formate : 0.04 Lactate : 0.05	0.4	H M	*N.M.*	*C. vulgaris*	4.8	0.38 0.80	0.45 0.7	Partial butyrate exhaustion S/X, light intensity, CO_2 sparging	Liu et al., 2013
	Pure (*Clostridium*) sucrose	Dilution: ¼ Sterilized	Acetate : 0.5 Butyrate : 1.13 Lactate: 0.78	0.4	M	*No*	*C. vulgaris*	9.6	0.09	0.21	Lactate was not consumed S/X	Liu et al., 2012
	Mixed Food waste	Dilution: 7/10	Acetate : 1.13 Butyrate : 0.83 Propionate:0.24 Formate, Ethanol : N.M.	1.4	M	N.M.	Mixed algae	15	0.41	1.22	Bacterial growth was not monitored.	V Mohan & P Devi, 2012
	Pure (*Ethanoligenens*) Glucose	Not diluted Sterilized	Acetate: 3 Butyrate: 0.15 Ethanol: 2.7 Prop, valerate: trace	20	H	*N.M.*	*Scenedesmus* sp.	58	0.34	1.88	Ethanol was not consumed.	Ren et al., 2014
Acidogenic fermentation	Mixed Soybean processing wastewater	Dilution: 1/2.7 Sterilized	Acetate: 0.67 Butyrate: 0.25 Propionate: 0.44 Others: < 0.11	2.7	M	*N.M.*	*C. pyrenoidosa*	4.9	1.23	1.85	VFAs represented 70% of initial COD.	Hongyang et al., 2011
	Mixed Secondary sludge	Not diluted Sterilized	Acetate: 4.5 Butyrate: 1 Propionate:1.3 Iso-valerate:1.1 Iso-butyrate & valerate: < 0.8	4.5	M	*Yes*	*C. vulgaris*	58	0.47	4.08	VFAs represented 75% of initial COD.	Cho et al., 2014
	Mixed Liquid swine manure	Dilution: 1/20 Sterilized [d] and Unsterilized	Acetate:0.30 Butyrate:0.03 Propionate: 0.24	10	M	*N.M.*	*Chlorella* sp.	2.2	0.79 [c] 0.79	0.35	Bacterial growth was not monitored.	Hu et al., 2012
	Mixed Liquid swine manure	Dilution: 1/8	Total: 1.5 – 1.8 g/L (composition N.M.)		M	*Yes*[e]	*Chlorella* sp.		0.37[f]		Bacterial growth was not monitored.	Hu et al., 2013

[a]: Acetate:butyrate ratio in g per g; [b]:biomass yield, g biomass per g total metabolites; [c]: Biomass produced in $g.L^{-1}$; [d]: 22% of total VFAs were lost through sterilization (probably autoclave); [e]: The mentioned experiment was carried out in Fed-batch mode. pH was maintained between 7 – 8 at steady state; [f]: biomass yield could not be calculated in g g-1 with the data available and was calculated as g per g of DCO;N.M.: Not Mentioned in the study; S/X: substrate:biomass ratio

1.3.2.1.2 Low S/X ratio and light supply used for low A:B ratios

Low S/X ratio (dilution of the effluent and/or increase of initial microalgae load) and adjusted mixotrophic conditions (light intensity and no CO_2 sparging) allowed microalgae to grow on effluents with A:B ratios lower than 1 (Liu et al., 2013, 2012; Venkata Mohan and Prathima Devi, 2012). As mentioned previously (sub-section 1.3.2.1.1), the main drawback of diluting effluents is the low microalgae production caused by low VFAs concentration (Table 1-7). Liu et al., (2013) showed that butyrate uptake rate by *Chlorella vulgaris* was 10 times faster under optimized mixotrophic conditions, with adjusted light intensity and without CO_2 sparging, than under heterotrophic conditions at low S/X ratio. Nevertheless, by sparging air enriched with 30% CO_2 or under saturating light intensities, the beneficial effect of mixotrophy decreased, butyrate uptake rate decreased by 20 to 30% as reported by Liu et al., (2013).

1.3.2.2 Challenges arising from the use of raw effluents

1.3.2.2.1 Possible presence of inhibitors or low nutrient availability

On top of VFAs composition and concentration, new challenges arise from the use of "real" raw AF and DF effluents to sustain microbial growth, with real wastes such as food waste as feedstock for fermentation (Figure 1-9). When using food waste as substrate for AF and further growth of the fungi *Cryptococcus albidus* on AF effluent, the presence of unknown inhibitors was suggested by the authors (Chi et al., 2011; Vajpeyi and Chandran, 2015). Indeed, growth rate was twice higher when fungi were grown on synthetic AF effluents (mimicking VFAs composition of the real effluents) than on real AF effluent (Vajpeyi and Chandran, 2015). Essential nutriments limitation (N, P, K^+, Mg^{2+} etc.) might also have caused microbial growth limitation. Nitrogen should not be limiting in DF effluents from protein-rich wastes fermentation such as food wastes (section 1.1 and (Chi et al., 2011). On the opposite, Chi et al. (2011) hypothesized that ammonium concentration (2.4 $g.L^{-1}$) in the DF effluent from food waste was too high, leading to a C:N ratio of 3.2:1, to induce lipids production from DF effluent. One of the main reasons to couple DF with microalgae growth is the availability of ammonium, orthophosphate and other nutrients in the effluent which are mineralized from feedstock (sub-section 1.1.3). Nevertheless, due to a lack of systematic screening of nutriments contents in DF effluents, there are still no data available to conclude on sufficient nutrient availability to sustain efficiently microalgae growth. Investigations on finding the optimal feedstock for both hydrogen and microalgae productions are required.

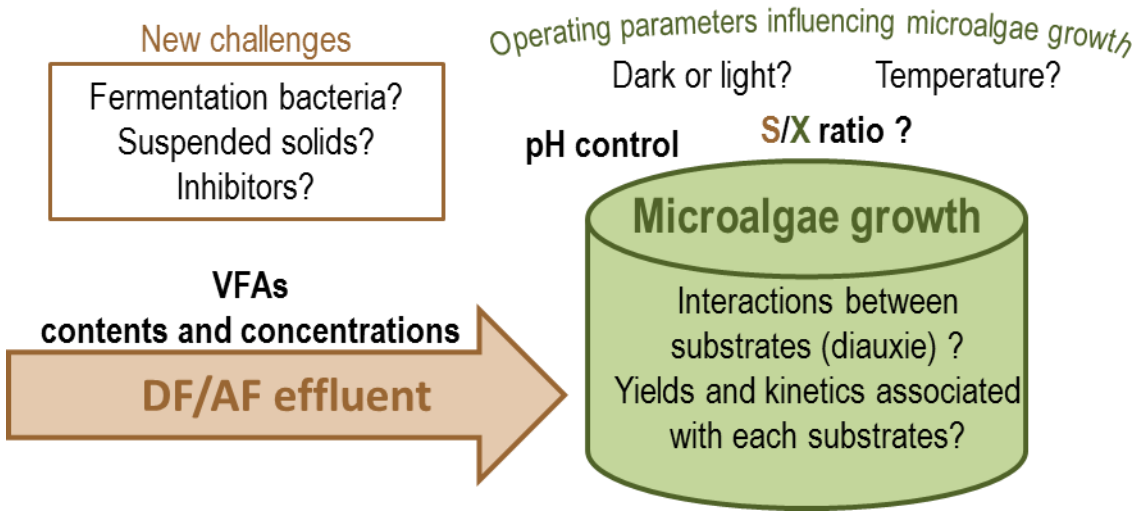

Figure 1-9. Known and possible parameters influencing microalgae growth on fermentation effluents.

1.3.2.2.2 Presence of suspended solids in untreated effluents

Another issue linked with the use of untreated effluent is the presence of suspended solids that darken the medium thus reducing the access to light to sustain mixotrophic growth (Hu et al., 2012b). By operating under heterotrophic conditions, the presence of suspended solids should not alter the microalgae growth. Dilution of AF or DF effluents might be necessary under mixotrophic conditions (Hu et al., 2012b).

1.3.2.2.3 Bacterial presence in unsterilized effluents

To couple DF and heterotrophic cultivation of microalgae efficiently, the cost of effluent sterilization has to be reduced. According to Park et al. (2014), sterilization of the medium accounts for more than one fourth of the investment cost of the process when coupling DF and oleaginous yeast cultivation. Bacterial contamination is one of the main challenges that must be resolved for upscaling heterotrophic cultivation (Rashid et al., 2014). During heterotrophic cultivation, the competition between microalgae and bacteria, for carbon, nitrogen, phosphate and oxygen, is usually found to be unfavorable for microalgae growth (Kamjunke et al., 2008; Zhang et al., 2012). Nevertheless, these authors suggested that some conditions may be favorable to microalgae growth, such as low initial bacterial density and high initial nutrient loads. In unsterilized municipal wastewater, with high NH_4^+ and PO_4^{3-} loads and low organic carbon loads, *Chlorella protothecoides* has been shown to grow efficiently under autotrophic conditions (Ramos Tercero et al., 2014). The impact of fermentation microorganisms on microalgae is still unknown. As described in section 1.1.1.2, microbial community in DF and AF effluents is composed mainly of strict anaerobic and

facultative anaerobic bacteria since protists and aerobic bacteria should not survive the heat pretreatment and the fermentation process.

Few studies worked with unsterilized fermentation effluents under mixotrophic conditions (Hu et al., 2013, 2012a; Venkata Mohan and Prathima Devi, 2012). However, the presence and the possible role of bacteria have not yet been studied. Bacterial growth has not been monitored and microalgae growth is commonly monitored by optical density or gravimetric methods which do not enable to distinguish between microalgae and bacteria. Thus, one cannot distinguish between VFAs uptake by microalgae or by bacteria. For ensuring VFAs uptake by microalgae, monitoring microbial community is mandatory.

1.4 Conclusion

Growing microalgae under heterotrophic or mixotrophic conditions on DF or AF effluents is feasible as was previously evidenced by several studies (Table 1-7). It was highlighted that DF and AF effluents are complex media. Indeed, the proportion of VFAs and the total concentration vary greatly according to the substrate for fermentation, the process used (AF or DF) and the fermentation bacteria. The main advantage of AF is the high A:B ratio generally obtained at the end of the fermentation. The main advantages of DF are (i) lower metabolites concentrations, which can be inhibitory to microalgae growth, (ii) narrower range of possible metabolites, since acetate and butyrate are the main end-products of DF, and (iii) the co-production of H_2. Using DF effluent from fermentation of *Ethanoligenens harbinense* seems very promising (Ren et al., 2014b). Nevertheless, *Ethanoligenens* species are non-sporulating, so they are not usually found in mixed cultures since they cannot survive the inoculum heat treatment used for DF and AF processes (section 1.1.1.2).

From the studies currently available on the coupling, it can be concluded that microalgae growth is favored on effluents with high acetate:butyrate ratio and high acetate concentration operated with pH control. At low acetate:butyrate ratio and/or high total metabolites concentrations, light and lowering the substrate:microalgae ratio appeared to be effective tools to enhance microalgae growth. More precisely, butyrate content in both synthetic and raw effluent seems to be a key parameter for the coupling. Butyrate presence and its concentration seemed to inhibit microalgae growth. So far, it can only be hypothesized that this inhibition relies on a slow uptake rate or a low biomass yield on butyrate. Butyrate and acetate might also interact through a diauxic phenomenon. The effects of abiotic parameters such as light and temperature might change the interactions between acetate and butyrate. Since butyrate is

very often found in DF effluent, butyrate uptake by microalgae and its effect on acetate uptake has to be further investigated to deal with butyrate-rich DF effluents.

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Chapter 2

Diauxic growth of heterotrophic microalgae on mixtures of VFAs: assessment of yields and kinetics by using a modelling approach

Growth behavior of two oleaginous microalgae, *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, on various mixtures of acetate, butyrate and lactate, under heterotrophic conditions, are investigated in this chapter. In a first part, the rationale of the study and of the choice of microalgae is explained. Then, the materials and methods are presented, with an emphasis on the mathematical model built to describe the experimental data. The results, including the evidence of a diauxic growth, are then described and discussed. The results of this chapter have been published in Bioresource Technology in an article entitled "Use of fermentation metabolites for heterotrophic microalgae growth: Yields and kinetics" (2015, volume 175, p342-349).

2.1 Introduction

2.1.1 Objective

According to the literature review (Chapter 1, section 1.3), it is generally hypothesized that the microalgae growth on mixtures on VFAs is inhibited at low acetate:butyrate ratio (<1) due to both the slow uptake of butyrate and a concentration-related inhibition of butyrate on microalgae growth and acetate uptake. The first objectives of the PhD were to study the interactions between acetate and butyrate uptakes and to clearly determine if these uptakes are diauxic and also to assess the threshold level of butyrate inhibition. To this end, microalgae were grown on acetate and butyrate as single substrates and on mixtures of acetate and butyrate. For each experiment, the kinetic parameters (growth rate and uptake rate) and the biomass yields were determined, using a mass balance model.

2.1.2 Choice of microalgae for the study

The choice of microalgae species used during the experiments was based on (i) their ability to grow heterotrophically and mixotrophically, (ii) their ability to compete with bacteria for nutrients, (iii) their resistance to drastic environmental conditions, (iv) their ability to produce high amounts of lipids for biofuel and, if possible, other interesting compounds such as pigments.

One evident choice could have been the fast growing *Crypthecodinium cohnii*. However, it was not chosen due to its lack of photosynthetic apparatus (no possible mixotrophy). Instead, *Chlorella* species were chosen. Indeed, they have been used and studied worldwide for 60 years because of their fast growth, their easy handling, their strong resistance to environmental changes (light availability, temperature, pH, etc…), and their competitiveness for high productivity of biomass and bio-based products such as proteins and lipids (Liu and Chen, 2014). The main morphological and physiological characteristics of the genus *Chlorella* are the round cell shape and the multiple division, one mother cell dividing itself into 2 to 8 daughters cells (Richmond and Hu, 2013). Within this genus, the two species *Chlorella sorokiniana* and *Auxenochlorella protothecoide*s were chosen as the model microalgae for the experiments of this PhD, due to their particular characteristics as described below.

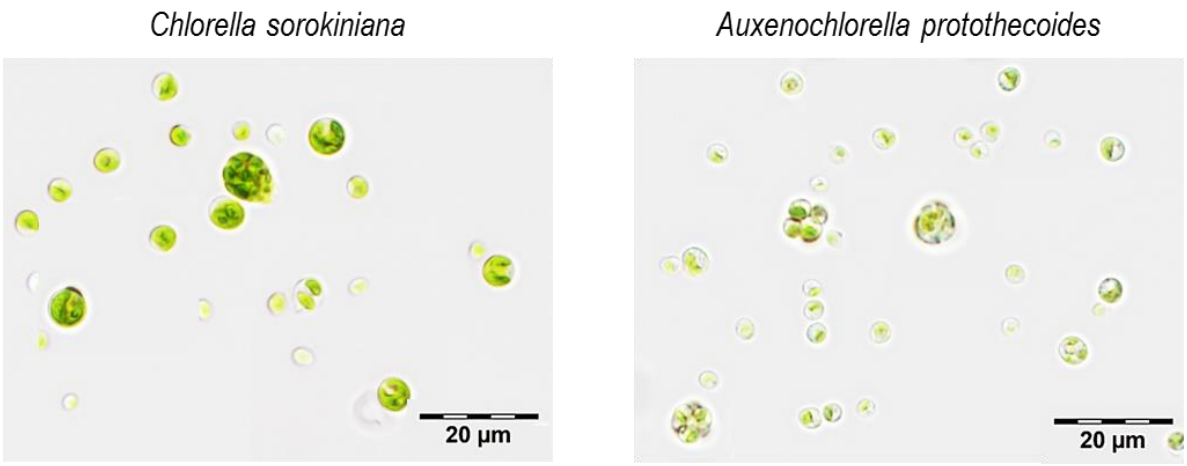

Figure 2-1. Microscopic observations of *Chlorella sorokiniana* and *Auxenochlorella protothecoides*.

Chlorella sorokiniana (Figure 2-1), previously known as *Chlorella pyrenoidosa*, is a small microalgae, 2 to 4.5 µm, thermotolerant, with an optimal growth temperature between 35 and 37 °C (Janssen et al., 1999; Li et al., 2014). It has a fast growth, with a growth rate as high as 6 d^{-1}, and is able to grow efficiently on acetate under both heterotrophic and mixotrophic conditions with a biomass yield close to 0.4 $g.g^{-1}$ (Lizzul et al., 2014; Ramanna et al., 2014; Van Wagenen et al., 2014a). In addition, it is oleaginous, with a lipid content, possibly as high as 61.5 % of DW (Ramanna et al., 2014). Another main advantage of *C. sorokiniana* is its high tolerance to NH_3, which is important to avoid inhibition when using effluents containing NH_4^+ (Godos et al., 2010). In addition, this particular species has also been used to study algal-bacterial consortium on wastewater treatments and was shown to be highly competitive for both nutrient (NH_4^+) removal and organic carbon (acetate) removal (Godos et al., 2010; Ogbonna et al., 2000). Recently, very high biomass and lipid productions by *C. sorokiniana* (103 $g.L^{-1}$ and 40 $g.L^{-1}$ respectively) have been achieved using a two-stage fed-batch cultivation strategy under heterotrophic conditions with glucose as substrate (Zheng et al., 2013). The lipids fraction was composed of 77 % of TAGs mainly composed of oleic (C18:1), palmitic (C16:0) and linoleic (C18:2) which, in combination, allows god quality of biodiesel in terms of oxidative stability and temperature resistance (Zheng et al., 2013).

Auxenochlorella protothecoides, also known as *Chlorella protothecoides*, is a microalga of 5 to 10 µm, with a high growth rate (2.4 d^{-1}) (O'Grady and Morgan, 2011) and high biomass productivity (1.02 $g.L^{-1}.h^{-1}$) (Xiong et al., 2008). It is also an oleaginous microalgae, with a lipid content as high as 57 % (Wang et al., 2013), and is able to grow efficiently on acetate under both heterotrophic and mixotrophic conditions (Fei et al., 2014; Heredia-Arroyo et al.,

2010). *A. protothecoides* is the most studied *Chlorella* sp. under heterotrophic conditions mainly for food, lutein and for biodiesel production (Liu and Chen, 2014). Very high biomass densities have been achieved during heterotrophic growth on glucose, as high as 116 $g.L^{-1}$ in fed-batch systems (Xiong et al., 2008). Recently, similar biomass concentration levels have been reached (97 $g.L^{-1}$), using hydrolyzed molasses, a low cost carbon sources (Yan et al., 2011). In addition, *A. protothecoides* lipids composition has been shown to be suitable for biodiesel production, since it contains long-chain monounsaturated fatty ester, such as oleic acids (C18:1), which allows a good oxidative stability and good low-temperature properties. *C. protothecoides* was also used as a model microalgae species to study heterotrophic growth on VFAs (Fei et al., 2014).

2.2 Materials and methods

2.2.1 Microalgae strains and stock culture conditions

Chlorella sorokiniana (CCAP 211/8K) and *Auxenochlorella 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 prepare stock cultures. 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 maintained in Petri dishes on solid medium (agar agar at 10 % w/v) and then one colony was transferred into 500 mL Erlenmeyer flasks, containing 200 mL of modified BG11 medium (Figure 2-2). Inoculum and Petri dishes were cultivated in autotrophic conditions to avoid a cometabolism effect (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 to 7 days of cultivation, the culture was used to further inoculate (10 % V/V) the different culture media (Figure 2-2). Axeny was daily checked by phase contrast microscopy and DAPI staining microscope observations as well as spreading cultures on ATCC5 solid media (ATCC, http://www.lgcstandards-atcc.org/).

2.2.2 Heterotrophic growth on mixtures of organic acids

In order to evaluate the cell viability of the inoculum, a positive control was cultivated under autotrophic conditions (with light and inorganic carbon as carbon source (bicarbonate). A negative control containing only bicarbonate as carbon source 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 2-1) but the C:N:P molar ratio was set at 48:16:1. This ratio avoided nitrogen limitation throughout the experiments (Hongyang et al., 2011). 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) (Figure 2-2).

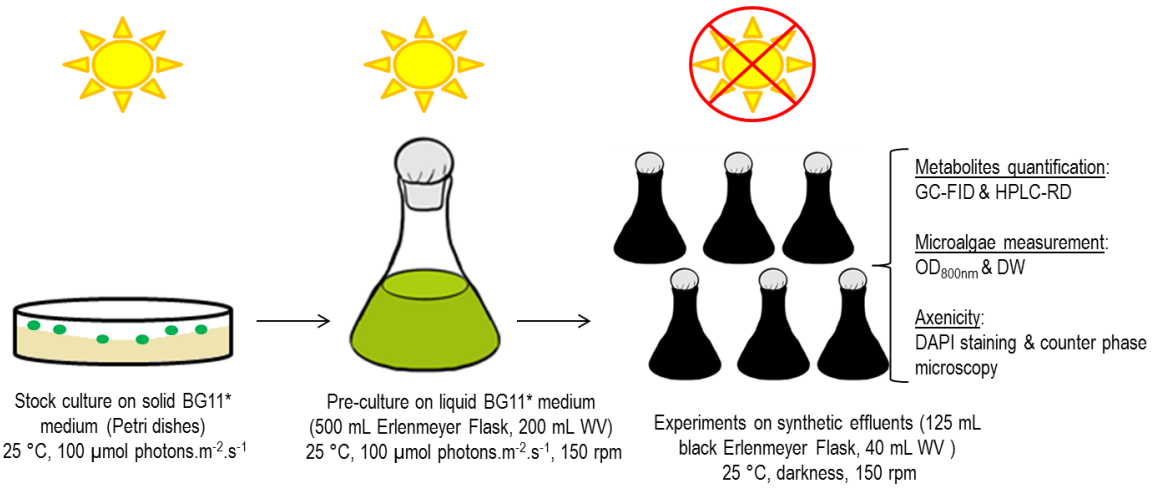

Figure 2-2. Schematic representation of the experiments.

BG11*: modified BG11 medium, WV: Working Volume

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

All experiments and controls were done in triplicates.

Table 2-1. List of all the conditions tested for *Chlorella sorokiniana* and *Auxenochlorella protothecoides* to estimate and validate model parameters.

Experiments tested	Acetate ($g_C.L^{-1}$)	Butyrate ($g_C.L^{-1}$)	Lactate ($g_C.L^{-1}$)	Estimation (E) or Validation (V)*
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 mixtures of acetate and butyrate	**0.25**	**0.25**	0	**E**
	0.4	**0.1**	0	**V**
	0.9	**0.1**	0	**E**
Growth on lactate and mixtures of metabolites	0	0	**0.5**	**
	0.25	0	**0.25**	**V**
	0.25	**0.45**	**0.16**	**V**

*: Data used for Estimation (E) or Validation (V) of model parameters

**: the experimental data were not used to build the model due to the absence of lactate removal during the experiments.

2.2.3 Analytical methods

2.2.3.1 Biomass measurement

Biomass growth was quantified by measurement of the Optical Density at 800 nm (OD_{800}) to minimize pigment interference (Schmidt et al., 2005). Culture samples of 300 µL were dispensed in a 96 well BD Falcon® microplate and analyzed using a Spectrophotometer Infinite Nanoquant M200 (Tecan®). Dry Weight (DW) was determined after filtering 15 mL of algal samples on pre-weighed GF/F Whatman® filters that were dried overnight at 105°C. DW was correlated to OD_{800} using a calibration curve. For *Chlorella sorokiniana* and *Auxenochlorella protothecoides* the equations were DW ($g_{DW}.L^{-1}$) = 1.24*OD_{800} (R^2 = 0.95) and DW ($g_{DW}.L^{-1}$) = 1.38*OD_{800} (R^2 = 0.99), respectively.

2.2.3.2 Organic acids measurements

Volatile fatty acids (VFAs), i.e. acetate and butyrate, were quantified using a gas chromatograph (Perkin Clarus 580), with capillary column maintained at 200°C and with N_2 as the gas vector (flow rate of 6 $mL.min^{-1}$) (Elite-FFAP crossbond®carbowax® (15 m))

equipped with a flame ionization detector maintained at 280°C (FID). This device is calibrated to quantify precisely acetate, butyrate, propionate, iso-butyrate, valerate and iso-valerate. Before use, 300 µL of filtered (0.45 µm) culture sample were mixed with 300 µL of a standard solution (ethyl-2-butyric acid, 1 $g.L^{-1}$).

Lactate was quantified using a high performance liquid chromatography (HPLC) equipped with an automatic sampler and coupled with a refractometric detector (Waters R410). A pre-column (Micro guard cation H refill cartridges, Bio-rad) was used to filter possible solids. The column used was an Aminex HPX-87H column, 300 x 7.8 mm (Biorad®). The column temperature was maintained at 35 °C and the flow rate of the eluent (H_2SO_4, 0.005 $mol.L^{-1}$) at 0.4 $mL.min^{-1}$. This device is calibrated to quantify precisely glucose, ethanol, lactate, valerate and caproate.

The errors associated with OD, DW and organic acids measurements were 2, 6 and 5 %, respectively.

2.2.4 Data analysis

2.2.4.1 Model design

A model was built to estimate kinetic parameters in batch cultures: (1) μ_{a_max} the maximum growth rate associated with acetate removal (d^{-1}), (2) K_{Sa} the half saturation constant for acetate ($g_C.L^{-1}$), (3) Y_a the yield coefficient associated with acetate removal ($g_{DW}.g_C^{-1}$), (4) μ_{b_max} the maximum growth rate associated with butyrate removal (d^{-1}), (5) S_{bopt} the concentration of butyrate when the growth rate is maximum ($g_C.L^{-1}$), (6) α the initial slope ($L.d.g_C^{-1}$), (7) Y_b the yield coefficient associated with butyrate removal ($g_{DW}.g_C^{-1}$) and (8) K_D the half inhibitory constant representing the inhibitory effect of acetate on butyrate removal ($g_C.L^{-1}$). Lag phases were graphically determined by logarithmic plotting of the DW versus time and were excluded from data analysis.

In its structure, the model considers that organic carbon, provided by acetate, S_a ($g_C.L^{-1}$), is assimilated by microalgae to produce biomass, X ($g_{DW}.L^{-1}$) at a rate μ_a (S_a) (d^{-1}) and with a yield coefficient, Y_a ($g_{DW}.g_C^{-1}$). This carbon assimilation can be described by the macroscopic reaction which represents the mass flux between substrate and biomass as following:

$$\frac{S_a}{Y_a} \xrightarrow{\mu_a(S_a)*X} X \qquad \text{Equation 2-1}$$

The growth rate μ_a (S_a), associated with acetate removal, was assumed as following a Monod function:

$$\mu_a(S_a) = \mu_{a\_max} * \left(\frac{S_a}{S_a + K_{S_a}}\right) \quad \text{Equation 2-2}$$

with S_a the concentration of acetate ($g_C.L^{-1}$), K_{Sa} the half saturation constant for acetate ($g_C.L^{-1}$) and μ_{a_max} the maximum growth rate associated with acetate assimilation (d^{-1}).

As for growth on acetate, the assimilation of carbon via butyrate removal can be described by the following macroscopic reaction:

$$\frac{S_b}{Y_b} \xrightarrow{\mu_b(S_b)*X} X \quad \text{Equation 2-3}$$

The growth rate $\mu_b(S_b)$, associated with butyrate removal, was assumed to follow a Haldane function, supplemented with a diauxic term, K_D ($g_C.L^{-1}$), representing the inhibitory effect of acetate on butyrate removal:

$$\mu_b(S_b) = \mu_{b\_max} * \frac{K_D}{K_D + S_a} * \frac{Sb}{S_b + \frac{\mu_{b\_max}}{\alpha} * \left(\frac{Sb}{S_{b\_opt}} - 1\right)^2} \quad \text{Equation 2-4}$$

with S_b the concentration of butyrate ($g_C.L^{-1}$), S_{b_opt} the concentration of butyrate when μ_b (S_b) is maximum ($g_C.L^{-1}$), α the initial slope ($L.d.g_C^{-1}$), μ_{b_max} the maximum growth rate associated with butyrate assimilation (d^{-1}), K_D the half inhibitory constant associated with the diauxic growth ($g_C.L^{-1}$).

Thanks to a mass-balance, the model was described by the following ordinary differential equations (ODE) system:

$$\frac{dS_a}{dt} = \mu_a(S_a) * \frac{1}{Y_a} * X \quad \text{Equation 2-5}$$

$$\frac{dS_b}{dt} = \mu_b(S_b) * \frac{1}{Y_b} * X \quad \text{Equation 2-6}$$

$$\frac{dX}{dt} = \mu_a(S_a) * X + \mu_b(S_b) * X \quad \text{Equation 2-7}$$

2.2.4.2 Parameters estimation

Kinetics parameters were estimated using six sets of experimental data: 0.1 and 1 $g_C.L^{-1}$ of acetate, 0.1 and 1 $g_C.L^{-1}$ of butyrate, 0.25 $g_C.L^{-1}$ of acetate and butyrate and 0.9 $g_C.L^{-1}$, 0.1 $g_C.L^{-1}$ of acetate, butyrate, respectively. The seven complementary data sets were used to validate the estimation (Table 2-1). To minimize the effect of inoculation and substrate addition differences between each flask of triplicates, each flask was analyzed individually. To estimate the kinetic parameters, squared-error between simulation and experimental data was minimized using the following formula:

$$error = \frac{\sum_x \sum_t \left(x_{mesured}(t) - x_{simulated}(t)\right)^2}{n} \quad \text{Equation 2-8}$$

where $x \in \{S_a; S_b; X\}$ and n is the total number of experimental data

To minimize this error, the Nelder-Mead algorithm (function *fminsearch* under Scilab (http://www.scilab.org) was used. To reduce the risk of finding a local minimum, several optimization sets were performed with random initial parameters.

2.2.4.3 Sensitivity analysis of the parameters

A range of 0-200% of the estimated value for each parameter was tested, the other parameters remaining constant. The results were analyzed by calculating the relative error of the model related to the variation of individual parameters. A parameter is considered as sensitive and accurately estimated when a small variation of its value induces a strong increase in the model error. The parameters were considered to be accurately estimated when a variation higher than 5 % induced more than a 5% increase in the error between the simulation and the experimental data.

2.3 Results and discussion

2.3.1 High cell growth using acetate

Both *Chlorella sorokiniana* and *Auxenochlorella protothecoides* growth were efficient and rapid for the four different concentrations of acetate (0.1 - 1 $g_C.L^{-1}$) (Figure 2-3). Acetate was completely exhausted in less than 1.5 days and less than 2 days, for *C. sorokiniana* and *A. protothecoides*, respectively (Figure 2-3). The end of the biomass growth occurred when acetate was completely exhausted. These results showed that acetate did not inhibit microalgae growth even for concentrations as high as 1 $g_C.L^{-1}$. This might be due to the

composition of the medium where nitrogen and phosphorus were provided in non-limiting concentrations using a very favorable C:N:P molar ratio of 48:16:1 (Stevenson et al., 1996).

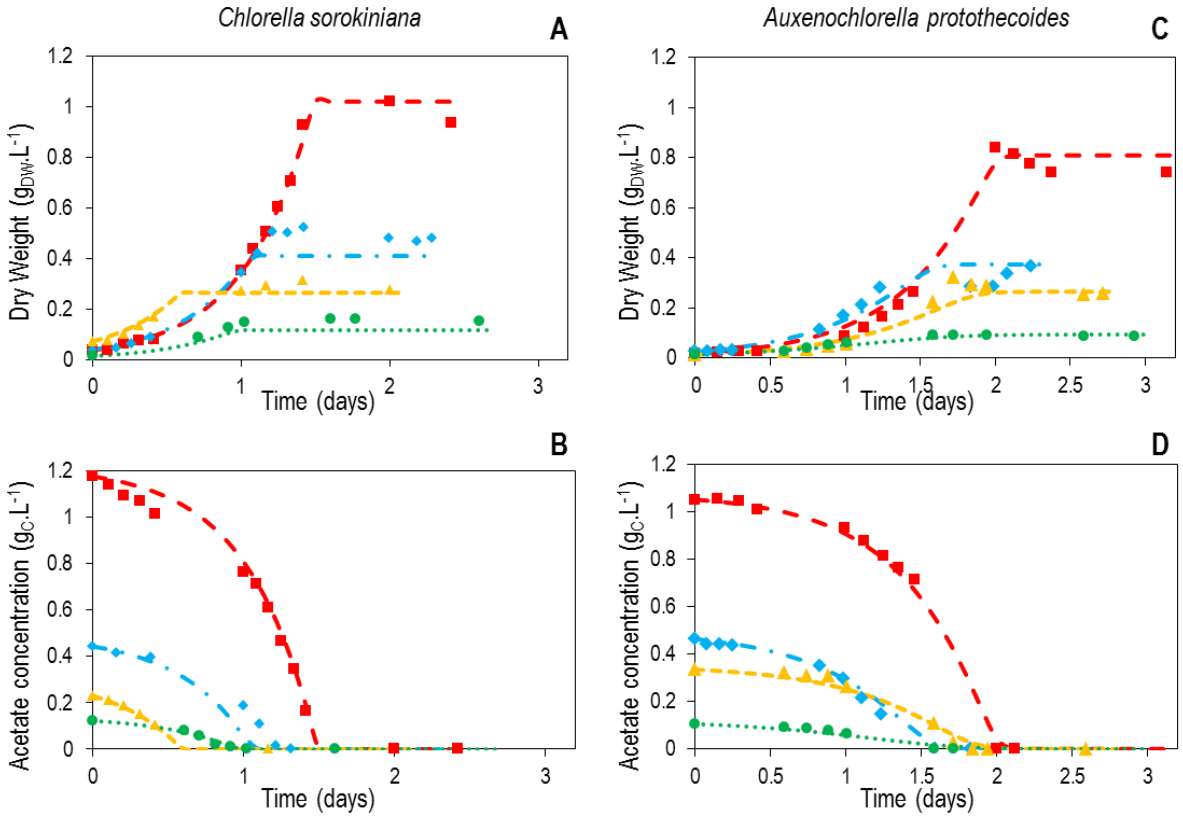

Figure 2-3. Growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on acetate in heterotrophic conditions.

A – B and C – D represent the growth of, and removal of VFA by, *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively. A and C, B and D, represent the biomass ($g_{DW}.L^{-1}$) growth, the substrate removal ($g_C.L^{-1}$), respectively, of microalgae on 1 $g_C.L^{-1}$ (■), 0.5 g_C $.L^{-1}$ (◆), 0.25 $g_C.L^{-1}$ (▲) and 0.1 $g_C.L^{-1}$ (●) of acetate. The associated model predictions (dashed lines) are also represented. One set of data per triplicate are represented, the model fitted the other two sets of data as well.

Positive controls (cultivation in light with inorganic substrate addition) showed that inoculum was always viable (Figure 2-4). In negative controls (cultivation in darkness without organic substrate addition but inorganic substrate), a slight growth was observed showing that microalgae carbon reserves were slightly able to support cell growth (Figure 2-4). It was therefore concluded that the biomass growth was only due to organic carbon assimilation by heterotrophic microalgae. These results are consistent with previous studies reporting that high concentration of acetate can be used to grow successfully *Chlorella sorokiniana* (up to 6 $g_C.L^{-1}$ of acetate) and *Auxenochlorella protothecoides* (up to 8.2 $g_C.L^{-1}$ of acetate) under heterotrophic conditions (Heredia-Arroyo et al., 2010; Ogbonna et al., 2000). Growth on

acetate was modelled with a Monod equation (equation 2). Dynamics of acetate removal S_a and biomass X were accurately predicted by the model for the two species studied (Figure 2-3).

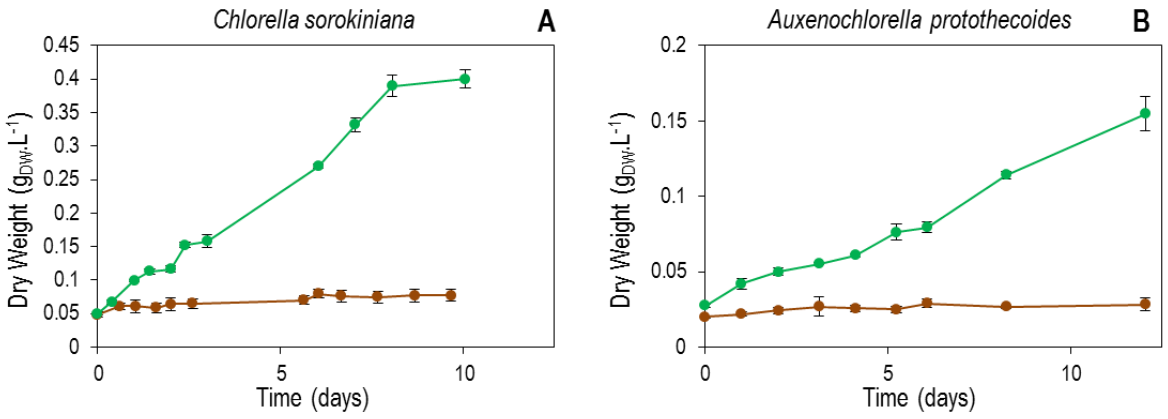

Figure 2-4. Controls.

Positive (autotrophic, ●) and negative (inorganic carbon under darkness, ●) controls for *Chlorella sorokiniana* (A) and *Auxenochlorella protothecoides* (B).

A sensitivity analysis of the model parameters was performed to assess the influence of parameter variation on biomass prediction and substrate removals. This analysis showed that the parameters μ_{a_max} and Y_a were highly sensitive and were accurately estimated for the two species (Figure 2-5). In contrast, K_{Sa} for *Chlorella sorokiniana* was not a sensitive parameter (Figure 2-5). More precisely, the values of K_{Sa} for *Chlorella sorokiniana* ranged between 0.025 and 2.10^{-10} $g_C.L^{-1}$. Therefore, these values were too small to be accurately quantified and identified.

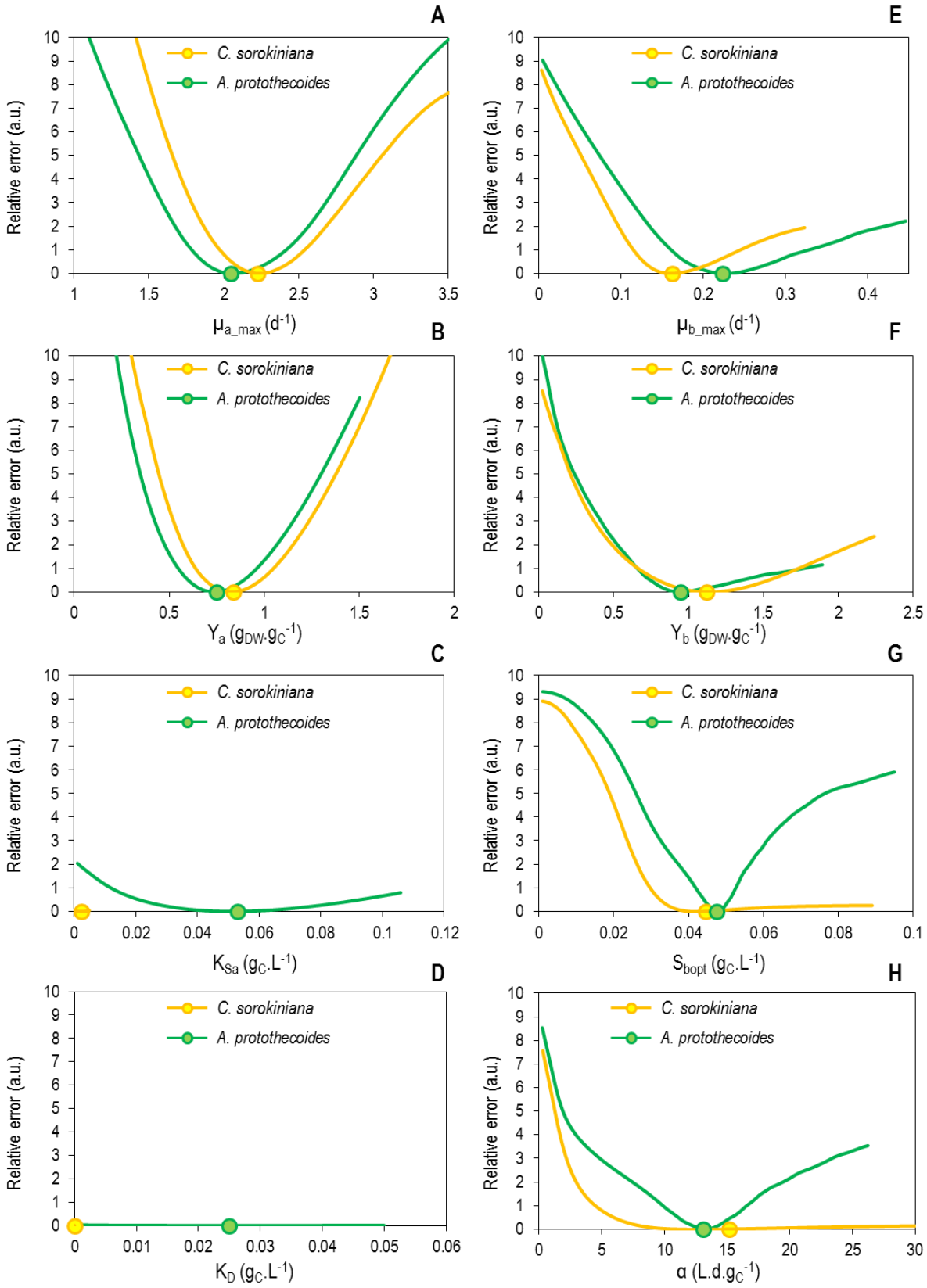

Figure 2-5. Sensitivity analysis of the model parameters.

The results of the different sensitivity analysis realized for the parameters associated with *Chlorella sorokiniana* (yellow line) and *Auxenochlorella protothecoides* (green line) are represented. The yellow

dots and the green dots represent the parameters values obtained for *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively.

The maximal growth rates on acetate, μ_{a_max}, were 2.23 d^{-1} and 2.05 d^{-1} for *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively (Table 2-2). The biomass yields on acetate (Y_a), the percentage of carbon that was assimilated into the biomass, were similar with 42% and 38% for *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively (Table 2-2). These values are very consistent with those reported previously by Ogbonna et al. (2000) for *Chlorella sorokiniana*. Samejina and Myers (1958) reported lower values with a maximal growth rate of 0.5 d^{-1} and a carbon yield of 26% for *Chlorella sorokiniana* growing on acetate (0.1 $g_C.L^{-1}$) and using nitrate as nitrogen source.

Table 2-2. Estimated parameters values for the growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on organic acids in heterotrophic conditions.

Values in italic indicate the range of parameters values for which the sensitivity analysis indicated that less than 5% variation of the error between simulation and data occurred.

Species studied	Growth on acetate			Growth on butyrate				Diauxie
	μ_{a_max}	K_{Sa}	Y_a	μ_{b_max}	α	S_{b_opt}	Y_b	K_D
	(d^{-1})	($g_C.L^{-1}$)	($g_{DW}.g_C^{-1}$ and %*)	(d^{-1})	($L.d.g_C^{-1}$)	($g_C.L^{-1}$)	($g_{DW}.g_C^{-1}$ and %*)	($g_C.L^{-1}$)
Chlorella sorokiniana	**2.23**	**0.002**	**0.84 – 42%**	**0.16**	**15.1**	**0.046**	**1.12 - 56%**	**2.10^{-10}**
	2.18 - 2.27	*5.10-5 - 0.005*	*0.79 - 0.87*	*0.15 - 0.17*	*9.4 - 19.4*	*0.038 - 0.049*	*1.03 - 1.23*	*N/A*
Auxenochlorella protothecoides	**2.05**	**0.05**	**0.75 - 38%**	**0.22**	**13.1**	**0.047**	**0.95 - 48%**	**0.025**
	2.01 - 2.13	*0.04 - 0.06*	*0.71 - 0.78*	*0.21 - 0.24*	*12.9 - 13.6*	*- 0.048*	*0.89 - 1*	*5.10-4 - 0.05*

*: estimated carbon assimilation for a cell composition of 50 % carbon (Chen and Johns, 1996).

2.3.2 Cell growth inhibition with butyrate

For *Chlorella sorokiniana*, cell growth and complete butyrate exhaustion were observed after 14 days of cultivation only for an initial butyrate concentration of 0.1 $g_C.L^{-1}$ (Figure 2-6). For butyrate concentrations above 0.1 $g_C.L^{-1}$, neither growth of *Chlorella sorokiniana* nor butyrate removal was observed after 23 days of cultivation (Figure 2-6). *Auxenochlorella protothecoides* was able to grow on butyrate for concentrations as high as 0.25 $g_C.L^{-1}$ over an incubation period of 23 days (Figure 2-6). At the initial concentration of 0.1 $g_C.L^{-1}$ and 0.25 $g_C.L^{-1}$, butyrate was exhausted in 7.5 days and 20 days, respectively. For initial butyrate concentration at 0.5 and 1 $g_C.L^{-1}$, neither growth nor butyrate removal was observed suggesting a strong inhibition of microalgae growth in the presence of butyrate at concentration higher than 0.1 $g_C.L^{-1}$, for *Chlorella sorokiniana* and 0.25 $g_C.L^{-1}$ for *Auxenochlorella protothecoides*. Growth on butyrate was modeled with a Haldane equation

including an inhibitory constant (Equation 4). Dynamics of butyrate removal S_b and biomass X were accurately predicted by the model for the two species (Figure 2-6).

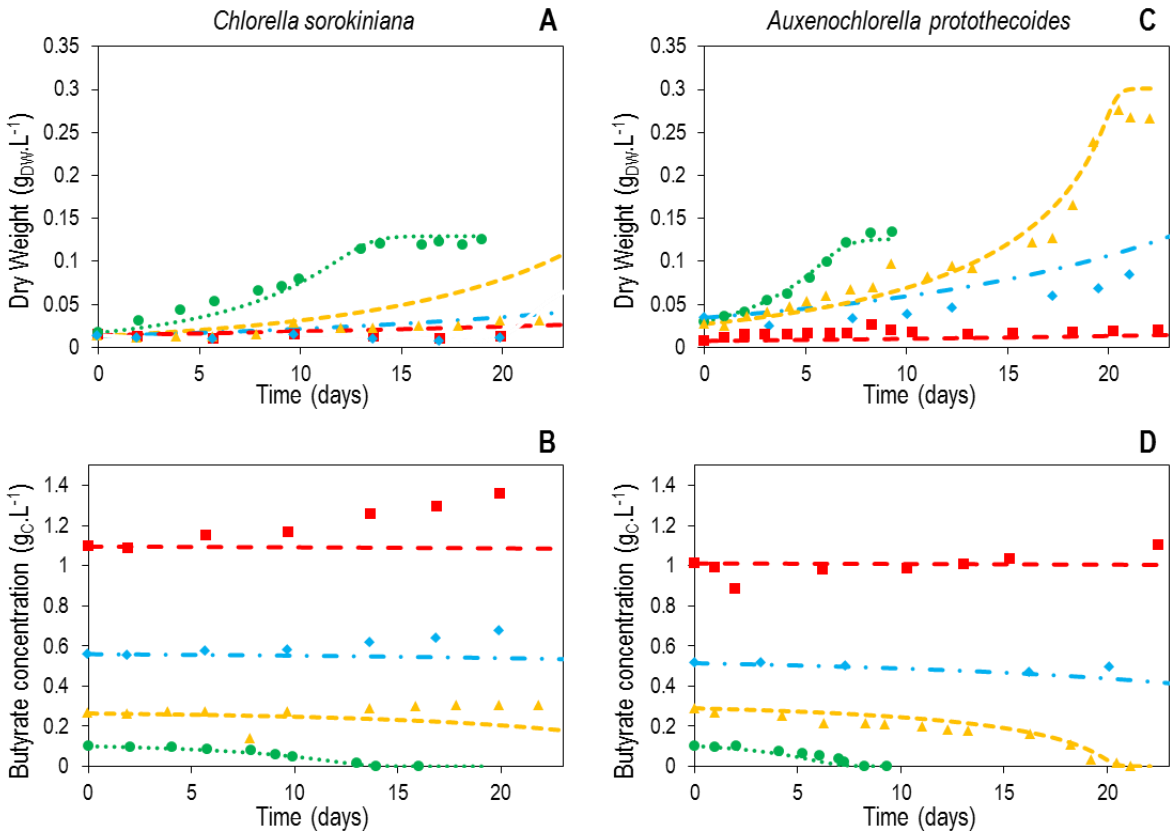

Figure 2-6. Growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on butyrate in heterotrophic conditions.

A – B and C – D represent the growth of, and removal of VFA by, *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively. A and C, B and D, represent the biomass ($g_{DW}.L^{-1}$) growth, the substrate removal ($g_C.L^{-1}$), respectively, of microalgae on 1 $g_C.L^{-1}$ (■), 0.5 $g_C.L^{-1}$ (◆), 0.25 $g_C.L^{-1}$ (▲) and 0.1 $g_C.L^{-1}$ (●) of butyrate. The associated model predictions (dashed lines) are also represented. One set of data per triplicate are represented, the model fitted the other two sets of data as well.

A sensitivity analysis of the model parameters was performed and showed that the parameters μ_{b_max}, S_{bopt} and Y_b were highly sensitive and were accurately estimated for the two species (Figure 2-5). In contrast, α was not a sensitive parameter (Figure 2-5). The maximal growth rate on butyrate, μ_{b_max}, was 0.16 d^{-1} and 0.22 d^{-1} for *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively (Table 2-2). The biomass yield, Y_b, on butyrate, was 56% and 48% for *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively (Table 2-2). The optimal butyrate concentration, S_{bopt}, was 0.046 and 0.047 $g_C.L^{-1}$ for *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively (Table 2-2). Similarly, Samejina and Myers (1958) and Chang et al. (2012) found that *Chlorella*

pyrenoidosa and *Chlorella vulgaris* growth in heterotrophic conditions were inhibited by butyrate concentrations higher than 0.05 $g_C.L^{-1}$. In this study, maximal growth rates on butyrate were 10 times lower than those found on acetate. It was therefore tested whether microalgae could acclimate to the presence of butyrate and improve their growth rates. *Auxenochlorella protothecoides* was cultivated in successive batches with 0.1 $g_C.L^{-1}$ of butyrate during 4 weeks (Figure 2-7).

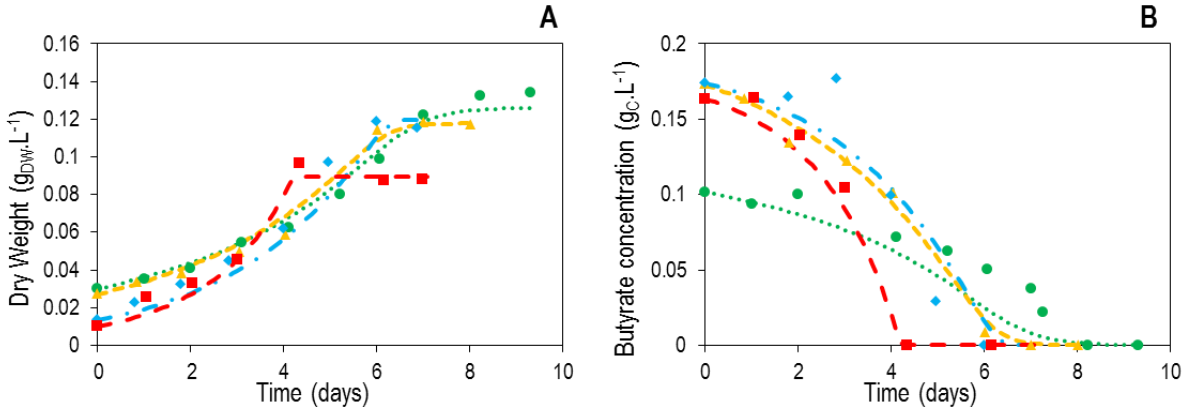

Figure 2-7. Growth of *Auxenochlorella protothecoides* on successive batches with butyrate.

A and B represent the growth and removal of butyrate, respectively, by *A. protothecoides*. Microalgae wer not acclimated (●), acclimated once (until complete butyrate exhaustion) (▲), twice (◆) and three times (■) on 0.1 gC.L-1 of butyrate, respectively. The associated model predictions (dashed lines) are also represented. One set of data per triplicate are represented, the model fitted the other two sets of data as well.

The experimental data were compared with a model simulation. The simulations did not fit the experimental data well. New estimation of the model parameters was therefore performed and the maximal growth rate was improved from 0.25, 0.36 to 0.58 d^{-1} after 1, 2 and 3 successive cultures on butyrate, respectively (Table 2-3). Therefore, the maximum growth rate was more than two times higher (0.58 d^{-1}) than the value found when the microalgae were not acclimated (0.25 d^{-1}). The biomass yields remained constant around 26%, 31% and 25% after 1, 2 and 3 successive cultures on butyrate, respectively (Table 2-3). The biomass yields were lower (25 – 30%) than what was observed when the microalgae were not acclimated (48%), suggesting a probable exhaustion of some internal carbon reserves through the acclimation process. A sensitivity analysis was carried out and indicated that μ_{b_max} and Y_b were sensitive parameters but α and S_{bopt} were not. Since S_{bopt} was not anymore a sensitive parameter, butyrate inhibition might have been partially reduced. The result showed the possibility of enhancing butyrate removal by acclimatizing specifically the microalgae to this inhibitory substrate.

Table 2-3. Estimated parameters values for the successive growth of *Auxenochlorella protothecoides* on 0.1 $g_C.L^{-1}$ of butyrate.

Values in italic indicate the range of parameters values for which the sensitivity analysis indicated that less than 5% variation of the error between simulation and data occurred.

Kinetic parameters	Culture 0*	Culture 1*	Culture 2*	Culture 3*
μ_{b_max}	**0.22**	**0.25**	**0.36**	**0.58**
(d^{-1})	*0.21 – 0.24*	-	*0.35 – 0.38*	*0.57 – 0.60*
Y_b	0.95 – 48%	0.52 – 26%	0.61 – 30.5%	0.49 – 24.5%
($g_{DW}.g_C^{-1}$ and %**)	*0.89 – 1*	*0.51 – 0.53*	*0.59 – 0.66*	*0.47 – 0.50*

*: Cultures 0, 1, 2, 3 refer to acclimation experiments. Culture 0, 1, 2 and 3, was not acclimated, acclimated once (until complete butyrate exhaustion),twice and three times on 0.1 $g_C.L^{-1}$ of butyrate, respectively.

**: estimated carbon assimilation for a cell composition of 50 % carbon (Chen and Johns, 1996).

2.3.3 Diauxic growth in the presence of mixtures of acetate and butyrate

Three different mixtures of acetate and butyrate, ranging from 0.25 to 0.9 $g_C.L^{-1}$ of acetate and 0.1 to 0.25 $g_C.L^{-1}$ of butyrate, were tested (Table 2-1). When acetate and butyrate were provided in equal amount, 0.25 $g_C.L^{-1}$ each, biomass growth and complete substrates exhaustion were observed for both species (Figure 2-8). Acetate was consumed in 1.5 and 1.85 days, by *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively. Butyrate removal started after complete acetate exhaustion for both species. It was completely consumed after 10 days and 7 days by *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively. For the two other tested mixtures, the same pattern was observed, rapid acetate removal followed by slow butyrate removal starting only after complete acetate exhaustion (Figure 2-8).

Interestingly, a sequential assimilation of acetate and butyrate was observed. Acetate appeared to be a preferred substrate than butyrate. This phenomenon is known as diauxie or diauxic effect and has been widely reported in many microbial species (Kovárová-kovar and Egli, 1998), but has never been clearly reported for microalgae grown on VFAs (Chapter 1, section 1.3). Narang and Pilyugin (2005) pointed out that when microorganisms are grown with two substitutable substrates, e.g. two carbon sources, they tend to preferentially exhaust the one that sustains a higher growth rate which is consistent with this study where acetate removal is faster and more efficient than butyrate removal.

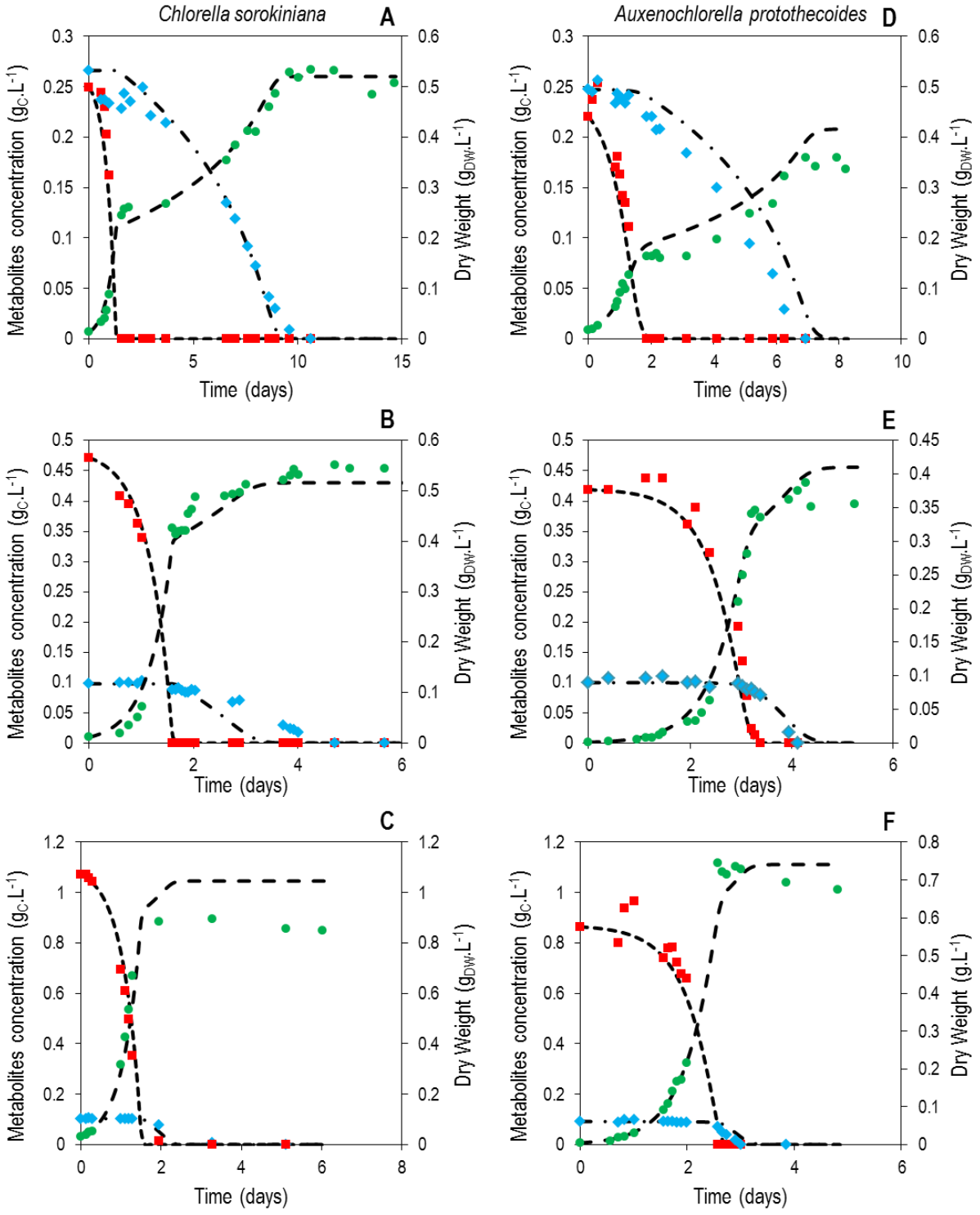

Figure 2-8. Growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on mixtures of acetate and butyrate in heterotrophic conditions.

A – C and D – F represent the growth of, and removal of organic acids by, *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively. Biomass ($g_{DW}.L^{-1}$) growth (●), acetate removal (■) and butyrate removal (◆), along with the associated model predictions (dashed lines) are represented. The data for microalgae grown on 0.25 $g_C.L^{-1}$ of acetate and 0.25 $g_C.L^{-1}$ of butyrate (A and D), on 0.4 $g_C.L^{-1}$ of acetate and 0.1 $g_C.L^{-1}$ of butyrate (B and E), on 0.9 $g_C.L^{-1}$ of acetate and 0.1 $g_C.L^{-1}$ of butyrate (C

and F) are represented. One set of data per triplicate are represented, the model fitted the other two sets of data as well.

In addition, one can notice that 0.25 $g_C.L^{-1}$ of butyrate was completely exhausted by *Chlorella sorokiniana* in only 10 days when 0.25 $g_C.L^{-1}$ of acetate was also added (Figure 2-8), whereas butyrate, as sole carbon source, was not consumed after 23 days (Figure 2-6). Similar results were observed for *Auxenochlorella protothecoides*. Acetate was first taken up by microalgae with a subsequent biomass increase that led to significant butyrate consumption even at inhibitory concentrations when added alone. In a previous study on mixotrophic cultivation of microalgae, Liu et al. (2012) reported that butyrate removal was enhanced by increasing the initial biomass concentration. Similarly, high acetate:butyrate ratios of 8:1 and 3:1 (in $g_{DW}.L^{-1}$) were used to enhance the biomass production of the oleaginous fungus, *Cryptococcus albidus*, and the oleaginous yeast, *Yarrowia lipolytica*, respectively (Fei et al., 2011; Fontanille et al., 2012).

The diauxic behavior was modelled by adding an inhibitory term in the Haldane equation (Equation 2-4). When acetate and butyrate were used in mixtures, the estimated maximal growth rates and biomass yields were similar to the ones estimated when acetate and butyrate were used as single substrates. This result supports that butyrate consumption is favored only by the increase of biomass concentration in a mixture of acetate:butyrate. The low values of K_D, which corresponds to the half inhibitory constant associated with the diauxic growth, 2.10^{-10} and 0.025 $g_C.L^{-1}$, showed that the diauxic effect was very strong for the two species. Moreover, a sensitivity analysis was carried out and showed that K_D was not a sensitive parameter (Figure 2-5). Thereafter, these kinetic parameters of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* growth were compared to literature data by simulating the growth of microalgae on a mixture of different carbon sources. In Liu et al. (2013), a mixture of acetate (0.12 $g_C.L^{-1}$) and butyrate (0.43 $g_C.L^{-1}$) was used to sustain the heterotrophic growth of *Chlorella vulgaris*. *Chlorella vulgaris* completely consumed acetate but only 12 % of the initial butyrate concentration in approximatively 5 days. According to the model simulations under similar conditions, about 27 % and 35 % of butyrate should be consumed in 5 days for *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively. Therefore, the two species used in the present study seemed more efficient than *Chlorella vulgaris* for heterotrophic cultivation on acetate and butyrate. The results of this study support that the addition of a second carbon source such as acetate may be a promising strategy to enhance butyrate removal under heterotrophic conditions.

2.3.4 **No** influence of lactate on growth

One concentration of lactate as single substrate, 0.5 $g_C.L^{-1}$, was tested for both species. Neither growth nor apparent lactate removal was observed. A mixture composed of 0.25 $g_C.L^{-1}$ of lactate and 0.25 $g_C.L^{-1}$ of acetate was tested. For both species, acetate was completely exhausted and was the sole substrate supporting the biomass growth (Figure 2-9). Lactate removal did not occur during the cultivation periods for the two species. A mixture composed of acetate (0.25 $g_C.L^{-1}$), butyrate (0.45 $g_C.L^{-1}$) and lactate (0.16 $g_C.L^{-1}$), a ratio similar to typical effluent composition of real dark fermenters (Rafrafi et al., 2013), was tested. Acetate and butyrate removals occurred during the cultivation periods, while lactate was not degraded (Figure 2-9). All results showed that lactate was not used as carbon source to sustain heterotrophic growth for the two species. Nevertheless, the presence of lactate did not influence neither acetate nor butyrate removals. It was therefore concluded that its presence in fermentation effluents would not have any influence on the final biomass growth in the range of the concentrations tested. In contrast, the presence of propionate might reduce butyrate removal efficiency. Indeed, in Fei et al. (2014), *Chlorella protothecoides* was grown on mixtures of acetate (Ac), butyrate (But) and propionate (Prop) with various Ac:But:Prop ratios, under similar growth conditions (darkness, 25 °C) (Chapter 1, Table 1.3-1). According to the model predictions made under similar conditions but without propionate, acetate exhaustion should be the same for *Auxenochlorella protothecoides*. Butyrate removal reached 75 % and 90 % in Fei et al. (2014), for the conditions with Ac:But:Prop ratios of 4:3:3 and 7:1:2, respectively. According to the model predictions, butyrate removal should reached 97 % and 100 %. Lactate removal and assimilation by *Chlorella*-like species does not reach a consensus in literature. Lactate removal is likely species-specific and depends highly on the culture conditions. As an illustration, it was previously reported that *Chlorella vulgaris* could not use lactate for growth in mixotrophic conditions, but its presence was inhibitory to biomass growth (Liu et al., 2012). In contrast, the use of lactate by the same microalgal species was observed in heterotrophic conditions by Perez-Garcia et al (2011a). Therefore, the influence of lactate on biomass growth and on other substrates removal remains unclear for *Chlorella* sp.

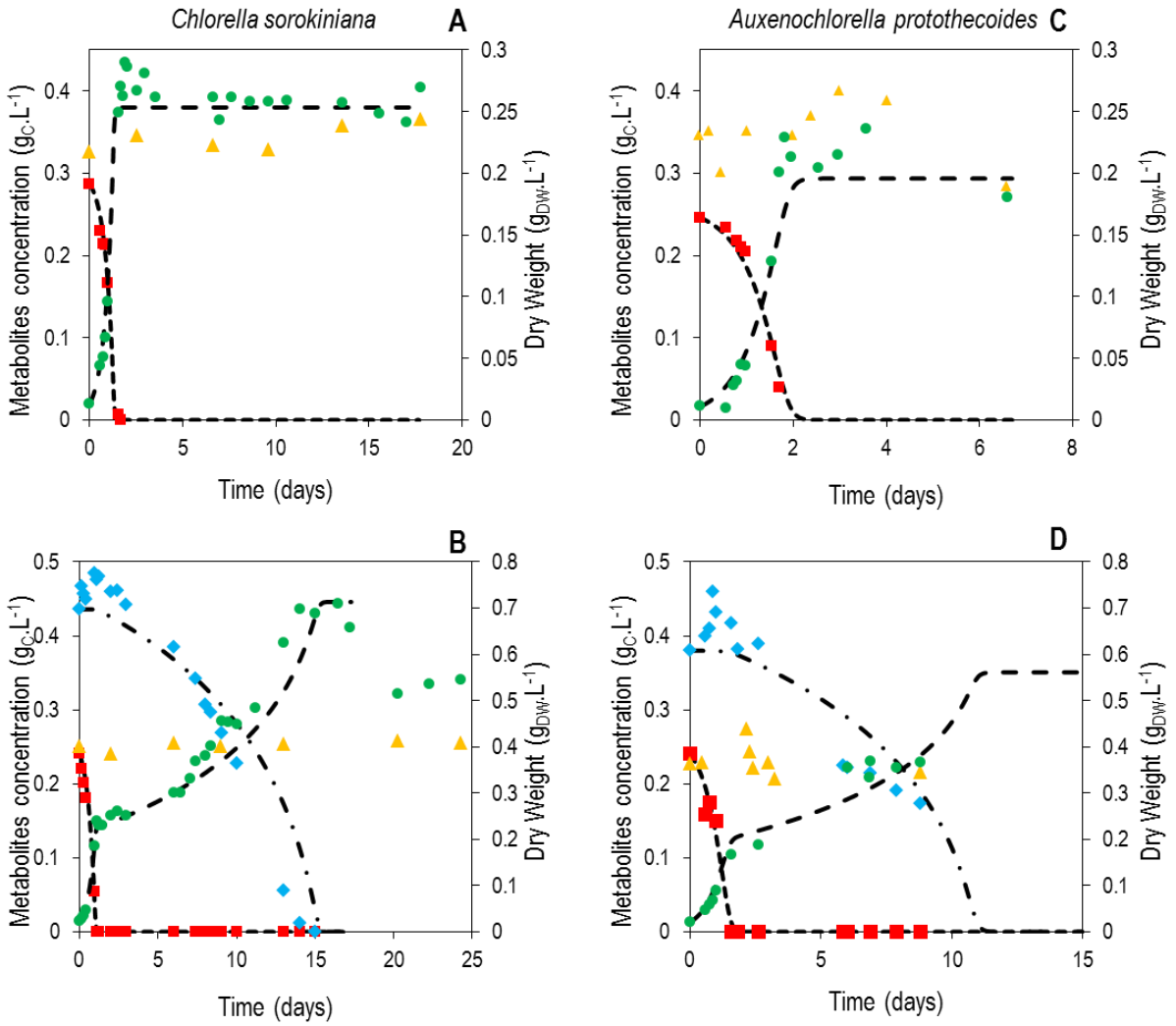

Figure 2-9. Growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on mixtures of acetate, butyrate and lactate in heterotrophic conditions.

A – B and C – D represent the growth of, and removal of organic acids by, *Chlorella sorokiniana* and *Auxenochlorella protothecoides*, respectively. Biomass ($g_{DW}.L^{-1}$) growth (●), acetate removal (■), butyrate removal (◆) and lactate removal (▲), as long with the associated model predictions (dashed lines) are represented. A and C represent the experimental and predicted data for microalgae grown on 0.25 $g_C.L^{-1}$ of acetate and 0.25 $g_C.L^{-1}$ of lactate. B and D represent the experimental and predicted data for microalgae grown on a mix of acetate (0.25 $g_C.L^{-1}$), butyrate (0.45 $g_C.L^{-1}$) and lactate (0.16 $g_C.L^{-1}$) according to published datas found in a fermentative digestate (Rafrafi et al., 2013). One set of data per triplicate are represented, the model fitted the other two sets of data as well.

2.4 Conclusions

The growth of microalgae on synthetic dark fermentation effluent, composed mainly of acetate, butyrate and lactate, was evaluated and modeled. For the two species studied, a diauxic phenomenon was observed during growth on acetate and butyrate. Inhibition of butyrate uptake by the presence of acetate highlighted the preference for acetate by microalgae. Indeed, reducing butyrate inhibition was identified as a key factor for coupling

dark fermentation with microalgal heterotrophy. Acclimation of microalgae (during four weeks) to the inhibitory butyrate may substantially improve biomass growth. Light and temperature are two abiotic parameters which have been suggested as tools to improve microalgae growth on butyrate. Assessing the influence of light and temperature will be the next step in further characterizing and understanding microalgae growth on dark fermentation effluent.

2.5 References

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Chapter 3

Light and temperature as tools to alleviate butyrate inhibition on growth of *Chlorella sorokiniana*

In this chapter, the effects of light, temperature and the combination of light and temperature on the growth of *Chlorella sorokiniana* supplemented with a mixture of acetate and butyrate were studied. The results of this chapter have been submitted to Bioresource Technology in an article entitled "Growth of *Chlorella sorokiniana* on a mixture of volatile fatty acids: The effects of light and temperature".

3.1 Introduction

From the literature review presented in Chapter 1 and the first results of this PhD (Chapter 2), heterotrophic growth of microalgae on a mixture of VFAs seems strongly dependent on the acetate:butyrate ratio as high concentrations of butyrate can inhibit algal growth (Fei et al.,

2014; Liu et al., 2012). However, the growth of the oleaginous *Chlorella sorokiniana* at high butyrate concentration could benefit from the supply of light. Indeed, during mixotrophic growth of *Chlorella vulgaris* on butyrate alone, it was suggested that microalgae could assimilate CO_2 first, with a subsequent increase in the total biomass resulting in faster uptake of butyrate (Liu et al., 2013, 2012). However, the interactions between acetate, butyrate and light and its effect on microalgae growth are still unknown. Furthermore, *Chlorella sorokiniana* is known to be thermotolerant. Indeed, At 35 – 37 °C, *C. sorokiniana* achieved high growth rates, between 3.4 d^{-1} and 6.5 d^{-1} under mixotrophic and autotrophic conditions, respectively (Janssen et al., 1999; Li et al., 2014; Van Wagenen et al., 2014b). Growing microalgae on a mixture of VFAs at high temperature (35 °C) could thus benefit from enhanced enzymatic activity and a reduction of cellular requirements for thermoregulation.

In the previous chapter (Chapter 2), *C. sorokiniana* has already been cultivated heterotrophically on a mixture of VFAs, giving a high growth rate on acetate, 2.2 d^{-1}, and a low growth rate on butyrate, 0.16 d^{-1}, at 25 °C. This study set out to determine the interaction between these two VFAs while growing *C. sorokiniana* in presence of light and at different temperatures. The effects of (i) light (with light and in the dark) (ii) temperature (25 °C, 30 °C, and 35 °C) and (iii) a combination of light and high temperature (35 °C) were tested on the growth rate and carbon yield of *C. sorokiniana* growing on a mixture of acetate and butyrate at an inhibiting butyrate concentration (both at 0.3 $g_C.L^{-1}$). Control experiments with either acetate or butyrate as single substrate (0.3 $g_C.L^{-1}$) were also performed to give a better understanding of the interactions between acetate and butyrate uptake mechanisms.

3.2 Materials and methods

3.2.1 *Chlorella sorokiniana* stock cultivation conditions

C. sorokiniana (CCAP 211/8K) was pre-cultivated axenically in 500 mL Erlenmeyer flasks with a working volume of 200 mL. A modified BG11 medium was used as described in Chapter 2. Sodium bicarbonate (10 mM) was used as an inorganic carbon (C) source, ammonium chloride (5 mM) as a nitrogen (N) source and dipotassium phosphate (0.31 mM) as a phosphorus (P) source. The flasks and components of the medium were sterilized by autoclaving at 121 °C for 20 min before use. Before starting the experiment, the axenic culture was cultivated under autotrophic conditions (light intensity of 100 µmol photons.m^{-2}.s^{-1}) at 25 °C for 7 days.

3.2.2 General cultivation conditions

The carbon concentration of each substrate was set to 0.3 $g_C.L^{-1}$, or to 0.2 $g_C.L^{-1}$ when specified, by adding sodium bicarbonate, for autotrophic growth conditions, or acetic acid (glacial acetic acid, 27221-Sigma-Aldrich®) and/or butyric acid (B103500- Sigma-Aldrich®) solutions at 500 mM, for heterotrophic and mixotrophic growth conditions. As high acetate concentrations have been shown to increase the lag phase of *C. sorokiniana* (Qiao et al., 2012), especially in heterotrophic conditions, relatively low concentrations of acetate (0.3 $g_C.L^{-1}$ equivalent to 0.75 $g.L^{-1}$ and 12.5 mM) and butyrate (0.3 $g_C.L^{-1}$ equivalent to 0.55 $g.L^{-1}$ and 6.25 mM) were used here.

The C:N:P molar ratio was set to 48:16:1. Ammonium chloride and dipotassium phosphate were used as N and P sources, respectively. To encourage heterotrophic metabolism, sodium bicarbonate was not added to the media for mixotrophic and heterotrophic growth conditions. Only CO_2 from the air dissolved in the media was available for mixotrophic growth. To maintain the same pH throughout the experiments, the media were buffered with 100 mM of 2-(*N*-morpholino) ethanesulfonic acid (MES). The initial pH was set to between 6 and 6.5. Prior to sterilization using a 0.2 µm pore filter, the working solutions of acetate and butyrate were adjusted to pH 6.5 with NaOH. The flasks and all components of the medium were sterilized by autoclaving at 121 °C for 20 min before use. The flasks were inoculated with *C. sorokiniana* stock cultures at 10% V/V.

C. sorokiniana was cultivated in 125 mL black (heterotrophy) or transparent (autotrophy and mixotrophy) Erlenmeyer flasks containing 40 mL of medium and sealed with cotton plugs. The flasks were incubated in the dark (Heterotrophy) or under a non-saturating light intensity of 123 ± 10 µmol $photons.m^{-2}.s^{-1}$ (autotrophy and mixotrophy) (Liu et al., 2012; Van Wagenen et al., 2014a) at different temperatures as described in sections 3.2.3, 3.2.4 and 3.2.5. The flasks were shaken on a rotary shaker (150 rpm) for a maximum of 10 days until the substrate was completely exhausted. All experiments and controls were performed in triplicate. During the experiment, axeny was checked daily by DAPI staining and phase contrast microscopy as well as by spreading the cultures on ATCC5 solid media (ATCC, http://www.lgcstandards-atcc.org/).

3.2.3 Cultivation at 25 °C

3.2.3.1 Using DCMU to inhibit autotrophic growth

A stock solution of 100 mM of 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), dissolved in ethanol, was used at a final nontoxic concentration of 10 µM for cultivation under mixotrophic, heterotrophic and autotrophic conditions (Zheng et al., 2014). The temperature was set to 25 °C and light to 123 µmol photons.m^{-2}.s^{-1} when required. For the three growth conditions, a control with no DCMU was also carried out in a single flask.

3.2.3.2 Cultivation on a mixture of VFAs in the presence of light

The mixotrophic growth of *Chlorella sorokiniana* on a mixture of acetate and butyrate at 25 °C was compared to the mixotrophic growth on either acetate or butyrate, as single substrates (acetate-control and butyrate-control) and to the autotrophic growth (autotrophic control). The results obtained from a predictive model, as previously described in Chapter 2, on VFAs in the dark at 25 °C were used to make assumptions about the heterotrophic growth. A Monod equation was used to describe the heterotrophic growth on acetate and a Haldane equation was used for butyrate. The diauxic growth pattern on acetate and butyrate was also included in the model. The acetate and butyrate concentrations tested in this study were in the range of concentrations used to build and validate the model. This model was developed to predict heterotrophic growth at 25 °C on acetate, butyrate or both acetate and butyrate. Since the lag phase was not considered when building the model, the microalgae biomass and the acetate and butyrate concentrations, measured at the start of the microalgal growth curve, were used to initialize the Scilab simulations (http://www.scilab.org).

3.2.4 Heterotrophic cultivation at 30 °C and 35 °C

The microalgae growth on acetate and butyrate, as single substrates, and on a mixture of acetate and butyrate in the dark at 30 °C and at 35 °C was compared to the heterotrophic growth simulated at 25 °C as described in sub-section 3.2.3.

3.2.5 Cultivation at 35 °C under light

The microalgae growth on acetate and butyrate, as single substrates, and on a combination of acetate and butyrate under light, set to 123 ± 10 µmol photons.m^{-2}.s^{-1}, at 35 °C was compared to autotrophic (with bicarbonate as the sole carbon source) and heterotrophic growth at 35 °C (sub-section 3.2.4), to mixotrophic growth at 25 °C (sub-section 3.2.3) and to predicted heterotrophic growth at 25 °C (sub-section 3.2.3).

3.2.6 Analytical methods

3.2.6.1 Biomass measurement

As described in Chapter 2, biomass growth was quantified by measurement of the Optical Density at 800 nm (OD_{800}) and was correlated to dry weight measures. Three calibration curves were determined to allow for the difference in microalgae cell shapes during heterotrophic and mixotrophic/autotrophic cultivation (Kumar et al., 2014). The equations were:

- DW ($g.L^{-1}$) = 1.24*OD_{800} (R^2 = 0.95) for heterotrophic cultivation,
- DW ($g.L^{-1}$) = 1.07*OD_{800} (R^2 = 0.94) for mixotrophic and autotrophic cultivation at 25 °C,
- DW ($g.L^{-1}$) = 1.15*OD_{800} (R^2 = 0.95) for mixotrophic and autotrophic cultivation at 35 °C.

The apparent growth rates, μ_{app} (d^{-1}), during exponential growth were calculated as follows (Equation 3-1):

$$\mu_{app} = \frac{\ln(B_f) - \ln(B_0)}{t_f - t_0} \qquad \text{Equation 3-1}$$

with t_0 and tf the start and end of the exponential growth phase and B_0 and B_f the DWs ($g.L^{-1}$) reached at t_0 and t_f, respectively.

The apparent linear production rates of biomass, r_{app_lin} ($g.L^{-1}.d^{-1}$), during linear growth were calculated as follows (Equation 3-2):

$$r_{app\_lin} = \frac{B_f - B_0}{t_f - t_0} \qquad \text{Equation 3-2}$$

where t_0 and tf are the start and end of the exponential growth phase and B_0 and B_f are the DWs ($g.L^{-1}$) at t_0 and t_f, respectively.

Under mixotrophic conditions, the mixotrophic carbon yields, Y_{Mixo}^{Mixo} (g_C of biomass per g_C of substrate), on acetate and butyrate separately were calculated as follows (Equation 3-3):

$$Y_{Mixo}^{Mixo} = \frac{(X_f - X_0) * \alpha}{S_i} \qquad \text{Equation 3-3}$$

where Xf and X_0 are the DWs ($g.L^{-1}$) at the start and the end of substrate exhaustion, α is the estimated content, 50%, of carbon in microalgae DW (Chen and Johns, 1996), S_i ($g_C.L^{-1}$) is the initial concentration of substrate.

Under mixotrophic conditions, the heterotrophic carbon yields, Y_{Het}^{Mixo} (g_C of estimated heterotrophic biomass per g_C of substrate), on acetate and butyrate separately were calculated as follows (Equation 3-4):

$$Y_{Het}^{Mixo} = \frac{(X_f - X_0 - X_{ctrl\_auto}) * \alpha}{S_i} \quad \textbf{Equation 3-4}$$

where Xf and X_0 are the DWs ($g.L^{-1}$) at the start and the end of substrate exhaustion, X_{ctrl_auto} is the DW in the strict autotrophic control at the same time as substrate exhaustion, α is the estimated content, 50%, of carbon in microalgae DW (Chen and Johns, 1996), S_i ($g_C.L^{-1}$) is the initial concentration of substrate.

Under mixotrophic conditions, the fraction of mixotrophic biomass due to heterotrophic growth on acetate and/or butyrate, X_{Het}^{Mixo} (%), was calculated as follows (Equation 3-5):

$$X_{Het}^{Mixo} = \frac{Y_{Het}^{Mixo}}{Y_{Mixo}^{Mixo}} * 100 \quad \textbf{Equation 3-5}$$

Under mixotrophic conditions, the fraction of mixotrophic biomass due to autotrophic growth on CO_2, X_{Auto}^{Mixo} (%), was calculated as follows (Equation 3-6):

$$X_{Auto}^{Mixo} = 100 - X_{Het}^{Mixo} \quad \textbf{Equation 3-6}$$

3.2.6.2 Organic acids measurements

Volatile fatty acids (VFAs), e.g. acetate and butyrate, were quantified using a gas chromatograph (GC 3900 Varian) equipped with a flame ionization detector as previously described in Chapter 2.

3.2.6.3 Statistical analysis

Pairwise comparisons of all results were performed with a one-way ANOVA and Tukey's post-hoc analysis. All statistical analyses were carried out using Rcmdr package 1.9-6 from R version 2.15.2 (R Development Core Team, 2012).

3.3 Results and discussion

3.3.1 Effect of light on *C. sorokiniana* growth

3.3.1.1 Mixotrophic conditions: a combination of autotrophic and heterotrophic conditions

DCMU is a specific inhibitor of electron transport between Photosystem I (PSI) and Photosystem II (PSII). DCMU was used to estimate the growth due to heterotrophic metabolism only, by organic carbon fixation from acetate, during mixotrophic growth by inhibiting autotrophic inorganic carbon fixation (Li et al., 2015). DCMU inhibits the transport of electrons from PSII to plastoquinone which further blocks the generation of NADPH and ATP in the chloroplast (Li et al., 2014). CO_2 fixation is subsequently hampered by the lack of both NADPH and ATP. The production of ATP via the cyclic electron flow in photosystem I is not affected (Li et al., 2014).

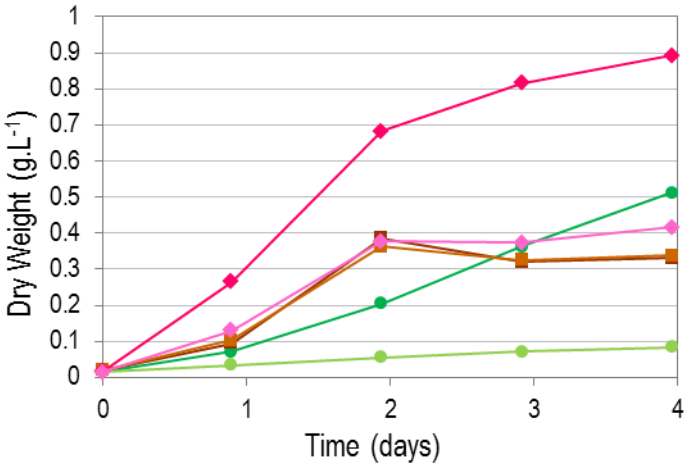

Figure 3-1. Effect DCMU on growth of *C. sorokiniana* at 25 °C in the presence of light.

Microalgae concentration ($g.L^{-1}$) during autotrophic (with 0.3 $g_C.L^{-1}$ of $NaHCO_3$ and under 123 ± 10 µmol photons.$m^{-2}.s^{-1}$) (●), mixotrophic (with 0.3 $g_C.L^{-1}$ of acetate and under 123 ± 10 µmol photons.$m^{-2}.s^{-1}$) (♦) and heterotrophic (with 0.3 $g_C.L^{-1}$ of acetate in darkness) (■) without DCMU (controls), microalgae concentration ($g.L^{-1}$) during autotrophic (with 0.3 $g_C.L^{-1}$ of $NaHCO_3$ and under 123 ± 10 µmol photons.$m^{-2}.s^{-1}$) (●), mixotrophic (with 0.3 $g_C.L^{-1}$ of acetate and under 123 ± 10 µmol photons.$m^{-2}.s^{-1}$) (♦) and heterotrophic (with 0.3 $g_C.L^{-1}$ of acetate in darkness) (■) with 10 µM DCMU.

As shown in Figure 3-1, almost no growth was observed when microalgae were cultivated autotrophically in the presence of DCMU, confirming that the autotrophic metabolism was inhibited and that no growth on cellular reserves was possible. Heterotrophic growth on acetate only (acetate-control) was not inhibited by DCMU (Figure 3-1). In the presence of DCMU under mixotrophic conditions, ie. acetate and light, the pattern of microalgae growth

was similar to the pattern under heterotrophic conditions (Figure 3-1). However, at day 1.9 (i.e., when the acetate was exhausted), the mixotrophic biomass (0.68 $g.L^{-1}$) was slightly higher (by 10%) than the sum of the heterotrophic (0.39 $g.L^{-1}$) and autotrophic (0.21 $g.L^{-1}$) biomasses. This suggests a synergistic interaction between the two metabolisms. Positive interactions could theoretically increase microalgae growth during mixotrophic metabolism: (i) through cellular energy (ATP), produced by photophosphorylation in the chloroplast that could be used to boost organic carbon uptake, (ii) by the O_2 released during photo-oxidation of water in the chloroplast that could increase the respiration rate in the mitochondrion and (iii) by the CO_2 released during respiration on organic carbon that could be recycled through the Calvin cycle and increase the biomass yield (Wan et al., 2011; Yang et al., 2000). Li et al. (2014) obtained similar results under mixotrophic conditions with light intensities ranging from 100 to 200 µmol $photons.m^{-2}.s^{-1}$ and glucose as the substrate. In their study, the *C. sorokiniana* mixotrophic growth rate was 20 to 40% higher than the sum of the growth rates obtained under heterotrophic and autotrophic conditions.

In order to provide further information on the heterotrophic fraction of the mixotrophic biomass, a strict autotrophic experiment (autotrophic control) was always run in parallel to the mixotrophic experiments. This control was used to assess the heterotrophic carbon yield, Y_{Het}^{Mixo}, associated with butyrate or acetate uptake during mixotrophic growth. The biomass reached under autotrophic conditions can be subtracted from the observed mixotrophic biomass to assess the fraction of microalgae growth due to organic carbon assimilation, as described in Van Wagenen et al. (2014a). The excess biomass due to the positive interaction between the two metabolisms was considered as a boost to the biomass generated by heterotrophic growth.

3.3.1.2 Increase in the butyrate uptake rate in the presence of acetate under mixotrophic conditions

The effect of light on *C. sorokiniana* cultivated on a mixture of acetate and butyrate was studied. The strict autotrophic control (without organic substrate) was used to give a better explanation for the mixotrophic growth observed in Figure 3-2. During the exponential phase (first two days), the apparent autotrophic growth rate was 1.04 ± 0.05 d^{-1}. During the linear phase (from day 2 to day 8), the biomass production rate was 0.11 ± 0.01 $g.L^{-1}.d^{-1}$. With limited light availability (low light intensities and cell self-shading) or CO_2 limitation (no air or additional CO_2), the exponential growth phase in autotrophic batch cultivation can be short and rapidly followed by linear growth (Ogbonna et al., 1995; Smith et al., 2015). The growth

rates during autotrophic growth were consistent with previously reported results obtained under similar conditions with *C. sorokiniana* (Kim et al., 2013a; Li et al., 2013; Rosenberg et al., 2014).

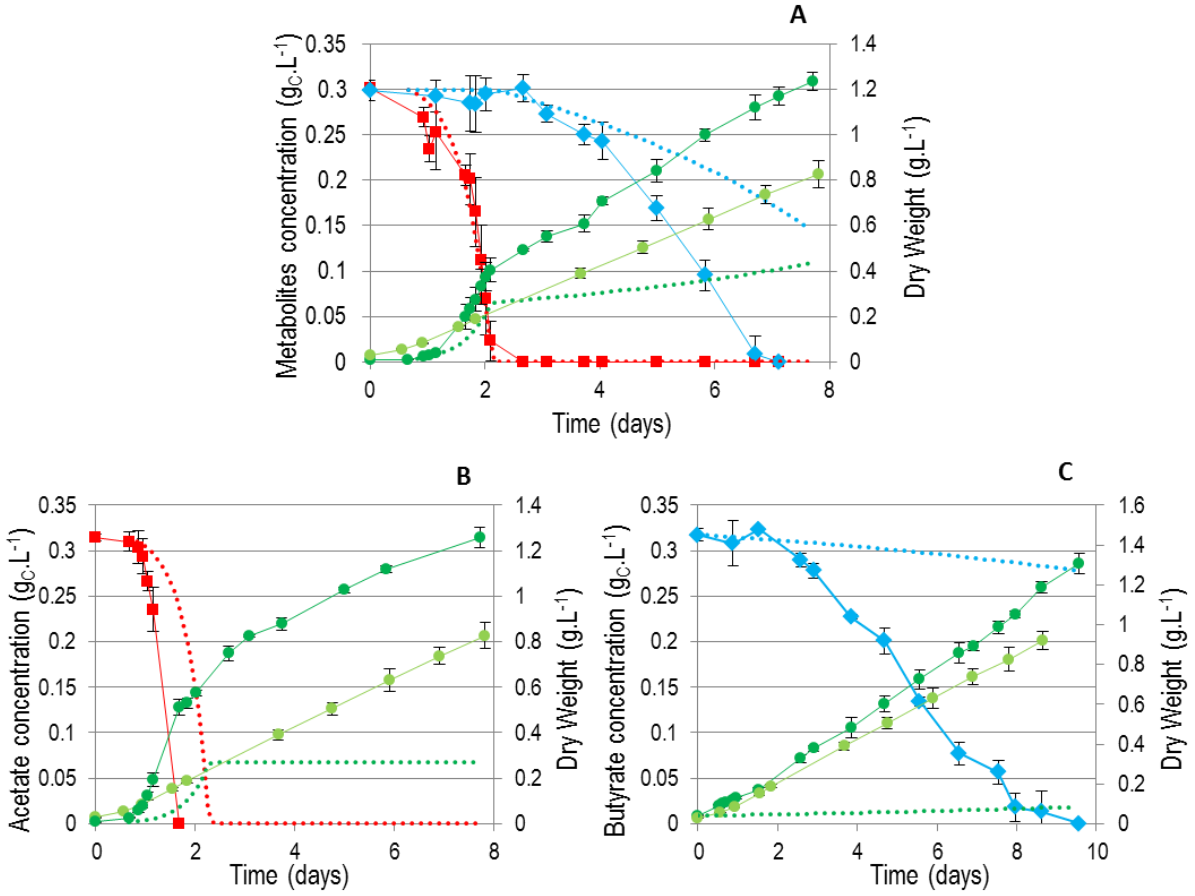

Figure 3-2. Effect on light on growth of *C. sorokiniana* cultivated on butyrate and acetate at 25 °C.

C. sorokiniana was grown under mixotrophic conditions at 25 °C. Microalgae ($g.L^{-1}$) (●), butyrate (◆) and acetate (■) concentrations during growth (A) on a mixture of butyrate and acetate, 0.3 $g_C.L^{-1}$ of each, (B) on 0.3 $g_C.L^{-1}$ of acetate as single substrate (acetate-control) and (C) on 0.3 $g_C.L^{-1}$ of butyrate as single substrate (butyrate-control). Microalgae concentration ($g.L^{-1}$) (●) during autotrophic growth. The simulated heterotrophic biomass growth (green dashed lines), acetate uptake (red dashed lines) and butyrate uptake (blue dashed lines) at 25 °C are shown.

During mixotrophic growth on a mixture of acetate and butyrate (Figure 3-2,A), assimilation of acetate and butyrate was diauxic under mixotrophic conditions since butyrate uptake started only after the acetate had been completely exhausted, as previously observed in heterotrophic conditions (Chapter 2). The growth rates on acetate and butyrate were, therefore, analyzed separately.

Table 3-1. Effect of light on growth and production rates (μ_{app} and r_{app_lin}) and yields of *C. sorokiniana* for cultivation at 25 °C on acetate (A), butyrate (B) and a mixture of butyrate and acetate (A + B).

Mean values and standard deviations calculated from triplicates are given.

	Growth on acetate				Growth on butyrate				
	μ_{app} (d^{-1})	Y_{Mixo}^{Mixo} ($g_C.g_C^{-1}$)	Y_{Het}^{Mixo} ($g_C.g_C^{-1}$)[a]	X_{Auto}^{Mixo} (%)[b]	r_{app_lin} ($g.L^{-1}.d^{-1}$)	Uptake rate ($mg_C.L^{-1}.d^{-1}$)	Y_{Mixo}^{Mixo} ($g_C.g_C^{-1}$)	Y_{Het}^{Mixo} ($g_C.g_C^{-1}$)[a]	X_{Auto}^{Mixo} (%)[b]
A	4.14 ± 0.35	0.8 ± 0.05	0.56 ± 0.06	30					
B					0.14 ± 0.00	47.5 ± 0.5	1.69 ± 0.02	0.44 ± 0.03	74
A + B	2.68 ± 0.12	0.79 ± 0.04	0.48 ± 0.05	39	0.16 ± 0.01	71 ± 2.7	1.19 ± 0.11	0.45 ± 0.05	62

[a]: The heterotrophic carbon yield (Y_{Het}^{Mixo}) was calculated by subtracting the carbon yield associated with autotrophic growth from the mixotrophic carbon yield (Y_{Mixo}^{Mixo}).

[b]: The fraction of mixotrophic biomass due to autotrophic growth on CO_2 (X_{Auto}^{Mixo}) was calculated as follow:

$$X_{Auto}^{Mixo} = \frac{Y_{Mixo}^{Mixo} - Y_{Het}^{Mixo}}{Y_{Mixo}^{Mixo}} * 100$$

The growth rate on acetate was slightly higher (2.7 ± 0.1 d^{-1}) under mixotrophic conditions than estimated by modeling under heterotrophic conditions (2.23 d^{-1} – see Table 3-2). The total biomass accumulated just after acetate exhaustion in mixotrophic conditions was higher than the biomass predicted by the model in heterotrophic conditions (Figure 3-2,A). Furthermore, the mixotrophic carbon yield on acetate, Y_{Mixo}^{Mixo}, (Equation 3-3), was almost twice as high (0.79 ± 0.04 $g_C.g_C^{-1}$) under mixotrophic conditions than predicted under heterotrophic conditions (0.42 $g_C.g_C^{-1}$) (Table 3-2). These results confirmed that the presence of light increased both the apparent growth rate and the mixotrophic carbon yield on acetate compared to those under heterotrophic conditions at 25 °C. Under mixotrophic conditions, the heterotrophic carbon yield, Y_{Het}^{Mixo} - see Equation 3-4, was calculated by subtracting the carbon yield for autotrophic growth (autotrophic control) from the mixotrophic carbon yield (Y_{Het}^{Mixo} = 0.48 ± 0.05 $g_C.g_C^{-1}$, see Table 3-1). Where there was uptake of both organic (acetate and butyrate) and inorganic carbon, only 39% of the microalgal biomass obtained after acetate exhaustion was due to CO_2 assimilation (X_{Auto}^{Mixo}, see Equation 3-6 and Table 3-1). In the acetate control (with no butyrate), the fraction of biomass due to CO_2 assimilation (X_{Auto}^{Mixo}, 30%) was statistically similar (p>0.05) (see Table 3-1 and Figure 3-2, B) but the mixotrophic growth rate on acetate reached 4.1 ± 0.4 d^{-1}. When using mixtures of VFAs, there may be a high ATP demand to deal with the inhibitory effects of butyrate, such as cytosolic pH acidification, resulting in lower ATP availability for fast growth on acetate (Tromballa, 1978). In conclusion, the growth rate and carbon yield on acetate were higher in the presence of light

than under heterotrophic conditions, suggesting that the mixotrophic growth on acetate probably relied on a synergy between heterotrophic and autotrophic conditions.

After a one-day delay after the acetate had been completely exhausted, there was linear butyrate uptake during the linear growth phase (Figure 3-2, A). Butyrate exhaustion in mixotrophic conditions was 3 days shorter than predicted for heterotrophic conditions. Based on the difference between the mixotrophic (Y_{Mixo}^{Mixo}, Equation 3-3), and heterotrophic (Y_{Het}^{Mixo}, Equation 3-4) carbon yields on butyrate, 62% of the biomass reached after butyrate exhaustion was probably due to CO_2 assimilation (X_{Auto}^{Mixo}, see Equation 3-6 and Table 3-1). Similarly, in the butyrate control (without acetate – see Figure 3-2, C), 74% of the biomass obtained after butyrate exhaustion was probably due to CO_2 assimilation (X_{Auto}^{Mixo} - see Table 3-1). The model predicted that at 25 °C no heterotrophic growth would have been observed at such initial butyrate concentration (with no acetate - see Figure 3-2, C). Furthermore, the linear butyrate uptake rate measured after acetate exhaustion was 1.5 times higher than measured for the butyrate control. It can, therefore, be concluded that mixotrophic conditions can substantially accelerate the apparent butyrate uptake through the production of algal biomass by CO_2 fixation.

3.3.2 Effect of temperature on heterotrophic growth on VFAs

3.3.2.1 Inhibition by butyrate on heterotrophic growth on acetate at high temperature (35 °C)

C. sorokiniana was grown heterotrophically on acetate as a single substrate (acetate control), on butyrate as single substrate (butyrate control) and on a mixture of acetate and butyrate, at 35 °C known to be the optimum temperature (Janssen et al., 1999; Li et al., 2014; Van Wagenen et al., 2014b). On acetate (Figure 3-3), the heterotrophic growth rate reached 5.88 d^{-1} which was consistent with previously reported values at 35-37 °C (Van Wagenen et al., 2014b).

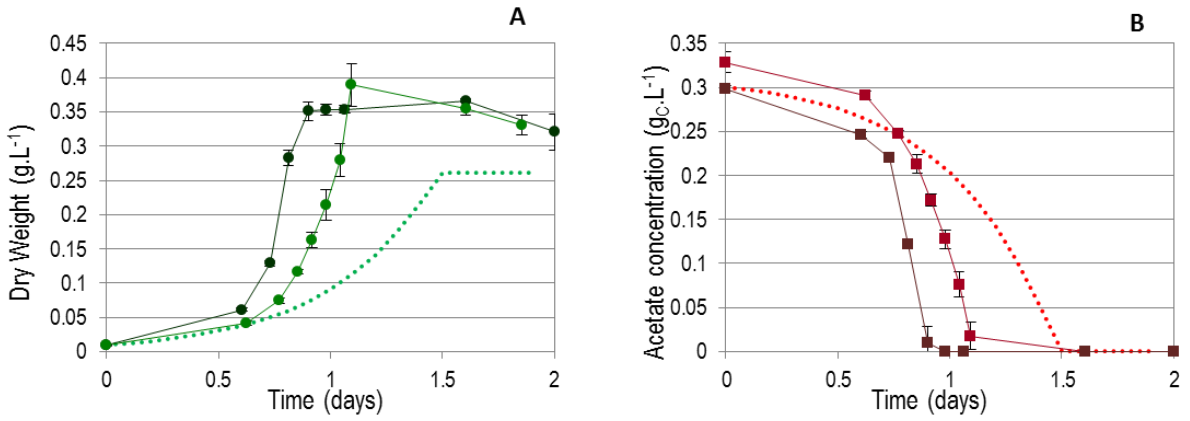

Figure 3-3. Effect of temperature on microalgae heterotrophic growth on acetate (0.3 $g_C.L^{-1}$).

Microalgae concentration, in $g.L^{-1}$, during heterotrophic growth on acetate at 30 °C (●) and 35 °C (●) are represented in subfigure A. Acetate concentrations, in $g_C.L^{-1}$, during growth at 30 °C (■) and 35 °C (■) are represented in subfigure B. The simulated heterotrophic microalgae concentration (green dashed lines) and acetate concentration (red dashed lines) at 25 °C are represented.

For heterotrophic growth on a mixture of acetate and butyrate (Figure 3-4, A and B), the apparent growth rate on acetate, at 35 °C (3.17 ± 0.45 d^{-1}) was higher than at 25 °C (2.23 d^{-1} - see Table 3-2). However, microalgae biomass concentrations after acetate exhaustion were similar at 25 °C and 35 °C (Figure 3-4, A and B). The carbon yields on acetate at 25 °C and at 35 °C were also similar (Table 3-2). However, the growth rate and carbon yield on acetate in

Table 3-2. Effect of temperature on apparent growth rate (μ_{app}) and heterotrophic carbon yield of *Chlorella sorokiniana* under heterotrophic conditions on acetate (A), butyrate (B) and a mixture of butyrate and acetate (B + A).

The figures at 25 °C are taken from a previous study for heterotrophic growth of *C. sorokiniana*. For 30 °C and 35 °C, the mean values and standard deviations calculated from triplicates are given. Values with different letters are statistically different ($p \leq 0.05$, one-way ANOVA and Tukey's post-hoc analysis). The carbon yield was estimated for a microalgae cell composition of 50% of carbon (Chen and Johns, 1996).

Temperature	Conditions tested	Growth on acetate		Growth on butyrate	
		μ_{app} (d^{-1})	Y_{Het}^{Het} ($g_C.g_C^{-1}$)	μ_{app} (d^{-1})	Y_{Het}^{Het} ($g_C.g_C^{-1}$)
25 °C	A; B and A + B	2.23	0.42	0.16*	0.56
30 °C	A	4.65 ± 0.16 ^{a}	0.58 ± 0.04 $^{a, b}$		
	B			0.13 ± 0.01 $^{a, b}$	0.42 ± 0.03 ^{a}
	A + B	4.12 ± 0.19 ^{a}	0.51 ± 0.01 ^{a}	0.16 ± 0.01 ^{b}	0.56 ± 0.01 ^{b}
35 °C	A	5.88 ± 0.39 ^{b}	0.64 ± 0.06 ^{b}		
	B			No growth	
	A + B	3.17 ± 0.45 ^{c}	0.41 ± 0.02 ^{c}	0.11 ± 0.02 ^{a}	0.28 ± 0.03 ^{c}

the acetate control (Figure 3-3) were almost 2 and 1.6 times higher than on the mixture of acetate and butyrate (Table 3-2). Even though the growth rate on acetate was highest at 35 °C in the acetate control, the presence of butyrate inhibited the increased growth rate on acetate at the higher temperature. At 25 °C, the presence of butyrate did not reduce the growth rate on acetate for butyrate concentrations up to 0.5 $g_C.L^{-1}$ (Chapter 2). Ugwu et al (2000) reported that when one abiotic parameter (irradiance) was set to the optimum, the negative effects of

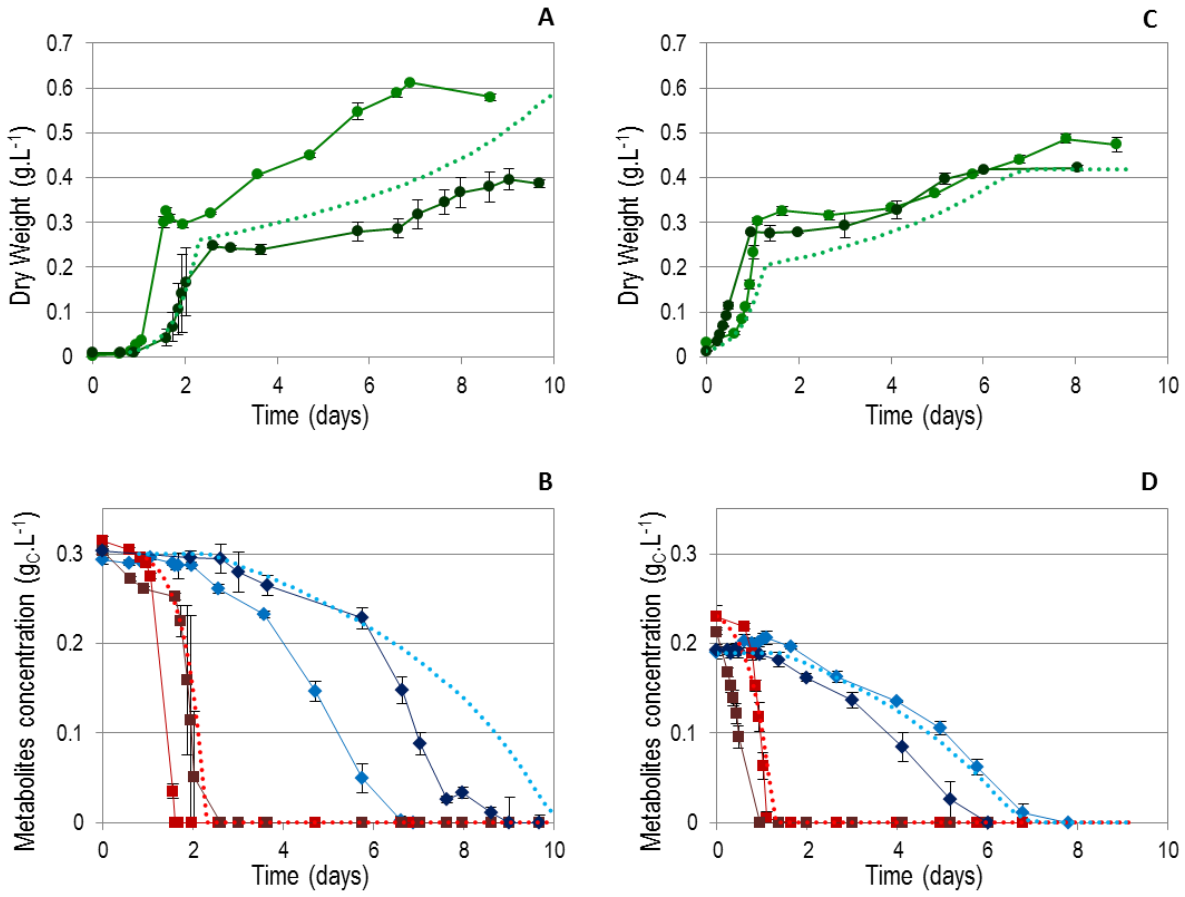

Figure 3-4. Effect of increasing temperature, from 25 °C to 35 °C, on heterotrophic growth of *Chlorella sorokiniana* cultivated on mixtures of acetate and butyrate.

(A and C) Microalgae concentration, in $g.L^{-1}$, during heterotrophic growth on mixtures of acetate and butyrate at 0.3 $g_C.L^{-1}$ each and 0.2 $g_C.L^{-1}$ each, respectively, at 30 °C (●) and 35 °C (●). (B and D) Acetate and butyrate removals, in $g_C.L^{-1}$, during growth on mixtures of acetate and butyrate at 0.3 $g_C.L^{-1}$ each and 0.2 $g_C.L^{-1}$ each, respectively, at 30 °C (■ and ◆) and 35 °C (■ and ◆). The simulated heterotrophic biomass growth (green dashed lines), acetate uptake (red dashed lines) and butyrate uptake (blue dashed lines) at 25 °C are shown.

another parameter (such as high dissolved oxygen concentration or temperature) were aggravated (Ugwu et al., 2007). Thus, when one growth factor is set at its optimum, the fast metabolism will, in particular, reduce energy storage and the microalgae might be less able to protect themselves from any adverse conditions. The negative effect of butyrate on

heterotrophic growth on acetate at 35 °C was reduced when the butyrate concentration was lowered to 0.2 $g_C.L^{-1}$ (Figure 3-4, C and D). At this concentration, the growth rate (4.71 ± 0.24 d^{-1}) and carbon yield (0.65 ± 0.02 $g_C.g_C^{-1}$) on acetate were higher than with 0.3 $g_C.L^{-1}$ of butyrate. As a consequence, these results confirmed that butyrate inhibition of heterotrophic growth depended on the concentration, as previously suggested in Chapter 2 and by Liu et al. (2012).

The apparent growth rate on butyrate was lower at 35 °C (0.11 d^{-1}) than the maximum growth rate at 25 °C (0.16 d^{-1}) (Table 3-2). However, when acetate was completely exhausted, the butyrate was assimilated and was exhausted after 9 days at 35 °C whereas butyrate was not predicted to be completely exhausted after 10 days at 25 °C (Figure 3-4, B). The growth rate associated with butyrate uptake, µb(Sb) (d^{-1}), at 25 °C, was described in Chapter 2 as following a modified Haldane equation (Equation 3-7).

$$\mu_b(S_b) = \mu_{b\_max} * \frac{K_D}{K_D + S_a} * \frac{Sb}{S_b + \frac{\mu_{b\_max}}{\alpha} * \left(\frac{Sb}{S_{b\_opt}} - 1\right)^2} \qquad \text{Equation 3-7}$$

where S_b is the concentration of butyrate ($g_C.L^{-1}$), S_{b_opt} (0.05 $g_C.L^{-1}$) is the concentration of butyrate when μ_b (S_b) is maximum and equivalent to μ_{b_max} (0.16 d^{-1}), the maximum growth rate associated with butyrate assimilation, α (15.1 $L.d.g_C^{-1}$) is the initial slope and K_D (2.10^{-10} $g_C.L^{-1}$) is the half inhibitory constant associated with the diauxic growth.

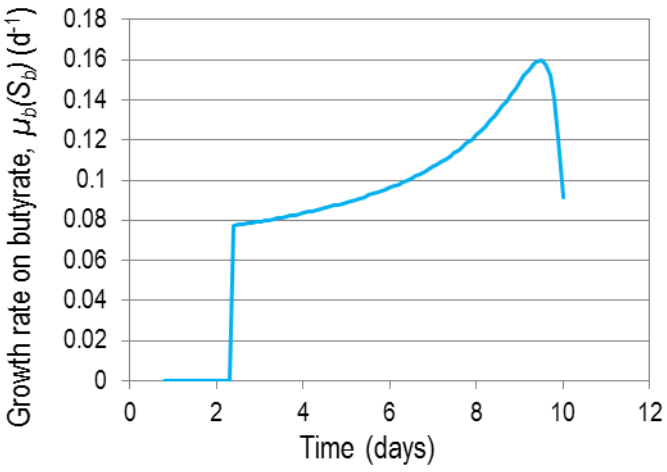

Figure 3-5. Variation of the growth rate on butyrate ($\mu_b(S_b)$) according to the simulations of the model representing heterotrophic growth at 25°C.

The predicted growth rate on butyrate at 25°C varied with the butyrate concentration and reached its maximum, μ_{b_max}, after 9.5 days of cultivation when the butyrate concentration

reached S_{b_opt} (0.05 $g_C.L^{-1}$) (Figure 3-5). At 35 °C, the apparent growth rate was calculated for a butyrate concentration of 0.23 $g_C.L^{-1}$ which was reached after 5.7 days of cultivation (Figure 3-4, B). Consequently, the time to reach butyrate exhaustion was shorter at 35 °C than at 25 °C despite a higher maximum growth rate at 25 °C than the apparent growth rate at 35 °C (Figure 3-4). The carbon yield on butyrate at 35 °C was half that at 25 °C. Contrary to the hypothesis suggesting that the butyrate inhibition might be reduced at 35 °C, butyrate inhibition was stronger at 35 °C than at 25 °C. Furthermore, no microalgae growth was observed at either 25 °C or 35 °C in the butyrate control (no acetate). As for growth on acetate in mixture, butyrate inhibition at 35 °C depended on the concentration since the butyrate uptake rate was faster at 35 °C than 25 °C when butyrate concentration was reduced to 0.2 $g_C.L^{-1}$ (Figure 3-4, C and D).

3.3.2.2 Butyrate inhibition was reduced at 30 °C

As shown in Figure 3-4 (A and B) and Table 3-2, the growth rate and carbon yield on acetate in mixture were both higher at 30 °C than at 25 °C or 35°C. However, there was no significant difference ($p>0.05$) between these growth rates and carbon yields and those in the acetate control (Table 3-2, Figure 3-3). The presence of butyrate did not appear to inhibit microalgae growth on acetate at 30 °C.

Similarly, when butyrate was taken up (in mixture), the apparent growth rate and the microalgae biomass yield were higher at 30 °C (0.16 d^{-1} and 0.56 $g_C.g_C^{-1}$, respectively) than at 35 °C (0.11 d^{-1} and 0.28 $g_C.g_C^{-1}$, respectively) (Table 3-2). The apparent growth rate at 30°C was calculated for a butyrate concentration of 0.29 $g_C.L^{-1}$ which was reached after 2 days of cultivation (Figure 3-4, B and Table 3-2). As explained in the previous paragraph (3.2.1), the maximum growth rate at 25 °C (0.16 d^{-1}) could only be reached at a low butyrate concentration (0.05 $g_C.L^{-1}$). These results suggest that there was less butyrate inhibition at 30 °C than at 25 °C. Furthermore, microalgae growth was observed in the butyrate control whereas no growth was observed at 25 °C or 35 °C. A cultivation temperature of 30 °C thus successfully reduced butyrate inhibition and consequently butyrate exhaustion occurred more than 3 days earlier than at 25 °C (Figure 3-4, B). At 30 °C, enzymatic reactions countering butyrate inhibition may have been encouraged.

Temperatures higher than 25 °C increased heterotrophic growth on both acetate and butyrate. However, the near-optimum temperature for acetate was 35 °C while for butyrate it was 30

°C. Cultivation on a mixture of acetate and butyrate at a suboptimum temperature for growth on acetate alone may have reduced butyrate inhibition.

3.3.3 Combined effects of temperature and light on growth of *C. sorokiniana* on mixture of VFAs

3.3.3.1 At 35 °C in the presence of light, microalgae growth on acetate or on butyrate relied more on heterotrophy than at 25 °C

A strict autotrophic control (bicarbonate as the sole carbon source) was carried out at 35 °C to assess the effect of temperature in autotrophic conditions. In the autotrophic control, the autotrophic production rate of biomass (0.09 $g.L^{-1}.d^{-1}$) at 35 °C (Figure 3-6) was similar to that observed at 25 °C (0.11 $g.L^{-1}.d^{-1}$ – see Figure 3-2). Temperature appeared to have no significant effect on autotrophic growth.

Under mixotrophic conditions for the acetate control (no butyrate) (Figure 3-6, A), the growth rate was significantly higher ($p < 0.05$) at 35 °C (5.65 d^{-1}) than at 25 °C (4.14 d^{-1}) in the presence of light but was not significantly different from the growth rate observed at 35 °C with no light (5.88 d^{-1}) ($p > 0.05$ - Table 3-1 and Table 3-3). About 85% of the biomass content (X_{Het}^{Mixo}, Equation 3-5) at the time of acetate exhaustion was due to acetate uptake (Table 3-3). These results suggest that *C. sorokiniana* followed a heterotrophic type of metabolism at 35 °C despite the presence of light.

The combined effects of temperature and light on microalgae growth for the butyrate control (no acetate) was also studied (Figure 3-6, B). During the first six days, the biomass in the butyrate control was lower than the biomass in the autotrophic control. The presence of butyrate seemed to inhibit autotrophic growth under mixotrophic conditions at 35 °C. This inhibition depended on the concentration since autotrophic growth was inhibited only during the first three days when the initial butyrate concentration was lowered to 0.2 $g_C.L^{-1}$ (Figure 3-6, D). However, the butyrate uptake rate was significantly higher ($p < 0.05$) at 35 °C (88 $mg_C.L^{-1}.d^{-1}$) than at 25 °C (47.5 $mg_C.L^{-1}.d^{-1}$) in the presence of light (Table 3-1 and Table 3-3). Moreover, the fraction of biomass production due to autotrophic growth (X_{Auto}^{Mixo}, Equation 3-6) was lower (55%) at 35 °C than at 25 °C (74%). As for growth on acetate, it was concluded that growth on butyrate at 35 °C with light relied more on heterotrophic growth than at 25 °C.

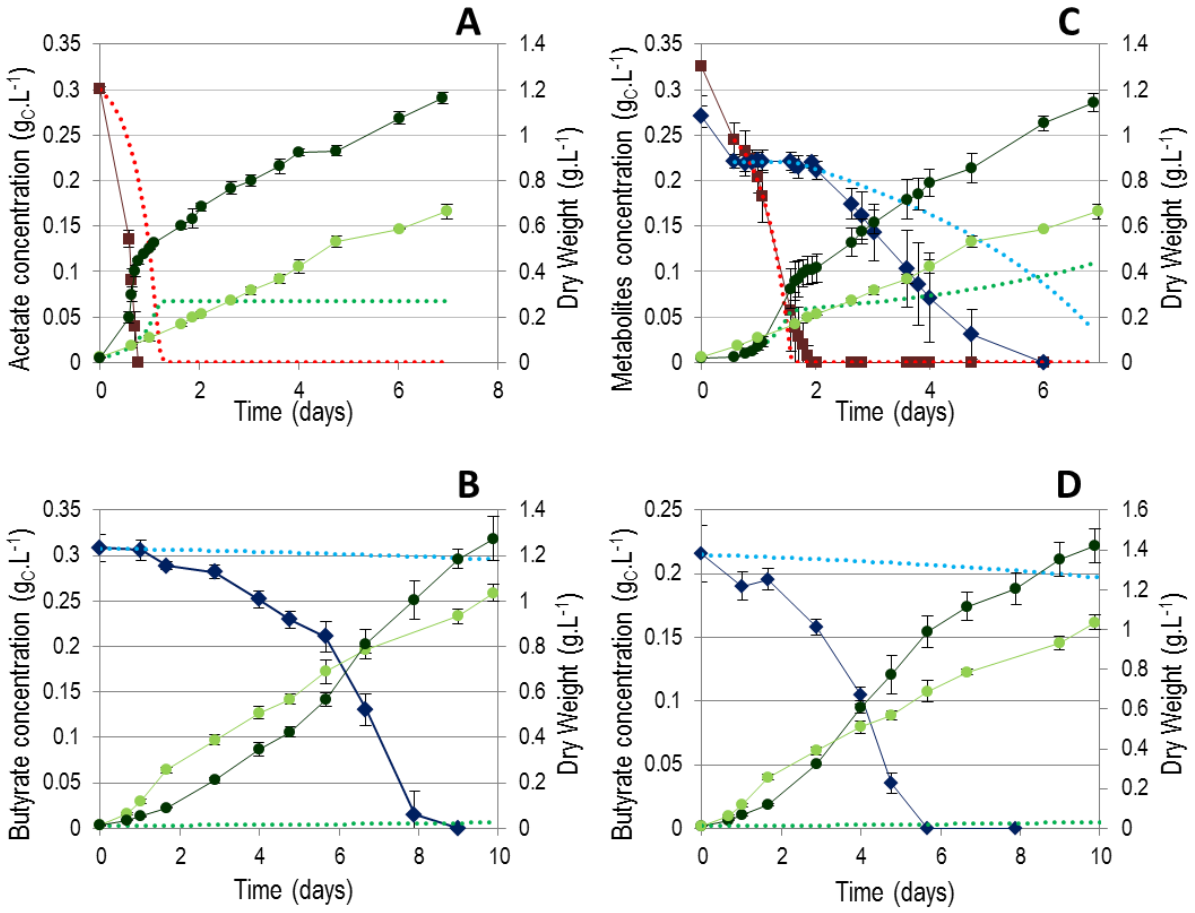

Figure 3-6. Combined effects of light and temperature (35 °C) on *C. sorokiniana* growth on acetate and butyrate.

Microalgae concentration (●), in $g.L^{-1}$, during mixotrophic growth on (A) acetate as single substrate at 0.3 $g_C.L^{-1}$, (B) butyrate as single substrate at 0.3 $g_C.L^{-1}$, (C) a mixture of acetate and butyrate at 0.3 $g_C.L^{-1}$ and (D) butyrate as single substrate at 0.2 $g_C.L^{-1}$. Acetate (■) and butyrate removals (♦), in $g_C.L^{-1}$. Microalgae concentration ($g.L^{-1}$) (●) during autotrophic growth. The simulated heterotrophic biomass growth (green dashed lines), acetate uptake (red dashed lines) and butyrate uptake (blue dashed lines) at 25 °C are shown.

3.3.3.2 At 35 °C, light reduced butyrate inhibition on growth on butyrate but not on acetate

The combined effect of temperature and light on *C. sorokiniana* growth on a mixture of acetate and butyrate, was studied to assess the interactions between acetate and butyrate (Figure 3-6, C). In the presence of butyrate, both the growth rate and the heterotrophic carbon yield on acetate (2.53 d^{-1} and 0.36 $g_C.g_C^{-1}$, respectively) were half those measured in the acetate control (5.65 d^{-1} and 0.60 $g_C.g_C^{-1}$, respectively – see Table 3-3). The growth rate on acetate was not statistically different ($p > 0.05$) from that measured with no light at 35 °C (3.17 d^{-1}) (Table 3-2 and Table 3-3). Consequently, butyrate inhibition of acetate uptake was not reduced by the presence of light at 35 °C. The fraction of biomass due to acetate uptake

(X_{Het}^{Mixo}, Equation 3-5) was estimated at 60% (Table 3-3). This suggests that *C. sorokiniana* growth on acetate in a mixture of acetate and butyrate relied mostly on heterotrophic growth as was also observed for the acetate control.

Table 3-3. Effect of light and temperature (35 °C) on growth rates and yields of *C. sorokiniana* for cultivation on acetate (A), butyrate (B) and a mixture of butyrate and acetate (B + A).

μ_{app} is the apparent growth rate, r_{app_lin} is the apparent linear production rate of biomass, $\boldsymbol{Y_{Mixo}^{Mixo}}$ is the mixotrophic carbon yield and $\boldsymbol{Y_{Het}^{Mixo}}$ is the heterotrophic carbon yield during mixotrophic cultivation. Mean values and standard deviations calculated from triplicates are given. Values with an asterisk (*) are statistically different from the results observed at 25 °C in the presence of light (Table 3-1) ($p \leq 0.05$, one-way ANOVA and Tukey's post-hoc analysis).

	Growth on acetate				Growth on butyrate				
	μ_{app} (d^{-1})	Y_{Mixo}^{Mixo} ($g_C.g_C^{-1}$)	Y_{Het}^{Mixo} ($g_C.g_C^{-1}$)[a]	X_{Auto}^{Mixo} (%)[b]	r_{app_lin} ($g.L^{-1}.d^{-1}$)	Uptake rate ($mg_C.L^{-1}.d^{-1}$)	Y_{Mixo}^{Mixo} ($g_C.g_C^{-1}$)	Y_{Het}^{Mixo} ($g_C.g_C^{-1}$)[a]	X_{Auto}^{Mixo} (%)[b]
A	5.65 ± 0.55*	0.71 ± 0.03	0.6 ± 0.01	15					
B					0.18 ± 0.01	88 ± 4.6*	1.61 ± 0.03	0.73 ± 0.02*	55
A + B	2.53 ± 0.16	0.6 ± 0.06*	0.36 ± 0.06	40	0.16 ± 0.00	62.4 ± 2.4	1.48 ± 0.02*	0.56 ± 0.01*	62

[a]: The heterotrophic carbon yield (Y_{Het}^{Mixo}) was calculated by subtracting the carbon yield associated with autotrophic growth from the mixotrophic carbon yield (Y_{Mixo}^{Mixo}).

[b]: The fraction of mixotrophic biomass due to autotrophic growth on CO_2 (X_{Auto}^{Mixo}) was calculated as follow:

$$X_{Auto}^{Mixo} = \frac{Y_{Mixo}^{Mixo} - Y_{Het}^{Mixo}}{Y_{Mixo}^{Mixo}} * 100$$

Inhibition of autotrophic growth on butyrate which was observed in the butyrate control (paragraph 3.3.3.1) did not appear after acetate exhaustion (Figure 3-6, C). The fraction of biomass due to autotrophic growth (X_{Auto}^{Mixo}, Equation 3-6) at 35 °C was estimated at 62% (Table 3-3). The time taken to exhaust butyrate completely was 3 days less than under heterotrophic conditions at 25 °C and 35 °C, probably because of the high biomass reached after acetate exhaustion and because of the autotrophic biomass growth at 35 °C in presence of light. Light increased butyrate uptake at 35 °C for cultivation on a mixture of acetate and butyrate. At 35 °C, the presence of butyrate reduced the apparent growth rate on acetate under both heterotrophic and mixotrophic conditions and also inhibited autotrophic growth in the butyrate control under mixotrophic conditions. Further investigation on the effect of butyrate on the respiration rate and/or photosynthetic activity may provide further information on the

negative effect of butyrate on mixotrophic and heterotrophic growth observed in this study at high temperature.

3.4 Conclusions

In this study, the previously accepted optimum cultivation temperature (35 °C) did not provide the best conditions for heterotrophic or mixotrophic growth of *C. sorokiniana* on a mixture of acetate and butyrate. Nevertheless, the apparent biomass growth was successfully enhanced under heterotrophic conditions at suboptimal temperature (30 °C) by increasing the acetate uptake (4.1 d^{-1}) and reducing the butyrate inhibition on growth. Moreover, a positive effect of light was observed on the apparent butyrate uptake (71 $mg_C.L^{-1}.d^{-1}$) that was favored in presence of acetate at 25 °C. This was due to an increase in biomass content (reaching 1.14 $g.L^{-1}$) through concomitant heterotrophic and autotrophic growth. In conclusion, *C. sorokiniana* may grow successfully on raw dark fermentation effluents containing acetate and butyrate, under suboptimal temperature (30 °C) and with light (123 µmol $photons.m^{-2}.s^{-1}$).

3.5 References

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Chapter 4

Heterotrophic growth of *Chlorella sorokiniana* on raw dark fermentation effluent: competition between microalgae and bacteria for VFAs

Microalgae growth on unsterilized dark fermentation effluent is investigated in this chapter in order to study the interactions between microalgae and fermentation bacteria. The results of this chapter have been published in Algal Research in a research article entitled "Raw dark fermentation effluent to support heterotrophic microalgae growth: microalgae successfully outcompete bacteria for acetate" (2015, 12: 119-125).

4.1 Introduction

As discussed in Chapter 1, only few studies have investigated the use of unsterile dark fermentation (DF) effluents, containing fermentation bacteria, to support microalgae growth (Hu et al., 2013, 2012a; Venkata Mohan and Prathima Devi, 2012). None of these few studies studied the bacterial growth. Thus no distinction between VFAs uptake by either bacteria or microalgae was done. In addition, all these experiments were carried out under mixotrophic conditions. Under such conditions, i.e. in the presence of fermentation bacteria and with light, it is difficult to know if microalgae grew using VFAs (heterotrophy) or CO_2 (autotrophy). Hence, to know whether microalgae growth on unsterile effluents is possible, a dedicated study performed in the dark while monitoring both bacterial and algal growth is necessary. It would allow to highlight the impact of the presence of bacteria which might compete for the substrate or excrete inhibiting compounds.

Microalgae growth response to the presence of fermentation bacteria originated from DF was thus investigated. Because of the microalgae fast growth on acetate (Chapter 2) and the fact that most of the bacteria from DF effluent should be strict anaerobic species (Chapter 1), it was hypothesized that microalgae could grow efficiently on raw DF effluent. Unsterilized raw DF effluents (obtained after dark fermentation of glucose), composed of acetate and butyrate, were used to assess the interaction between microalgae and bacteria from DF effluent and compared to axenic algal growth on sterilized effluents.

4.2 Materials and methods

4.2.1 Dark fermentation batches

Five identical test batches of "DF effluent" were produced simultaneously in 600 mL glass bottles with a working volume of 200 mL. No culture medium was added or removed during the fermentation. The culture medium consisted of 100 mM of 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, 5 $g.L^{-1}$ of glucose, 0.8 $g.L^{-1}$ of NH_4Cl, 0.5 $g.L^{-1}$ of K_2HPO_4 and 5 $mL.L^{-1}$ of a micronutrient solution. The composition of the micronutrient solution is described in Table 4-1. The medium was supplemented with 1 $mL.L^{-1}$ of F/2 medium vitamin solution (CCAP, http://www.ccap.ac.uk/) as described in Chapter 2. The flasks were inoculated with 1 mL of heat-treated (15 min at 90 °C) anaerobic sludge from an anaerobic digester treating waste from a sugar plant (Marseille, France). The initial substrate to biomass ratio S:X was 40, S representing the initial chemical oxygen demand (COD) of the substrate (in $gCOD.L^{-1}$) and X representing the initial inoculum (in g of total volatile solids per L). To

ensure anaerobic conditions, the flasks were sealed and flushed with nitrogen gas as described by Pierra et al. (2013). The pH was adjusted to 6 and the bottles were incubated at 37 °C until the glucose was completely exhausted. At the end of the growth phase, after glucose exhaustion and hydrogen accumulation, the five anaerobic cultures batches were mixed to produce the "DF effluent". The pH of the DF effluent was increased to 6.5 with 1 M NaOH. VFAs, ammonium and phosphate concentrations were also measured. Half of the DF effluent was centrifuged three times at 15,000 rpm for 15 min. A fraction of the supernatant was sterilized using Acrodisc® PF syringe filter with 0.8/0.2 µm pores (PALL). The sterilized and unsterilized DF effluents samples were then stored at 4 °C until the start of the experiments.

Table 4-1. Composition of the micronutrients solution.

Components	Concentration in micronutrient stock solution
$FeCl_2$, H_2O	1.5 $g.L^{-1}$
H_3BO_3, H_2O	60 $mg.L^{-1}$
$MnSO_4$, H_2O	117 $mg.L^{-1}$
$CoCl_2$, $6H_2O$	25 $mg.L^{-1}$
$ZnCl_2$	70 $mg.L^{-1}$
$NiCl_2$, $6H_2O$	25 $mg.L^{-1}$
$CuCl_2$, $2H_2O$	15 $mg.L^{-1}$
$NaMoO_4$, $2H_2O$	25 $mg.L^{-1}$
HCl (liquid, 25 %)	6.5 $mL.L^{-1}$

4.2.2 Axenic microalgae strain and preparation of the microalgae stock culture

Chlorella sorokiniana stock culture was prepared as described in Chapter 2. Briefly, *Chlorella sorokiniana* (CCAP 211/8K) was cultivated axenically in 500 mL Erlenmeyer flasks with a working volume of 200 mL. A modified BG11 medium was used as described in Chapter 2. Sodium bicarbonate (10 mM), ammonium chloride (5 mM) and dipotassium phosphate (0.31 mM) were used as inorganic carbon (C), nitrogen (N) and phosphorus (P) sources, respectively. All the components of the medium as well as the flasks were sterilized by autoclaving at 121 °C for 20 min before use. The flasks were incubated under autotrophic conditions (light intensity of 100 µmol photons m^{-2} s^{-1}) at 25 °C for 7 days.

4.2.3 Heterotrophic microalgae growth on dark fermentation effluent

A fixed volume of either the sterilized or the unsterilized DF effluent (36 mL) was placed in sterile 125 mL black Erlenmeyer flasks sealed with a cotton wool plug. Four mL of microalgae culture, 0.2 $g.L^{-1}$, were added to each flask (Figure 4-1). The flasks were then

incubated on a rotary shaker (150 rpm) at 25 °C for 10 days in complete darkness. A 1 mL sample of the culture was taken every day to measure the optical density (OD), VFA concentration, microbial concentration and diversity. The experiment was carried out in triplicate. During the whole experiment, the microalgae cultures in the sterilized DF effluent were daily checked for other living organisms by DAPI counterstaining and contrast phase microscopy.

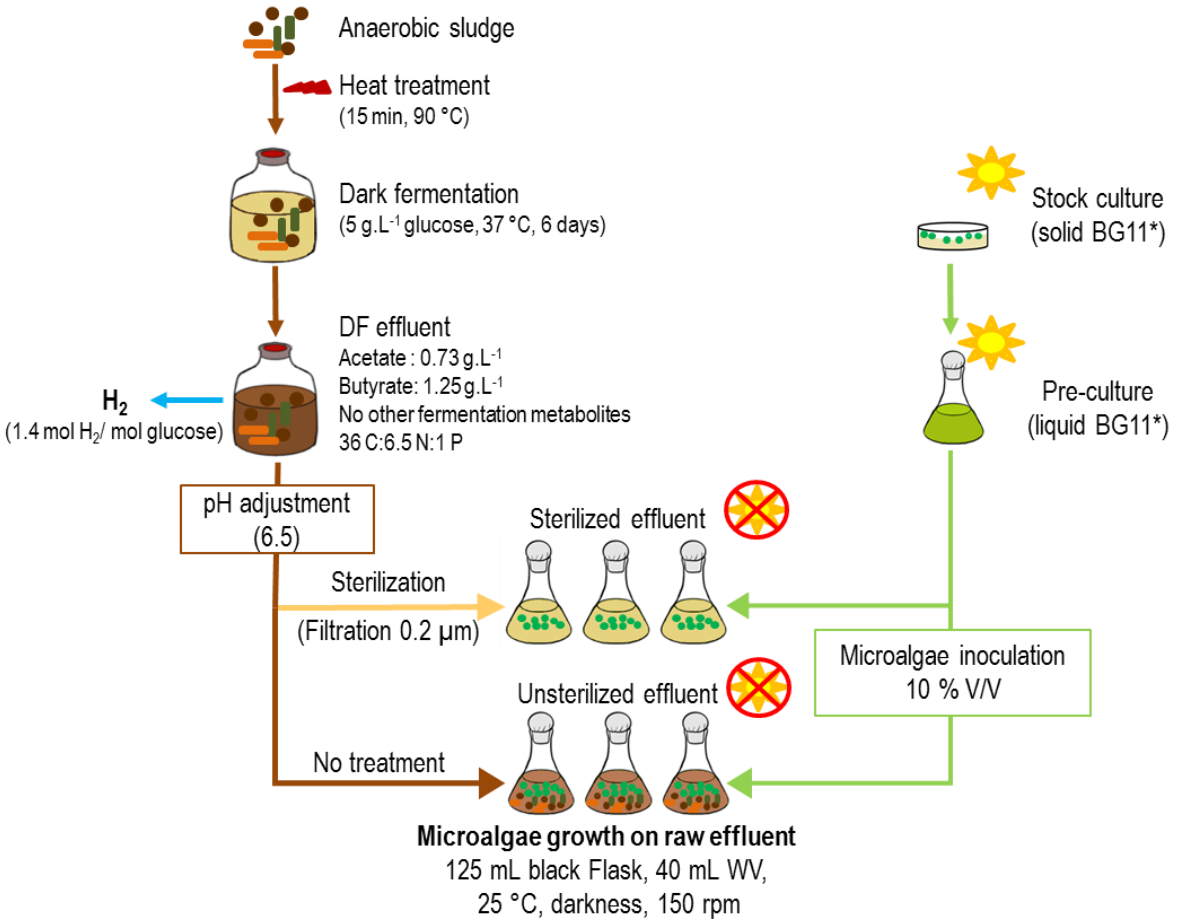

Figure 4-1. Schematic representation of the experiments.

BG11*: modified BG11 medium, WV: Working Volume.

4.2.4 Microbial analysis

4.2.4.1 Microalgae biomass measurement

The microalgae growth was quantified on sterilized DF effluent by measuring the OD at 800 nm (OD_{800}) and the associated calibration function (DW ($g.L^{-1}$) = 1.24*OD_{800} (R^2 = 0.95)) as described in Chapter 2. For calculating the biomass yield, the carbon content was estimated at 50% of microalgae biomass (Chen and Johns, 1996).

The dynamics of microalgae growth were monitored by amplification of 18S rDNA gene copies, in cultures carried out using with both sterilized and unsterilized DF effluents. Indeed, because of the presence of small suspended solids in the unsterilized DF effluent, optical density measurement could not be used for monitoring the algal biomass. In addition, as the chlorophyll content of microalgae during heterotrophic cultivation can change, it was also not suitable for monitoring the microalgae growth accurately (Rosenberg et al., 2014).

4.2.4.2 DNA extraction and purification

700 µL of the culture sample was centrifuged at 10,000 rpm for 10 min and genomic DNA was extracted using the PROMEGA Wizard® Genomic DNA Kit and then purified using the QIAamp DNA Mini Kit (Qiagen).

4.2.4.3 Quantification using Quantitative real-time PCR

The microalgae biomass was quantified by quantitative PCR (qPCR) targeting a partial sequence of 18S rDNA from *Chlorophyta* and using specific primers INT-4F and INT-5R (Table 4-2). The quantitative amplification reaction was carried out with 5 µL of DNA sample, 12.5 µL of Universal SYBR® Green Supermix (Biorad), 1 µL of forward primer INT-4F, 1 µL of reverse primer INT-5R and 5.5 µL of H_2O, for a total volume of 25 µL. The PCR was run in a 100 Touch™ thermal cycler equipped with a CFX96™ Real-Time System (Bio-rad). There was an initial incubation of 3 min at 95 °C followed by 40 cycles of denaturation-amplification (10 s at 95 °C and 30 s at 56 °C). Data analysis was carried out with the Bio-rad CFX Manager software, version 3.0. A linear standard curve was generated for each assay by amplification of eight 10-fold dilutions of pEX-A2 plasmids (Eurofins MWG Operon) containing the targeted gene sequence. X62441.2 (ENA, http://www.ebi.ac.uk/ena). The amplicon corresponded to a 77 bp sequence in position from 1666 to 1742 NT in *Chlorella sorokiniana* 18S gene. In order to detect PCR inhibition, two different dilutions of each DNA sample were amplified and the initial calculated concentrations were compared in pairs. Inhibited PCR reactions produced lower values which were eliminated from the analysis. The quantification limit defined using the lowest concentration of standard in the linear range was 10 copies per qPCR reaction for all qPCR systems. The total number of 18S rDNA gene copies per sample was expressed as the logarithm of the number of target copies per mL of culture sample. For SYBR® Green assays, the specificity of the PCR products was checked by a melting curve analysis using the dissociation protocol from the Bio-rad CFX Manager software. An initial incubation of 3 min at 95 °C and 40 cycles of denaturation (10 s at 95 °C and 30 s at 56 °C) were performed.

For quantification of total bacteria, bacterial primers targeting 16S rDNA gene, BAC338F and BAC805R, and the associated probe, BAC16F, were used (Table 4-2). The quantitative amplification reaction was carried out in a total volume of 12.5 µL with 2 µL of sample DNA, 6.5 µL of Universal probes Supermix (Biorad), 0.5 µL of each primers and the probe and 2.5 µL of H_2O. The thermal cycler and analysis method were the same as for qPCR of *Chlorophyta*. An initial incubation of 2 min at 95 °C followed by 40 cycles of denaturation-amplification (7 s at 95 °C and 25 s at 56 °C) were performed. The linear standard curve was obtained as described by Abbassi-Guendouz et al. (2013). The total number of 16S rDNA gene copies per sample was expressed as the logarithmic value of the number of target copies per mL of culture sample.

The amplification efficiency in total bacterial 16S rRNA and *Chlorophyta* standard curves was between 97% and 100%, with a regression coefficient value (R^2) systematically above 0.98.

Table 4-2. Primers for qPCR.

Fluorescent probes: YY: Yakima Yellow, TAMRA*:* 6-carboxytetramethylrhodamine.

Micro-organisms	Primers	Sequences (5'-3')	Positions	References
Chlorophyta	INT- 4F	TGGTG AAGTG TTCGG ATTGG	*C. sorokiniana* -1666	(Hoshina et al., 2004)
	INT- 5R	ARGTG GGAGG GTTTA ATGAA	*C. sorokiniana* -1723	
Bacteria	BAC338F	ACTCC TACGG GAGCPG AG	*E. coli* - 338	(Yu et al., 2005)
	BAC16F	YY-TCPGCA CPGACPG CCPGGG TAATA C- TAMRA	*E. coli* - 516	
	BAC805R	GACTA CCAGG GTATC TAATC C	*E. coli* - 785	

4.2.4.4 Sequencing bacterial 16S rDNA

Samples taken on day 0, at day 2.7 and day 10 (9 samples in total) were used for sequencing. The V4-V5 region of the 16S rDNA gene was amplified over 30 amplification cycles at an annealing temperature of 65 °C, with the forward primer 5'-CTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA-3' and the reverse primer 5'-GGAGTTCAGACGTGTGCTCTTCCGATCTCCCCGYCAATTCMTTTR AGT-3' with their respective linkers. In a second PCR reaction of 12 cycles, an index sequence was added using the primers AATGATACGGCGACCACCGAGATCTACACTC TTTCCCTACACGAC and CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGA

GTTCAGACGTGT. The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions for sequencing 250 bp reads. Sequencing was carried out at the GeT PlaGe sequencing center of the Genotoul life science network in Toulouse, France (get.genotoul.fr). 595799 forward and reverse sequences were retained after assembly and quality checking using a slightly modified version of the standard operation procedure described by Kozich et al. (Kozich et al., 2013). Mothur version 1.33.0. SILVA release 102 provided by Schloss et al. (Schloss et al., 2009) was used for alignment and as a taxonomic outline. Using mothur, representative sequences of bacterial operational taxonomic units (OTUs) were identified at the 2% level while representative sequences of OTUs with less than 2% difference were grouped into a single OTU. The known species that were phylogenetically the closest to OTUs with a relative abundance of more than 2% at the start of the experiment (day 0), at acetate exhaustion (day 2.7) or at the end of the experiment (day 10) were identified using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The sequences identified as *C. sorokiniana* chloroplast were removed before analysis of bacterial abundance.

4.2.5 Chemical analysis

Acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate were measured using a gas chromatograph (GC 3900 Varian) equipped with a flame ionization detector (see Chapter 2). Other non-volatile molecules such as glucose, ethanol, lactate and formate were quantified using HPLC with a refractive index detector and an Aminex HPX-87H column (Biorad®). The column temperature was maintained at 35 °C and the flow rate at 0.4 $mL.min^{-1}$ (see Chapter 2).

Biogas production of the DF test batches was monitored daily by measuring the gas pressure in the headspace. The biogas composition (CO_2, O_2, H_2, N_2 and CH_4) was measured using a gas chromatograph (GC) (Clarus 580, Perkin Elmer) coupled to a thermal conductivity detector (TCD), maintained at 150 °C (Rafrafi et al., 2013). The GC was composed of one injector (250 °C) and two capillary columns maintained at 60 °C and with argon as gas vector (31.7 $mL.min^{-1}$ at 350 kPa): a RtUBond column, to separate CO_2 from the gas, and a RtMolsieve column which decomposes the gas into O_2, H_2, N_2 et CH_4.

Ammonium (NH_4^+) and phosphate (PO_4^{3-}) ions were quantified using an ion chromatography system (ICS 3000 Dionex, USA) composed of two pre-columns (NGI 2mm and CG 11 2mm) and separation columns CS 16 3mm and AG 15 2mm for cations and anions, respectively

(Uggetti et al., 2014). Hydroxymethanesulfonic acid (HMSA) at 25-40 mM (cations) and KOH at 10–74 mM were used as eluents at a flow rate of 0.3 $mL.min^{-1}$.

4.3 Results and discussion

4.3.1 Dark fermentation test batches

DF anaerobic test batches were carried out to provide a culture medium for subsequent heterotrophic microalgae growth. The fermentation ended with complete glucose exhaustion, after 6 days of fermentation. The final acetate and butyrate concentrations were 1.09 ± 0.09 $g.L^{-1}$ and 1.43 ± 0.07 $g.L^{-1}$, respectively. No other fermentation metabolites, lactate, ethanol, propionate, valerate and caproate, were found. The hydrogen yield, i.e. the molar ratio of hydrogen produced per mol of glucose consumed, was 1.37 ± 0.18 mol H_2.mol $glucose^{-1}$. The hydrogen yield was consistent with previously reported experimental results obtained in similar conditions with mixed cultures (Rafrafi et al., 2013). The molar ratio of H_2 per mol of VFAs (the sum of acetate and butyrate produced) was 1.1 ± 0.14 mol H_2.mol $VFAs^{-1}$. This value is lower than the theoretical value of 2 mol H_2 $mol.VFAs^{-1}$ produced through the acetate pathway (Equation 4-1) or the butyrate pathway (Equation 4-2) (Chapter 1). In general, the H_2 yield can be lowered either by the consumption of glucose through non-hydrogen-producing pathways, such as ethanol or lactate pathways, or by the direct consumption of H_2 through homoacetogenesis, i.e. the production of acetate via CO_2 and H_2 consumption (Equation 4-3) (Rafrafi et al., 2013) (Chapter 1). Since neither lactate nor ethanol were found, only homoacetogenesis could have occurred.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \text{Equation 4-1}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad \text{Equation 4-2}$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad \text{Equation 4-3}$$

Three samples of DF effluent were sequenced for microbial community characterization. The closest phylogenetically known sequences of the most representative Operational Taxonomic Units (OTUs) are given in Table 4-3. About 53 ± 1.5% of initial bacteria were strict anaerobes, with 36.8 ± 1.3% related to *Clostridium* sp. and 16.2 ± 0.6% to *Sporolactobacillus* sp. It has been established that bacteria belonging to *Clostridium* genus are responsible for hydrogen fermentation (Chapter 1). *Sporolactobacillus* sp. are known for their ability to degrade glucose into lactate in anaerobic conditions (Saady, 2013). Since no lactate was found in the medium and since *Clostridium tyrobutyricum* was the most abundant species, it can be

assumed that lactate has been converted into butyrate and hydrogen, as previously reported (Wu et al., 2012). *C. tyrobutyricum* is also known to use the butyrate pathway preferentially during DF. The presence of this species can explain the high butyrate:acetate molar ratio of 1.14 found at the end of DF (Zhu and Yang, 2004).

Table 4-3. Classification, relative abundance (%) and physiological characteristics of bacteria at the end of the dark fermentation test batches.

ORDER */species (closest known sequence)*[1]	Relative abundance (%)[2]	Anaerobic or aerobic metabolism	Specific characteristics	Reference
BACILLALES				
Paenibacillus chibensis	3.17 ± 0.1	Strict aerobe	Spore producer Unable to grow on acetate	(Shida et al., 1997)
Paenibacillus cookii	8.21 ± 0.73	Facultative anaerobe	Spore producer	(Logan et al., 2004)
Paenibacillus stellifer	10.66 ± 0.91	Facultative anaerobe	Spore producer Unable to grow on acetate	(Suominen et al., 2003)
Sporolactobacillus laevus	16.18 ± 0.65	Strict anaerobe	Spore producer, lactate producer	(Saady, 2013)
BURKHOLDERIALES				
Achromobacter aegrifaciens	3.12 ± 0.32	Strict aerobe	Acetate and butyrate consumer	(Vandamme et al., 2013)
Ralstonia pickettii	9.6 ± 0.36	Facultative anaerobe / Strict aerobe	Can grow under anaerobic conditions	(Boutros and Gonullu, 2002)
CLOSTRIDIALES				
Clostridium magnum	8.12 ± 0.65	Strict anaerobe	Spore producer, homoacetogene	(Schiel-Bengelsdorf and Dürre, 2012)
Clostridium tyrobutyricum	28.7 ± 0.66	Strict anaerobe	Spore producer, hydrogen producer	(Assih et al., 2002)
OTHERS[3]	12.23 ± 1.65			

[1]: Names in bold letters correspond to the bacterial orders identified. Names in italics correspond to the closest genetically known sequences, for all sequences, percentages of identity to reference sequence were greater than or equal to 97%.
[2]: Relative abundances were calculated by dividing the number of sequences for the taxon by the total number of sequences per sample. The values are the mean and standard deviation of the sequences from the three samples.
[3]: Taxa with less than 2% relative abundance were grouped under "Others".

The remaining bacteria were either facultative or strict aerobic species. Strict aerobes were previously found in an anaerobic digester, even non-spore producing species (Assih et al., 2002). Even though the anaerobic sludge was heat-treated before the fermentation, facultative and strict aerobic bacteria may have survived, as reported by Saady (2013). They in fact play an important role in removing residual-oxygen in anaerobic systems, which subsequently favors the growth of *Clostridium* species and hydrogen production (Hung et al., 2011).

4.3.2 Microalgae growth on raw fermentation effluent

4.3.2.1 Characterization of the dark fermentation effluent

The initial acetate concentrations in the sterilized and unsterilized DF effluent samples were 0.30 ± 0.0 $g_C.L^{-1}$ (0.74 ± 0.02 $g.L^{-1}$) and 0.29 ± 0.00 $g_C.L^{-1}$ (0.73 ± 0.02 $g.L^{-1}$), respectively. The initial butyrate concentrations in the sterilized and unsterilized DF effluent sample were 0.68 ± 0.03 $g_C.L^{-1}$ (1.25 ± 0.06 $g.L^{-1}$) and 0.69 ± 0.02 $g_C.L^{-1}$ (1.26 ± 0.03 $g.L^{-1}$), respectively. The acetate:butyrate mass ratio ($g.g^{-1}$) was about 0.6 in both cases. These concentrations as well as the acetate:butyrate ratio were consistent with previous studies using raw DF effluents to sustain microalgae growth (Liu et al., 2013, 2012; Venkata Mohan and Prathima Devi, 2012). Sterilization by microfiltration had no effect on the carbon, nitrogen and phosphorus contents of the effluent. The C:N:P molar ratios in the sterilized and unsterilized DF effluents were 35:6.7:1 and 36:6.5:1, respectively. These ratios were lower than the Redfield C:N:P ratio for phytoplankton cellular composition, i.e. 106:16:1. Hence, only the carbon substrate was assumed to be the limiting element for *C. sorokiniana* growth here.

As detailed in Chapter 1, other authors used the effluents from acidogenic fermentation (AF) which contained high amounts of VFAs ranging from 5 to 14 $g.L^{-1}$, to sustain microalgae growth instead of the effluents from DF (Hu et al., 2013, 2012a). Indeed, in AF, VFA accumulation is targeted rather than biohydrogen production as hydrogen conversion to acetate through homoacetogenesis is promoted. Nevertheless, AF effluents have to be diluted between 8 and 20 fold before use to avoid excess initial VFA concentrations inhibiting microalgae growth (Hu et al., 2013, 2012a). Thus, the final concentrations of VFAs were in the range, between 1.5 – 1.8 $g.L^{-1}$ (Hu et al., 2013), or even below, 0.03 – 0.3 $g.L^{-1}$ (Hu et al., 2012a), than the ones from the DF effluent used in this study.

4.3.2.2 Microalgae growth on sterilized and unsterilized dark fermentation effluent

C. sorokiniana was grown for 10 days on sterilized and unsterilized DF effluent in heterotrophic conditions (Figure 4-2 and Figure 4-3, respectively). Microalgae grew during the first 2.7 days in both experiments, which concurs with acetate consumption (Figure 4-2 and Figure 4-3). There was very similar growth in microalgae, expressed as the logarithm of the number of 18S rDNA copies per mL of culture sample reaching 8.13 ± 0.05 and 8.20 ± 0.04 log(18S copies mL^{-1}) during growth on sterilized and unsterilized DF effluent, respectively (Figure 4-3). The maximum DW during heterotrophic cultivation on sterilized DF effluent reached 0.33 ± 0.01 $g.L^{-1}$. In both conditions, microalgae concentration did not

increase during the last 7 days. Acetate was completely exhausted after 2.7 days in both experiments. Butyrate was not consumed when axenic *C. sorokiniana* was grown on sterilized DF effluent (Figure 4-2). On the other hand, butyrate degradation started after complete acetate exhaustion and ended after 8 to 9 days when *C. sorokiniana* was grown on unsterilized DF effluent (Figure 4-3). From these results, it was concluded that *C. sorokiniana* grew in both experiments using acetate until it was completely exhausted and that the bacterial community initially present in unsterilized DF effluent was responsible for butyrate degradation. Since very similar microalgae biomass yields were reached during both experiments, *C. sorokiniana* was probably responsible for the complete exhaustion of acetate, despite the presence of the DF bacterial community. This suggested that *C. sorokiniana* successfully outcompeted DF bacteria for acetate uptake. The abrupt shift from anaerobic to aerobic culture conditions might have hampered DF bacterial growth and enabled microalgae to degrade acetate winning the competition with bacteria.

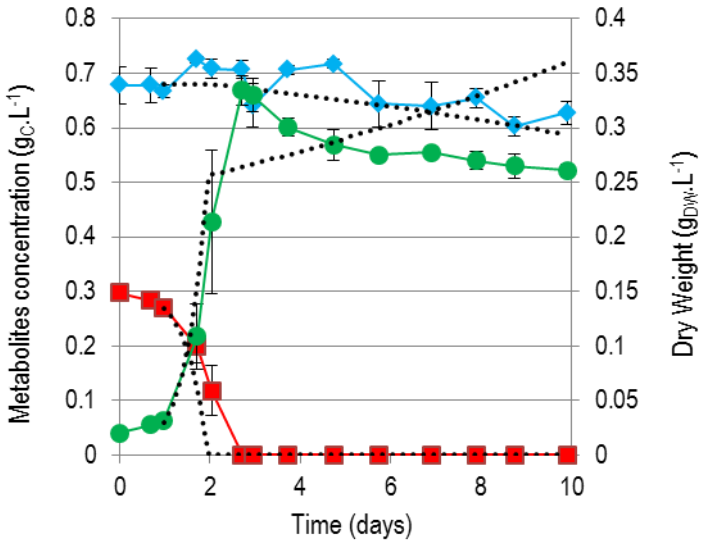

Figure 4-2. Heterotrophic growth of *Chlorella sorokiniana* on sterilized dark fermentation effluent.

Biomass growth in $g.L^{-1}$ (●), acetate concentration in $g_C.L^{-1}$(■) and butyrate concentration in $g_C.L^{-1}$ (◆) during heterotrophic growth on sterilized effluents. Dashed lines: simulations, using the model described in Chapter 2, of heterotrophic biomass growth, acetate uptake and butyrate uptake on synthetic media for comparison.

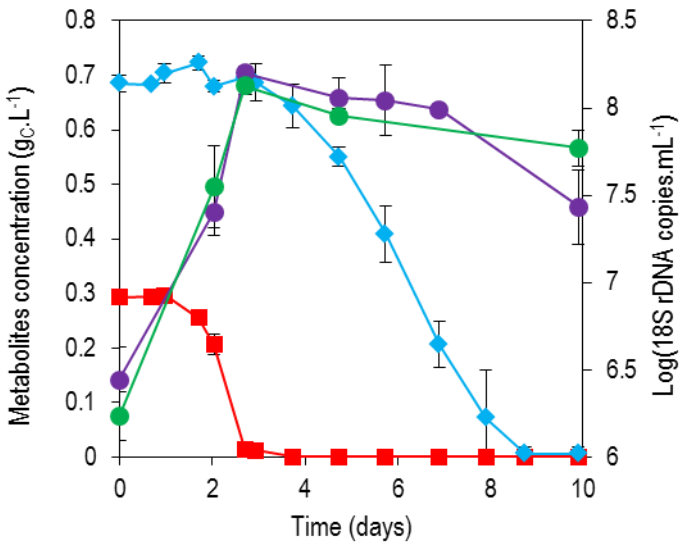

Figure 4-3. Heterotrophic growth of *Chlorella sorokiniana* on unsterilized dark fermentation effluent.

Logarithm of the number of target copies per mL of culture, using Chlorophyta specific primers for 18S rDNA, during heterotrophic growth on sterilized (●) and unsterilized (●) effluents with the acetate concentration in $g_C.L^{-1}$(■) and butyrate concentration in $g_C.L^{-1}$ (◆) during heterotrophic growth on unsterilized effluents.

The maximum growth rate, μ_{max}, of the microalgae was assessed during the exponential growth phase and was 1.75 ± 0.14 d^{-1} on sterilized DF effluent. This μ_{max} value was consistent with the growth rates of *C. sorokiniana* on acetate obtained during heterotrophic growth on synthetic media at 25 °C (2.2 d^{-1}) (Chapter 2). Indeed, the model developed in Chapter 2 was found to fit the data associated with acetate uptake and biomass growth (Figure 4-2). The main difference was that the biomass reached at the end of acetate uptake on DF effluents (0.33 $g.L^{-1}$) was higher than the one predicted by the model (0.26 $g.L^{-1}$). During growth on sterilized DF effluent, the biomass yield was 55 ± 4%. A similar carbon yield of 52% was also reported with a mixotrophic culture of *Chlorella vulgaris* on raw acidogenic fermentation effluent (Cho et al., 2015). Interestingly, this yield is significantly higher than previous values reported on synthetic DF effluents, 42% for *Chlorella sorokiniana* (Chapter 2) and 44% for *C. protothecoides* (Fei et al., 2014). In raw DF effluents, compounds other than VFAs, such as amino acids and proteins, are available for microalgae growth (Singhania et al., 2013). The uptake of such compounds by microalgae for their own growth could explain the higher yield on acetate found in this study.

4.3.3 Bacterial growth and diversity during cultivation on raw fermentation effluent

Bacterial growth was monitored using quantitative PCR during the experiment on unsterilized DF effluent (Figure 4-4). The bacterial primers were tested on axenic *C. sorokiniana* samples. DNA amplification was observed, probably because the primers matched chloroplast rDNA sequences. However, due to the high initial load of bacteria, the number of 16S rDNA copies due to microalgae (10^7 copies per mL) was insignificant compared with the copies due to bacteria (10^9 copies per mL). Therefore, the results presented in Figure 4-4 were considered to be the result of bacterial growth. Bacterial growth had two phases. The first growth period occurred during acetate exhaustion from the beginning of the experiment to 2.7 days, during the *C. sorokiniana* growth phase (Figure 4-3). The logarithm of the number of 16S rDNA copies per mL of culture sample started at 9.06 ± 0.22 and ended at 10.04 ± 0.22 after 2.7 days. As it was suggested that acetate degradation resulted mainly from microalgae activity (sub-section 4.3.2.2), the bacterial community probably used other organic compounds initially present in the raw DF effluent or released by microalgae. The second bacterial growth period started with butyrate degradation, and bacterial biomass reached 11.01 ± 0.4 log (16S rDNA copies per mL) on day 7. During butyrate degradation, the butyrate concentration was probably too high to support the microalgae growth, as previously suggested (Liu et al., 2012 and Chapter 2).

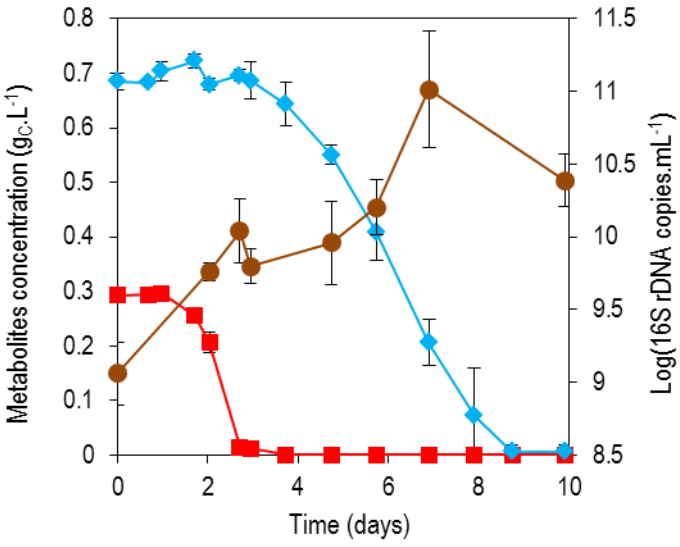

Figure 4-4. Bacterial growth on unsterilized dark fermentation effluent.

Logarithm of the number of target copies per mL of culture, using bacterial primers for 16S rDNA, during growth unsterile (●) effluent with acetate concentration in g.L-1(■) and butyrate concentration in g.L-1 (◆).

No competition was observed between microalgae and bacteria for butyrate and the bacterial community was responsible for the entire butyrate degradation. Butyrate exhaustion by bacteria could be very useful for the efficient cultivation of microalgae on DF effluent. Indeed, since high concentration of butyrate can prevent microalgae growth (Chapter 2), butyrate removal by bacteria could be favorable for microalgae. In addition, under fed-batch operating conditions which lead to best microalgae production (Chapter 1), butyrate would accumulate as a result of the inability of *C. sorokiniana* to consume butyrate rapidly and would eventually lead to growth inhibition (Chapter 2). Indeed, to produce high densities of microalgae and/or lipids, fed-batch cultivation has been suggested for which sequential additions of medium are performed after exhaustion of the substrate or when the growth plateau is reached (Bumbak et al., 2011). For biomass production, this technique avoids growth inhibition due to high initial substrate concentration and substrate concentration is maintained at less than the inhibitory concentration during the process (Bumbak et al., 2011). This strategy has been successfully used to produce high concentrations of lipids (40 $g.L^{-1}$) by heterotrophic cultivation of *C sorokiniana* on glucose (Zheng et al., 2013). Therefore, butyrate degradation by bacteria may be beneficial for lipid production by heterotrophic microalgae, suggesting that bacteria may have a positive effect, if well managed, for upscaling using unsterilized DF effluent.

The closest phylogenetically known sequences found for the representative OTUs, with a relative abundance of more than 2%, present at the start of the experiment (day 0), at acetate exhaustion (day 2.7) and at the end of the experiment (day 10) were identified (Figure 4-5). The bacterial community in the raw effluent mainly comprised 13 OTUs each with a relative abundance of more than 2% at the start of the experiment, at acetate exhaustion or at the end of the experiment. As shown on Figure 4-5, there were significant shifts in the bacterial community during the experiment. The dominant bacterial species shifted from strict anaerobes (*Clostridium* sp. and *Sporolactobacillus* sp.) to facultative anaerobes (*Paenibacillus* sp.) and then to strict aerobes (*Stenotrophomonas maltophilia*). The dominant bacteria at the beginning of the experiment (over 73% of the total bacteria abundance) accounted for less than 3.5% of the bacteria present at day 2.7 when a species close to *Paenibacillus chibensis* was dominant (over 60% of total bacteria) in two of the replicates (Figure 4-5). In only one of the three replicates (flask 3), a high abundance (42.5%) of a species close to *Lysinibacillus xylanilyticus* was observed with a reduction in the abundance of a species close to *Paenibacillus chibensis, (*36.2%). According to Shida et al. (1997), *Paenibacillus chibensis* is

unable to grow on acetate. The presence of a dominant species closely related to *Paenibacillus chibensis* (99% identity) suggested that acetate exhaustion by this species was unlikely. Because acetate degradation was very similar in all replicates when unsterilized DF effluent was used (Figure 4-4), the emergence of a different species closely related to *Lynsinibacillus xylaniticus* in only one of the replicates, suggested that this species was probably not involved in the exhaustion of acetate. These two observations were consistent with and reinforced our previous suggestion that *C. sorokiniana* was highly competitive and mainly responsible for acetate exhaustion.

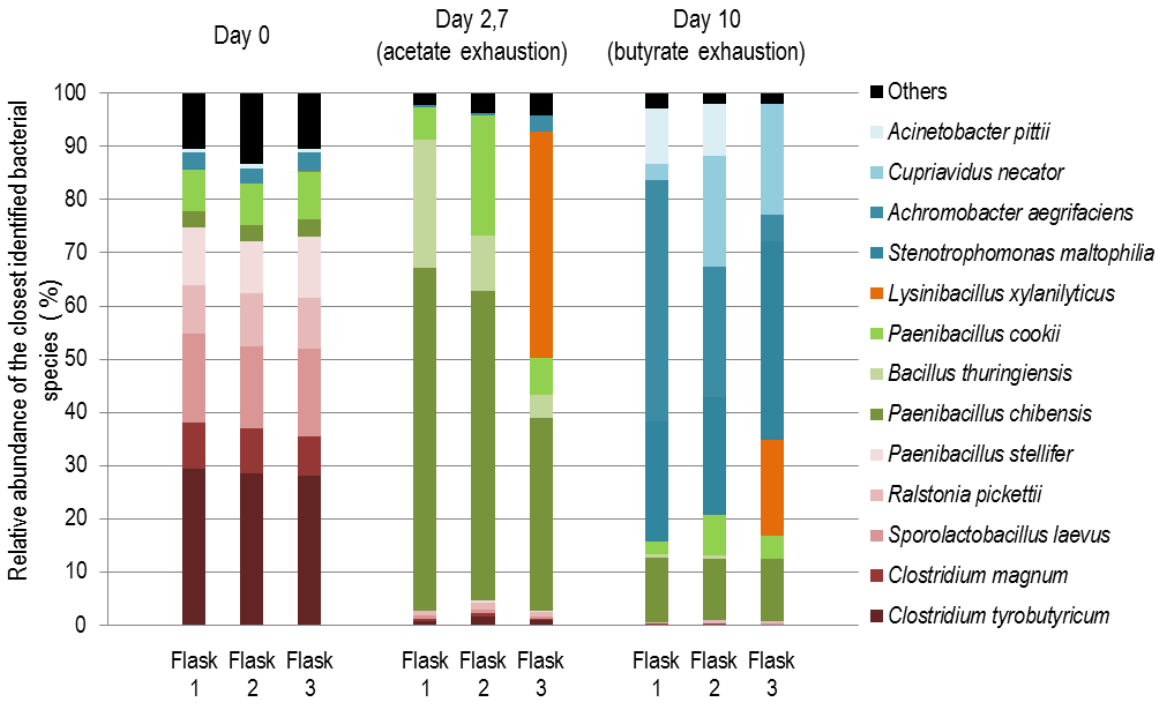

Figure 4-5. Bacterial community diversity during aerobic growth on unsterilized dark fermentation effluent. Taxa with less than 2% of individual relative abundance were grouped under "Others".

At the end of the experiment, after complete butyrate exhaustion in the three replicate flasks, species close to *Stenotrophomonas maltophilia* and *Cupriavidus necator*, both strict aerobic species (Assih et al., 2002), were largely dominant (Figure 4-5). *Cupriavidus necator* is known to assimilate butyrate and then produce polyhydroxyalkanoates (Grousseau et al., 2013). The emergence of these bacterial species during this period, during which no growth of *C. sorokiniana* was observed, confirmed that strict aerobic bacterial species initially present in the raw DF effluent were responsible for the butyrate uptake.

4.4 Conclusions

The results of this chapter showed that the two main obstacles to industrial microalgae cultivation in heterotrophic conditions, glucose and medium sterilization costs, could be easily overcome by using unsterilized DF effluents. Firstly, acetate uptake by *C. sorokiniana* was fast and sufficiently efficient to enable microalgae to outcompete bacteria. However, microalgae achieved a carbon growth yield on acetate of 55% which is higher than the one observed on synthetic media probably due to the uptake of undetermined organic compounds (Chapter 2). Secondly, the abrupt change in the operating parameters between DF and heterotrophic cultivation, from anaerobic culture conditions at 37 °C to aerobic culture conditions at 25 °C, favored microalgae growth and may be a solution to avoid sterilization.

The butyrate concentration was too high to support microalgae growth but can be degraded by the aerobic bacterial species initially present in the raw DF effluent. Further research to find means of reducing butyrate inhibition would have considerable potential. Operating parameters such as temperature (30 °C) could be further optimized to reduce butyrate inhibition on microalgae growth (Chapter 3). The use of a fed-batch mode for heterotrophic cultivation of microalgae using raw DF effluent, with the medium being added at periods set to allow DF bacteria to exhaust the butyrate, could prevent butyrate accumulation and thus allowing microalgae to grow on acetate and accumulate lipids.

4.5 References

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Chapter 5

General discussion and perspectives

The results of this PhD addressed mainly two points: (i) microalgae behavior when growing on synthetic mixtures of VFAs and (ii) microalgae growth on raw fermentation effluent with the objective of coupling bioprocesses. Based on these results, perspectives for further research are here discussed in terms of both fundamental knowledge and further applications.

5.1 Mixtures of VFAs as carbon sources for microalgae growth and lipids production

The first objective of this PhD was to characterize the heterotrophic and mixotrophic growth of microalgae on synthetic mixtures of VFAs (yields and kinetics associated with each VFAs uptakes in mixtures) in the context of coupling dark fermentation (DF) and microalgae production. Indeed, microalgae behavior on mixtures of acetate and butyrate had not been clearly described (Chapter 1). For instance, the interactions between acetate and butyrate uptakes and butyrate inhibition on growth were poorly characterized (Chapter 1).

The main results of this PhD concern the butyrate inhibition on heterotrophic and mixotrophic algal growth and are further discussed here (sub-section 5.1.1). A kinetics model, based on Monod (acetate) and Haldane (butyrate) equations, was built to describe the heterotrophic

growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on VFAs at 25°C and is further discussed (sub-section 5.1.2). The two aforementioned oleaginous microalgae species, known to accumulate lipids, were used as model species. This PhD focused on microalgae growth and not directly on lipids accumulation. Lipids production by microalgae grown on DF effluents is thus discussed in sub-section 5.1.3.

5.1.1 Challenges associated with the butyrate inhibition on microalgae growth

5.1.1.1 Towards a better understanding of the butyrate effect on microalgae physiology

In Chapter 2, it was shown that the heterotrophic growth of *C. sorokiniana* and *A. protothecoides* on butyrate (i) was inhibited for initial butyrate concentrations higher than 0.05 $g_C.L^{-1}$ and (ii) when the growth occurred (mainly due to the presence of acetate), the growth rate on butyrate was 10 times slower than on acetate (the maximal growth rate reached 0.22 d^{-1} for *A. protothecoides*). As further explained in Chapter 4, butyrate inhibition on microalgae growth reduced the efficiency of using raw dark fermentation (DF) effluents to sustain the microalgal growth. From these results and a literature review, it was hypothesized that under heterotrophic conditions either (i) butyrate could down-regulate the cellular activities such as cytosolic pH regulation or oxidative respiration, leading to an inhibition of the growth at high concentrations or (ii) butyrate assimilation by microalgae is slow because of intrinsic limits of the enzymatic reactions. To better understand and control such butyrate inhibition, identifying the mechanisms associated with the effects of butyrate on microalgae cell is crucial.

The variations of cytosolic pH (pHc) possibly induced by butyrate accumulation could be quantified. Cytosolic pH of microalgae, as for most microorganisms and cells, can be monitored using labeled weak acids as chemical probes (El-Ansari and Colman, 2014). Radiolabeled benzoic acid and 5,5-dimethyl-2,4-oxazolidinedione (DMO) are usually used as chemical probes. As explained in Chapter 1 sub-section 1.2, undissociated weak acids diffuse through the cellular membrane. According to the pKa of the acid and pHc, the acid dissociates in the cytosol. An equilibrium of the concentration of undissociated acids inside and outside the cells is reached after 1 to 1.5 hours. Knowing the concentration of labelled acids inside and outside the cells, the pKa of the acid and external pH, the pHc can be estimated by calculation. Moreover, ATP used to expulse protons (H^+), thus buffering pHc, could also be assessed using a mathematical model estimating the H^+ flow in cells (Bethmann and Schönknecht, 2009). These approaches would help us to confirm whether the slow growth

rate observed during microalgae growth on butyrate is due to cellular energy redirection towards pH regulation. If the butyrate inhibition comes from an acidification through the pHc, glycerol addition to the DF effluent and subsequent facilitated transport through the cell membrane might help to suppress this inhibition. Glycerol is indeed known to protect microalgae cells against drastic changes in cytosolic osmotic pressure which could come from pHc acidification (Perez-Garcia et al., 2011b). Since glycerol is a by-product of biodiesel production through transesterification, glycerol addition would not increase the production costs of the process. In addition, respiration might be stressed during heterotrophic growth on butyrate to cope with the inhibitory effect on cytosolic pH (Bethmann and Schönknecht, 2009). The measurements of the respiration rate by monitoring the O_2 level variations during the growth could also highlight a cytosolic pH inhibition (Smith et al., 2015).

The hardest task would be to test that slow butyrate uptake is directly related to slow enzymatic reactions associated to butyrate assimilation. Given the fact that acetate and butyrate assimilation is diauxic, acetate might possibly inhibit an enzyme of the β-oxidation pathway (chapter 1 section 1.2). The enzymatic reactions of the β-oxidation might also be slow. An experiment aiming at identifying directly the enzymes associated with butyrate assimilation is hard to suggest. Indeed, the butyrate transport chain is still unknown and the metabolism of butyrate in algae is also unknown. Furthermore, *Chlorella sorokiniana* and *Auxenochlorella protothecoides* genomes are not yet fully sequenced. As a consequence, omics studies (genomics, transcriptomics and proteomics) to identify genes or proteins which are up-regulated or down-regulated in presence of butyrate would probably be very difficult to analyze but constitute the first step for better understanding. Among the *Chlorella*, only the genome of *Chlorella variabilis*, a model species to study symbiosis between microalgae and *Paramecium*, is fully sequenced (Blanc et al., 2010). Investigating butyrate pathway based on this genome could also be the first step in understanding butyrate slow assimilation by microalgae.

In addition, it was shown in Chapter 3 that the presence of butyrate, as single substrate at 0.3 $g_C.L^{-1}$, inhibited the autotrophic production of microalgae under mixotrophic conditions at 35 °C. Indeed, strict autotrophic growth (without any organic carbon), was higher than mixotrophic growth on butyrate at 35 °C during the first six days of cultivation (Figure 3-6). This inhibition was concentration-related and was thus reduced with lower initial butyrate concentration (0.2 $g_C.L^{-1}$). Such inhibition of autotrophic metabolism in presence of butyrate was not observed at 25 °C. The negative influence of butyrate on light-dependent reactions of

photosynthesis could be assessed by measuring some of the usual parameters assessing photosynthetic activity in microalgae: (i) O_2 produced by from photosystem II (Smith et al., 2015), (ii) pulse-amplitude modulation (PAM) fluorescence to assess photosynthetic activity as efficiency of absorbed light energy (Li, 2014) and (iii) chlorophyll content of microalgae (Smith et al., 2015). Concerning the dark reactions of photosynthesis (Calvin cycle), butyrate inhibition could be assessed, for example, by following the CO_2 assimilation thanks to labeled bicarbonate ($^{13}C/^{14}C$) in presence of varying concentrations of butyrate. In addition, such an experiment would allow to know precisely the part of biomass growth due to CO_2 fixation during mixotrophic conditions (sub-section 5.1.2). Otherwise, the level of expression of the RuBisCO genes could be quantified, such as the RNA from the RuBisCO large subunit gene, rbcL, to assess the regulation of the Calvin cycle in the presence of butyrate (Li, 2014).

5.1.1.2 Possible Reduction of butyrate inhibition: abiotic and biotic parameters

To reduce the butyrate inhibition on the microalgae growth during cultivation on VFAs mixtures, two abiotic parameters were tested in this PhD: the light and the temperature (Chapter 3). At 25 °C, the presence of light efficiently reduced the butyrate inhibition due to simultaneous autotrophic (via CO_2 uptake) and heterotrophic (via VFAs uptake) growths. Nevertheless, in presence of light, the presence of butyrate in VFAs mixtures reduced the growth rate on acetate when compared to acetate used alone.

Variation of the temperature (25, 30 and 35 °C) showed contrasted effects on heterotrophic and mixotrophic growth of the thermotolerant *Chlorella sorokiniana* (Chapter 3). At the best temperature (35 °C) for acetate uptake as single substrate, the presence of butyrate in VFAs mixtures reduced the growth rate on acetate (under both heterotrophic and mixotrophic conditions) and the autotrophic growth (under mixotrophic conditions). Nevertheless, when compared to heterotrophy at 25 °C, the butyrate inhibition was reduced at 30°C under heterotrophic conditions. As pointed out in the previous sub-section, a better understanding of the butyrate effect on microalgae metabolism would allow us to find the ideal abiotic conditions to minimize its inhibition and to maximize its assimilation. Even without new insights on the cellular mechanisms of butyrate inhibition, an optimal condition could be found based on the results of this PhD and on complementary experiments using experimental design to cover a broad range of mixtures VFAs and their concentrations.

The main abiotic parameter which was not studied so far is the pH of the medium (Chapter 1 sub-section 1.2). This parameter might show promising results since very recently, Béligon et

al. (2015) showed that a pH increase from 6 to 7 leads to an increase of the maximal growth rate (50%) and biomass yield (20%) of the oleaginous yeast *Cryptococcus curvatus* on acetate. Increasing pH indeed allows the reduction of the concentration of the undissociated forms of VFAs, which are very toxic (Chapter 1). A slight increase of the pH of the medium to 7, which was set between 6 and 6.5 for the experiments of this PhD, might lead to similar increase of microalgae growth.

Butyrate inhibition could also be reduced through the association of microalgae-bacteria which could lead to either butyrate removal by bacteria or synthesis by bacteria of microalgae-promoting substances, such as vitamins and phytohormones (Tate et al., 2013). As an illustration, Imase et al. (2008) built an artificial symbiosis between *Chlorella sorokiniana* and propionate-degrading bacteria and showed that propionate inhibition on microalgae was successfully lowered. Such strategy could be similarly used to lower the butyrate inhibition. In addition, phytohormones, e.g. auxins, cytokinins and gibberellins, have been used to help microalgae to cope with cellular stress, such as hydrogen peroxide toxicity (Piotrowska-Niczyporuk and Bajguz, 2013). Providing mixtures of phytohormones or growing microalgae with phytohormones-producing bacteria, such as *Azospirillum brasilense* (Tate et al., 2013), could be another biological strategy to reduce the butyrate inhibition. In addition, a stable and engineered or artificial community composed of microalgae and bacteria could prevent from further fermentation bacteria colonization when unsterilized fermentation effluents are used (sub-section 5.2.2). However, if a strong competition for acetate between microalgae and microalgae-promoting bacteria arises, the use of microalgae-bacteria consortium in heterotrophy or mixotrophy would be less beneficial. As an illustration, the positive effects of the growth-promoting bacteria *Azospirillum brasilense* on the growth rate and the nutrient removal rate of *Chlorella vulgaris* were higher under autotrophic and mixotrophic conditions because of a strong competition for glucose between microalgae and bacteria under heterotrophic conditions (Perez-Garcia et al., 2010).

5.1.2 Prediction of microalgae growth on VFAs mixtures according to the operating parameters

At 25 °C under heterotrophic conditions, a mathematical model, based on Monod and modified Haldane (to represent the butyrate inhibition and the diauxic growth) equations, was successfully built to describe the kinetics parameters and yields associated with microalgae growth on mixtures of acetate and butyrate. However, because adverse effects of butyrate on growth on acetate and on inorganic carbon were observed in the presence of light and at high

temperature (Chapter 3), but were not observed at 25 °C without light, the model could not be used to represent the experimental data under these conditions To model the experimental data obtained under mixotrophic conditions, quantifying the proportion of acetate, butyrate or CO_2 assimilated by microalgae (e.g., by performing experiments using $^{13}C/^{14}C$ labeled acetate, butyrate or bicarbonate (sub-section 5.1.1.1), is required. Indeed, without such information, the fraction of microalgae biomass due to either VFAs uptake or CO_2 uptake can only be hypothesized based on the corrected carbon yield obtained by subtracting the autotrophic biomass of the control from mixotrophic biomass (Chapter 3). For *Chlorella sorokiniana* growth related to the temperature (specifically at 30 °C where no butyrate inhibition was observed), complementary experiments are required before adjusting the model parameters. Indeed, in order to have enough data to estimate and validate new kinetics parameters, different mixtures VFAs and a wide range of concentrations have to be tested, as it was done in this PhD for growth at 25 °C (Chapter 2).

Due mainly to a difference in biomass yield on acetate (Chapter 4), the model did not fit well the experimental data obtained during the heterotrophic growth of *Chlorella sorokiniana* on sterilized dark fermentation (DF) effluent. It was suggested that other organic compounds than VFAs were present in the effluent such as vitamins or polysaccharides that might have been assimilated by the microalgae. A deeper analysis of the effluent composition (saccharides, lipids) would thus be necessary to improve the model performances. In addition, the model predicted a significant microalgae growth on butyrate whereas such growth was not observed in the experiment on sterilized DF effluent (Chapter 4). This absence of growth might have been caused by a strong requirement of maintenance energy to allow the microalgae to survive at high butyrate concentration. By considering the maintenance energy in the biomass equation (Equation 5-1), the model fitness might be improved (Chen and Johns, 1996).

$$\frac{dX}{dt} = \mu_a(S_a) * X + \mu_b(S_b) * X - m * X \qquad \textbf{Equation 5-1}$$

with X ($g.L^{-1}$) the biomass, $\mu_a(S_a)$ (d^{-1}) the growth rate, associated with acetate (S_a) removal, μ_b (S_b) (d^{-1}) the growth rate, associated with butyrate (S_b) removal, and m (d^{-1}) the loss of growth due to the energy maintenance requirement.

So far, the model provides a global view of the microalgae growth on raw effluent but does not predict precisely the microalgae yield since it is not considering other organic compounds. Further investigations are necessary in this field.

During all the experiments of this PhD, the microalgae growth was highly reproducible. Differences between triplicates of one set of experiments were very low most of the time. The microalgae growth appeared to follow either a Monod equation or a Haldane equation or a linear equation. With complementary experiments, as suggested above, a wider model could possibly be built to describe both heterotrophic and mixotrophic growth of *Chlorella sorokiniana* and *Auxenochlorella protothecoides* on VFAs. With this model, microalgae growth could be likely better controlled according to the fluctuating composition of the dark fermentation effluent. Indeed, according to the VFAs concentration and the acetate:butyrate ratio, operating conditions (temperature, light, microalgae initial load (S/X)) could be adjusted to maximize the microalgae growth.

5.1.3 Production of biomolecules of industrial interest from microalgae grown on VFAs

The final goal of coupling dark fermentation (DF) with microalgae growth is to extract molecules of interest from microalgae, such as lipids for biofuels or such as pigments and proteins for feed or food industries. Carbon is used by the microalgae for its own growth under favorable conditions or lipids accumulation under stress conditions, e.g., nitrogen (N) starvation. Here, only the growth of microalgae, and not lipids production, was investigated. Obviously, one of the next steps would be to study lipids and/or pigments accumulation by *Chlorella sorokiniana* or *Auxenochlorella protothecoides* grown on VFAs mixtures. Recently, the influence of acetate and butyrate, used as single substrates, on the mixotrophic growth followed by lipids production under N starvation was studied by Chandra et al. (2015). Lipids yield reached 35% of DW with acetate and only 12% with butyrate due to a low growth. Nevertheless, since the biomass was composed of many microalgae species and bacteria (and possibly fungi) which were not differentiated (only measurements of the total weight were performed), it seems difficult to conclude on the use of VFAs for either microalgae growth or lipids production under mixotrophic conditions. High lipid yield on acetate (40% of dry weight) has already been achieved, without applying a drastic N starvation, during heterotrophic growth of *Scenedesmus* sp. on sterilized raw DF effluent composed mainly of acetate and ethanol (Ren et al., 2014b). Indeed, to alleviate the trade-off between microalgae growth and lipids accumulation, the current trend is to favor growth under N limitation which allows concurrent production of biomass and lipids (Adams et al., 2013). This could be achieved under specific continuous conditions with adapted influent C, N and P

concentrations or in fed-batch cultures and would deserve dedicated experiments to be conducted.

5.2 Dark fermentation effluent as potential culture medium for microalgae

Another objective of the PhD was to assess the potential interaction between microalgae and fermentation bacteria for VFAs. Indeed, the economic feasibility of coupling DF and microalgae production can only be reached with unsterilized effluents. *Chlorella sorokiniana* was thus grown heterotrophically on sterilized (control) and unsterilized DF effluents (Chapter 4). As main results, it was found that:

- the biomass yield on acetate was higher than on synthetic medium
- microalgae outcompeted bacteria for acetate
- butyrate removal was entirely due to bacteria uptake.

Due to the difficulty to fully characterized C, N and P compounds from the effluents and also since quantifying microalgae in the presence of bacteria may be difficult, the microalgae growth on DF effluent can be difficult to analyze. Increasing microalgae competitiveness for butyrate in presence of bacteria is further discussed.

5.2.1 Assessment and understanding microalgae growth on effluents: technical difficulties

Organic carbon content, as well as nitrogen and phosphorus contents of dark fermentation (DF) effluents is not always fully determined. As an illustration, Hongyang et al. (2011) showed that 30% of the chemical oxygen demand (COD) of the effluent used to sustain *Chlorella pyrenoidosa* growth was not due to fermentation metabolites but was not characterized. Microalgae growth on this uncharacterized part of organic matter cannot be analyzed. For N and P contents of the DF effluents, they are usually never described even though the C:N:P ratio of the effluent may strongly influence the microalgae growth. In addition, the C:N ratio of the effluent would have a major impact on lipids production under N limitation (or N starvation) (Fei et al., 2011). A thorough analysis of C, N and P compounds of the effluent is thus required to precisely study the coupling of DF and microalgae production.

Quantifying accurately the microalgae growth (dry weight or cells number or equivalents) in presence of fermentative bacteria and suspended solids (originating from anaerobic sludge and the feedstock for fermentation) is another major technical issue. Usually, the microalgae

biomass ($g.L^{-1}$) is estimated by direct weight measurement or indirectly either by optical density (OD) (turbidity), pigment extraction (Chlorophyll a), or via the bio-volume (using flow cytometry for example). The number of algal cells (measured directly by microscopic counting) is not necessarily linked to the dry weight since among a same species a great range of sizes is possible (daughter cells vs mother cells) (Richmond and Hu, 2013) and the algal production of exudates (polysaccharides) may be also considered into the dry weight measurement. Since OD measurements do not discriminate bacteria, suspended solids and microalgae, the microalgae biomass cannot be quantified by using OD in raw effluents. Flow cytometry is a powerful tool to differentiate microalgae from bacteria thanks to the autofluorescence of chlorophyll, cells size and nucleus staining. Nevertheless, the chlorophyll fluorescence might vary according to the heterotrophic cultivation conditions (Rosenberg et al., 2014). In addition, samples would have to be filtered to remove the suspended solids before analysis. Similarly, counting microalgae using contrast phase or fluorescence microscopy would be very difficult due to the high bacterial load and/or suspended solids. In this PhD and in several other studies (Coyne et al., 2005; Fowler, 2011; Lakaniemi et al., 2012b), microalgae were quantified in presence of bacteria and suspended solids using quantitative PCR with primers specific to microalgae. Although this method is accurate and does not require any filtration of the samples, results from qPCR analysis cannot be directly correlated to the dry weight. Indeed, results from qPCR are always analyzed using the logarithmic values of the number of copies of the targeted genes which is due to the precision of the method. From a biological point of view, there is no correlation between the dry weight (or the cells number) and this logarithmic value. The only possible correlation would be between the dry weight (or the cells number) and the number of genes copies. Lakaniemi et al. (2012a) pointed out that the number of rDNA copy per cells may vary according to the growth phase. Even though monitoring the microalgal growth by qPCR has several advantages compared to the other techniques, several drawbacks exist and cannot be avoided. To monitor daily the microalgae and bacterial growths in reactors, no method is today readily available except microscopic counting, which also has several drawbacks.

Under heterotrophic conditions and in presence of fermentation bacteria, the microalgae growth was assumed to be only due to acetate uptake (Chapter 4). Nevertheless, bacterial growth was observed during acetate removal. It was then suggested that bacteria might have grown on microalgae exudates or other unquantified organic compounds. Future research on carbon partitioning from VFAs, between microalgae and bacteria, would be necessary to

precisely assess carbon assimilation by microalgae. In addition, under mixotrophic conditions, it is difficult to differentiate between VFAs uptake by microalgae or bacteria. Indeed, microalgae growth could be due to CO_2 assimilation and not VFAs uptake in presence of bacteria. After incubation with labeled carbon ($^{13}C/^{14}C$), flow cytometry coupled with cell sorting could be used to differentiate microalgae and bacteria and measure the incorporation of labeled carbon (Hartmann et al., 2009). Nevertheless, as pointed out by You et al. (2015), the traditional methods to differentiate according to the cell size (filtration, density gradient centrifugation and cell sorting) give poor results for bacteria and microalgae with similar abundance and cells sizes, as for *Chlorella sorokiniana* (2 and 6 µm). Very recently, You et al. (2015) developed a new method based on monitoring the assimilation of labeled carbon into photosystem I (PSI), which is specific of microalgae since PSI is not present in heterotrophic bacteria. Obviously, if synthesis of PSI is down-regulated in the presence of VFAs under heterotrophic conditions, this labelled protein should not be used but another one, such a histone protein for example.

5.2.2 Perspectives and challenges on coupling DF and microalgae heterotrophy

As a main result of this PhD, *Chlorella sorokiniana* was fast enough to be competitive for acetate uptake with facultative bacteria originated from dark fermentation (DF) effluents (Chapter 4). Nevertheless, butyrate was taken up only by bacteria due to inhibitory initial concentrations preventing its uptake by microalgae. As discussed in sub-section 5.1, operating parameters, such as temperature, light, pH, S/X ratio and maybe using *Auxenochlorella protothecoides* instead of *C. sorokiniana*, could improve microalgae competitiveness for butyrate uptake. Indeed, *A. protothecoides* was slightly less inhibited by butyrate than *Chlorella sorokiniana* even though its growth rate on acetate was lower (Chapter 2). Obviously, increasing acetate content in the effluent would also probably promote the microalgae growth on DF effluent. Theoretically, the H_2 production through the acetate pathway is maximal (Chapter 1). Therefore, increasing the acetate content could be linked with higher H_2 production. Nevertheless, a tradeoff between reducing butyrate inhibition on microalgae growth and not enhancing bacterial growth would have to be investigated. Temperature and pH adjustments, as well as acetate enrichment, might promote bacterial growth. In addition, due to competition with bacteria for organic carbon, microalgae might shift their metabolism towards autotrophy under mixotrophic conditions as a mean for survival. Finding an optimal S/X ratio to ensure the microalgae dominance over bacteria might be the most likely and reliable option.

Despite the very promising results of this PhD, the influence of the bacterial community structure on microalgae growth and competitiveness for VFAs should be further investigated to generalize the use of unsterilized effluent to sustain microalgae growth. Indeed, the bacterial community structure in DF processes is varying mainly according to the inoculum sources (anaerobic digester or anaerobic sludge) and the type of pretreatment of the inoculum (heat versus no pretreatment). As a consequence, the emergence or dominance of facultative anaerobic species, including H_2-producers, more competitive for acetate, might occur. Thus, microalgae competitiveness for acetate might be bacterial inoculum-dependent. In addition, other fermentation metabolites, such as lactate and ethanol, could be present in the effluent. Given the diversity of the bacterial community in DF effluents, it is very likely that at least one species would be able to grow on those metabolites. Microalgae growth on ethanol is very species-specific (Perez-Garcia et al., 2011b; Ren et al., 2014b). Preliminary experiments showed that both *Chlorella sorokiniana* and *Auxenochlorella protothecoides* could grow heterotrophically on ethanol but microalgae competitiveness for ethanol with bacteria remains unknown. In contrast, no microalgae growth on lactate was observed in this PhD for both *C. sorokiniana* and *A. protothecoides* as well as for mixotrophic growth of *C. vulgaris* as shown by Liu et al. (2012). Perez-Garcia et al. (2011a) observed a very slow growth of *Chlorella vulgaris* on lactate under heterotrophic conditions. Lactate might therefore not be a suitable carbon source for either heterotrophic or mixotrophic growth.

To minimize the competition with bacteria and also the sterilization cost (autoclave) of effluents, methods such as Ultra Violet (UV) radiation have been recently suggested as low-cost methods for sterilization of large volume (Passero et al., 2014). As main advantages of this method, not only the bacterial load is reduced but also the suspended organic matter is modified which could enhance the light availability in the case of mixotrophic growth conditions.

5.3 Conclusions

The aim of this PhD was to unravel several aspects of the microalgae growth associated with the use of mixtures of VFAs such as diauxic growth, the effect of light and temperature and the presence of fermentative bacteria. To understand the microalgae response to the presence of butyrate, several experiments, such as the monitoring of cytosolic pH, are suggested. According to the future results, microalgae growth on VFAs composed of butyrate and acetate could be increased thanks to the addition of glycerol (to minimize cytosolic pH variation) or the control of abiotic parameters such as pH, temperature and light. Lipids production

according to the content of acetate and butyrate in raw fermentation effluents also has to be assessed. Furthermore, the economic viability of coupling DF with microalgae production will not be achieved by optimizing separately each process and additional investigations will have to consider the two processes once coupled. From the microalgae production point of view, an ideal coupling should involve high acetate production during DF, to lower the butyrate-related inhibition, with abundant *Clostridium sp* (or strict anaerobic species) as H_2-producers to minimize competition for acetate.

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Conclusions

This PhD focused on understanding and characterizing microalgae growth on synthetic and raw fermentation effluents in order to further assess the feasibility of coupling processes of dark fermentation and microalgal heterotrophic growth.

In a first part, a literature review was performed providing a state of the art of i) dark fermentation processes, ii) heterotrophic and mixotrophic growth of microalgae on acetate and butyrate (as single substrates) and iii) microalgae growth on raw fermentation effluents (consisting mainly of mixtures of acetate and butyrate). From the few papers published recently, highlighting the innovative aspects of this PhD, it results that butyrate as a major component of dark fermentation effluents, may affect the microalgae growth and is the key compounds limiting the feasibility of the coupling. Indeed, butyrate concentration may inhibit microalgae growth. In addition, based on this review, a diauxic phenomenon implying a strong interaction between acetate and butyrate, was suggested. According to this review, light and temperature were also suggested to potentially alleviate butyrate inhibition on microalgae growth. Moreover, the interactions between microalgae and fermentative bacteria originating from the effluents and their possible competition for VFAs have never been investigated although such interaction can hinder microalgae growth and reduce the feasibility of the coupling.

In this context, it was first hypothesized that the slow uptake of butyrate and the butyrate inhibition, suggested as being concentration-related, hampered microalgae growth on dark fermentation effluents. The growth of two lipid-producing *Chlorella* species on a mixture of fermentation end-products (i.e., acetate, butyrate and lactate) was investigated using a dynamic mass balance 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, but did not use lactate. The threshold level of butyrate inhibition on microalgae growth was found at 0.05 $g_C.L^{-1}$. It was also shown that acetate and butyrate uptakes were diauxic for the two microalgae species. However, during the heterotrophic microalgae growth on mixtures of VFAs, butyrate uptake might be favored by the increase of biomass concentration induced by the initial use of acetate. In addition, acclimation of microalgae (successive batches at a low initial concentration (0.1 $g_C.L^{-1}$) of butyrate) was suggested as a mean to lower the strong butyrate inhibition on

microalgae growth. A model combining Monod and Haldane functions was then built and fitted the experimental data for both species. The butyrate concentrations and the ratio of acetate:butyrate were identified as key parameters for heterotrophic growth of microalgae on fermentative metabolites.

Then, the influences of light and temperature on microalgae growth on VFAs were assessed in order to cope with butyrate inhibition on the growth of *Chlorella sorokiniana*. Indeed, it was hypothesized that the presence of light could trigger autotrophic biomass production, through CO_2 fixation, and thus could increase the overall butyrate uptake rate. In addition, growing *Chlorella sorokiniana* at high temperatures (30 °C and 35 °C) was suggested as another way to reduce the butyrate inhibition when compared to heterotrophy at 25 °C. Indeed, this species is known to be thermotolerant so high temperature could enhance its enzymatic activity and reduce its requirements for cellular thermoregulation. As main results, the time to reach complete butyrate exhaustion, during growth on mixtures of VFAs, was substantially reduced because of (i) the presence of light and subsequent autotrophic biomass production (at 25 °C) and (ii) temperature at 30°C (in darkness). Unexpectedly, at the optimal temperature for heterotrophic growth on acetate (35 °C), the presence of butyrate (i) reduced substantially the growth rate and the carbon yield on acetate and (ii) inhibited autotrophic growth under light conditions. For successful microalgae growth on dark fermentation effluent, the use of a temperature set point at sub-optimal values (30 °C) combined with light was thus suggested to reduce the inhibition of butyrate.

Finally, the possibility of using unsterilized raw dark fermentation effluents to support heterotrophic growth of *Chlorella sorokiniana* was investigated. Raw dark fermentation effluents were obtained from fermentation of glucose by anaerobic sludge and were mainly composed of acetate and butyrate. All the acetate in sterilized and unsterilized DF effluents was exhausted in less than three days of heterotrophic cultivation, whereas butyrate was not used by the microalgae. The microalgae biomass reached 0.33 $g.L^{-1}$ with a carbon yield on acetate of 55%. The algal yield was higher than previously reported values for synthetic DF effluent. It was concluded that compounds other than volatile fatty acids were present in the DF effluent and that they could be consumed by the microalgae. After the acetate had been exhausted, butyrate was consumed by facultative and strict aerobic bacteria originating from the DF effluents. The concentration of the bacterial community increased during the experiment but did not have any significant impact on heterotrophic microalgae growth. A high microalgal biomass yield was achieved without requiring the DF effluent to be sterilized.

To further assess the feasibility of coupling dark fermentation with microalgae growth, to produce lipids, several perspectives were highlighted. To control and maybe alleviate the butyrate inhibition on microalgae growth, future research on understanding butyrate impact on cellular mechanisms, such as cytosolic pH acidification, will have to be carried on. In addition, modifying the predictive model built in the PhD for heterotrophic growth in order to take into account the presence of light and the variations of temperature would be an important step before upscaling microalgae production in fermenters. Moreover, this PhD focused on microalgae growth on fermentation effluents but the lipids accumulation by microalgae was not studied. Assessing lipids production by microalgae according to the composition of dark fermentation effluents (metabolites compositions and ratios, presence of fermentation bacteria) would also be crucial. Furthermore, future investigations related to the integration and engineering of these two processes would be necessary to find their best combination

COUPLING DARK FERMENTATION WITH MICROALGAL HETEROTROPHY: INFLUENCE OF FERMENTATION METABOLITES MIXTURES, LIGHT, TEMPERATURE AND FERMENTATION BACTERIA ON MICROALGAE GROWTH

Growing microalgae in heterotrophic mode present several advantages over autotrophic mode such as a higher productivity in terms of biomass and lipids for biofuels production. Nevertheless, this process is limited by the production cost associated with the organic substrate (i.e. glucose) and fermenters sterilization costs. Dark fermentation effluents, mainly composed of acetate and butyrate, could be used as a low-cost medium to grow microalgae heterotrophically or mixotrophically. The aims of this PhD were i) to optimize microalgae growth on various mixtures of fermentations metabolites using the presence or absence of light and different cultivation temperatures and ii) to assess the feasibility of using unsterilized fermentation effluents.

First, a model based on mass balance was built to characterize heterotrophic growth rates and yields when Chlorella sorokiniana and Auxenochlorella protothecoides were supplemented with different mixtures of acetate and butyrate. Results showed that the acetate:butyrate ratio and the butyrate concentration per se were two key parameters for promoting heterotrophic growth. Then, further studies showed that the presence of light and the use of suboptimal temperature (30 °C) could reduce the butyrate inhibition on growth by either triggering autotrophic production of biomass or enhancing growth on acetate. Finally, it was shown that microalgae could outcompete fermentation bacteria for acetate when growing on raw dark fermentation effluents, thanks to a fast algal growth on acetate (1.75 d-1) and a drastic change of culture conditions to the detriment of bacterial growth.

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