Exploring alternative feedstocks and engineering butanol tolerance to optimise biofuel production by *Clostridium saccharoperbutylacetonicum*

By

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Abstract

Acetone-butanol-ethanol (ABE) fermentation is an established industrial process that uses Clostridium bacteria for the conversion of plant-derived 'feedstocks' into solvents (acetone, butanol and ethanol) that can be used as biofuels. These solventogenic clostridial strains are naturally adapted to access energy/carbon from complex sugars found in common feedstocks made from corn and rice, but it is of interest to explore a variety of waste biomass to provide a stable supply of feedstock for industrial biofuel production. It was of particular interest to investigate microalgae as a feedstock for ABE fermentation: microalgae are currently used by project partners Algaecytes® to produce Omega 3, which results in large amounts of low-value spent biomass following product extraction. In this study, commercially-available *Chlorella vulgaris* was used as well as a Eustigmatophyceae proprietary strain obtained from the project partner. Feedstocks were processed in a variety of ways and fermentations were performed in serum bottles and 500 mL fermenters to optimise optical detection of clostridial growth and solvent production. The highest solvent yield of 3.27 g/L (acetone: 0.40 g/L; butanol: 1.40 g/L; ethanol: 1.47 g/L) was achieved with non-autoclaved and non-centrifuged 10 % Eustigmatophyceae spent biomass supplemented with 1 % glucose, whereas a 10 % feedstock of *C. vulgaris* supplemented with 1% glucose had a lower yield of 1.20 g/L (acetone: 0.20 g/L; butanol: 1.00 g/L). These yields are significantly lower than those obtained with industrial feedstocks (in excess of 20 g/L) where butanol toxicity becomes limiting, so further work will be necessary to refine the use of algal biomass as a feedstock.

In addition to investigation of alternative feedstocks, there is clear biotechnological value in producing a Clostridium strain with increased butanol tolerance. Previously, overexpression of the FocA formate transporter has been shown to enhance butanol tolerance in *Escherichia coli*. Cloning/overexpression of *E. coli focA* and the clostridium homologue *fdhC* were done, with the aim of generating a butanol-tolerant strain of *C. saccharoperbutylacetonicum*. This work has the potential to generate higher solvent yields that could improve process economics for industrial biofuel production by ABE fermentation.

Declaration

No part of this thesis has been submitted in support of any other application for a degree or qualification of the University of Kent or any other University or Institute of learning.

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Abbreviations

aad	Aldehyde/alcohol dehydrogenase
ABE	Acetone-butanol-ethanol
ack	Acetate kinase
adc	Acetoacetate decarboxylase
adh	Alcohol dehydrogenase
aldh	Aldehyde dehydrogenase
bcd	Butyryl-CoA dehydrogenase
buk	Butyrate kinase
CoA-T	CoA- transferase
cPCR	colony PCR
crt	Crotonase
ctfAB	Acetoacetyl-CoA transferase
CV	10% *C. vulgaris,* autoclaved and centrifuged
CVG	10% *C. vulgaris* supplemented with 1% glucose, autoclaved and centrifuged
EtBr	Ethidium bromide
EG	10% Eustigmatophyceae whole cell supplemented with 1% glucose, autoclaved and centrifuged
EG-NT	10% Eustigmatophyceae whole cell supplemented with 1% glucose, non-autoclaved and non-centrifuged
ESBG	10% Eustigmatophyceae spent biomass supplemented with 1% glucose, autoclaved and centrifuged
ESBG-ApH	10% Eustigmatophyceae spent biomass supplemented with 1% glucose, autoclaved, non-centrifuged and pH a djusted to 6.0
ESBG-NT	10% Eustigmatophyceae spent biomass supplemented with 1% glucose, non-autoclaved and non-centrifuged
ESBG-pH	10% Eustigmatophyceae spent biomass supplemented with 1% glucose; non-autoclaved, non-centrifuged and pH adjusted to 6.0
fdhC	Putative formate trasporter

FNT	Nitrate transport family
FocA	Formate channel
GCMS	Gas chromatography-mass spectrometry
hbd	3-hydroxybutyryl-CoA dehydrogenase
IBE	isopropanol/butanol/ethanol
LB	Luria-Bertani
MQ water	Milli-Q® water
NHE	Normal Hydrogen Electrode
OD	Optical density
ORP	Oxidation / reduction potential
P_{fdx}	*Ferredoxin* gene *promoter*
P_{thl}	*Thiolase gene promoter*
pta	Phosphotrans acetylase
ptb	Phosphotrans butyrylase
PTS	Phosphotransferase system
pfl	pyruvate formate lysate
pflb	Pyruvate formate-lysate
RCM	Reinforced Clostridial Medium
SMAB	Spent microalgae biomass
sadh	Secondary alcohol dehydrogenase
thl	Thiolase
TYE	Tryptone yeast extract

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Chapter 1

Introduction

The world is facing an energy shortage, primarily resulting from increasing energy demands associated with an increase in global population, which is expected to reach 9.7 billion by 2050 (United Nations Department of Economic and Social Affair 2017). The combination of these key data points shall further escalate the emission of greenhouse gases, and the rapid depletion of fossil fuels will inevitably lead to an energy crisis. This significant challenge has attracted many key stakeholders, businesses, and researchers to the development of sustainable and more environmentally friendly energy sources. Bacterial biofuels are one potential solution.

Acetone, **b**utanol and **e**thanol (ABE) can be produced by solventogenic *Clostridium* species via the process of ABE fermentation. The main species that are employed for ABE production include *C. acetobutylicum, C. beijerinckii, C. saccharobuytlicum* and *C. saccharoperbutylacetonicum* (Wang *et al.* 2017). ABE fermentation was first discovered by Louis Pasteur in 1861 (Ndaba *et al.* 2015). *Clostridium* species have been used to produce solvents since the early 20th century: during the first world war, *C. acetobutylicum* was used to produce acetone, which was used to produce cordite (gun powder). After the war, the demand switched to butanol as a solvent for lacquers (Moon *et al.* 2016; Sreekumar *et al.* 2015).

1.1. Acetone

Acetone is a least undesirable solvent because it cannot be used as fuel. It corrodes the rubber and plastic components of the engine. Furthermore, it reduces the butanol production (per unit mass of substrate utilised). Hence, the reduction of acetone production has been an important aspect of clostridial metabolic engineering (Xin *et al.* 2017).

1.2. Butanol

Butanol (aka 1-butanol) is a four-carbon alcohol. The properties and the application of butanol are shown in Table 1.1. In comparison to other contemporary biofuels (e.g. ethanol), butanol is known as a "superior biofuel". The advantages of *n*-butanol are: (1) high heating value. Butanol ($C_4H_{10}O$) has twice as many carbon atoms as ethanol (C_2H_6O), hence, butanol has a better mileage and higher energy content (per unit mass); (2) a lower volatility and lower vapour pressure. *n*-butanol is less likely to cause vapour lock (i.e. an interruption in pipeline flow), and hence, the need of a special blend during summer and winter is not necessary. Also, *n*-butanol is less hazardous and emits fewer volatile organic compounds; (3) fewer ignition problems. The ignition temperature of *n*-butanol is low (ignition temperature: 35 °C; flash point: 29 °C), and hence, an engine that runs on *n*-butanol has fewer problems during a cold start; (4) lower corrodibility. Thus, distribution and storage are easier via existing infrastructure (e.g. pipelines and fuel tanks); (5) lower hygroscopicity (low affinity for water). Hence, butanol is better able to tolerate water contamination; (6) higher viscosity. The viscosity of *n*-butanol is higher than gasoline, hence, it reduces the chances of problems associated with wear in fuel pumps caused by insufficient lubricity; and (7) less flammable. Hence, butanol can be blended with gasoline in any proportion. Furthermore, butanol has similar characteristics to gasoline (Table 1.1) and therefore fewer engine modifications are required (Abdehagh *et al.* 2014; Gao *et al.* 2016; Jang *et al.* 2012a; Jin *et al.* 2011; Lee *et al.* 2008; Wang *et al.* 2016).

The key constraints for sustainable butanol production include: (1) A limitation of sustainable feedstock (expensive and competition with human food sources); (2) A low butanol titre due to the limitation of bacterial tolerance; and (3) A high product recovery cost due to low yield of butanol (IPCS 2005; Moon *et al.* 2016; Xin *et al.* 2016; Xin *et al.* 2017).

Table 1.1. Properties of butanol, ethanol and gasoline as biofuel.

	Butanol	Ethanol	Gasoline
Energy density (MJ/L)	29.2	19.6	32
Air-fuel ratio	11.2	9	14.6
Heat of vaporization (MJ/kg)	0.43	0.92	0.36
Octane number	96	108	80-99

1.3. Ethanol

Ethanol is the most widely used biofuel in USA and Brazil but it is not as ideal as butanol, mainly due to the fact that ethanol corrodes pipelines, and hence it must be transported via barge, lorry or rail, and yields lesser energy (Jin *et al.* 2011). Furthermore, food feedstocks such as corn and sugarcane are used for the production of ethanol (Lopez *et al.* 2016).

The properties and applications of acetone, butanol and ethanol are summarized in Table 1.2.

Table 1.2. Properties and applications of acetone, butanol and ethanol (adapted from ChemicalSafetyFacts.org., International Program on Chemistry Safety; Jin *et al.* 2011; Lee *et al.* 2008; National Centre for Biotechnology Information).

Properties	Solvents		
	Acetone	Butanol	Ethanol
Melting point	-94.9 °C	-89.3 °C	-114.1 °C
Boiling point	56.3 °C	117.7 °C	78.2 °C
Molecular formula	C_3H_6O	$C_4H_{10}O$	C_2H_6O
Molecular weight	58.1 g/mol	74.1 g/mol	46.1 g/mol

Applications

Acetone	Butanol	Ethanol
Primary ingredient in nail polish remover, solvent of manufacturing lacquers	Diluent for brake fluid, replacement of gasoline, solvent for the manufacturing of antibiotics, vitamins and hormones	Disinfectant, biofuel, food additive, common ingredient in cosmetics and beauty products, solvent for manufacturing of paint, lacquers, varnish

1.4. Commonly used solventogenic clostridial species

Solventogenic clostridial are non-pathogenic, Gram-positive, low GC content, spore forming obligate anaerobe that is rod-shaped (0.4-0.9 μm x 1.6-6.4 μm)

and motile (tumbling motion in a forward direction) due to its peritrichous flagella. Solventogenic clostridial have a single circular chromosome (Bao *et al.* 2011; Keis *et al.* 2001; Wu *et al.* 2012).

1.4.1. *Clostridium acetobutylicum*

C. acetobutylicum is well-known as a "Weizmann organism" (Johnson *et al.* 1997). Between 1912 to 1914, *C. acetobutylicum* was first identified and isolated by Weizmann (Strain: DSM 1732; British patent no. 4845) (Lu 2014; Weizmann and Rosenfeld 1937). *C. acetobutylicum* is the most commonly used bacteria for the butanol production and it is well known as a hyper-butanol producer (Lu 2014; Zheng *et al.* 2009). Furthermore, the scale of its ethanol production, *C. acetobutylicum* is ranked second after yeast (Bao *et al.* 2011).

Studies had been shown that almost 50% of the previous *C. acetobutylicum* available cultures were actually *C. beijerincki.* Phenotypic traits were used to differentiate solventogenic clostridial. For example, *C. acetobutylicum* is susceptible to rifampicin and produces yellow pigment riboflavin in milk. Whereas, *C. beijerinckii* and *C. saccharoperbutylacetonicum* is resistant to rifampicin and does not produce riboflavin in milk (Johnson *et al.* 1997; Keis *et al.* 2001).

Besides being a crucial bacterium for industrial used, *C. acetobutylicum* is also used as a model for the study of endospore formation, which was compared with *Bacillus subtilis* (Nölling *et al.* 2001).

1.4.2. *Clostridium beijerinckii*

C. beijerinckii was first isolated by Marins Beijerinck (Durre 2008). The most striking feature of *C. beijerinckii* (previously known as *C. butylicum*) is that it is able to produce either acetone or further reduce the acetone to isopropanol with the help of secondary alcohol dehydrogenase (s*adh*) (Figure 1.2A) (Alalibo *et al.* 2014; Millat and Winzer 2017). However, the amount of isopropanol produced by *C. beijerinckii* is very low. The isopropanol can be added to biodiesel production, which reduces the crystallisation at low temperature (Alalibo *et al.* 2014).

C. beijerinckii is more favourable as a solvent producer when compared with *C. acetobutylicum* due to a number of factors such as: (1) higher tolerance to

fermentation inhibitors released from the hydrolysis of fibre-rich agriculture biomass such as weak acids (e.g. formic acid) and furan derivatives (hydroxymethylfurfural and furfural); (2) less vulnerable to solventogenic degeneration as the genes for solventogenesis in *C. beijerinckii* is located on the chromosome. Whereas, *C. acetobutylicum* carries the gene on the plasmid; (3) exhibits wider substrate range (Table 1.4); (4) broader optimum pH for growth and solvent production; and (5) higher metabolic capability as the genome size of *C. beijerinckii* is 50% bigger than *C. acetobutylicum* (Table 1.3) (Cho *et al.* 2012; Ezeji *et al.* 2006; Lu 2014; Wang *et al.* 2011).

1.4.3. *Clostridium saccharoperbutylacetonicum*

In 1959, Hongo *et al.* (1968) was first to isolate a strain of *C. saccharoperbutylacetonicum* from soil (strain 97; US patent no. 2945786), which was subsequently used by the Sanraku Distillers company in the early 1960s to produce butanol, although production was ceased by phage issues (Poehlein *et al.* 2014). The species name *saccharoperbutylacetonicum* (*saccharin*: sugar juice; *per*: throughout; *butylum*: butanol) refers to the hyper-production of solvents especially butanol from a wide range of carbohydrates (Table 1.4). The ability to ferment a wide range of carbohydrates is because the bacterium produces a diverse complement of hydrolytic enzymes. *C. saccharoperbutylacetonicum* produces more hydrolytic enzymes than the yeast, *Saccharomyces cerevisiae*, which is a universal ethanol producer (Patakova *et al.* 2013), and is therefore able to grow on a greater range of substrates. Substantial actives of hydrolytic enzymes occur during the growth phase, which will hydrolyse complex sugars to fermentable sugars that can be used for solvent production.

The desirable traits of *C. saccharoperbutylacetonicum* are: (1) capability to produce a high level of butanol (approximately 85% of the total solvent production); (2) as low sporulation frequency; (3) enzymatic and saccharolytic properties; (4) it is extremely good at reutilising formed acid as well as supplied acids (Kosaka *et al.* 2007; Patakova *et al.* 2013; Poehlein *et al.* 2014).

Table 1.3. The commonly used three species of solvent-producing clostridial (adapted from Bao *et al.* 2011; Gérando *et al.* 2018; Poehlein *et al.* 2014).

Species and strain	Genomic sequence size (bp)	Extrachromosomic elements. Size (bp)	Nucleotide accession number
C. acetobutylicum DSM 1731	3,942,462	Two plasmids. pSMBa: 191,996 pSMBb: 11,123	CP002660, CP002661, CP002662
C. beijerinckii DSM6423	6,383,364	Two plasmids. pNF1: 10,278 pNF2: 4,282 One linear double stranded DNA bacteriophage; ϕ6423: 16,762	PRJEB11626
C. saccharoperbutylacetonicum DSM 14923	6,530,257	One megaplasmid; Csp_135p: 136,188	CP004121, CP004122

Table 1.4. Differences in carbohydrate utilization in three species of solvent-producing clostridial (adapted from Keis *et al.* 2001).

Species	Amygdalin	Arabinose	Cellobiose	Dextrin	Glycogen	Glycerol	Glucose	Inulin	Lactose	Maltose	Mannose	Mannitol	Melezitose	Melibiose	Methyl-glucopyranoside	Raffinose	Salicin	Saccharose	Sorbitol	Starch	Trehalose	Turanose	Xylose
C. acetobutylicum	+	+	+	+	+	w	+	d	+	+	+	+	(-)	(-)	+	+	+	+	d	+	(-)	(w)	+
C. beijerinckii	+	+	+	+	(+)	w	+	+	+	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+
C. saccharoperbutylacetonicum	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	d	+	+	+	+

+: positive; -: negative; (+): positive in most strain; (-) negative in most strain; w: weak; (w) weak in most strains; d: 40-60% of strains are positive

1.5. ABE fermentative metabolism

ABE fermentation produces solvents in the ratio of 3:6:1 for acetone: butanol: ethanol (Gutierrez *et al.* 1998) and involves biphasic growth consisting of acidogenesis and solventogenesis (Kosaka *et al.* 2007). According to the ABE ratio (3:6:1), more butanol is produced because during acidogenesis phase, more butyrate is formed than acetate as the NADHs formed during glycolysis are only taken up in butyrate pathway but not in acetate pathway. Then, more of the butyrate and less of the acetate convert to butanol and ethanol, respectively (Zheng *et al.* 2009).

Optimal temperature for solvent production is 30-35 °C. At high pH ranges from 6.0 to 6.5 triggers organic acids production (Maddox 1989). Whereas, at low pH ranges from 4.5 to 5.0 initiates solvent production. On the other hand, the solventogenesis will be unproductive if the pH is lower than 4.5 (Al-Shorgani *et al.* 2015; Ellis *et al.* 2012; Jones and Woods 1986; Keis *et al. 2001;* Kosaka *et al.* 2007; Lee *et al.* 2008). The *sol* operon of *C. saccharoperbutylacetonicum* (Figure. 1.1) is required for solventogenesis and consists of genes coding for aldehyde dehydrogenase (*aldH*), CoA transferase (*ctfAB*), and acetoacetate decarboxylase (*adc*). The general architecture of the *sol* operon of *C. beijerinckii* and *C. saccharoperbutylacetonicum* are the same, but differs from the *sol* operon of *C. acetobutylicum* where the aldehyde/alcohol dehydrogenase gene (*aad*) replaces *aldH*, and *adc* is part of a separate operon. Transcription of the *sol* operon is highly up-regulated during solventogenesis in a polycistronic manner. Nakayama *et al.* (2011) reported that quorum-sensing controls the transcription of *sol* operon and induces solventogenesis. Most of the Gram-positive bacteria such as clostridial communicate via quorum-sensing, which the bacteria monitor their population density by sensing the diffusible signalling molecules. These bacteria use these signalling molecules to regulate genes expression. Kosaka *et al.* (2007) reported that "low-solvent" mutant of *C. saccharoperbutylacetonicum* generates signal compounds to induce solvent production. However, further investigation is needed to identify the signal compound (Berezina *et al.* 2009; Cerror *et al.* 2013; Kosaka *et al.* 2007; Nakayama *et al.* 2011; Poehlein *et al.* 2014; Steiner *et al.* 2012).

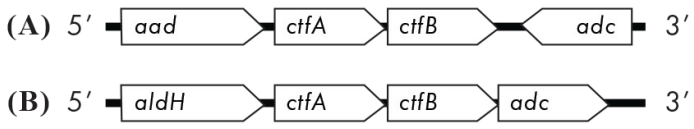

Figure 1.1. *sol* operon in three species of solvent-producing clostridial. (A) *C. acetobutylicum.* The *sol* operon structure of *C. acetobutylicum* is different from *beijerinckii*, and *C. saccharoperbutylacetonicum,* where the *aad* replaces *aldH*, and *adc* is part of a separate operon; (B) *C. beijerinckii,* and *C. saccharoperbutylacetonicum.* The *sol* operon structure of *C. beijerinckii*, and *C. saccharoperbutylacetonicum* are the same (adapted from Berezina *et al.* 2009; Kosaka *et al.* 2007).

Figure 1.2A depicts the ABE fermentation. During anaerobic ABE fermentation, clostridial species hydrolyse carbohydrate by the action of amylase (Figure 1.2, 1). Sugars in the form of pentoses and hexoses (in the form of mono-, di-, tri-, and polysaccharides) are then metabolized through glycolysis (Embden-Meyerhof pathway) to produce pyruvate that is converted to acetyl CoA with the release of carbon dioxide (CO_2) (Figure 1.2, 2 and 3). Acetyl CoA is further converted to other intermediates including acetoacetyl CoA, acetyl-P, and acetaldehyde (Figure 1.2, 4 and 5) (Ndaba *et al.* 2015).

Acidogenesis happens under specific growth conditions such as during the log phase of growth (2 to 12 h of fermentation), pH value more than 5, and limitation of iron. The vegetative cells produce a large amount of organic acids (e.g. acetate, butyrate and lactate), hydrogen, carbon dioxide and accumulate ATP. The increased production of organic acids, causes a significant drop of pH. For example, the undissociated butyric acid diffuses into the cells. Hence, proton gradient between the inside and the outside of the cell is destroyed, which can inhibit the cell growth. Clostridial increase the internal pH by switching to solventogenesis from acidogenesis (commonly occurs at pH 5.5 or lower), which occurs during the late log phase and early stationary of growth (Abdehagh *et al.* 2014; Cheng *et al.* 2015; Jin *et al.* 2011; Ndaba *et al.* 2015; Tashiro *et al.* 2004; Wang *et al.* 2017). Jones and Woods (1986) suggested that the initiation of solvent production appears to be a detoxification mechanism, which prevents the cells from inhibitory effects that would happen when the organic acids (end products of acidogenesis) reach a toxic level.

During the solventogenesis (early stationary phase; after 12 hrs of fermentation), the organic acids are assimilated together with the consumption of additional carbon source to produce acetone, butanol and ethanol. Hence, a low pH condition is prerequisite for solvent production (Cheng *et al.* 2015; Jin *et al.* 2016; Tashiro *et al.* 2004).

Besides the cleavage of pyruvate to acetyl-CoA, clostridial can convert pyruvate to lactate under unfavourable conditions such as the inhibition of hydrogenase activity by carbon monoxide or the limitation of iron. This operation is less effective for energy generation and allows the continuation of the oxidation of

NADH. This pathway is inactive during solventogenesis (Jones and Woods 1986; Millat and Winzer 2017).

Electron flow in clostridial is governed by ferredoxin, which acts as an electron carrier and has a role in electron distribution at a very low redox potential (-410 mV *vs.* NHE). Under ideal condition(s), the reduced ferredoxin transfers electron to hydrogenase, which uses proton as a final electron accepter. At this step, the ferredoxin is reoxidized, resulting in the release of hydrogen gas from the cell (Figure 1.2, 3). During acidogenesis, there is a sharp drop of redox potential due to the rapid flow of electrons, which is mainly derived from the cleavage of pyruvate. During acidogenesis phase, the electron and carbon flow are directed to hydrogen and organic acid production, respectively (Jones and Woods 1986). During solventogenesis, the production of hydrogen is lesser than the expected amount from the oxidation of pyruvate, as the majority of the electron and carbon flow are directed to solvent production (Jones and Woods 1986; Rao and Mutharasan 1987).

Although the pH-acid effect from acidogenesis act as a key role in the onset of solventogenesis, but acid crush might occur when the pH of the medium is lower than 4.5. For example, in pH-uncontrolled fed-batch fermentations, when the concentration of the acids (mainly acetic and butyric acids, undissociated and dissociated) in the medium is more than 57-60 mmol/l. Hence, the excess acids are produced together with insignificant switching of acidogenesis to solventogenesis. As a result, the switching of acidogenesis to solventogenesis fails, which then lead to the failure of solvent production and a cessation of glucose uptake. The solventogenesis can be inhibited when the total acid concentration reaches 240-350 mmol/L (Chen and Blaschek 1999; Maddox *et al.* 2000).

There is a significant change in gene expression pattern during the metabolic switch, resulting in the suppression of acidogenic enzymes along with the induction of solventogenic enzymes. During the acidogenesis phase, phosphotransacetylase (pta) and acetate kinase (ack) play a role for the production of acetic acid from acetyl-CoA (Figure 1.2, 4). The conversion of acetyl-CoA to butyryl-CoA, were done by four enzymes, which are thiolase (thl), 3-hydroxybutyryl-CoA dehydrogenase (hbd), crotonase (crt), and butyryl-CoA dehydrogenase (bcd) (Figure 1.2, 7). Then, butyryl-CoA is catalysed by phosphotrans butyrylase (ptb) and butyrate kinase (buk) to produce butyric acid (Figure 1.2, 8). During the

solventogenesis phase, ethanol and butanol are generated by aldehyde dehydrogenase (aldh), and alcohol dehydrogenase (adh) (Figure 1.2, 5 and 9). Acetic and butanoic acid are re-assimilated by acetoacetyl-CoA transferase (ctfAB), which produce acetyl CoA and butyryl-CoA, respectively (Figure 1.2, dashes lines). Then, acetyl CoA is converted to acetone and ethanol (Figure 1.2, 5 and 6) and butyryl-CoA is converted to butanol (Figure 1.2, 9) (Kosaka *et al.* 2007; Patakova *et al.* 2013; Wang *et al.* 2017).

Butanol can be produced in two pathways, which are the "hot and cold channel(s)" (Figure 1.2B). The "cold channel" is the process of generating butanol via the reassimilation of acetate and butyrate into acetyl-CoA and butyryl-CoA, respectively through a CoA- transferase (CoA-T) pathway. Then, acetyl-CoA is converted to butyryl-CoA or reduced to ethanol. Butyryl-CoA is then reduced to butanol. Whereas the "hot channel" prevents organic acid reassimilation and it is the only direct route for the conversion of acetyl-CoA to butyryl-CoA followed by reduction to butanol. Production of butanol via "hot channel" prevents the yield losses to CO_2 and acetone as 1 mol of acetoacetate is generated from every mol of reassimilated organic acid. The acetoacetate is then decarboxylated into CO_2 and acetone (Jang *et al.* 2012b; Ou *et al.* 2015). However, the study of Jang *et al.* (2012b) showed that reducing the "cold channel" reduced the acetate and butyrate production but the butanol yield was rather low. Hence, decreasing "cold channel" does not increase the butanol yield unless all the organic acid pathways are halted simultaneously (Jang *et al.* 2012b).

Sporulation can occur during solventogenesis, which poses a problem as sporulation compromises solvent production as the cells fall into a state of dormancy. The Spo0A regulator is responsible for sporulation and solventogenesis by regulating the expression of metabolic enzymes that are crucial for solvent production. Kosaka *et al.* (2007) reported that *Spo0A* may regulate the *sol* operon indirectly as no binding site for *Spo0A* was found near the *sol* operon. Hence, further investigation is required to confirm the regulation of *spo0A* (Abdehagh *et al.* 2014; Jin *et al.* 2011; Patakova *et al.* 2013).

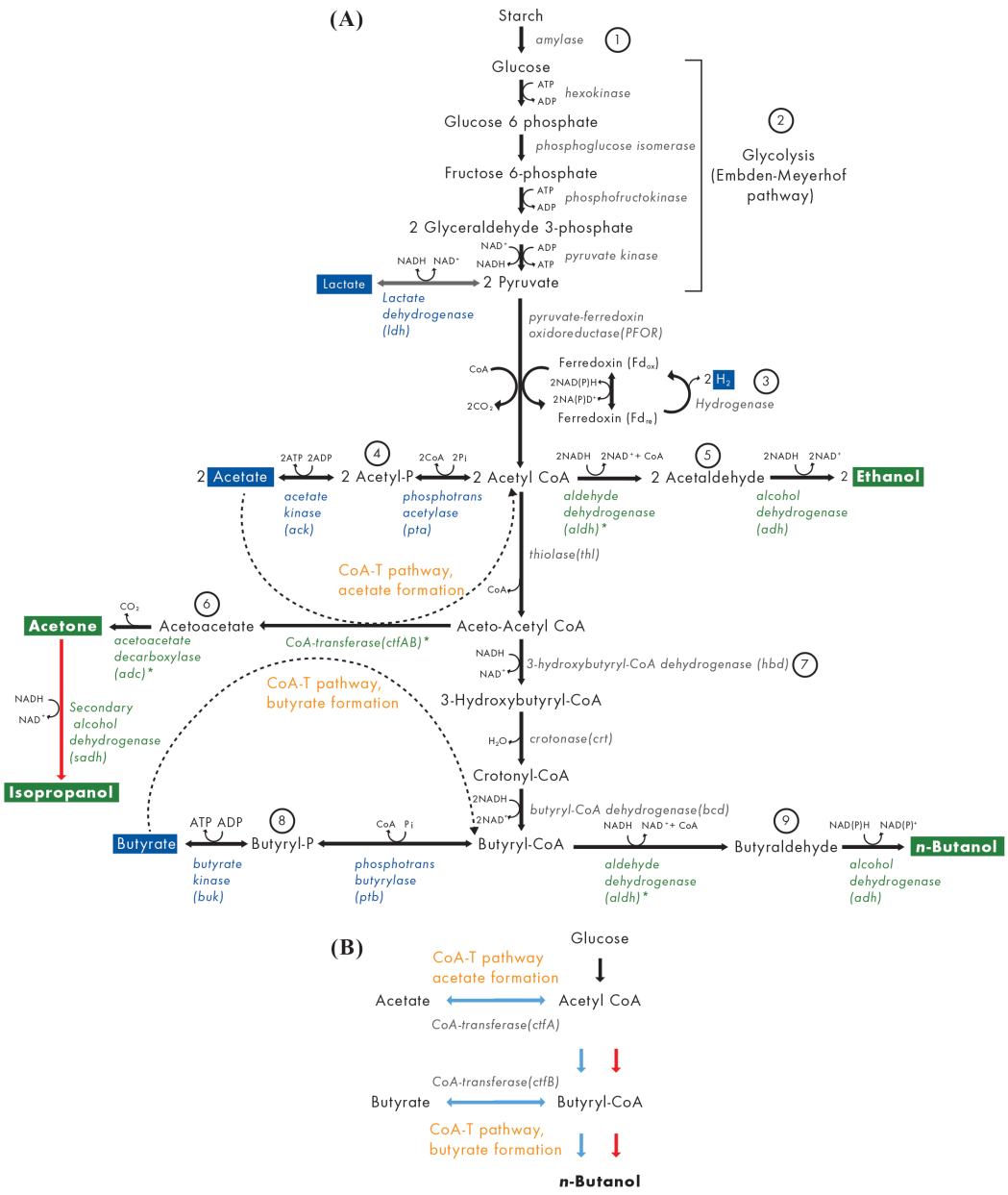

Figure 1.2. ABE fermentation pathway. (A) The major products during acidogenesis are shown in the blue boxes, while those mainly produced during solventogenesis are shown in the green boxes. The dashes lines show the CoA-T pathway, where organic acids are re-assimilated during solventogenic growth. Enzymes are shown in italics. Asterisk (*) indicates genes and enzymes encoded by the *sol* operon. Pathway in red arrow indicates isopropanol production naturally occurs *C. beijerinckii*; Pathway in grey arrow indicates lactate production under unfavourable condition. Numbers are used for easy reference; (B) Butanol formation routes via "hot and cold channel(s)" in clostridial. Blue arrows indicate the cold channel, which is the organic acid assimilation route. Red arrows indicate the hot channel, which is the direct route (Adapted from Alalibo *et al.* 2014; Jang *et al.* 2012b; Kosaka *et al.* 2007; Lee *et al.* 2008; Moon *et al.* 2016; Ndaba *et al.* 2015; Ou *et al.* 2015).

1.6. Strategies to improve solvent production

The condition and the age of the inoculum plays a crucial role in improving the efficiency of ABE fermentation. In order to increase productivity, the best stage to seed culture is at the final stage of the acid decreasing phase during the ABE fermentation (Ezeji *et al.* 2013).

In anaerobic fermentation, agitation is needed to homogenize the solid-liquid suspension, which is important for the nutrients to transfer into the cells and the metabolites to transfer out from the cells (Yerushaimi and Volesky 1985). Yerushalmi *et al.* (1985) reported that solvent production can be increased when the agitation rate is set between 190 to 340 rpm. Further increases of agitation resulted in an adverse effect and inhibition of cell growth occurred at agitation rate of 560 rpm due to mechanical cell injury (Jones and Woods 1986; Yerushaimi and Volesky 1985). In contrast to above, Doremus *et al.* (1985) reported that low agitation (100 rpm) together with head-space pressure (100 kPa) using hydrogen gas improved butanol productivity. Doremus *et al.* (1985) suggested that head-space pressurized (105 to 1,479 kPa) using hydrogen gas supersaturates the medium with hydrogen favours the production of reduced products such butanol and ethanol by expensing oxidized products such as acetate and butyrate. Whereas, in non-pressurized fermentation, the head-space pressure generated naturally by the hydrogen gas produced by clostridial is too low to have a marked improvement in solvent productivity because during acidogenesis the hydrogen gas acts as a reducing agent, which is used for butanol production (Maddox 1989).

Iron at an appropriate concentration is crucial for solvent production as the iron-sulphur protein ferredoxin oxidoreductase is required for the conversion of pyruvate to acetyl-CoA. However, by limiting the iron concentration in the medium to 0.2 mg/L, butanol production is elevated (Kótai *et al.* 2013). Under these conditions of iron limitation, the activity of *adc* and hydrogenase is decreased by 25% and 40%, respectively. In addition, supplementation of ammonium acetate (CH_3COONH_4) is also required to induce solvent synthesis with a high butanol content (Jin *et al.* 2011; Kótai *et al.* 2013).

Studies have shown that butanol production can be enhanced by supplementation with organic acids such as butyric acid in pH-controlled fed-batch culture as butyric acid is intermediate products of ABE fermentation (Al-Shorgani *et*

al. 2012; Oshiro *et al.* 2010*;* Tashiro *et al.* 2004). Al-Shorgani *et al.* (2012) demonstrated that *C. saccharoperbutylacetonicum* is able to produce butanol with 5-10 g/L butyric acid supplementation without glucose. However, only butanol is produced as the fermentation pathway requires butyric acid for butanol production. In contrast, Tashiro *et al.* (2004) also used butyric acid supplementation to increase butanol yield, although no butanol was produced without supplementation of glucose as the NADH-dependent dehydrogenase enzymes (e.g., *aldh* and *adh*), which are responsible for the conversion of butyric acid to butanol require NADH, which is obtained from glycolysis. Besides butyric acid supplementation, Sonomoto *et al.* (2010) reported that the supplementation of 5 g/L lactic acid also can increase butanol production. The effect of organic acid supplementation is greater when the acids are added before fermentation, as this can lower the pH which in turn activates solventogenic growth (Kótai *et al.* 2013).

Inclusion of acetic acid has positive effects upon solventogenesis such as: (1) promoting growth of solventogenic *Clostridium* species; (2) increasing the buffering capacity, which can prevent "acid crush", in which the medium can lead to a sharp drop of pH to 4.0-4.5 (Chen *et al.* 1999); and (3) enhancing solvent production. The production of acetone is enhanced the most as acetic acid increases CoA transferase activity, which is responsible for the production of acetone. However, solvent production ceases when acetic acid in the medium exceeds 9.7 g/L (Cho *et al.* 2012; Maddox *et al.* 2000).

Acetate is a valuable substrate for ABE production, as supplementation of acetate (2-4 g/l) during acidogenesis and early solventogenesis aids glucose uptake and consequently increases solvent production. Furthermore, acetate is sometimes viewed as a commercially viable supplement as cost is 5-fold lower than butanol (Gao *et al.* 2015).

1.7. Solvent toxicity

Solvent toxicity is a ubiquitous issue with ABE fermentation. During solventogenesis, Clostridia continues to produce butanol reaches inhibitory levels. Butanol is the only solvent produced to reach the toxic levels during ABE fermentation. Butanol is the most toxic solvent due to its lipophilicity. Early studies reported that the butanol toxicity exhibits at concentration of 5 g/L or higher and the cell

growth was inhibited by 99.7% when butanol level reaches 15 g/L (Al-Shorgani *et al.* 201; Jones and Woods 1986). Butanol increases cell membrane fluidity by disrupting the phospholipid components found in the membrane. The high butanol concentration also destroys the membrane-associated functions (e.g. glucose uptake and membrane-bound ATPase activity), disrupts fatty acid and protein. These disruptions impair pH regulation, destroy protein-lipid interaction, and decrease energy nutrient transport (Jin *et al.* 2011; Lee *et al.* 2008; Moreira *et al.* 1981). Furthermore, Xiao *et al.* (2011) reported that 8 g/L of butanol inhibits clostridial utilising xylose. The culprit of the inhibition is most likely due to the disruption of transmembrane enzyme, which responsible for transporting xylose into the cell. On the other hand, the levels of acetone and ethanol produced by clostridial do not appear to reach the inhibitory level (Jones and Woods 1986).

1.8. Strategies to elevate butanol tolerance

Research into butanol tolerance has utilised heterologous host such as *Escherichia coli* (*E. coli*) for butanol production, as *Clostridium* species are less amenable to genetic manipulation, have complex ABE metabolism and a requirement for anaerobic conditions (Herman *et al.* 2017; Ou *et al.* 2015). Introduction of membrane transporters that can potentially export butanol has been shown to be a promising approach to enhance butanol tolerance in *E. coli,* including the FocA formate transporter. Formate (HCO_2^-) is produced by *E. coli* during anaerobic mixed-acid fermentation. Glucose is decomposed into pyruvate, which then convert into formate and acetyl CoA (Figure 1.3). Formate is an electron donor as well as an energy source for the cells. *E. coli* converts as much as one third of the carbon atom from carbohydrate to formate. As a result, formate accumulates rapidly and leads to sharp decrease of pH in the cytoplasm. Furthermore, formate has a low pK_a (3.77), which can cause acidification. Hence, formate is either quickly oxidised to CO_2, or exported from the cell (Suppmann and Sawers 1994; Beyer *et al.* 2013).

FocA (formate channel) transporter plays a role in regulating intracellular fomate pool in *E. coli*. FocA belongs to the nitrite transporter family (FNT). FocA is pH dependent and bidirectional transporter (Figure 1.3). At high pH (pH 7), FocA acts as an anionic formate-specific channel. At low pH (pH 5) FocA works as a H^+/formate symporter. The *focA* gene is co-transcribed with pyruvate formate-lyase

activating enzyme (*pflA*) and pyruvate formate-lyase (*pflB*), which responsible for formate formation. These genes are encoded in an inducible *pfl* operon, which can be induced under anaerobic condition (Beyer *et al.* 2013; Lü *et al.* 2011; Suppmann and Sawers 1994; Waight *et al.* 2010). Reyes *et al.* (2011) reported that overexpression of *focA* enhances the butanol tolerance in *E. coli*, possibly by increasing the efflux of butanol as focA transporter can be a non-specific transporter.

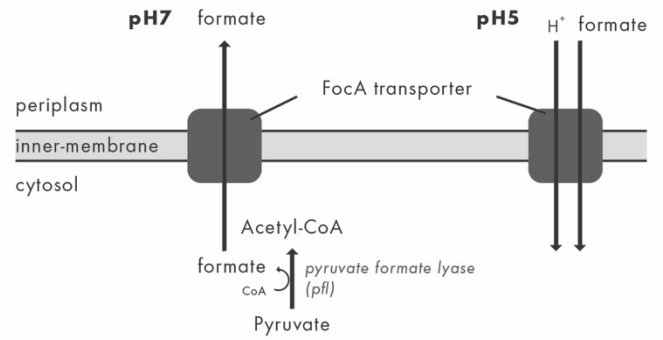

Figure 1.3. FocA transporter activity in high and low pH environment (adapted from https://biocyc.org/).

1.9. Feedstock

Feedstock is one of the main factors that influences the price of the solvent production, especially butanol. Hence, using a renewable, inexpensive, and abundant feedstock has become a desirable part of the economic model for ABE fermentation. The cost of solvent production also can be reduced by optimising the upstream (metabolic engineering and the use of inexpensive feedstock), midstream (improve fermentation strategies), and downstream (*in situ* recovery) processes (Jang *et al.* 2012a).

First-generation biofuels are produced with feedstocks derived from food crops. Sugar-based feedstocks, such as sugarcane, are grown mainly in tropical climates, whereas starch-based feedstocks are (i.e. mainly grains such as corn) can be produced in more temperate climates. Furthermore, corn and wheat are the main ingredients of livestock feed. Hence, large scale butanol production for fuel by using sugar- and starch-based food feedstock is not sustainable (Chen *et al.* 2013; Xue *et al.* 2013).

The second-generation biofuels utilize lignocellulosic material or non-food feedstock such as wheat straw, manure, wood, or other agricultural by-products and

forest residues. However, using these feedstocks are only beneficial if the biomass is produced sustainably. Furthermore, these feedstocks are difficult to degrade and unable to convert to biofuels in an economical way (Xue *et al. 2013*). The main components of lignocellulosic material are cellulose, hemicelluloses and lignin, which form the lignin-carbohydrate complex that prevent cellulose degradation. Hence, energy intensive pre-treatment is necessary to destroy the lignin-carbohydrate complex. Lignin is very difficult to break down, and prevents access of cellulases and hemicellulases. Lignin also acts as an inhibitor during hydrolysis, and phenolic inhibitory compounds derived from lignin can affect the cell growth (Chen *et al.* 2013). Hence, feedstocks with a low lignin content are preferable.

Biofuels that use microalgae as a feedstock are third-generation biofuels, and are an attractive feedstock due to ample availability. Microalgae are photosynthetic microorganisms that grow in aquatic environments where they use sunlight to convert water and CO_2 to biomass (Abomohra *et al.* 2016; Demirbas 2011; Ndaba *et al.* 2015). There is a precedent for using microalgae as a feedstock for ABE fermentation, as Castro *et al.* (2015) used pre-treated wastewater microalgae to grow *C. saccharoperbutylacetonicum*. Microalgae are also cultivated to produce oil for foods, energy or products, yielding large amount of spent microalgae biomass (SMAB). SMAB is the microalgae biomass collected from the primary use, which can take up as much as 70% of whole microalgae biomass and consists of carbohydrates, lipids and proteins (Rashid *et al.* 2013; Snow *et al.* 2015), which could also potentially be used as a feedstock. The advantages of using microalgae as a feedstock are: (1) a short harvesting cycle (approximately 14 days cultivation); (2) the ability to grow in marine, wastewater (domestic/municipal/industrial wastewater) and brackish water. In addition, the cultivation of microalgae with wastewater is a technique of bioremediation (e.g., removing nitrogen, phosphorus, urea and CO_2 sequestration); (3) the cultivation of land is not required, and hence there is no further pressure to increase deforestation; (4) the ability to produce higher biomass per square meter compare with terrestrial plants; (5) non-food carbon substrate; (6) it requires minimal nutrition; (7) continuous harvesting whereas most crops can only be harvested seasonally; and (8) the absence of recalcitrant lignin (Abomohra *et al.* 2016; Castro *et al.* 2015; Chen *et al.* 2013; Demirbas 2011; Jang *et al.* 2012a; Safi *et al.* 2014; Tashiro *et al.* 2004).

1.10. Microalgal species

There are a diverse range of microalgal species, the taxonomy/phylogeny of which is beyond the focus of this thesis. This section therefore focusses solely on the species used in this study.

1.10.1 *Chlorella vulgaris*

C. vulgaris (*chloros*: green; *ella*: green) is unicellular green eukaryotic microalgae without flagella. It has a spherical cellular morphology with a diameter of 2-10 μm (Ho *et al.* 2013b; Safi *et al.* 2014), and rapidly reproduces asexually. Previous studies have demonstrated that *C. vulgaris* is able to accumulate up to 37-55% carbohydrates per dry weight (Chen *et al* 2013). The carbohydrates mainly reside as structural polysaccharides in the cell wall (Table 1.5) as well as starch found in the chloroplast of *C. vulgaris* (Abomohra *et al.* 2016; Castro *et al.* 2015; Ellis *et al.* 2012; Wakasugi *et al.* 1997; Wang *et al.* 2014; Wang *et al.* 2016). Finally, microalgae-based carbohydrates are not associated with lignin, which makes saccharification easier as the pre-treatment with heat/chemicals is not required (Chen *et al* 2013; Gao *et al.* 2016).

Another beneficial trait of *C. vulgaris* is that after 4 days of cultivation nitrogen-depletion can lead to sharp increase in the content carbohydrates and lipids, as this forces the cells to transform proteins to carbohydrate and lipids, which are high in energy. Furthermore, Gerken *et al.* (2012) reported THAT when *C. vulgaris* was grown under extreme nitrogen-DEPRIVED conditions, the cells SCAVENGED the amino sugar found in the cell wall as an alternative source of nitrogen. As a result, the cell walls appear similar to AN lysozyme-digested cell wall. Also, *C. vulgaris* utilizes urea as a nitrogen source which is very cost effective (approximately $2.00 per kilogram of biomass) compared to other nitrogen sources such as nitrate and ammonium ions (Ho *et al.* 2013b).

Table 1.5. Simple sugars composition in the cell wall of *C. vulgaris* (adapted from Chen *et al.* 2013).

Simple sugars	Percentage (%)
Rhamnose	45-54
Galactose	14-26
Xylose	7-19
Arabinose	2-9
Mannose	2-7
Glucose	1-4

C. vulgaris also contains a large amount of protein, and the total protein content per dry weight is 42-58%. More than 20% and 50% of the protein can be found in the cell wall and internal, respectively. The remaining 30% migrates in and out the cell. Proteins promote cell growth, maintain, and repair the cells (Safi *et al.* 2014). Amino acid such as isoleucine, valine, and glutamic acid, asparagine, serine, threonine, alanine, and glycine can provide an additional nitrogen source, which might promote cell growth and accelerate solvent production. However, cysteine and tyrosine may cause some negative effects such as inhibiting fermentation and decreasing butanol production. Other amino acids such as leucine, phenylalanine, tryptophan, proline, lysine, histidine and arginine are not required for growth (Kótai *et al.* 2013; Lee *et al.* 2008; Wang *et al.* 2016).

Iron is one of the important minerals require for solvent production in ABE fermentation. Approximately 0.38 g/100g of iron can be found in *C. vulgaris.* Furthermore, *C. vulgaris* also contains minerals such as sodium, potassium calcium, magnesium, phosphorus, chromium, copper, zinc, manganese, selenium, and iodine (Chen *et al.* 2013).

1.10.2. Eustigmatophyceae proprietary strain from Algaecytes®

The current study involved collaboration with a company called Algaecytes® (http://algaecytes.com/), which focusses mainly upon on growth of microalgae for extraction of valuable natural products (e.g. Omega 3). During this process, a huge amount of spent biomass is generated, so it was hypothesised that this could be used as a feedstock for ABE fermentation. A Eustigmatophyceae proprietary strain was obtained from Algaecytes®, with the carbohydrate and protein content being 27% and 43%, respectively. Eustigmatophyceae is a small class of eukaryotic algae (30 species), which has a green coccoid morphology (2-25 μm in

dimension). Eustigmatophytes thrive in freshwater and terrestrial habits except in marine and blackish water, and receive much attention from the sustainable industries owing to an ability to synthesize valuable omega-3 fatty acids such as eicospentaenoic acid (EPA) (Eliáš *et al.* 2017; Ma *et al.* 2016).

1.11. Cell disruption of microalgae

Microalgae have a strong cell wall which requires a cell wall disruption process to extract the intracellular contents. Physical (autoclaving, electroporation, French press, homogenization, lyophilization, microwave, thermal, ultrasonication, osmotic shock), chemical (acid, alkaline treatment) and biological (enzymatic polysaccharide, protein degradation) treatments have been used, although the most promising techniques is an enzymatic lysis approach (Dalatony *et al.* 2017; Naghdi *et al.* 2016; Safi *et al.* 2014) albeit one of the most expensive and challenging processes. Hence, minimum pre-treatment or non-pre-treatment feedstock can greatly optimize production cost.

1.12. Aims and experimental strategies for the current study

1) Assess the efficacy of microalgae as an alternative feedstock for clostridial biofuel production.

Strategy: Measure solvent yields from *C. saccharoperbutylacetonicum* grown on feedstocks prepared from commercially available *C. vulgaris* and from Algaecytes®, Eustigmatophyceae whole cell and spent biomass.

2) Engineer a butanol-tolerant bacterial strain.

Strategy: Clone and overexpress the membrane transporters FocA and FdhC in *E. coli* and *C. saccharoperbutylacetonicum*. Grow in the presence of various concentrations of butanol.

Chapter 2

Materials and Methods

2.1. Bacterial strains

The bacterial strains that were used in study are listed in Table 2.1.

Table 2.1. List of microorganisms. MS numbers are used as a numerical ordering system for strains in the Shepherd lab.

MS number	Species/Strain	Plasmids	Note	Reference or source
MS256	*C. saccharoperbutylacetonicum* DSM 14923	Megaplasmid found in wild type cells	Solventogenic strain and template for amplification of *fdhC*	American Type Culture Collection (ATCC)
MS449	*E. coli* JM109	pMTL83353, spectinomycin resistant	-	New England Biolabs
MS2	*E. coli* MG1655	Template for amplification of *focA*	K12 wild type and template for amplification of *focA*	Bachmann 1996
-	*E. coli* DH5α	Competent cells	-	NEB

2.2. Microalgal biomass

C. vulgaris FACHB-31 dry biomass was purchased from Seven Hills Wholefood, supplied as fragmented cells. Eustigmatophyceae proprietary strain ALG01-CL1 whole cells and spent biomass were obtained from Algaecytes®. Eustigmatophyceae culture was harvested, dewatered and spray-dried. Followed by esterification and oil extraction. Eustigmatophyceae was in dry powder with an intact cell wall.

2.3. Clostridial growth medium

The culture media were made anaerobic either by purging with 0.2 μm filtered N_2 gas (BOC, UN1002) for 10 mins or autoclaving for 15 min at 121 °C and 15 psi (Quirumed, Prestige Medical, 2100 classic 9L without manometer). The serum bottles containing medium were sealed with disposable Butyl rubber septa,

which is designed to allow air to be released out from the serum bottle during autoclaving and prevents the reentry of air when the temperature is dropped (Behbehani *et al.* 1982). The media were sterilized by autoclaving. The media were stored in an incubator at 32 °C.

2.3.1. Reinforced Clostridial medium

Reinforced Clostridial medium (RCM) per litre distilled water consisted of 13 g yeast extract, 10 g peptone, 5 g glucose, 1 g soluble starch, 5 g sodium chloride, 3 g sodium acetate, 0.5 g cysteine hydrochloride, and 0.5 g agar, pH 6.8±0.2.

2.3.2. Tryptone-yeast-extract medium

Tryptone-yeast-extract (TYE) medium contained the following substances per litre of distilled water: 50 g glucose, 2.5 g yeast extract, 2.5 g tryptone, 0.5 g ammonium sulphate, 0.025 g iron (II) sulphate, and 19.52 g 0.1 M MES free acid. Sodium hydroxide was used to adjust the pH to 6.2 to 6.3.

2.3.3. Luria-Bertani medium

Luria-Bertani **(**LB) medium contained the following substances per litre of distilled water: 10 g tryptone, 5 g yeast extract and 5 g sodium chloride.

2.3.4. Super Optimal broth with Catabolite repression medium

Super Optimal broth with Catabolite repression **(**SOC) medium contained the following substances per litre of distilled water: 20 g tryptone**,** 5 g yeast extract**,** 0.584 g NaCl**,** 0.186 g KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose were added to the autoclaved medium.

2.3.5. 10% microalgal medium

The process flowchart for preparation of 10% microalgal medium subjected to autoclaving (15 min at 121 °C and 15 psi) and centrifugation is depicted in Figure 2.1A. 20 g of dry microalgal biomass was weighed and diluted with 100 mL distilled water to get a final concentration of 20% microalga. The medium was mixed until homogenous by using magnetic stirrer. Then, the medium was centrifuged at 5000 rmp for 20 min at 20 °C. The supernatant was then diluted with distilled water with the dilution factor of 1:1 to create a final concentration of 10% microalga. To

prepare a supplemented microalgal medium, 1% glucose was added after dilution. 50 mL of the diluted medium then pipetted into 100 mL serum bottle followed by autoclaving. 5 M HCl was used to adjust the Eustigmatophyceae spent biomass to pH 6 prior purging (Figure 2.1B and C).

Table 2.2 List of microalgal medium used in this study.

Abbreviation	Microalga	1% glucose supplementation	Autoclaving (15 min at 121 °C and 15 psi)	Centrifugation (5000 rpm for 5 min)	pH adjustment (to pH 6)
CV	10% *C. vulgaris*	N	Y	Y	N
CVG	10% *C. vulgaris*	Y	Y	Y	N
EG	10 % Eustigmatophyceae whole cell	Y	Y	Y	N
EG-NT	10 % Eustigmatophyceae whole cell	Y	N	N	N
ESBG	10 % Eustigmatophyceae spent biomass	Y	Y	Y	N
ESBG-NT	10 % Eustigmatophyceae spent biomass	Y	N	N	N
ESBG-pH	10 % Eustigmatophyceae spent biomass	Y	N	N	Y
ESBG-ApH	10 % Eustigmatophyceae spent biomass	Y	Y	N	Y

Y: received indicated treatment or supplementation.
N: did not received indicated treatment or supplementation.

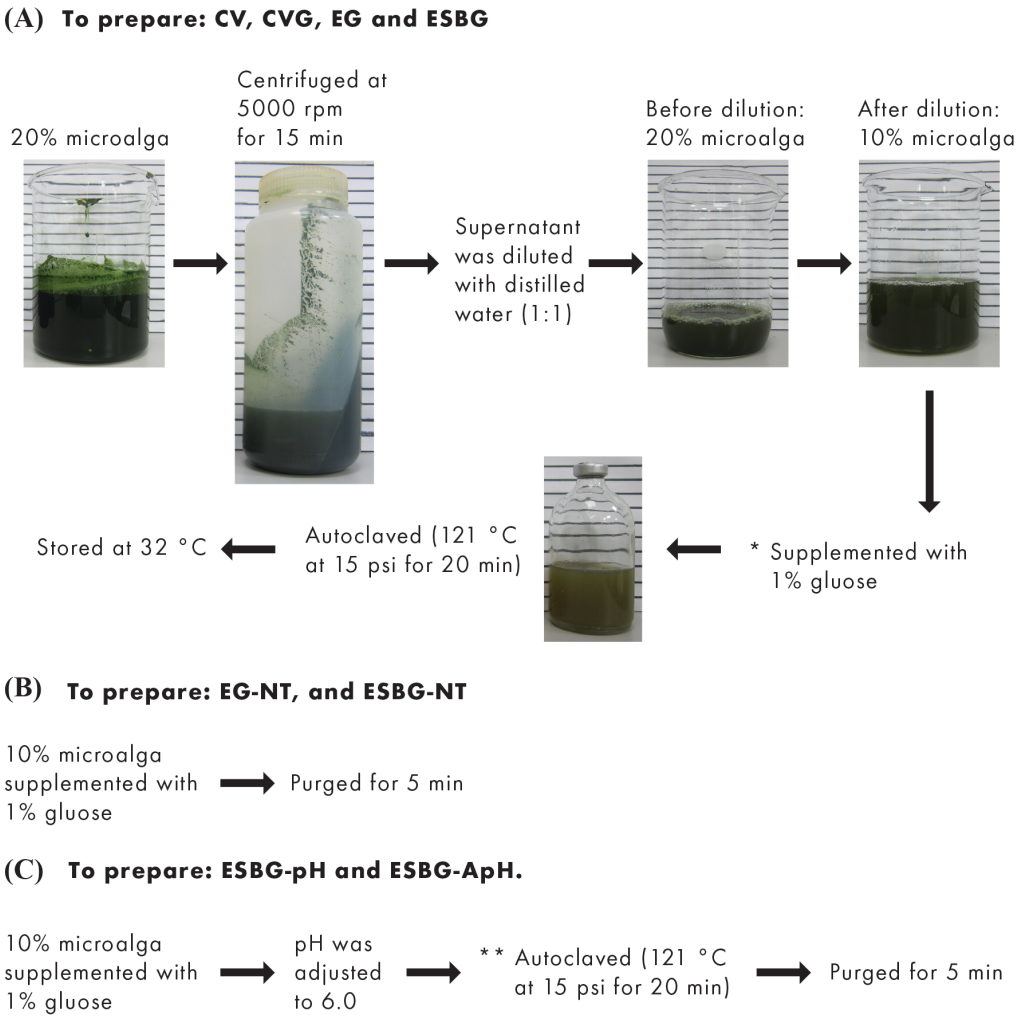

Figure 2.1. Process flowchart for preparation of 10% microalgal medium. (A) preparation of 10% microalgal medium treated with autoclaving and centrifugation. *: this step was omitted when preparing CV; (B) preparation of 10% microalgal medium without autoclaving and centrifugation; (C) preparation of 10% microalgal medium with pH adjustment. **: this step was omitted when preparing ESBG-pH.

2.4. Clostridial growth in serum bottles

To prepare glycerol stock, 750 µl of the overnight liquid culture was added to 250 µl of 60% glycerol (v/v) in a 2 mL cryovial and mixed gently. The glycerol stock tube was store at -80 °C. The bacteria were recovered by thawing without mixing. The remaining thawed culture was discarded.

20 mL of autoclaved (121 °C at 15 psi for 20 min) RCM pH 6.8±0.2 was prepared in a 50 mL serum bottle, and was inoculated with 0.5 mL of *C. saccharoperbutylacetonicum* glycerol stock. The serum bottle was sealed and then incubated anaerobically at 32 °C for approximately 18 h until cells had reached stationary phase. Prior to the inoculation of the selective fermentation medium, the optical

density (OD) of stationary phase cultures was measured to ensure the OD_{600} was in the range 1.5-1.8. The health of cells was assessed under the microscope (GT Vision, GXML 2800) at x400 magnification (Appendix Figure D1) to ensure the cells were not aggregating and were moving in a tumbling motion. 50 mL of selected fermentation media, in a 100 mL serum bottle, was then inoculated with 5 mL of the stationary phase culture and incubated anaerobically at 32 °C.

2.5. Fermenter setup

Fermentation experiments were designed such that three biological replicates were performed (Figure 2.2) in 1000 mL culture vessels with 500 mL of growth media. To form a tight seal between culture vessel and flat flange lid, a gasket (PTFE seal) was coated with a thin layer of petroleum gel and secured by a retaining clip. Rubber turn-over closure (Suba-seal®) and rubber stopper (2 hole) were used to seal the flanges of lid. The longer tube of the rubber stopper was connected to 10 mL syringe, which was used to draw samples. The shorter tube was connected to a Minisart® filter (pore size: 0.2 μm) for gas outlet during fermentation (Figure 2.2C). The culture vessel and medium were sterilized by autoclaving at 121 °C and 15 psi for 20 min and kept at 32 °C by submerging in 2 L beakers with 900 mL of distilled water and magnetic bead. After autoclaving, a sterilized thermometer was immersed in the medium through a suba-seal® with hole. The apparatus was placed on the hotplate stirrer with temperature set at 32 °C, and magnetic beads were used to gently agitate the medium. To create an anaerobic environment, the medium was purged with filtered N_2 gas for 20 min. The fermenters were inoculated initiated with 1% (v/v) of actively proliferating cells in RCM (Section 2.4). The medium was purged with N_2 for another 5 min to achieve optimal anaerobic conditions. 5 mL samples were collected periodically and centrifuged at 8000 rpm for 5 min, and supernatants were stored at -20 °C for subsequent solvent analyses by using GCMS (Section 2.6.3).

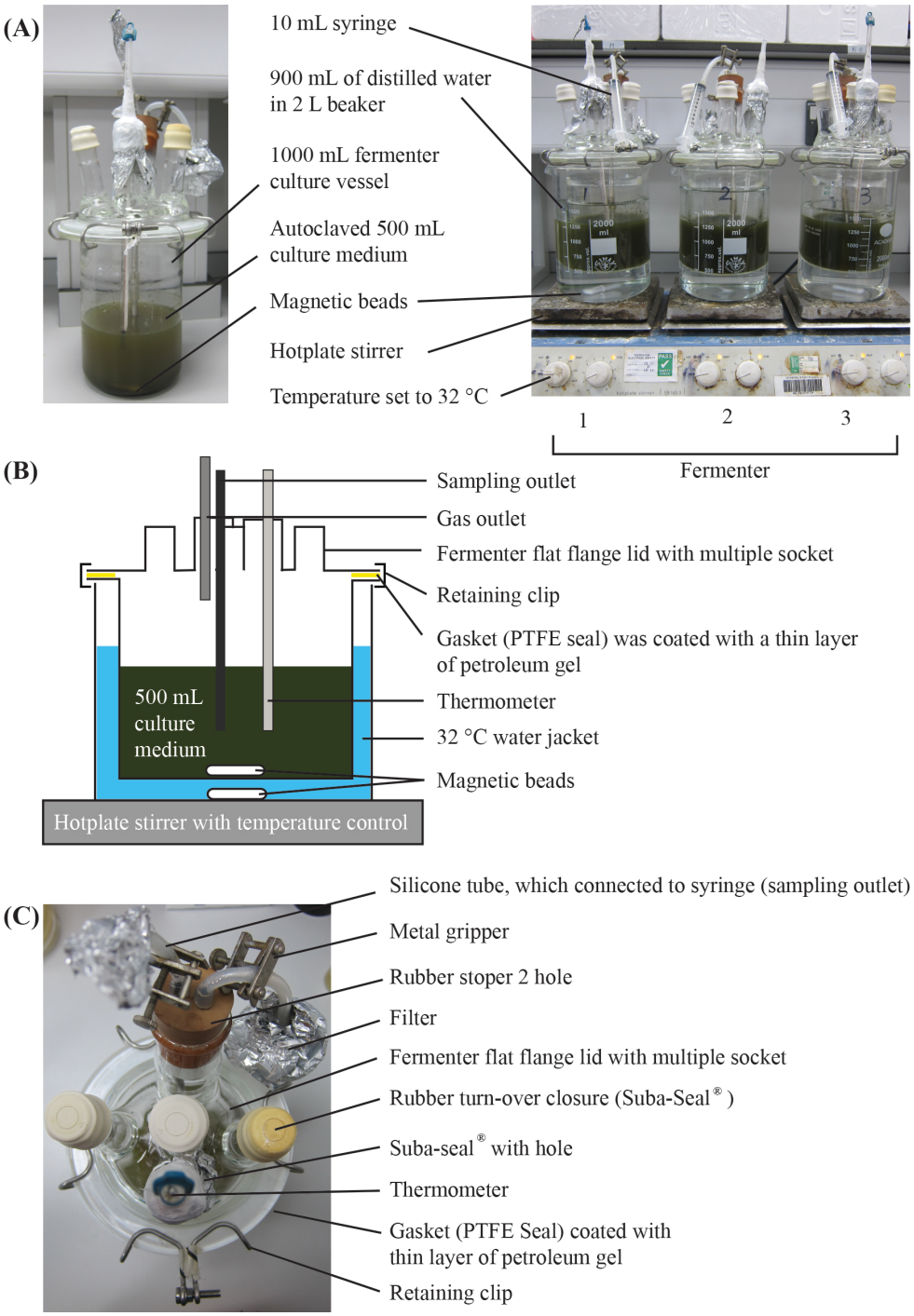

Figure 2.2. Fermenter apparatus setup. (A). Front view. Thermometer and syringe were added after autoclaving; (B) Schematic diagram of fermenter apparatus setup. The unused sockets of the flat flange lid were covered with subseals; (C) Top view. Aluminium foils were removed after autoclaving, which exposed the filter for gas outlet and silicone tube, which was attached to syringe for sampling outlet.

2.6. Analytical procedures

2.6.1. Optical density measurements

Cell growth was measured using a UV-visible spectrophotometer (Agilent Technologies, Cary 60 UV-Vis) at OD_{600}. The measurement volume was 1 mL. The spectrophotometer was blanked with culture medium. Samples were diluted as necessary to achieve OD_{600} readings below 1.0. The OD_{600} of microalgal medium without centrifugation were not monitored due to the high amount of fragmented microalgal biomass.

2.6.2. pH and redox measurements

The pH and redox poise were measured by pH (Mettler Toledo InLab® Semi-Micro-L) and Ag/AgCl redox (Mettler Toledo InLab® Redox Micro) electrodes. The probes were rinsed with IMS before and after use, as well as between measurements. The redox electrode was stored in 3M KCl and calibrated using saturating solutions of quinhydrone at pH4 (expected 264 mV) and pH7 (expected 87 mV). Correction factors were applied for slight deviations from the expected readouts, and data were converted to *vs.* Normal Hydrogen Electrode (NHE) by addition of 210 mV. Hence. the presented redox data was adjusted by adding 210.

2.6.3. Solvent quantitation

0.5 mL of culture supernatants were transferred to GCMS vials for solvent analysis. Concentrations of acetic acid, butanoic acid, acetone, butanol, and ethanol were measured by using an Agilent Technologies, 6890N GCMS system equipped with 7HG-G013-11 Zebron column. Helium (>99.999%) was used as the carrier gas (mobile phase) and a flow rate of 1 mL/min. Methanol was used as a polar solvent. 0.2 µl of water sample was injected with a 100:1 split. The injection temperature was set to 150 °C. The GCMS transfer line temperature was set to 280 °C, ion source 230 °C, and quadrapole 150 °C. After injection column temperature was held at 30 °C for 5 min, then increased to 150 °C for 20 min.

Solvents identification was based on retention times (Table 2.3). The concentration of each solvent was calculated by comparing the peak area of the analyte in the sample with the peak areas obtained for standard curves.

Table 2.3. Solvent retention times.

Solvent	Retention time (min±0.2)
Acetic acid	11.23
Butanoic acid	12.75
Acetone	2.60
Butanol	8.60
Ethanol	5.03

2.7. Metabolic engineering

2.7.1. Preparation of competent cells

50mL falcon tubes, Eppendorf tubes, and $CaCl_2$ were pre-chilled to 4 °C. 10 mL of LB broth was inoculated with a single colony of *E. coli* DH5α and incubated overnight at 37 °C and 210 rpm. On the following day, 200 mL of LB was inoculated with 2 mL of the overnight started culture and incubated at 37 °C and 210 rpm. When the OD_{600} reached 0.4-0.6, the cells were placed on ice. The culture was split into four 50 mL falcon tubes and centrifuged for 15 min at 4 °C and 4000 rpm. The supernatants were discarded. Pellets were resuspended in 10 mL of 100mM $CaCl_2$. The suspensions were then transferred into a single 50 mL falcon tube followed by incubation on ice for 1 h. After incubation, the suspension was centrifuged for 15 min at 4 °C and 3000 rpm, then, the supernatant was discarded. The pellet was resuspended with 100 mL of 100 mM $CaCl_2$, centrifuged for 15 min at 4 °C and 3000 rpm, then, the supernatant was discarded. The pellet was resuspended in 2 mL of 100 mM $CaCl_2$ and 100 µl aliquots were transferred to Eppendorf tubes on dry ice. The cells were stored at -80 ºC for future use.

2.7.2. Vector preparation

LB broth (10 mL) supplemented with 10 µl of spectinomycin (50 mg/mL) was inoculated with a single colony of *E. coli* JM109 cells and grown overnight at 37 °C and 200 rpm. The cells were harvested by centrifugation at 8000 rpm for 3 min at 20 °C. Plasmids were extracted and purified by using QIAprep Spin Miniprep Kit according to the manufacturer's instructions. Plasmid concentrations were measured using a NanoPhotometer (Implen) and the purified plasmid DNA was stored at -20 °C for future use.

2.7.3. Primer design

Gibson Assembly® was performed to clone *focA* and *fdhC* genes into the pTML83353 shuttle vector (Heap *et al.* 2009). Primers were designed to amplify inserts and plasmid to generate a 18-25 bp overlap (Table 2.4). Primers were manufactured by Integrated DNA Technologies®.

Table 2.4. Primers used in this study.

MS primer number	Name	Sequence (5′ - 3′)	Usage	Direction
360	FocFWD	GGAGGTGTGTTACATATGATGAAAGCTGACAACCCTTTTGATCTTTTAC	Amplification of *focA* from *E. coli* MG1655 genomic DNA	Forward
361	FocRev	GCTTCTTATTTTTATGCTAGCTTAGTGGTGATGGTGATGATGATGGTGGTCGTTTTCACGCAG		Reverse
333	FdhCGAFwd	GGAGGTGTGTTACATATGATGATGAGTACAAAGAATTATTTAAC	Amplification of *fdhC* from *C. saccharoperbutylacetonicum* genomic DNA	Forward
334	FdhCGARev	GCTTCTTATTTTTATGCTAGCTTAGTGGTGATGGTGATGATGTATGTCTATTTTCTTATCCAAAC		Reverse
364	83353GA_focA_F1	CTGCGTGAAAACGACCACCATCATCATCACCATCACCACTAAGCTAGCATAAAAATAAGAAGCCTG	Amplification of pMTL83353 with *focA* overhang	Forward
365	83353GA_focA_R1	GTAAAAGATCAAAAGGGTTGTCAGCTTTCATCATATGTAACACACCTCCTTAAAAATTAC		Reverse
366	83353GA_fdhC_F1	GTTTGGATAAGAAAATAGACATACATCATCACCATCACCACTAAGCTAGCATAAAAATAAGAAGCCTG	Amplification of pMTL83353 with *fdhC* overhang	Forward
367	83353GA_fdhC_R1	GTTAAATAATTCTTTGTACTCATCATCATATGTAACACACCTCCTTAAAAATTAC		Reverse
368	83353_cPCR_SCRN_FWD	TACAATTTTTTTATCAGGAAACAGC	cPCR screening	Forward
369	83353_cPCR_SCRN_REV	CCCGTAATTGAATACATAACAAGTA		Reverse

2.7.4. Preparative polymerase chain reaction

PCR was performed to amplify *focA* and *fdhC* from *E. coli* K-12. and *C. saccharoperbutylacetonicum*, respectively. For amplification of *focA*, colonies of *E. coli* MG1655 (MS2) were picked and resuspended in 50 µL of sterile water in a PCR tube. 2 µl of the supernatant was used as DNA template for PCR. For amplification of *fhdC*, genomic DNA was prepared from *C. saccharoperbutylacetonicum* by using a GenElute^{TM} Bacterial Genomic Kit according to the manufacturer's instructions, and 2 µl was used as a template in the PCR reaction. Vector fragments for pTML833353 were amplified using colony PCR with template prepared from strain MS449 (*E. coli* JM109 pTML833353) as described above for MS2.

The vector fragments and genes were amplified using Applied Biosystems PCR machine in 50 µl reactions containing 25 µl of Q5® High-fidelity 2X master mix (NEB), 2.5 µl of each primer (300 nM final), 2 µl of insert (1ng - 1µg) or 0.5 µl of vector (1 ng – 100 ng) and the reactions were topped up with autoclaved milli-Q® water (MQ water) to 50 µl final volume. Q5® High-fidelity 2X master mix contained the following components: 4.0 mM Mg^{++}, additives, 400 µM of each dNTP, and Q5® High Fidelity DNA polymerase. Table 2.5 indicates the program used in the thermal cycler.

Table 2.5. Thermocycling conditions for PCR using Q5® High-fidelity 2X master mix.

Step		Temperature (°C)	Time
Initial denaturation		98	30 s
Denaturation	x35	98	10 s
Anneal		50	30 s
Extension		72	inserts: 40 s; vector: 3 min 30 s
Final extension		72	2 min
Hold		10	∞

2.7.5. Gibson Assembly®

PCR products were purified using a QIAquick® PCR purification kit according to the manufacturer's instructions. PCR reactions and 1kb DNA ladder (Promega) were mixed with loading dye (6X) and loaded on 1.0% agarose gel and separated by electrophoresis (80 V, 300 mA, 40 min) in 1X Tris-acetate buffer. Gels were stained and soaked with ethidium bromide solution (10 mg/mL) for 30 min on an orbital shaker. Gels were analysed using a GeneSys gel imager.

The purified vectors and inserts were quantitated using a NanoPhotometer (Implen). Cloning was performed using a Gibson Assembly® kit (NEB)**.** Gibson assembly master mix consists of T5 exonuclease, Phusion DNA polymerase and Taq DNA ligase. The reactions contained 0.02-0.5 pmols vector with 3-fold of excess insert, 10 µl Gibson assembly master mix (2X) and the reactions were topped up with autoclaved miliQ water to a final volume of 20 µl. Reactions without insert were used as a negative control. The reactions were incubated in a thermocycler at 50 °C for 60 min. Following incubation, the reactions were stored at -20 °C for subsequent transformation.

2.7.6. Transformations

Transformations were performed via the heat shock method using chemically competent *E. coli* DH5α cells that were thawed on ice. 10 µl of Gibson Assembly reaction was added to 100 µl competent cells and mixed gently by flicking the tubes 4-5 times, and the tubes were placed on ice for 30 min. Thereafter, the tubes were exposed to heat shock for 30 s at 42 °C and were transferred to ice for 2 min. 950 µl of SOC medium was added to each tube and incubated 37 °C and 250 rpm for 60 min. 100 µl of cell suspension was spread on pre-warmed (37 °C) LB plates containing 50 µg/mL spectinomycin. The remaining cells were harvested at 5000 rpm for 5 min, resuspended in 100 µl of supernatant, and spread on pre-warmed (37 °C) LB plates containing 50 µg/mL spectinomycin. Plates were then incubated overnight at 37 °C.

2.7.7. Screening PCR

Colonies from the transformation plates were patched onto LB plates containing 50 µg/mL spectinomycin and subjected to colony PCR screening using primers 83353_cPCR_SCRN_FWD (368) and 83353_cPCR_SCRN_REV (369) (Table 2.3) designed to amplify plasmid inserts, 243 bp at 5′ and 249 bp at 3′ flanking regions of pTML833353. Colonies were resuspended in 50 µl of sterile water for colony PCR. Plates were incubated overnight at 37 °C to confirm specinomycin resistance. Colony PCR screening reactions contained 12.5 µl of 2X PCRBIO Taq Mix Red (PCR Biosystems), 1 µl of each primer (300 nM final), 1 µl of resuspended colony, and were topped up with autoclaved MQ water to 25 µl final volume. 2X

PCRBIO Taq Mix Red contains PCBIO Taq DNA Polymerase, 6 mM $MgCl_2$, 2 mM dNTPs, enhancers, stabilizers and red dye for tracking during gel electrophoresis. Colony suspensions from competent cells (*E. coli* DH5α) and MS449 (*E. coli* JM109 pTML833353) were used as controls. Table 2.6. indicates the program used in the thermal cycler. The reactions were analysed on 1% agarose gel (80 V, 300 mA, 45 min).

Table 2.6. Thermocycling conditions for cPCR using 2X PCRBIO Taq Mix Red.

Step		Temperature (°C)	Time
Initial denaturation		95	1 min
Denaturation	x35	95	15 s
Anneal		50	15 s
Extension		72	40 s
Hold		10	∞

2.7.8. Plasmid Restriction Digestion

Colonies that yielded PCR products with fragments corresponded to the correct insert sizes were used to inoculate 10 mL of LB medium supplemented with 10 µl of spectinomycin (50 mg/mL) and were grown overnight 37 °C and 210 rpm. The cells were harvested by centrifugation at 8000 rpm for 3 min at 20 °C, and plasmids were purified using a QIAprep Spin Miniprep kit according to the manufacturer's instructions. Restriction digestion reaction contained purified plasmid DNA (1 µg), 0.5 µl of NdeI (20,000 U/mL), 1 µl of NheI (10,000 U/mL), and 5 of µl 10X CutSmart® buffer (NEB). The reactions were topped up with autoclaved MQ water to a final volume of 20 µl. The reactions were incubated at 37 °C for 1 h and the entire reaction mixtures were analysed on 1% agarose gels (80 V, 300 mA, 45 min).

Chapter 3

Results

3.1. Development of a spectrophotometric approach for measuring clostridial growth in turbid microalgal growth medium

One of the main aims of this work was to test the efficacy of growth medium prepared from microalgal biomass for the culture of solventogenic clostridial strains. However, growth medium prepared from microalgal biomass contains fragmented cells, which could pose a problem for measuring clostridial cells density using spectrophotometric approaches. It was therefore necessary to measure OD_{600} values for known cell densities of *C. saccharoperbutylacetonicum* in microalgal medium, and to ensure that a linear relationship exists between OD_{600} and cell density. The process flowchart depicted in Figure 3.1 describes the final approach undertaken following extensive trial and error with several medium compositions and dilutions**.** Briefly, a *C. saccharoperbutylacetonicum* culture was grown in RCM (i.e. not turbid medium) to an OD_{600} of 1.5- 2.0, and was divided into two equal volumes (25 mL each), followed by centrifugation at 5000 rpm for 10 min at 20 °C. The pellets were then resuspended with either 5 mL of RCM or 10% microalgal medium, and dilutions of 1/5, 2/5, 3/5, 4/5 were prepared (using RCM or 10% microalgal medium a dilutant). To ensure that cell densities were in an appropriate range for spectrophotometric analysis, samples were diluted with 1/10 with distilled water. The spectrophotometer was blanked with distilled water and the OD_{600} of cell suspensions was measured. While the microalgal medium predictably resulted in more background light scattering, Figure 3.2A shows that increasing cell density had a linear relationship with OD_{600} in both RCM and 10 % *C. vulgaris*. Hence, it was possible to subtract 'zero clostridial cell' OD_{600} value from subsequent growth curve data to accurately monitor cell growth. Since the data shown in Figure 3.2A are for the cell cultures diluted 1/10 in the cuvette, the undiluted 5 mL cell cultures had OD_{600} values of approximately 6. Hence, it is possible to measure clostridial cells densities of up to $OD_{600} = 6$ in 10% microalgal growth medium using this approach.

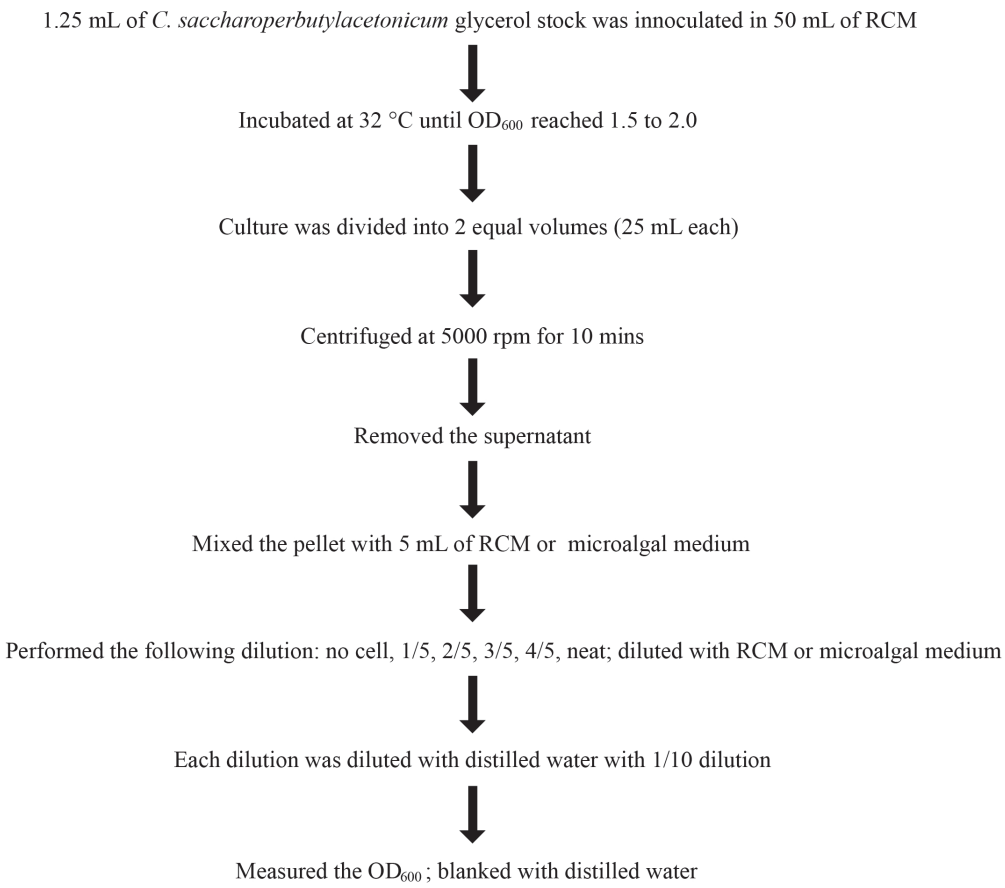

Figure 3.1. Flow chart to depict an experimental approach for measuring clostridial cell density in microalgal medium.

To further investigate the relationship between OD_{600} readings and clostridial cell densities, a growth curve of *C. saccharoperbutylacetonicum* in 10% *C. vulgaris* medium supplemented with 1% glucose was performed with light microscopy analysis performed in tandem. Figure 3.2B depicts the growth curve for *C. saccharoperbutylacetonicum* (performed in triplicate in 50 mL serum bottles), and Figure 3.2C depicts the microscopy analysis of one of these cultures at various timepoints. These data are both consistent with a low number of cells during the lag phase followed by a rapid increase in cell density during log phase, and finally, no net growth during stationary phase.

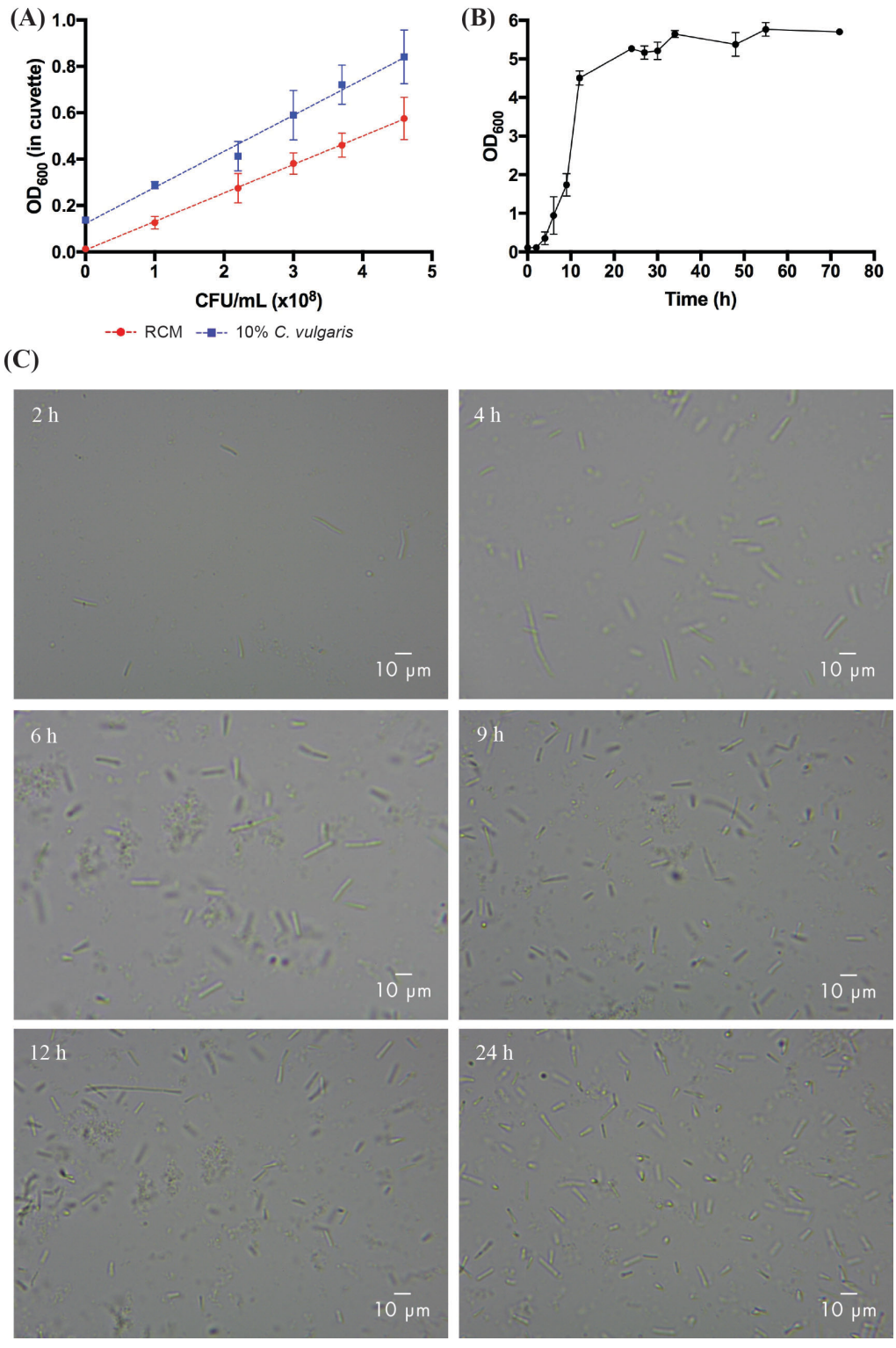

Figure 3.2. Spectrophotometric analysis of clostridial cell density in 10% *C. vulgaris* medium. (A) OD_{600} values of varying *C. saccharoperbutylacetonicum* cell densities in RCM and 10% *C. vulgaris* media. OD_{600} values are of the cultures in the cuvette (diluted 1/10 water), so the original 5 mL undiluted culture had an OD_{600} ~ 6. Conversion factor of 8.0 x 10^8 for an OD_{600} of 1.0; (B) Growth curve of *C. saccharoperbutylacetonicum* in 10% *C. vulgaris* supplemented with 1% glucose. Performed in triplicate, with error bars showing SD values; (C) Direct observation of bacterial growth from panel B under the microscope. The magnification was x400. Clostridium cells have a green hue when grown in microalgal medium.

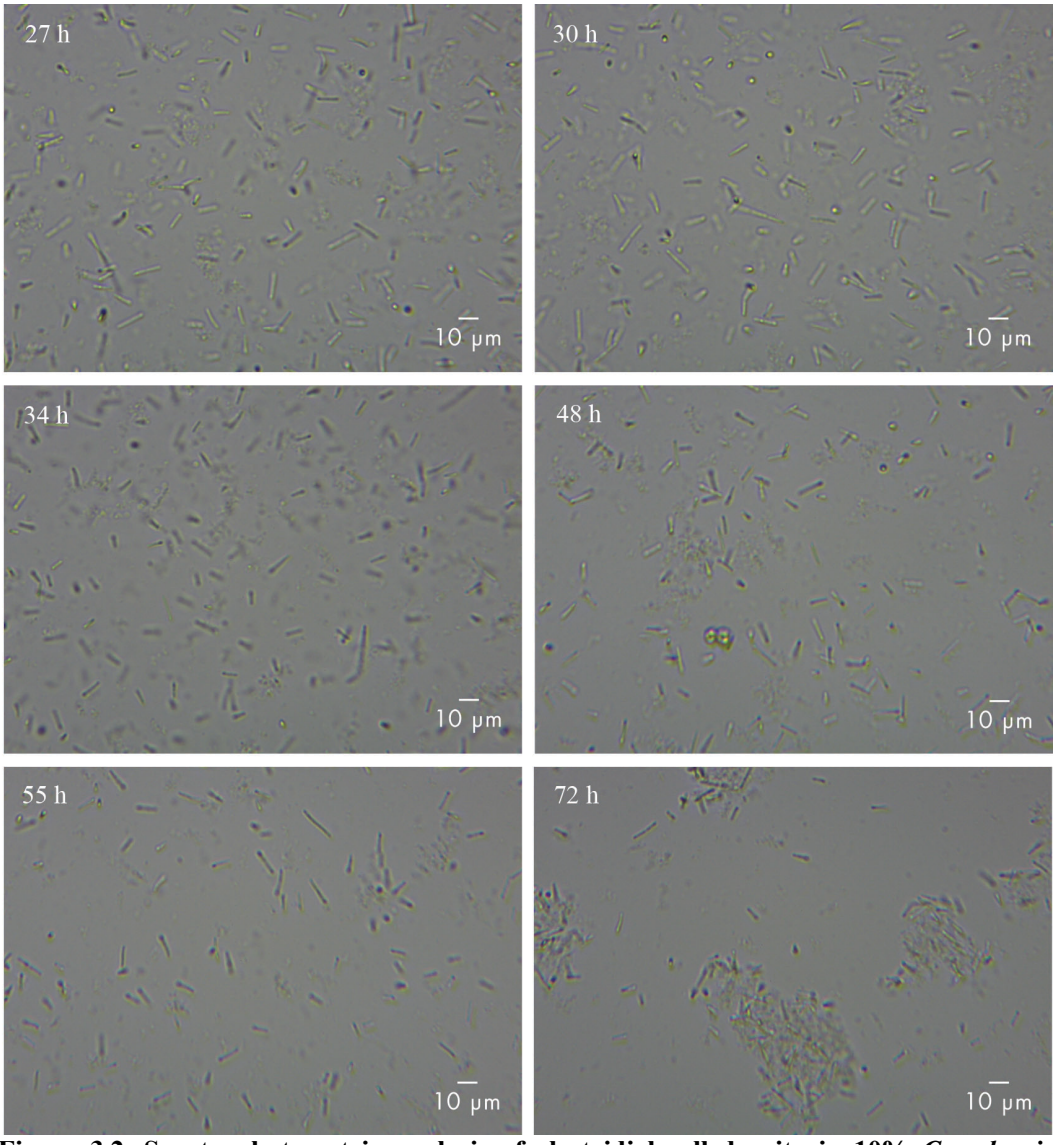

Figure 3.2. Spectrophotometric analysis of clostridial cell density in 10% *C. vulgaris* medium (continued).

3.2. Assessment of ABE fermentation using microalgal medium in serum bottles

Batch fermentations using a 10% microalgal growth medium were conducted to investigate solvent production by *C. saccharoperbutylacetonicum.* As indicated in the literature (Castro *et al.* 2015, Ellis *et al.* 2012, Gao *et al.* 2016), growth medium made from 10% microalga was found to be suitable for ABE fermentation. Solutions containing 10 % microalga were subjected to centrifugation (5000 rpm for 15 min) and autoclaving. ABE fermentation was carried out in serum bottles for 72 h. This study assessed the fermentation profile in the following growth media: 10% *C. vulgaris* (CV), 10% *C. vulgaris*

supplemented with 1% glucose (CVG), 10% Eustigmatophyceae whole cell supplemented with 1% glucose (EG), 10% Eustigmatophyceae spent biomass supplemented with 1% glucose (ESBG), RCM, TYE, 1% glucose and MQ water. 50 mL of growth media was inoculated with 5 mL of overnight culture in RCM with *C. saccharoperbutylacetonicum*. Since these inocula contained nutrients, a medium containing 10% RCM (in water) was used as a negative control. And RCM used as positive control. TYE with MES buffer (pH 6.2 to 6.3) is an ideal medium for solvent production by *C. saccharoperbutylacetonicum* as it for contains the sufficient amount of carbon and other nutrient (glucose, yeast extract, tryptone, ammonium sulphate, iron (II) sulphate), and it was therefore used as second positive control for growth. 1% glucose (in water) was used as second negative control to assess the extent to which the 1% glucose or the 10% microalga support growth and solvent production.

Figure 3.3 shows that acidogenesis lasted for 12 h in all the media except for ESBG, where acidogenesis lasted for approximately 24 h. The pH and redox decreased dramatically during this growth phase, which was accompanied by exponential increase in biomass (OD_{600}). Following this, the pH either decreased slightly or plateaued, which usually coincides with entry to the solventogenic phase. In the latter stages of the growth curves the cells entered stationary phase and the ORP reading (i.e. redox poise) reached a plateau.

Figure 3.3A depicts the fermentation profile of *C. saccharoperbutylacetonicum* using CV as a growth medium. During acidogenesis, the pH dropped from 6.95±0.01 to 6.14±0.01, and the OD_{600} increased from 0.110±0.01 to 3.612±0.05. At 6 h, the ORP reached the lowest point, which is 86±38 mV *vs.* NHE. At the end of acidogenesis (12 h), acetic and butanoic acids levels were 0.24±0.02 and 0.11±0.02 g/L, respectively. During solventogenesis, the pH had increased to 6.41±0.02 (72 h). In this period the OD_{600} started to decrease from 3.612±0.05 to 3.461±0.06. At 72 h, acetic and butanoic acid decreased to 0.32±0.10 and 0.13±0.03 g/L, respectively. And butanol had reached the maximum concentration, which was 0.20±0.03 g/L. Table 3.1. shows that the organic acids and solvent production found in CV were lower than both of the positive controls (TYE and RCM).

The fermentation profile of *C. saccharoperbutylacetonicum* using CVG had slightly different behaviours compared to CV (Figure 3.3B). During the acidogenesis phase, the pH dropped from 7.07±0.01 to 5.72±0.03 and the OD_{600} increased from 0.107±0.01 to 4.507±0.19. At the end of acidogenesis, acetic and butanoic acid concentrations were 0.31±0.14 and 0.17±0.05 g/L (Figure 3.4B), respectively. During solventogenesis (after 12 h), the pH decreased slightly from 5.72±0.03 to 5.62±0.06 and the ORP had reached the lowest point (-139±40 mV *vs.* NHE). In this period, the OD_{600} increased slightly (4.507±0.19 to 5.700±0.08). Acetone and butanol production were observed at 24 and 12 h, respectively. In comparison with CV, there was a 12 h delay of butanol production in CV (12 *vs.* 24 h). At 72 h, the organic acids and solvent production found in CVG were lower than both of the positive control (TYE and RCM), which for CVG were as follows: acetic acid=0.80±0.06 g/L; butanoic acid= 0.39±0.04 g/L; acetone=0.18±0.04 g/L; and butanol=0.98±0.04 g/L (Table 3.1).

Figure 3.3C shows that at the end of acidogenesis (12 h), the OD_{600} of *C. saccharoperbutylacetonicum* using EG medium increased from OD_{600} 0.361±0.01 to 1.775±0.10. The pH decreased from 5.87±0.06 to 4.61±0.04. The ORP reached the lowest point at -298±7 mV *vs.* NHE after 2 h of fermentation. During solventogenesis (12 to 72 h), there was a moderate increase of OD_{600} from 1.761±0.10 to 2.285±0.17. At 48 h, organic acids and solvent production had reached as follows: acetic acid=1.39±0.01 g/L; butanoic acid= 0.77±0.24 g/L; acetone=0.24±0.11 g/L; and butanol=1.53±0.51 g/L (Table 3.1), in which EG had the highest yield of butanol. Table 3.1 shows that the organic acids and solvent production found in EG were higher than those produced during growth in RCM (positive control) but lower than values obtained for TYE (positive control).

The fermentation profile of *C. saccharoperbutylacetonicum* using ESBG was very different. Figure 3.3D shows that acidogenesis lasted for 24 h, which was 12 h longer than other media. The OD_{600} remained low for the first 12 h (0.312±0.04). Following this, the OD_{600} increased to 2.864±0.09 at 24 h of fermentation and decreased slightly to 2.798±0.33 at the end of the fermentation (72 h). The ORP had reached the lowest point (-332±15 mV *vs.* NHE) at 72 h. The initial pH was high, which was 8.73±0.02 and decreased to 6.21±0.06 at the of acidogenesis (24 h). The pH then plateaued during solventogenesis (6.21±0.06 at

24 h to 6.23±0.10 at 72 h). At 48 h cells grown on ESBG medium had the highest level of acetic acid (4.39±1.59 g/L). However, the concentration of butanoic acid was 0.23±0.09 g/L, which was lower than both of the positive controls (TYE and RCM). The most striking differences in the ESBG fermentation profile were that neither acetone nor butanol were detected at 48 h, and high levels (8.41±0.56 g/L) of ethanol were detected (Table 3.1). Ethanol was not detected in positive controls (TYE and RCM), CV, CVG and EG.

Throughout the fermentation time course, no formation of foam was observed in cells grown on MQ water and only minor foam production was observed in cultures grown on 1% glucose, CV and ESBG. Foam was formed vigorously in cells grown on CVG, EG, TYE and RCM (Appendix Figure B1).

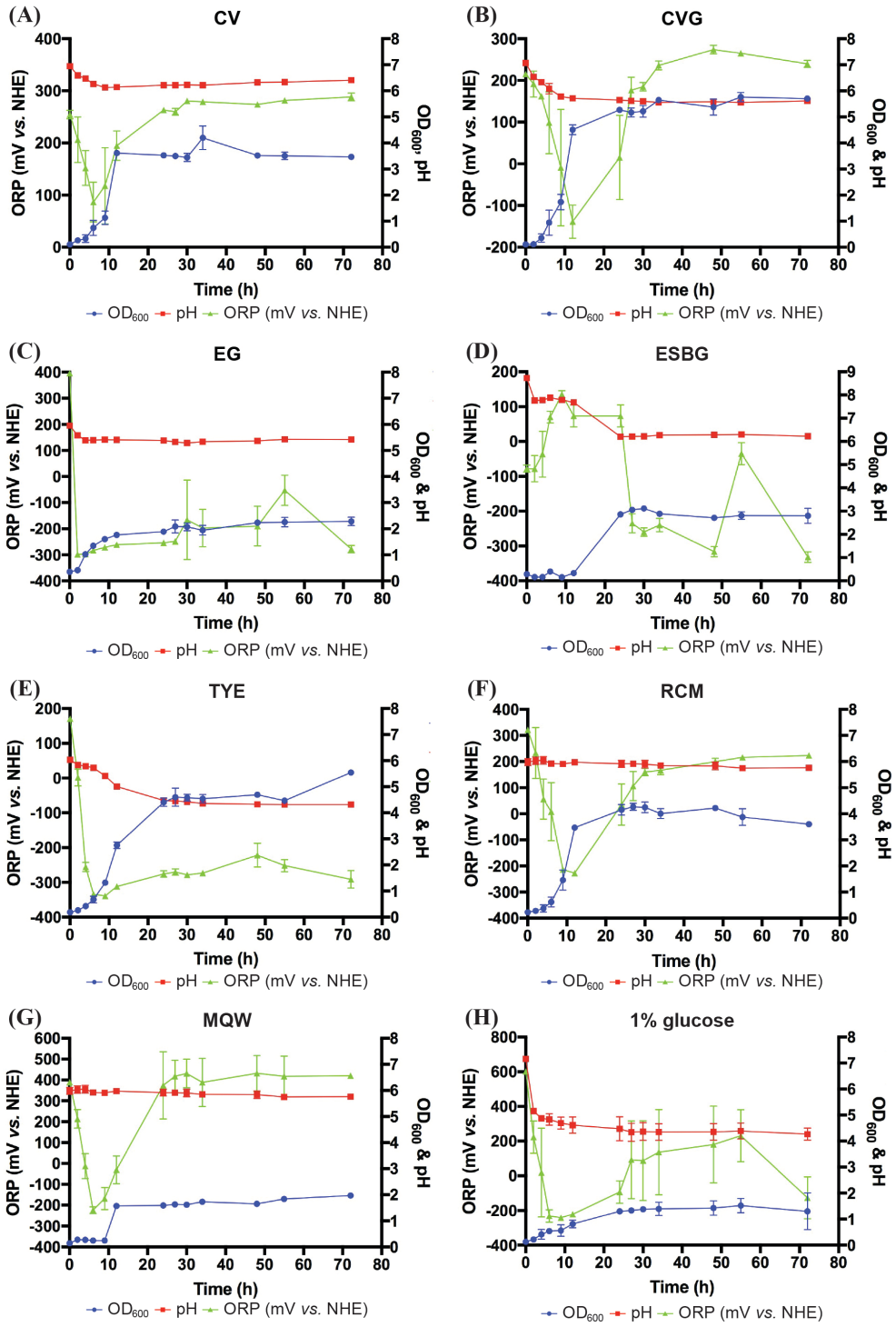

Figure 3.3. The OD_{600}, pH, and ORP changes of the selected fermentation media fermented by *C. saccharoperbutylacetonicum*. Fermentations were performed in serum bottles in a variety of different growth media: (A) CV: 10% *C. vulgaris*; (B) CVG: 10% *C. vulgaris* supplemented with 1% glucose; (C) EG: 10% Eustigmatophyceae whole cell supplemented with 1% glucose; (D) ESBG: 10% Eustigmatophyceae spent biomass supplemented with 1% glucose; (E) TYE; (F) RCM; (G) MQ water; and (H) 1% glucose. Positive controls: RCM and TYE. Negative controls: 1% glucose and MQ water. CV, CVG, EG and ESBG were centrifuged and autoclaved. Operating conditions; temperature: 32 °C, anaerobic environment without agitation; without pH control; 5 ml of overnight culture in liquid RCM with *C. saccharoperbutylacetonicum* (OD_{600} range 1.5-1.8) inoculated into 50 mL of selected media. Data points are averages of three biological repeats, with error bars showing SD values.

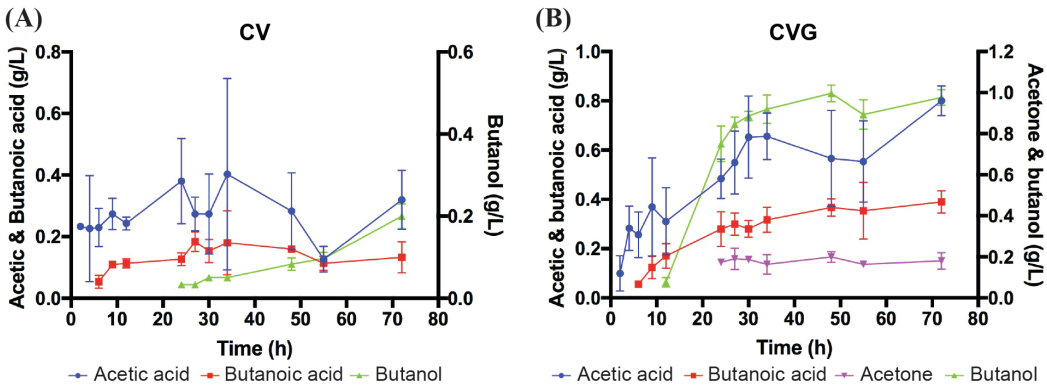

Figure 3.4. The organic acids and solvent production by *C. saccharoperbutylacetonicum* grew in (A) CV; and (B) CVG. Performed in triplicate, with error bars showing SD values. Only CV and CVG were subjected to complete GCMS analysis.

Table 3.1. Organic acids and solvent production by *C. saccharoperbutylacetonicum* after 24 and 48 h of fermentation in different media.

Media	Acetic acid (g/L±SD)		Butanoic acid (g/L±SD)	
	24 h	48h	24 h	48h
CV	0.38±0.14	0.28±0.13	0.13±0.02	0.16±0.02
CVG	0.48±0.08	0.56±0.19	0.28±0.07	0.36±0.04
EG	0.82±0.27	1.39±0.01	0.87±0.29	0.77±0.42
ESBG	4.39±1.59	6.86±0.60	0.23±0.09	0.42±0.03
TYE	2.97±0.16	3.36±0.25	1.80±1.14	5.83±0.50
RCM	0.51±0.27	0.92±0.03	0.25±0.19	0.60±0.17

Media	Acetone (g/L±SD)		Butanol (g/L±SD)		Ethanol (g/L±SD)	
	24 h	48 h	24 h	48 h	24 h	48 h
CV	-	-	0.03±0.00	0.09±0.02	-	-
CVG	0.17±0.01	0.20±0.02	0.75±0.09	1.00±0.04	-	-
EG	0.04±0.03	0.24±018	0.10±0.07	1.53±088	-	-
ESBG	-	-	-	-	6.57±1.00	8.41±0.56
TYE	0.22±0.01	0.33±0.03	2.63±0.25	3.74±2.16	-	-
RCM	0.06±0.06	0.21±0.15	0.29±0.15	0.50±0.27	-	-

- undetectable

Data are averages of three biological controls, and error are SD values.

Positive controls: RCM and TYE

GCMS analysis was not done on negative controls (MQ water and 1% glucose).

3.3. Assessment of ABE fermentation using 10% *C. vulgaris* in fermenters

A further ABE fermentation study was performed using CV media in a larger scale fermenter system which enabled in-line monitoring of pH and redox poise, and perhaps is a better approximation of industrial batch fermentations. The main differences between the fermentation using CV in fermenters and serum bottles were: (1) size of reaction vessel (1000 mL culture vessels *vs.* 100 mL serum bottle); (2) medium volume (fermenter: 500 ml; serum bottle: 50 ml); (3) inoculum dilution. The inoculum for the fermenter was a 100-fold dilution, whereas the inoculum for serum bottles was a 10-fold dilution; (4) agitation. The rate of agitation in fermenters was 100 rpm. No agitation in serum bottles; (5) gas outlet. A filter was connected to the fermenter for gas outlet. whereas, no gas outlet in serum bottle; (6) headspace. The fermenter had 500 mL of headspace, whereas serum bottles had 50 mL of headspace; (7) fermentation duration. Growth in fermenters was terminated at 48 h, whereas growth using serum bottles was terminated at 72 h.

Figure 3.5A shows that the acidogenesis phase lasted for 9 h when ABE fermentation was performed in a fermenter, which was 3 h shorter than the acidogenesis phase in serum bottles (9 h *vs.* 12 h). The pH decreased from 6.81±0.01 to 6.05±0.01. The growth had increased exponentially from OD_{600} 1.895±0.52 to 4.926±1.00. In this period, acetic acid was produced and reached a maximum concentration of 0.15±0.13 g/L. However, butanoic acid production reached a maximum concentration of 0.06±0.01 g/L occurring at 27 h (Figure 3.5B). After 6 h of fermentation, the ORP reached the lowest point (-220±61 mV *vs.* NHE), and the pH reached the lowest point at 9 h (6.05±0.01). Then, pH gradually increased to 6.32±0.05 at 48 h, and the OD_{600} continued to increase at a lower rate (from 4.926±1.00 to 6.332±1.17). The amount of acetic acid reached the highest point after 12 h of fermentation (0.08±0.04 g/L) and decreased slightly at 48 h (0.06±0.02 g/L). The butanoic acid concentration reached the highest level at 27 h (0.06±0.01 g/L) and decreased slightly at 48 h (0.04±0.03 g/L). Butanol production was observed after 24 h of fermentation and reached the maximum level at 48 h (0.15±0.04 g/L) (Figure 3.5B).

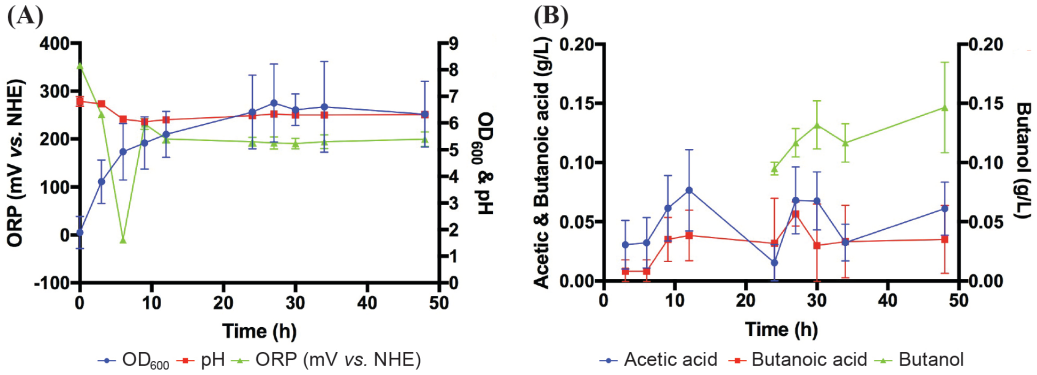

Figure 3.5. Fermentation profiles of *C. saccharoperbutylacetonicum* grown in 10% *C. vulgaris* using fermenters. (A) Time course of the changes in the OD_{600}, pH and redox changes in; (B). Organic acids and solvent production by *C. saccharoperbutylacetonicum*. Operating conditions; temperature: 32 °C, anaerobic environment with 100 rpm agitation; without pH control; 50 ml of overnight culture in liquid RCM with *C. saccharoperbutylacetonicum* (OD_{600} range 1.5-1.8) inoculated into 500 mL of CV. The fermentation lasted for 48 h. Experiment ran in three biological replicates were performed in triplicate, with error bars showing SD values.

3.4. ABE fermentation in serum bottles using medium made from Eustigmatophyceae waste biomass

The viability of ABE fermentation is greatly dependent on the inexpensive fermentation medium used. Hence, use of waste products such as SMAB for ABE fermentation has the potential to reduce the cost tremendously and improve product sustainability. SMAB was obtained from Algaecytes®, following their lipid extraction procedure and was used for the growth of *C. saccharoperbutylacetonicum*: the medium was not autoclaved to mimic conditions that might be encountered during industrial ABE fermentation. In addition, the Eustigmatophyceae media used in Section 3.2 was also centrifuged, and therefore whole cells and larger cell fragments could be lost in this process, which may be a valuable source of polysaccharides to fuel ABE fermentation. It was therefore of interest to test Eustigmatophyceae media that had not been centrifuged/autoclaved. Due to the high turbidity of these media, it was not possible to measure the growth of *saccharoperbutylacetonicum* spectrophotometrically so bacterial cell proliferation was monitored using light microscopy.

Fermentation in non-autoclaved/non-centrifuged 10% Eustigmatophyceae whole cell medium supplemented with 1% glucose (EG-NT) was performed to investigate the ability of *C. saccharoperbutylacetonicum* to access intracellular material from the Eustigmatophyceae cells. During acidogenesis, the pH decreased from 5.92±0.03 to 4.09±0.04. During the first 24 h of fermentation, foam was

formed vigorously and reached the top of the serum bottle. Once the fermentation entered the solventogenic phase, the pH continued to decreased to 3.94±0.04 (72 h). The ORP reached the lowest point (-287±1 mV *vs.* NHE) at the end of the fermentation (72 h) (Figure 3.6A). Despite this obvious proliferation of *C. saccharoperbutylacetonicum,* the cell wall of the Eustigmatophyceae appeared to remain intact throughout the entire fermentation (Figure 3.7A).

In addition to non-autoclaved/non-centrifuged whole Eustigmatophyceae cells, it was of interest to investigate Eustigmatophyceae spent biomass that was prepared in a similar way. Eustigmatophyceae spent biomass (non-autoclaved/non-centrifuged) supplemented with 1% glucose (ESBG-NT) was used as a medium for ABE fermentation with *C. saccharoperbutylacetonicum* (Figure 3.6B). These data show that the ESBG-NT medium had a high initial pH (10.52±0.05), which was probably responsible for inhibition of *C. saccharoperbutylacetonicum* growth (Figure 3.7B). The pH had decreased to 9.96±0.01 at the end of acidogenesis and continued to decrease slightly during solventogenesis (9.89±0.01 at 72 h). No formation of foam was observed throughout the entire fermentation (Appendix Figure B1). The overall ORP was higher than other Eustigmatophyceae media and reached the lowest value at 2 h (-250±15 mV *vs.* NHE). Due to the high initial pH and lack of growth, it was therefore of interest to adjusted the pH of ESBG-NT medium to 6.0 (to produce ESBG-pH). The fermentation profile showed a very different behaviour (Figure 3.6C). The most significant differences were: (1) foam was formed vigorously; and (2) ethanol was detected. During the first 12 h (acidogenesis), the pH decreased from 6.00±0.00 to 4.87±0.08. The ORP reached the lowest point after 4 h (-347±5 mV *vs.* NHE). When viewed under the microscope, the ESBG-pH culture had the highest density of *C. saccharoperbutylacetonicum* (Figure 3.7C). During solventogenesis, the pH continued to decrease to 4.45±0.12 (72 h). Solvent analysis was performed after 24 and 48 h of fermentation. Organic acids and solvent production after 48 h of fermentation had reached as follows: acetic acid=2.11±0.17 g/L; butanoic acid= 0.73±0.08 g/L; acetone=0.40±0.15 g/L; ethanol=1.47±0.57; and butanol=1.40±0.40 g/L (Table 3.2).

The ESBG-pH medium (non-autoclaved and non-centrifuged, pH adjusted) clearly supported much higher levels of butanol compared to the

centrifuged/autoclaved ESBG medium in Section 3.2 (Table 3.1). Clearly, pH is a major factor but it was also of interest to examine whether the autoclaving also diminishes solvent yields. Hence, fermentations with autoclaved 10% Eustigmatophyceae spent biomass supplemented with 1% glucose, without centrifugation and pH adjusted to 6.0 (ESBG-ApH) were performed. The ESBG-ApH medium yielded a similar growth profile to ESBG-pH (Figure 3.6D). Acidogenesis lasted for 12 h, during which the pH decreased from 6.02±0.04 to 4.97±0.02. During solventogenesis, the pH continued to decrease slightly to 4.82±0.03 (at 72 h). Foam was observed throughout the entire fermentation (Appendix figure B1). The ORP reached the lowest point at 4 h of fermentation (-327±4 mV *vs.* NHE). In comparison with ESBG at 48 h of fermentation (Figure 3.3D), the main differences were: (1) the acidogenesis in ESBG-ApH was 12 h shorter than ESBG (12 *vs.* 24 h); (2) much lower level of acetic acid was detected in ESBG-ApH (2.73±1.44 *vs*. 4.39±1.59 g/L); (3) much higher level of butanoic acid was detected in ESBG-ApH (2.84±0.82 *vs*. 0.73±0.14 g/L); (4) Acetone (0.23±0.02 g/L) and butanol (1.44±0.09 g/L) were detected in ESBG-ApH; and (5) much lower level of ethanol was detected in ESBG-ApH (0.99±0.27 *vs*. 8.41±0.56 g/L) (Table 3.1 and 3.2). However, when compared with ESBG-pH, autoclaved ESBG-ApH did not further improve the solvent production, although the production of organic acids improved significantly (Table 3.2).

When comparing data for CV, CVG, EG, ESBG, ESBG-pH, and ESBG-ApH, at 48 h, the cultures grown on ESBG had the highest acetic acid levels (6.86±0.60 g/L). As for butanoic acid production, ESBG-ApH had the highest yield at 48 h, (2.84±0.82 g/L). Acetone production was not detected in CV only, which was not supplemented with 1% glucose. ESBG-ApH had the highest yield of acetone at 48 h (0.40±0.15 g/L at 48 h). EG had the highest butanol concentration (1.53±088 g/L). At 48 h of fermentation, ethanol production was only observed in cultures grown on ESBG (8.41±0.56 g/L), ESBG-pH (1.47±0.57 g/L) and ESBG-ApH (0.99±0.27 g/L) (Table 3.1 and 3.2). The total ABE solvent concentrations at 48 h in *C. saccharoperbutylacetonicum* cultures grown on different media were as follows (in order from low to high): ESBG (8.41 g/L) > TYE (4.07 g/L) > ESBG-pH (3.27 g/L) > ESBG-ApH (2.66 g/L) > EG (1.77 g/l) > CVG (1.20 g/L) > RCM (0.71 g/L) > CV in fermenter (0.15 g/L) > CV in serum bottle (0.09 g/L).

Interestingly, ESBG had the highest total ABE concentration, which is solely from the production of ethanol.

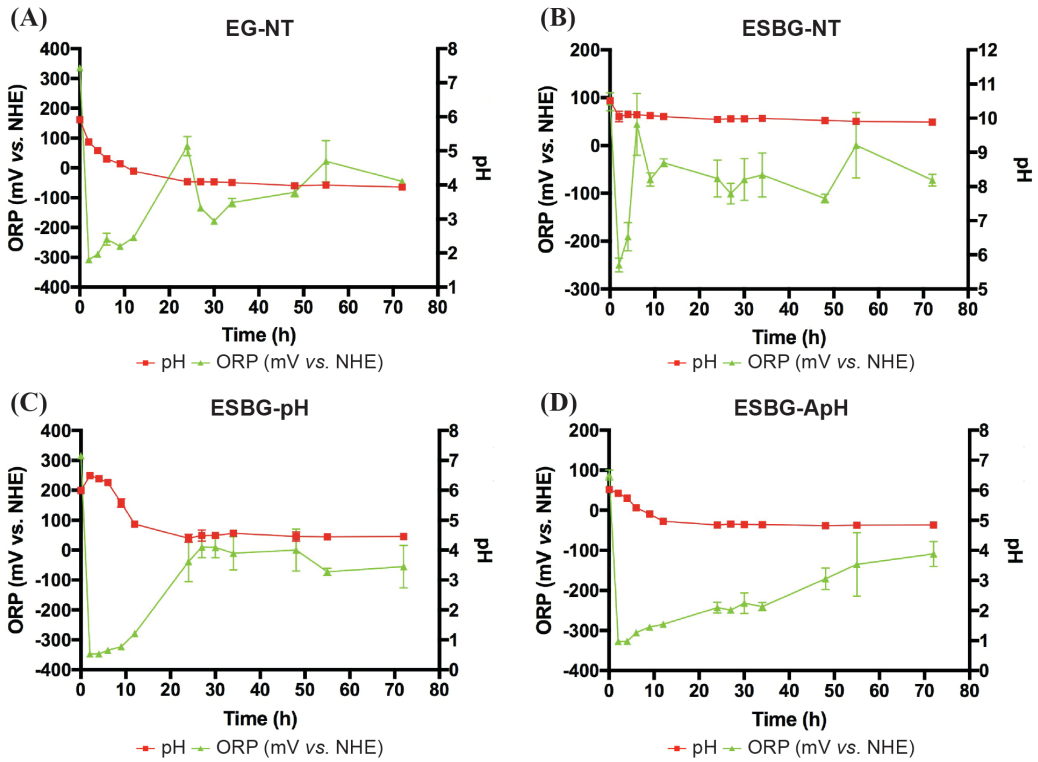

Figure 3.6. Fermentation profiles of *C. saccharoperbutylacetonicum* grown in 10% Eustigmatophyceae spent biomass. (A) EG-NT: 10% Eustigmatophyceae whole cell supplemented with 1% glucose, non-autoclaved and non-centrifuged; (B) ESBG-NT: 10% Eustigmatophyceae spent biomass supplemented with 1% glucose, non-autoclaved and non-centrifuged; (C) ESBG-pH: 10% Eustigmatophyceae spent biomass supplemented with 1% glucose; non-autoclaved, non-centrifuged and pH adjusted to 6.0; (D) ESBG-ApH: 10% Eustigmatophyceae spent biomass supplemented with 1% glucose, autoclaved, non-centrifuged and pH adjusted to 6.0; OD_{600} were not monitored due to the high amount of fragmented microalgal biomass as the medium was not subjected to centrifugation. Operating conditions; temperature: 32 °C, anaerobic environment without agitation; 5 ml of overnight culture in liquid RCM with *C. saccharoperbutylacetonicum* (OD_{600} range 1.5-1.8) inoculated into 50 mL of selected media. The fermentation lasted for 72 h. Performed in triplicate, with error bars showing SD values.

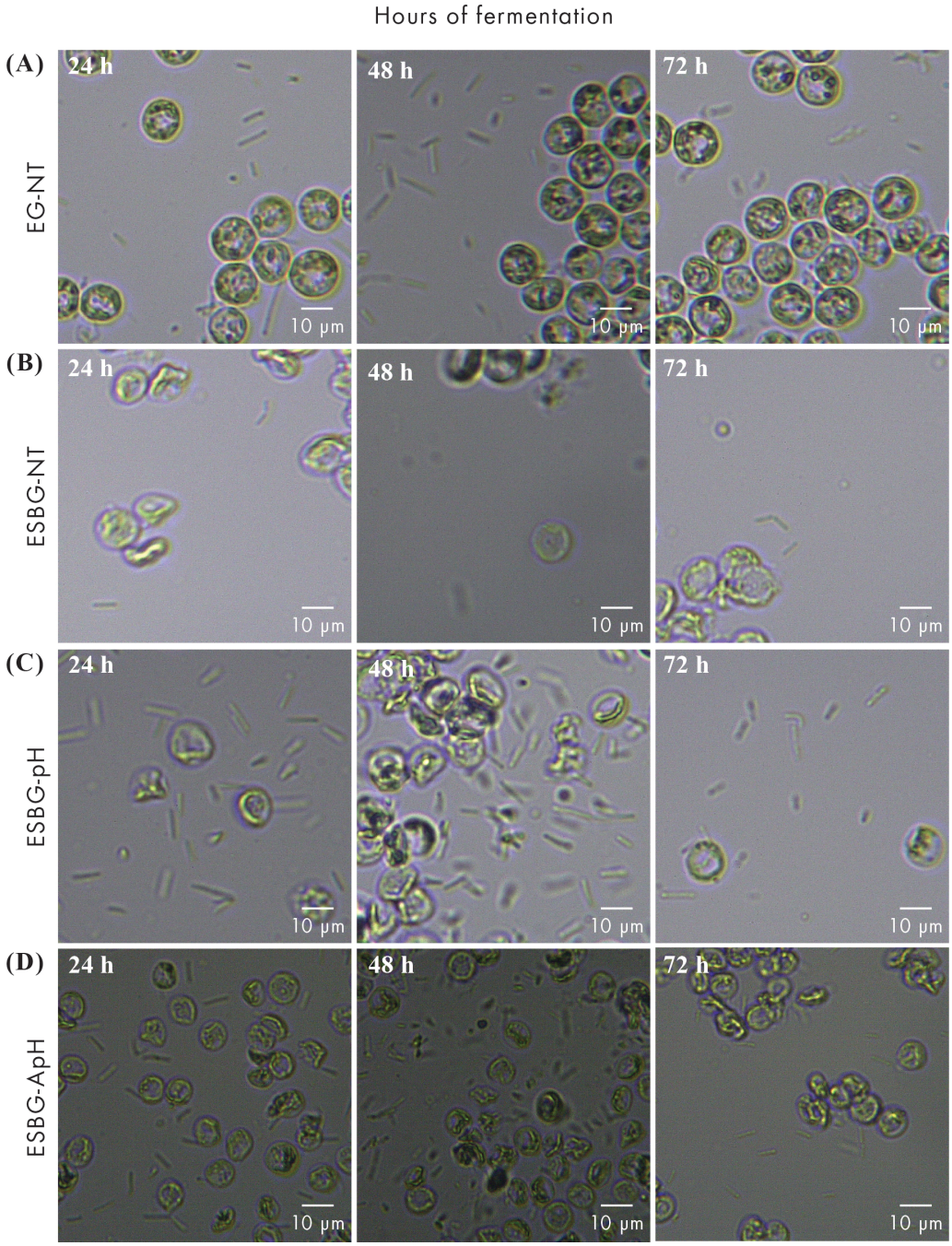

Figure 3.7. Direct observation of *C. saccharoperbutylacetonicum* when grown in Eustigmatophyceae-derived media. (A) EG-NT: 10% Eustigmatophyceae whole cell supplemented with 1% glucose, non-autoclaved and non-centrifugated. The cell wall of the Eustigmatophyceae whole cell remained intact during fermentation; (B) ESBG-NT: 10% Eustigmatophyceae spent biomass supplemented with 1% glucose, non-autoclaved and non-centrifuged. Low number of bacterial cells was observed; (C) ESBG-pH: 10% Eustigmatophyceae spent biomass supplemented with 1% glucose; non-autoclaved, non-centrifuged and pH adjusted to 6.0; (D) ESBG-ApH: 10% Eustigmatophyceae spent biomass supplemented with 1% glucose, autoclaved, non-centrifuged and pH adjusted to 6.0. The magnification was x400. Clostridium cells have a green hue when grown in microalgal medium.

Table 3.2. Organic acids and solvent production by *C. saccharoperbutylacetonicum* after 24 and 48 h of fermentation in ESBG-pH and ESBG-ApH.

Media	Acetic acid (g/L±SD)		Butanoic acid (g/L±SD)	
	24 h	48h	24 h	48h
ESBG-pH	0.39±0.14	2.11±0.13	0.17±0.16	0.73±0.14
ESBG-ApH	2.73±1.44	3.42±1.08	2.00±1.13	2.84±0.82

Media	Acetone (g/L±SD)		Butanol (g/L±SD)		Ethanol (g/L±SD)	
	24 h	48 h	24 h	48 h	24 h	48 h
ESBG-pH	0.31±0.13	0.40±0.26	0.89±0.32	1.40±0.69	0.57±0.28	1.47±0.99
ESBG-ApH	0.22±0.01	0.23±0.02	1.20±0.27	1.44±0.09	1.11±0.40	0.99±0.27

Data are averages of three biological controls, and error are SD values.
The solvent production of EG-NT and ESBG-NT were not analysed by GCMS.

3.5. Development of butanol-tolerant strains of *C. saccharoperbutylacetonicum* and *E. coli*

During solventogenesis, cellular metabolism can be perturbed when the butanol concentration reaches an inhibitory level (greater than 15 g/L). Accumulation of butanol disrupts membrane stability as well as inhibiting the transportation of nutrients into the cells. *E. coli* is a good candidate for solvent production due to rapid growth, facultative anaerobic nature, lack of spores, and ease of genetic modification (Jones and Woods 1986; Zheng *et al.* 2009). On the other hand, clostridial species remain a preferable organism for ABE fermentation as they perform this naturally and can utilise a wide range of lignocellulosic carbon sources (Keis *et al.* 2011). Given that butanol toxicity is the major limiting factor for solvent production, it was of interest to use genetic approaches to improve butanol tolerance in *E. coli* and *C. saccharoperbutylacetonicum.*

It has previously been reported that overexpression of the *focA* transporter from *E. coli*, which has a primary role in formate transport, enhances butanol tolerance in the native host (Reyes *et al.* 2011). The current work identified the *fdhC* gene of *C. saccharoperbutylacetonicum* as a homologue of *focA* and is therefore a focus of the current study. Amino sequence alignment of *E. coli* FocA and *C. saccharoperbutylacetonicum* FdhC proteins suggests a potential common function (Figure 3.8).

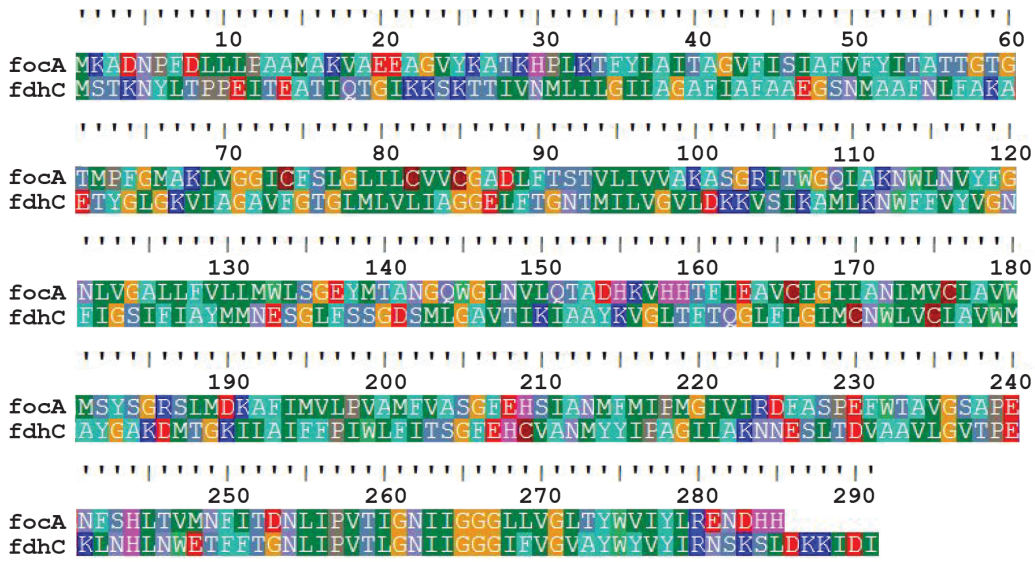

Figure 3.8. Alignment of *focA* and *fdhC* amino sequences. *focA* and *fdhC* amino sequence were taken from *E. coli* and *C. saccharoperbutylacetonicum,* respectively. Sequences were aligned using Bioedit free software (v7.2.5). Amino acids are coloured to allow differences in amino acid sequence to be easily visualised. The sequence identity is 35%, which was calculated by using the BLASTp.

The *fdhC* and *focA* genes from *C. saccharoperbutylacetonicum* and *E. coli* K-12, respectively, were amplified by using the Q5® high-fidelity polymerase. A fragment of the *E.coli*/clostridium shuttle vector pMTL83353 was also amplified as the plasmid backbone for Gibson assembly. This vector incorporates a ferredoxin promoter that is designed for high levels of expression in clostridium species. The primers were designed with 18-25 bp overhangs at both 5′ and 3′ ends for annealing of plasmid and insert, and codons for a 6X-Histag were included at the 3′ end of the *fdhC* and *focA* genes. An overview of the Gibson reaction process is shown in Figure 3.9, and detailed plasmid maps of the desired pMTL83353-*focA* and pMTL83353-*fdhC* expression vectors are shown in Figure 3.10.

Figure 3.11A lane 1 shows that PCR amplification of *focA* with primers FocFWD (MS primer #360) and FocRev (MS primer #361) resulted a band around 1,000 bp, which was close to the expected size of 936 bp. Lane 2 shows that PCR amplification of *fdhC* with primers FdhCGAFwd (MS primer #333) and FdhCGARev (MS primer #334) resulted a band around 1000 bp, which was close to the expected size of 915 bp. In Figure 3.11B lane 1 shows the PCR of pMTL83353 with *focA* overhangs, which was amplified with primers 83353GA_focA_F1 (MS primer #364) and 83353GA_focA_R1 (MS primer #365). This yielded a band size of approximately 5,000 bp, which was closed to the

expected size of 4,758 bp. Lane 2 shows the PCR of pMTL83353 with *fdhC* overhangs, which was amplified with primers 83353GA_fdhC_F1 (MS primer #366) and 83353GA_fdhC_R1 (MS primer #367). This resulted in a band size around 5,000 bp, which was close to the expected size of 4,755 bp.

Following Gibson assembly reactions, transformations of super-competent *E. coli* cells were performed using the heat shock method (Section 2.7.6.). To screen colonies for the desired plasmids, colony PCR was performed with primers 83353_cPCR_SCRN_FWD (MS primer #368) and 83353_cPCR_SCRN_REV (MS primer #369) that bind to the pMTL83353 vector with 215 bp upstream and 218 downstream from the insert. Band sizes of 1,371 bp and 1,392 bp were expected for colonies containing pMTL83353-*focA* and pMTL83353-*fdhC,* respectively. Whereas the control colony PCR with vector alone (pMTL83353) gave the expected band size of 751 bp (Figure 3.12), the screening process did not identify any plasmids containing *focA* or *fdhC*.

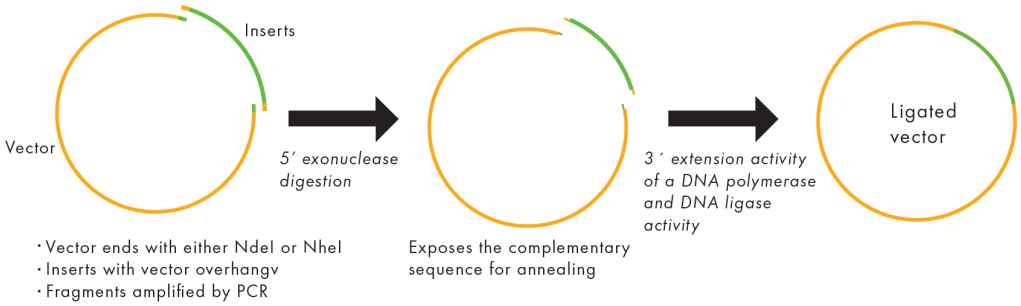

Figure 3.9. Overview of Gibson assembly for cloning of *focA* and *fdhC* genes. 4 primers were designed for each Gibson assembly. The primers were designed with 18-25 bp overhangs at the 5′ and 3′ ends. Gibson assembly performed in a single tube reaction, in which T5 exonuclease creates single-strand DNA 3′ overhangs by digesting the DNA 5′ end and exposing the complementary sequence for annealing. Then, Phusion DNA polymerase fills in the gaps on the annealed regions. Taq DNA ligase then seals the nick and covalently links the DNA fragments together. The reactions were incubated at 50 °C for 1 h (adapted from Gibson *et al.* 2009).

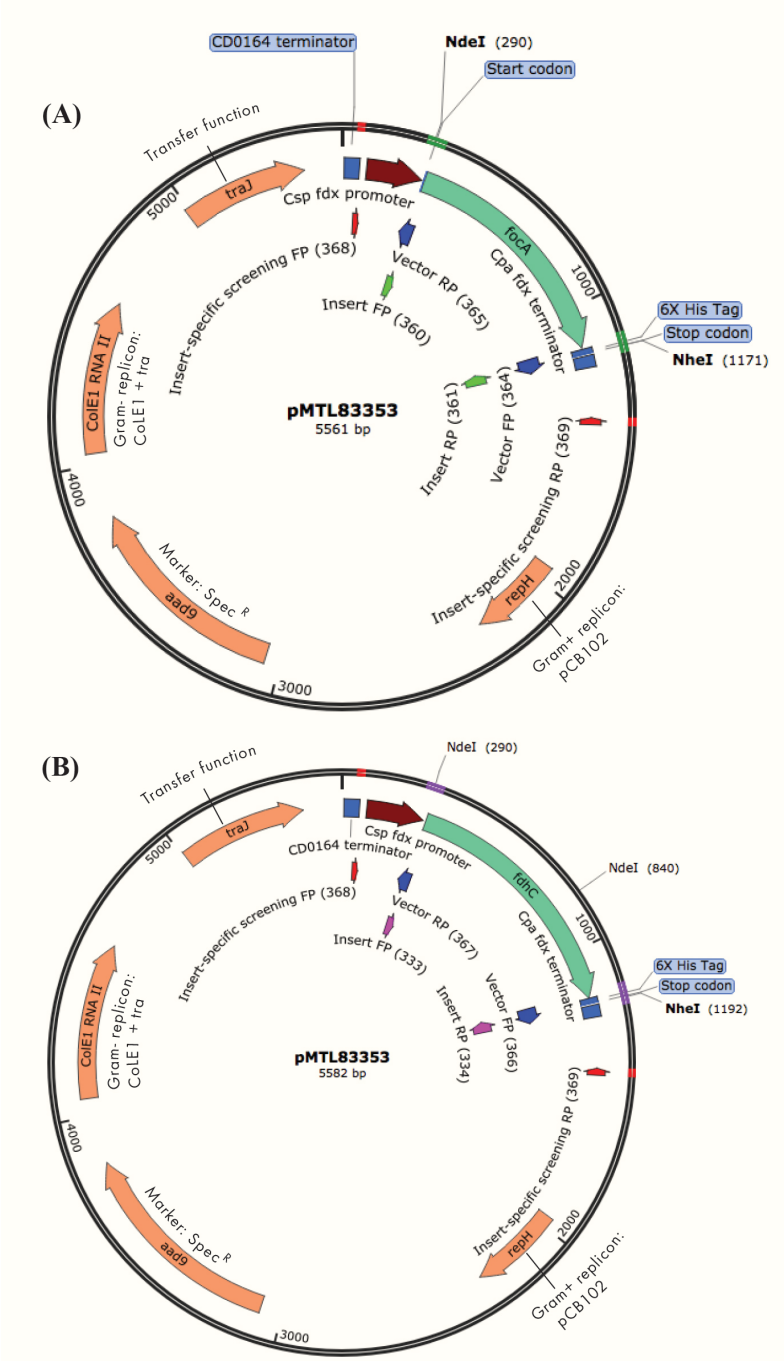

Figure 3.10. Vector maps of the desired plasmid constructs for *focA* and *fdhC*. (A) Map of pMTL83353-*focA* with 6X-Histag is shown, with binding sites for the cloning primers FocFWD (MS primer #360) and FocRev (MS primer #361), 83353GA_focA_F1 (MS primer #364) and 83353GA_focA_R1 (MS primer #365); (B) Map of pMTL83353-*fdhC* with 6X-Histag is shown, with binding sites for cloning primers FdhCGAFwd (MS primer #333) and FdhCGARev (MS primer #334). pMTL83353 with *fdhC* overhang was amplified by primer 83353GA_fdhC_F1 (366) and 83353GA_fdhC_R1 (MS primer #367). Primers 83353_cPCR_SCRN_FWD (MS primer #368) and 83353_cPCR_SCRN_REV (MS primer #369) were used for cPCR screening with 215 bp upstream and 218 downstream from the insert. The antibiotic resistance marker for pMTL83353 is spectinomycin.

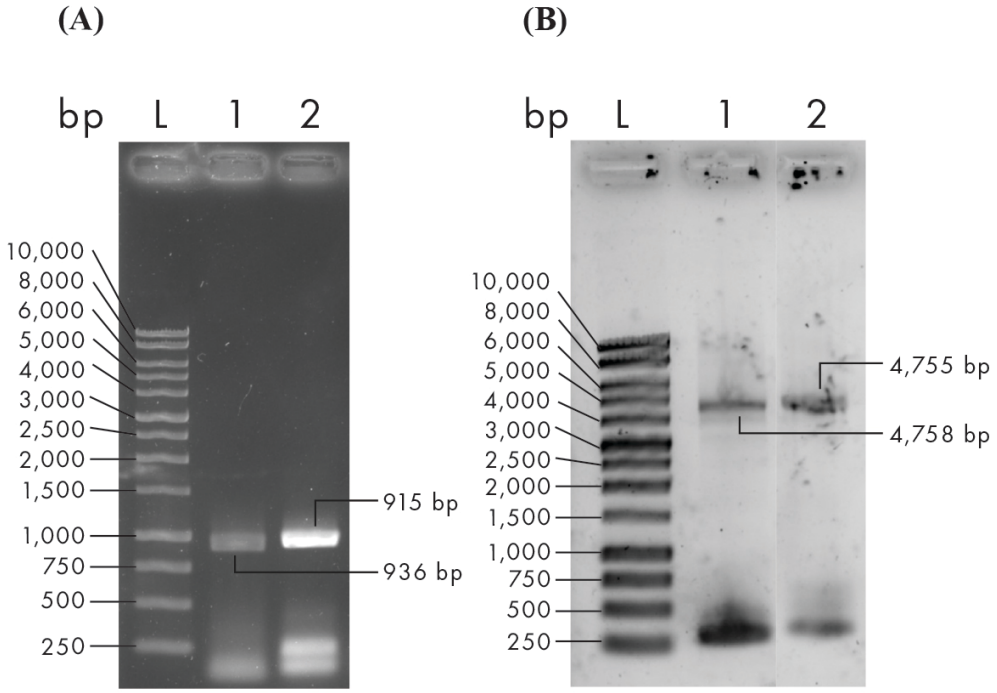

Figure 3.11. PCR amplification of DNA fragments for Gibson assembly. (A) Lane 1: *focA* with pMTL83353 overhang (MS primer #915 bp) was amplified with primers FocFWD (MS primer #360) and FocRev (MS primer #361); Lane 2: *fdhC* with pMTL 83353 overhang (MS primer #936 bp) was amplified with primers FdhCGAFwd (MS primer #333) and FdhCGARev (MS primer #334); (B) Lane 1: pMTL83353 with *focA* overhang (4,758 bp) was amplified by primer 83353GA_focA_F1 (364) and 83353GA_focA_R1 (MS primer #365) ; Lane 2: pMTL83353 with *fdhC* overhang (4,755 bp) was amplified by primer 83353GA_fdhC_F1 (MS primer #366) and 83353GA_fdhC_R1 (MS primer #367). Ladder: 1kb ladder (Promega). Gibson assembly fragments were amplified using Q5® High-fidelity 2X master mix. 5 µL of PCR reactions were loaded onto 1.0% agarose gel and separated by electrophoresis in 1X Tris-acetate buffer (80 V, 300 mA, 40 min).

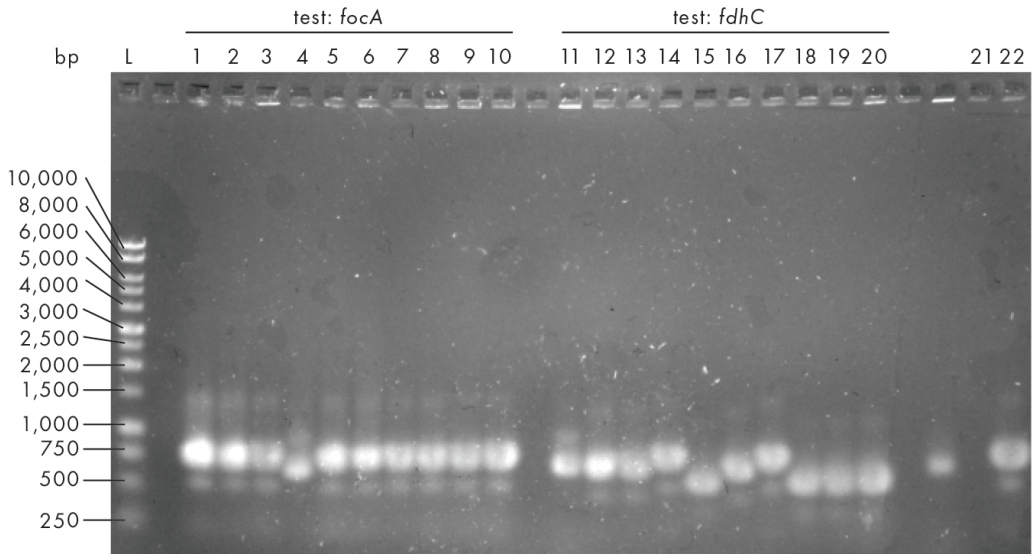

Figure 3.12. Colony PCR screen of Gibson Assembly transformants. Colony PCR was performed using screening primers 83353_cPCR_SCRN_FWD (MS primer #368) and 83353_cPCR_SCRN_REV (MS primer #369) to screen for transformants with either *focA* or *fdhC* incorporated into pMTL83353. Lanes 1 to 10: colonies obtained for *focA* transformation (desired transformant should show a band size of 1,371 bp). Lanes 11 to 20: colonies found for *fdhC* transformation (desired transformant should show a band size of 1,392 bp). Lane 21: competent cells only; Lane 22: vector (pMTL83353) only, which gave a band close to the expected band size of 751 bp. A faint band around 1,500 bp can be seen in vector only as well as transformants.

Chapter 4

Discussion

The overarching goals of this study were to assess the efficacy of waste algal biomass as a feedstock for ABE fermentation, and to enhance butanol tolerance in *Clostridium* species. However, the initial challenge faced during this project was to develop a technique to measure the growth of Clostridium in turbid cultures. After considerable optimisation, a protocol for dilution and spectrophotometric measurement was devised (Figure 3.1) and comparison with duplicate cultures in non-turbid RCM medium (Figure 3.2A-B) confirmed that this approach can be used to accurately measure the cell density of *C. saccharoperbutylacetonicum* cultures up to 5 x 10^9 CFU/mL (i.e. up to 5 x 10^8 cells in the cuvette with a 1:10 dilution). Increases in bacterial cell density were verified using light microscopy (Figure 3.2C), and this approach was then used to monitor the growth of *C. saccharoperbutylacetonicum* in growth media made from microalgal biomass.

Initial growth experiments (Figure 3.3) with media made from microalgal cells (autoclaved and centrifuged) showed that while the nutrients transferred with the RCM inoculum could support bacterial growth independently (i.e. MQ water medium), the media produced from *C. vulgaris* (i.e. CV) provided a significant contribution to the final bacterial biomass. Furthermore, supplementation with 1% glucose provided an additional increase the growth rate and final biomass of *Clostridium* cells (i.e. CVG *vs.* CV), as previously observed for clostridial growth where addition of low levels of glucose have also been shown to aid the solvent production (Ellis *et al.* 2012; Castro *et al.* 2015). Indeed, addition of 1% glucose had a dramatic effect upon butanol production in CV medium (Figure 3.4). Similar experiments with medium produced from Eustigmatophyceae cells supplemented with 1% glucose (EG) did support growth much beyond the MQ water negative control experiments. Waste algal biomass from Algaecytes (following oil extraction) was also tested as a feedstock using this approach: this glucose-supplemented medium did provide a modest contribution to clostridial biomass, although it could be argued that this growth effect was due to the 1% glucose in this medium.

As the medium produced from *C. vulgaris* seem to be the most promising in terms of supporting bacterial growth, this CV medium (without glucose) was used for growth of 500 mL cultures in 1 L fermentation vessels to better approximate conditions found in industrial ABE fermentation. This approach did support the growth of *C. saccharoperbutylacetonicum* (Figure 3.5A), although like the serum bottle experiments, low yields of butanol were obtained in the absence of exogenous glucose (Figure 3.5B).

The experiments described above with autoclaved/centrifuged microalgal media did not yield significant levels of butanol (Table 3.1) compared to the TYE positive control (3.74 g/L at 48h), although the ESBG medium did produce an impressive 8.41 g/L of ethanol after 48 h but without the production of butanol and acetone. Besides, the ESBG medium also produced the highest level of acetic acid, which suggested that the reassimilation of the accumulated acetic acid was solely converted to ethanol. This is supported by literature, which reported that the reassimilation of acetic acid is obligate for the production of ethanol (Richter *et al.* 2013). The absence of butanol and acetone in this sample is consistent with previous studies where high ethanol levels decrease acetone and butanol production (Brosseau *et al.* 1985). In an attempt to improve solvent yields for medium produced using Eustigmatophyceae cells, the protocol for preparation of growth medium was changed. Autoclaving and centrifuging was no longer performed to make the process more industrially relevant and to avoid the loss of whole cells or cell fragments that could provide complex polysaccharides for ABE fermentation. Also, the pH of the medium was adjusted to 6.0 to avoid alkaline conditions observed in ESBG-NT medium previously. Furthermore, to put this work into context, solvent yields are compared to literature values in Table 4.1. This shows that untreated ESBG-pH medium (i.e. non-autoclaved, non-centrifuged, pH adjusted) was found to be optimal microalgal medium used in the current study for ABE production, with elevated nutrient availability relative to 'pretreated media' presumably being a major factor. The influence of external pH is also a known key factor in in influencing solvent yields for ABE fermentation (Al-Shorgani *et al.* 2015; Keis *et al.* 2001): this is consistent with this study, with improved bacterial growth and better solvent yields found in both pH adjusted media (ESBG-pH and ESBG-ApH). In comparison to other studies, relatively low butanol yields were

obtained in this study (Table 4.1). This could be explained by low glucose concentrations, where more than 15 g/L of glucose is optimal during acidogenesis as demand for ATP is high (Oshiro *et al.* 2010). Furthermore, clostridia can utilise a range of low molecular weight carbohydrates such as glucose, fructose, sucrose, lactose, mannose and dextrin, while the main carbohydrates found in microalgae are galactose and xylose (Jones and Woods 1986). The modest growth a low solvent yields may also be influenced by carbon catabolite repression (CCR), where clostridial species rapidly utilise glucose and repress the catabolism of alternative sugars such as xylose and galactose (Essalem and Mitchell 2016; Noguchi *et al.* 2013). Indeed, Noguchi *et al.* (2013) reported that CCR was observed in *C. saccharoperbutylacetonicum* when grown using mixed sugar carbon sources such as glucose and xylose. Furthermore, Xiao *et al.* (2011) reported that *C. acetobutylicum* utilises xylose poorly due to two main reason: (1) weak affinity of the transporter (encoded by XylT) for the xylose substrate; and (2) weak xylose-dissimilation enzymes activity (e.g. xylose isomerase and xylulose kinase, which are encoded by *xylA* and *xylB*). One of the reasons *C. saccharoperbutylacetonicum* was used in this study was its ability to utilise a wide range of carbon source, including xylose. However, xylose and other carbon sources found in microalgae are stored within the cells and may not be readily accessible for *C. saccharoperbutylacetonicum* to utilise. Future work in this area may focus on the phosphotransferase system (PTS), which is the environmental sensor for CCR and phosphorylates sugar substrates such as glucose. Hence, by manipulating the PTS system one could potentially engineer clostridial species to preferentially utilise alternative sugars such as xylose (Mitchell 2015).

Another factor for low butanol yields in the current study could be due to the low acetone levels (0.20 - 0.40 g/L at 48 h). Diminished acetone production could be caused by sub-optimal pH of the cultures. The optimum pH for enzyme *adc*, which is responsible for production of acetone is approximately pH 5 (Jones and Woods 1986), and this study also reported that high yields of butanol are impossible without the production of acetone. Furthermore, Jang *et al.* (2012b) reported that a decrease in acetone production during solventogenesis minimised the production of butanol in *C. acetobutylicum*. Studies have been performed to

increase acetone production, for example through overexpression of enzymes involved in acetone production (encoded by *adc, cfA,* and *cfB*) (Zheng *et al.* 2009).

Concentrations of organic acids are a crucial factor in influencing solvent yields during ABE fermentation, as acetate and butyrate are important intermediates in this metabolic pathway. Furthermore, acetic acid aids cell survival by increasing the pH buffering capacity, and also enhances CoA-transferase activity, which is responsible for the conversion of aceto-acetyl CoA to acetoacetate which is subsequently converted to acetone by acetoacetate decarboxylase (Figure 1.2) (Chen and Blaschek 1999; Cho *et al.* 2012). Reports have also shown that an increase in acetic acid concentration from 3.7 to 9.7 g/L increases solvent production by 21% (44%, 6% and 42% for butanol, acetone and ethanol, respectively). However, 11.7 g/L of acetic acid greatly reduced solvent production (Cho *et al.* 2012; Maddox *et al.* 2000). This is consistent with the results obtained in this study, in which both ESBG-pH and ESBG-ApH produced much more acetic acid at 48 h compared to CV and CVG (ESBG-pH: 2.11±0.13 and ESBG-ApH: 3.42±1.08 g/L *vs.* CV: 0.28±0.13 and CVG: 0.56±0.19 g/L). Consequently, butanol production in EG and ESBG-pH was higher than for CV and CVG (EG: 1.40±0.69 and ESBG-pH: 1.44±0.09 g/L *vs.* CV: 0.09 and CVG: 1.00 g/L). Acetic acid levels measured in this study were lower than the minimum amount (3.7 g/L) of acetic acid previously shown to improved butanol production.

Physical factors such as low agitation (100 rpm) together with head-space pressure (100 kPa) using hydrogen gas have previously been shown to improve butanol productivity (Doremus *et al.* 1985). This could potentially contribute to the low butanol yields found in this study, as the serum bottle experiments were not subjected to agitation. However, the microalgal media in the fermenter was agitated with 100 rpm, which did not result in a significant improvement in solvent yield.

Another factor that could result in low butanol yields is the presence of high levels of nitrogenous compounds (e.g. amino acids) from microalgal cells (Wang *et al.* 2016). Previous studies report that low concentrations of nitrogen-containing compounds are optimal for solvent production (Jones and Woods 1986; Maddox 1989). Furthermore, Roos *et al.* (1985) demonstrated that the rate of solvent production increases when the ratio of nitrogen source:carbon source (glucose) decreases. The total protein content per dry weight of *C. vulgaris* is 42-58%, and the

protein content found in Eustigmatophyceae is approximately 43%. Wang *et al.* (2016) reported that butanol production started to decrease when protein concentrations in the medium exceed 500 mg/L, which could be provided by approximately 1 g/L dry microalgal biomass. The 10% microalgal medium used in this study would be equivalent to approximately 100 g/L protein, which could potentially cause detrimental effects on cell growth and solvent production. To prevent the negative effect caused by excess protein/amino acids, alkali treatment (wash with 1% NaOH, then neutralised with 3% H_2SO_4) could be employed in future (Wang *et al.* 2016).

Another obvious reason for the low solvent yields compared to literature values (Table 4.1) is that previous studies have employed extensive pre-treatment of the growth media. Treatments such as acid hydrolysis, alkaline treatment, enzymatic digestion, centrifugation and microwaving were employed to increase fermentable sugar yields from microalgae and to remove protein-related materials found in microalgae. Furthermore, some of the microalgal media used in other studies were supplemented either with TYE medium, T6 medium, or enzymes to further improve the butanol yield. As for this study, pre-treatment of media was deliberately avoided where possible so as to produce a baseline assessment of each growth medium, and to attempt avoidance of time-consuming and expensive processes from the outset. Future experiments might include the development of more efficient strategies to remove proteinaceous components from microalgal medium, and cheaper methods for digestion of the microalgal cell wall.

Table 4.1. Comparison of solvent production by clostridial species using microalgal biomass as the feedstock.

Microalgal medium	Bacterial strain	Total ABE production in g/L (acetone: butanol: ethanol)	Growth Vessel	Hours of fermentation (h)	Source
10% untreated Eustigmatophyceae ALG01-CL1 spent biomass supplemented with 1% glucose[a] (ESBG-ApH)	*C. saccharoperbutylacetonicum* DSM 14923	2.66 (0.23: 1.44: 0.99)	serum vial	48	This study

10% untreated Eustigmato-phyceae ALG01-CL1 spent bio-mass supple-mented with 1% glucose[b] (ESBG-pH)	*C. saccharoper-butylacetonicum* DSM 14923	3.27 (0.40: 1.40: 1.47)	serum vial	48	This study
10% untreated Eustigmato-phyceae ALG01-CL1 spent bio-mass supple-mented with 1% glucose[c] (ESBG)	*C. saccharoper-butylacetonicum* DSM 14923	8.41 (0.00: 0.00: 8.41)	serum vial	48	This study
10% pretreated Eustigmato-phyceae ALG01-CL1 whole cell supplemented with 1% glucose[c] (EG)	*C. saccharoper-butylacetonicum* DSM 14923	1.77 (0.24: 1.53: 0)	serum vial	48	This study
10% pretreated *C. vulgaris* FACHB-31 supplemented with 1% glucose[d] (CVG)	*C. saccharoper-butylacetonicum* DSM 14923	1.20 (0.20: 1.00: 0)	serum vial	48	This study
10% pretreated *C. vulgaris* FACHB-31[d] (CV)	*C. saccharoper-butylacetonicum* DSM 14923	0.15 (0: 0.15: 0)	fer-menter	48	This study
10% pretreated *C. vulgaris* FACHB-31[d] (CV)	*C. saccharoper-butylacetonicum* DSM 14923	0.20 (0: 0.20: 0)	serum vial	72	This study
10% pretreated mixed microalgae with T-6 medi-um[e]	*C. saccharoper-butylacetonicum* N1-4	5.23 (0.96: 3.74: 0.53)	serum vial	NA	Castro *et al.* 2015
300 g/l of *C. so-rokiniana* CY1 supplemented with 400 mg-N/L of sodium ammo-nia; 360 mg/l of peptone and 0.175 mg /l of resazurin[f]	*C. acetobutylicum* ATCC 824	6.32 (NA: 3/86: NA)	glass bottle	NA	Cheng *et al.* 2015
10% pretreated wastewater algae[g]	*C. saccharoper-butylacetonicum* N1-4 (ATCC 27021)	2.74 (0.45: 2.26: 0.03)	serum vial	96	Ellis *et al.* 2012
10% pretreated wastewater algae supplemented with 1% glucose[g]	*C. saccharoper-butylacetonicum* N1-4 (ATCC 27021)	7.27 (1.36: 5.61: 0.30)	serum vial	96	Ellis *et al.* 2012

Table 4.1. (Continued)

10% pretreated wastewater algae supplemented with 10 U of endo-1,4- β xylanlase and 100 U of endo-1,4- β-D cellulase[g]	*C. saccharoperbutylacetonicum* N1-4 (ATCC 27021)	9.75 (1.43: 7.79: 0.53)	serum vial	96	Ellis *et al.* 2012
10% pretreated *C. vulgaris* UTEX 2714 with TYE medium[h]	*C. saccharobuytlicum* DSM 13864	12.44 (3.45: 8.05: 0.94)	shaker flask	36	Gao *et al.* 2016
Pretreated *C. vulgaris* JSC-6[i]	*C. acetobutylicum* ATCC824	19.65 (4.37: 13.1: 2.18)	serum vial	NA	Wang *et al.* 2016

Table 4.1. (Continued)

[a] pH adjusted to 6 with 5M HCl; autoclaved (121 °C at 15 psi for 20 min) and non-centrifuged

[b] pH adjusted to 6 with 5M HCl; non-autoclaved and non-centrifuged

[c] Autoclaved (121 °C at 15 psi for 20 min) and centrifuged (5000 rpm for 15 min)

[d] Broken cell wall; centrifuged (5000 rpm for 15 min) and autoclaved (121 °C at 15 psi for 20 min)

[e] Mixed microalgae= *Scenedesmus, Chlorella, Ankistrosdemus, Micromonas,* and *Chlamydomonas.* Treated with acid hydrolysis using 1M H_2SO_4 for 120 min at 80-90 °C, followed by centrifugation (1200 rpm), neutralization ($Ca(OH)_2$), second centrifugation (1200 rpm for 30 min) and sterilization (120 °C for 15 min)

[f] Mixed with methanol and microwaved for 10 min. Then, subjected to 2% H_2SO_4 acid hydrolysis, followed by 2% NaOH; each step was heated at 121 °C for 60 min; pH was maintained above 4.5 with $CaCO_3$

[g] Treated with 1M H_2SO_4 followed by 5M NaOH; each step was heated at 90 °C for 30 min

[h] Subjected to 2% H_2SO_4 acid hydrolysis followed by autoclaving (121 °C at 15 psi for 20 min) and neutralized to a pH of 6 with 4M NaOH. Then, centrifuged at 3500 rpm

[i] Treated with cellulose-hydrolysing enzyme. Then, treated with 1% NaOH followed by centrifugation (9000 rpm for 10 min), rinsed several times and finally treated with 3% H_2SO_4. Treatments were carried out under autoclaved conditions (121 °C for 20 min).

NA= not available

The level of butanol detected in all growth media used in this study was lower than the minimal level shown to elicit inhibition growth and solvent production (> 5.0 g/L) (Jones and Woods 1986). Hence, the low butanol yields in this study were not caused by the toxicity of butanol. However, maximising the yield of butanol is clearly important for industrial ABE fermentation, so it was therefore of interest to engineer *C. saccharoperbutylacetonicum* to tolerate higher levels of butanol. Given that overexpression of the *E. coli* FocA transporter had previously been shown to elicit butanol tolerance in the native host (Reyes *et al.* 2011), the current project aimed to clone *focA* and *fdhC*, a homologue from *C. saccharoperbutylacetonicum*, for subsequent overexpression in *E. coli* and *Clostridium* species. The cloning strategy was to insert *focA* and *fdhC* downstream of the ferredoxin promoter of the pTML83353 vector (Chain Biotech 2010) via Gibson assembly (Gibson *et al.* 2009). PCR of the vector and insert fragments was successfully

performed and following Gibson assembly reaction and transformation into *E. coli* DH5α, several colonies were obtained for both ligations. However, PCR screening showed that none of the transformants contained the *focA* or *fdhC* genes: the majority were consistent with empty pTML83353 vector. One explanation for the false positives is that a small amount of pTML83353 template DNA present during PCR amplification of the vector fragment was transferred to the Gibson reaction. A potential explanation for the lack of positive clones containing *focA* or *fdhC* could be that high levels of transporter overexpression from the relatively high copy number plasmid (~15-20) and strong and constitutive promoter P_{fdx} may exert a toxic burden over the host cells. Hence, future cloning strategies might include using a different vector such as pMTL84422, which harbours a p15a Gram- replicon (copy number ~10) and a weaker promoter, $P_{thl.}$ This combination should result a lower expression, which might help with the cloning of these transporters.

If the cloning of *focA* and *fdhC* would have been successful, the butanol tolerance of cells will evaluate by growing in the RCM containing various concentrations of butanol (0, 5, 9, 13 and 17 g/L) then follow by measuring the OD_{600}.

Chapter 5

Conclusion

The use of inexpensive feedstocks and high butanol titre are major considerations for industrial butanol production by ABE fermentation. This study demonstrated that butanol could be successfully produced by *C. saccharoperbutylacetonicum* grown on media produced from the microalgae *C. vulgaris* and Eustigmatophyceae. While the butanol yields from this work were low (< 5 g/L), this work provides a benchmark to test the efficacy of largely untreated growth media for ABE fermentation. Interestingly, the most promising growth medium in terms of solvent production was ESBG-pH (3.27 g/L solvent), which produced all three solvents with the highest total ABE production. It was produced from an industrial waste product of no real value. Future work on these microalgal growth media will focus upon pre-treatments to minimise protein content and maximise sugar release to optimise solvent production towards economically-feasible levels.

References

Abdehagh, N., Tezel, F. H. and Thibault, J. (2014) Separation techniques in butanol production: Challenges and developments. *Biomass and Bioenergy.* 60: 222-246.

Abomohra, A. E., Jin, W., Tu, W., Tu, R., Han, S. F., Eid, M. and Eladel, H. (2016) Microalgal biomass production as a sustainable feedstock for biodiesel: Current status and perspectives. *Renewable and Sustainable Energy Reviews.* 64: 596-606.

Alalibo, T. T., Purwantini, E., Lomax, J., Setubal, J. C., Muckhopadhyay, B. and Tyler, B. M. (2014) Genetic resources for advanced biofuel production described with the Gene Ontology. *Frontiers in Microbiology.* 581 (5): 1-17.

Al-Shorgani, N.K., Kalil, M.S. and Yusoff, W.M. (2011) The effect of different carbon sources on biobutanol production using *Clostridium saccharoperbutylacetonicum* N1-4. *Biotechnology.* **10** (3): 280-285.

Al-Shorgani N.K., Kalil, E. A. Yussoff, W. M. (2012) Bioconversion of butyric acid to butanol by *Clostridium saccharoperbutylacetonicum* N1-4 in a limited nutrient medium. *Bioenergy Research.* 5: 287-293.

Al-Shorgani, N.K., Shukor, H. Abdeshahian, P., Nazir, M. Y., Kalil, M. S., Hamid, A. A. and Yussoff, W. M. (2015) Process optimization of butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564 using palm oil mill effluent in acetone–butanol–ethanol fermentation. *Biocatalysts and Agricultural Biotechnology.* 4: 244-249.

Bachmann, B. J. (1996) Derivatives and genotypes of some mutant derivatives of *Escherichia coli* K-12. *Escherichia coli and Salmonella: Cellular and Molecular Biology. ASM Press.* 2460-2488.

Bao, G., Wang, R., Zhu, Y., Dong, H., Mao, S., Zhang, Y., Chen, Z., Li, Y. and Ma, Y. (2011) Complete genome sequence of *Clostridium acetobutylicum* DSM 1731, a solvent-producing strain with multireplicon genome architecture. *Genome Announcements.* 193 (**18**): 5007-5008.

Behbehani, M. J., Jordan, H. V. and Santoro, D. L. (1982) Simple and Convenient Method for Culturing Anaerobic Bacteria. *Applied and Environmental Microbiology.* 43(**1**) 255-256.

Berezina, O. V., Brandt, A., Yarotsky, S., Schwarz, W. H., and Zverlov, V. V. (2009) Isolation of a new butanol-producing *Clostridium* strain: High level of hemicellulosic activity and structure of solventogenesis genes of a new *Clostridium saccharobutylicum* isolate. *Systematic and Applied Microbiology*. 32: 449-459.

Beyer, L. Doberenz, C., Falke, D., Hunger, D., Suppmann, B. and Sawers (2013) Coordination of FocA and Pyruvate Formate-Lyase Synthesis in *Escherichia coli* demonstrates preferential translocation of formate over other mixed-acid fermentation products. *Journal of bacteriology.* 195 (**7**): 1428-1435.

Brosseau, J. D. Yan, J. Y. and Victor Lo, K. (1985) The relationship between hydrogen gas and butanol production by *Clostridium saccharoperbutylacetonicum*. *Biotechnology and Bioengineering.* 28: 305-310.

Castro, Y. A., Ellis J., Miller, C. D. and Sims, R. (2015) Optimization of wastewater microalgae scarification using dilute acid hydrolysis for acetone, butanol, ethanol fermentation. *Applied Energy.* 140: 14-19.

Cerror, C., Santero, C. F., Rojas, A., Tortajada, M., Ramón, D. and Gracía, J. L. (2013) Genome Sequence of the Butanol Hyperproducer *Clostridium saccharoperbutylacetonicum* N1-4. *Genome A.* 1(**2**): 1-2.

Chain Biotech (2010) pMTL80000 modular plasmids. Available at: http://clostron.com/pMTL80000.php. *Chain Biotech.* Assessed on August 16, 2018.

ChemicalSafetyFacts.org. Acetone. Available at: https://www.chemicalsafetyfacts.org/acetone/. *ChemicalSafetyFacts.org.* Assessed on November 19, 2018.

ChemicalSafetyFacts.org. Ethanol. Available at: https://www.chemicalsafetyfacts.org/ethanol/. *ChemicalSafetyFacts.org.* Assessed on November 19, 2018.

Chen, C. K. and Blaschek, H. (1999) Effect of acetate on molecular and physiological aspects of *Clostridium beijerinckii* NCIMB 8052 solvent production and strain degeneration. *Applied Environmental Microbiology.* 65: 499-505.

Chen, C. Y., Zhao, X. Q., Yen, H. W., Ho, S. H. Cheng, C. L., Lee, D. J., Bai, F. W. and Chang, J. S. (2013) Microalgae-based carbohydrates for biofuel production. *Biochemical Engineering Journal.* 78: 1-10.

Cheng, H. H., Whang, L. M., Chan, K. C., Chung, M. C., Wu, S. H., Liu, C. P., Tien, S. Y., Chen, S. Y., Chang, J. S. and Lee, W. J. (2015) Biological butanol production from microalgae-based biodiesel residues by *Clostridium acetobutylicum*. *Bioresource Technology.* 184: 379-385.

Cho, D. H., Shin, S. J. and Kim Y. H. (2012) Effects of acetic and formic acid on ABE Production by *Clostridium acetobutylicum* and *Clostridium beijerinckii. Biotechnology and Bioprocess Engineering.* 17: 270-275.

Dalatony, M. M., Salama, E. S., Kurade, M. B., Hassan, S. H., Oh, S. E., Kim, S. and Jeon, B. H. (2017) Utilization of microalga biofractions for bioethanol, high alcohols, and biodiesel production: A review. *Energies.* 10: 1-19.

Demirbas (2011) Biofuels from algae for sustainable development. *Applied Energy.* 88: 3473-3480.

Doremus, M. G., Linden, J. C. and Moreira, A. R. (1985) Agitation and pressure effects on acetone-butanol fermentation. *Biotechnology and Bioengineering.* 27: 852-860.

Durre, P. (2008) Fermentative butanol production: bulk chemical and biofuel. *Annals of the New York Academy of Sciences.* 1125: 353-362.

Eliáš, M., Amaral, R., Fawley, K. P., Fawley, M. W.m Nemcová, Y., Neustupa, J., Prilbl, P., Santos, M. A. and Ševčíková, T. (2017) Chapter 11. Eustigmatophyceae. *Handbook of protists.* 367-406.

Ellis, J. T., Hengge, N. H., Sims, R. C. and Miller, C. D. (2012) Acetone-butanol-and-ethanol-production-from-wastewater-algae. *Bioresource Technology.* 111: 491-495.

Essalem, M.E. and Mitchell, W. J. 2016. Identification of a glucose–mannose phosphotransferase system in *Clostridium beijerinckii*. *FEMS Microbiology Letters.* 363 (**8**): 1-5.

Ezeji, T. C., Qureshi, N., and Blaschek, H. P. (2013) Microbial production of a biofuel (acetone–butanol–ethanol) in a continuous bioreactor- impact of bleed and simultaneous product removal. *Bioprocess and Biosystem Engineering*. 36: 109- 116.

Gao, M., Tashiro, Y., Yoshida, T, Jin, Z., Wang, Q., Sakai, K. and Sonomoto, K. (2015) Metabolic analysis of butanol production from acetate in *Clostridium saccharoperbutylacetonicum* N1-4 using 13C tracer experiments. *Royal Society of Chemistry.* 5: 8486-8495.

Gao, K., Orr, V. and Rehmann, L. (2016) Butanol fermentation from microalgae-derived carbohydrates after ionic liquid extraction. *Bioresource Technology.* 206: 77-85.

Gérando, M., Wasels, F., Bisson, A., Clement, B., Budard, F., Jourdier, E., López-Conteras, A. M. and Ferreira, N. L. (2018) Genome and transcriptome of the natural isopropanol producer *Clostridium beijerinckii* DSM6423. *BMC Genomics.* 19 (**242**): 1-12.

Gerken, H. G., Donohoe, B. and Knoshaug, E. P. (2012) Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. *An International Journal of Plant Biology.* 237 (**1**): 239-253.

Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A. and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods.* 6 (**5**): 343-345.

Gutierrez, N. A., Maddox, I. S., Schuster, K. C., Swoboda, H., and Gapes, J. R. (1998) Strain comparison and medium preparation for the acetone-butanol-ethanol (ABE) fermentation process using a substrate of potato. *Bioresource Technology.* 66 (**3**): 263-265.

Heap, J T., Pennington, O. J., Cartman, S. T. and Minton, N. P. (2009) A modular system for Clostridium shuttle plasmids. *Journal of Microbiology Methods.* 78 (**1**): 79-85.

Herman, N. A., Li, J., Bedi, R., Turchi, B., Liu, X., Miller, M. J. and Zhang, W. (2017) Development of a high-efficiency transformation method and implementation of rational metabolic engineering for the industrial butanol hyperproducer *Clostridium saccharoperbutylacetonicum* strain N1-4. *Applied and Environmental Microbiology.* 83 (**2**): 1-17.

Ho, S. H., Huang, S. W., Chen, C. Y., Hasunuma, T. and Kondo, A. (2013b) Characterization and optimization of carbohydrate production from an indigenous microalga *Chlorella vulgaris* FSP-E. *Bioresource Technology.* 135: 157-165.

Hongo, M., Murata, A., Kono, K. and Kato, F. (1968) Lysogeny and bacteriocinogeny in strains of Clostridium Species. *Agricultural and Biological Chemistry.* 32 (**1**): 27-33.

International Program on Chemistry Safety (IPCS) 1-butanol. Available at: http://www.inchem.org/documents/icsc/icsc/eics0111.htm. *IPCS.* Assessed on September 7, 2018.

Jang, Y. S., Malaviya, A., Cho, C., Lee, J. and Lee, S. Y. (2012a) Butanol production from renewable biomass by clostridia. *Bioresource Technology.* 123: 653-663.

Jang, Y. S., Lee, J. Y., Lee, J., Park, J. H., Im, J. A., Eom, M. H., Lee, J., Lee, S. H., Song, H., Cho, J. H., Seung, D. Y. and Lee, S. Y. (2012b) Enhanced butanol production obtained by reinforcing the direct butanol-forming route in *Clostridium acetobutylicum*. *MBIO.* 3(**5**): 1-9.

Jin, C., Yao, M., Liu, H., Lee, C.F. and Ji, J. (2011) Progress in the production and application of n-butanol as a biofuel. *Renewable and Sustainable Energy Reviews.* 15: 4080-4106.

Johnson, J. L., Santiwatanakul, T. and Chen, J. S. (1997) Cultures of "*Clostridium acetobutylicum*" from Various Collections Comprise *Clostridium acetobutylicum, Clostridium beijerinckii*, and two other distinct types based on DNA-DNA reassociation. *International Journal of Systematic Bacteriology.* 47(**2**): 420-424.

Jones, D. T. and Woods, D. R. (1986) Acetone-butanol fermentation revisited. *Microbiology and Molecular Biology Reviews.* 50 (**4**): 484-524.

Keis, S., Shaheen, R. and Jones, D. T. (2001) Emended descriptions *of Clostridium acetobutylicum* and *Clostridium beijerinckii*, and descriptions of *Clostridium saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov. *International Journal of Systematic and Evolution Microbiology.* 51(**6**): 2095-2103.

Kosaka, T., Nakayama, S., Nakaya, K., Yoshino, S. and Furukawa, K. (2007) Characterization of the sol operon in butanol-hyperproducing *Clostridium saccharoperbutylaceronicum* strain N1-4 and its degeneration mechanism. *Biosciences, Biotechnology and Biochemistry.* 71 (**1**): 58-68.

Kótai, L., Szépvölgyi J., Szilágyi, M., Zhinbin, L., Baiquan, C., Sharma, V. and Sharma, P. (2013) Biobutanol from renewable agricultural and lignocellulose resources and its perspectives as alternative of liquid fuels. *Intech.* 199-262.

Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J. and Jung, K. S. (2008) Fermentative butanol production by clostridia. *Biotechnology and Bioengineering.* 101 (**2**): 209-228.

Lopes, M. L., Paulillo, S. C., Godoy, A., Cherubin, R. A., Lorenzi, M. S., Giometti, F. H., Bernardino, C. D., Amorim Neto, H. B., and Amorim, H. V. (2016) Ethanol production in Brazil: a bridge between science and industry. *Brazilian Journal of Microbioly*. 47: 64-76.

Lü, W., Du, J., Wacker, T., Smentek, E. G., Andrade, L. A., Einsle, O. (2011) pH-dependent gating in a FocA formate channel. *Science.* 332: 352-353.

Ma, X. N., Chen, T. P., Yang, B., Liu, J. and Cen, F. (2016) Lipid production from *Nannochloropsis*. *Marine Drugs.* 14 (**61**): 1-18.

Maddox, I. S. (1989) The acetone-butanol-ethanol fermentation: Recent progress in technology. *Biotechnology and Genetic Engineering Reviews.* 7(**1**): 189-220.

Maddox, I. S., Stelner, E., Hlrsch, S. Wessner, S., Gutlerrez, N. A., Gapes, J. R. and Schuster, K. C. (2000) The cause of "acid crash" and "acidogenic fermentations" during the batch ABE fermentation process. *Journal of Molecular Microbiology and Biotechnology.* 2 (**1**): 95-100.

Madihah, M. S., Ariff, A. B., Suraini, A. A. and Karim, M. I. A. (2001) Direct fermentation of gelatinized sago starch to acetone-butanol-ethanol by *Clostridium acetobutylicum. World Journal of Microbiology and Biotechnology.* 27 (**6**): 567-576.

Millat, T. and Winzer, K. (2017). Mathematical modelling of clostridial acetone-butanol-ethanol fermentation. *Applied Microbiology and Biotechnology.* 101: 2251-2271.

Mitchell, W. J. (2015) The phosphotransferase system in solventogenic Clostridia. *Journal of Molecular Microbiology and Biotechnology.* 25: 129-142.

Moon, H. G., Jang, Y. S., Cho, C., Lee, J., Binkley, R. and Lee, S. Y. (2016) One hundred years of clostridial butanol fermentation. *FEMS Microbiology Letters.* 363 (**3**): 1-15.

Moreira, A. R., Ulmer, D. C. and Linden, J. C. (1981) Butanol toxicity in the butyric fermentation. *Biotechnology and Bioengineering Symposium.* 11: 567-579.

Naghdim F. G., González, L. M., Chan, W. and Schenk, P. M. (2016) Progress on lipid extraction from wet algal biomass for biodiesel production. *Microbiology and Biotechnology.* 9 (**6**): 718-726.

Nakayama, S., Kiyoshi, K., Kadokura, T. and Nakazato, A. (2011) Butanol production from crystalline cellulose by cocultured *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* N1-4. *Applied and Environmental Microbiology*. 77 (**18**): 6470-6475.

National Centre for Biotechnology Information. Acetone. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/180. *PubChem Compound Database.* Assessed on November 19, 2018.

National Centre for Biotechnology Information. Ethanol. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/702. *PubChem Compound Database.* Assessed on November 19, 2018.

Ndaba, B., Chiyanzu, I., and Marx, S. (2015) n-Butanol derived from biochemical and chemical routes: A review. *Biotechnology Reports.* 8: 1-9.

Noguchi, T., Tashiro, Y., Yoshida, T., Zheng, J., Sakai, K. and Sonomoto, K. (2013) Efficient butanol production without carbon catabolite repression from mixed sugars with *Clostridium saccharoperbutylacetonicum* N1-4. *Journal Bioscience and Bioengineering.* 116 (**6**): 716-721.

Oshiro, M., Hanada, K., Tashiro, Y. and Sonomoto, K. (2010) Efficient conversion of lactic acid to butanol with pH-stat continuous lactic acid and glucose feeding method by *Clostridium saccharoperbutylacetonicum*. *Applied Microbiology and Biotechnology.* 87: 1177-1185.

Ou, J. Ma, C., Xu, N., Du, Y., Liu, X. (2015) High butanol production by regulating carbon, redox and energy in Clostridia. *Frontier of Chemical Science and Engineering.* 9 (**3**): 317-323.

Papoutsakis, T. E. (1983) Equations and calculations for fermentations of butyric acid bacteria. *biotechnology and Bioengineering.* 26: 174-187.

Patakova, P., Linhova, M., Rychtera, M., Paulova, L. and Meizoch, K. (2013) Novel and neglected issues of ABE fermentation by Clostridia- Clostridium metabolic diversity tools for process mapping and continuous fermentation systems. *Biotechnology Advances.* 31: 58-67.

Poehlein, A., Krabben, P., Dürre, P. and Daniel, R. (2014) Complete genome sequence of the solvent producer *Clostridium saccharoperbutylacetonicum* strain DSM 14923. *Genome A*. 2 (**5**): 1-2.

Rao, G. and Mutharasan, R. (1987) Altered Electron Flow in Continuous Cultures of *Clostridium acetobutylicum* Induced by Viologen Dyes. *Applied and Environmental Microbiology.* 53: 1232-1235.

Rashid, N., Rehman, M. S. and Han, J. I. (2013) Recycling and reuse of spent microalgal biomass for sustainable biofuels. 75: 101-107.

Reyes, L. H., Almario, M. P. and Kao, K. C. (2011) Genomic library screens for genes involved in n-butanol tolerance in *Escherichia coli*. *PLoS ONE.* 6 (**3**): 1-9.

Richter, H., Molitor, B., Wei, H., Chen, W., Aristilde, L. and Angenent, L. T. (2013) Ethanol production in syngas-fermenting *Clostridium ljungdahlii* is controlled by thermodynamics rather than by enzyme expression. *Royal Society of Chemistry.* 00: 1-3.

Safi, C., Zabib, B., Merah, O., Pontalier, P. Y. and Garcia, C. V. (2014) Morphology composition production processing and applications of *Chlorella vulgaris. Renewable and Sustainable Energy Reviews.* 35: 265-278.

Show, P. L., Tang, m. S., Nagarajan, D., Liang, T. C., Ooi, C. W. and Chang, J. S. (2016) A holistic approach to managing microalgae for biofuel applications. *International Journal of Molecular Sciences.* 18 (**215**): 1- 34.

Sreekumar, S., Baer, Z. C., Pazhamalai, A., Gunbas, G., Grippo, A., Blanch, H. W., Clark, D. S. and Toste, F. D. (2015) Production of an acetone-butanol-ethanol mixture from *Clostridium acetobutylicum* and its conversion to high-value biofuel. *Nature Protocols.* 10 (**3**): 528-537.

Steiner, E., Scott, J., Minton, N. P., and Winzer, K. (2012) An *agr* quorum sensing system that regulates granulose formation and sporulation in *Clostridium acetobutylicum. Applied and Environmental Microbiology.* 78 (**4**): 1113-1122.

Suppmann, B. and Sawers, G. (1994) Isolation and characterization of hypophosphite-resistant mutants of *Escherichia coli:* Identification of the FocA protein encoded by the pfl operon, as a putative formate transporter. *Molecular Microbiology.* 11 (**5**): 965-982.

Tashiro, Y., Taketa, K., Kobayashi, G., Sonomoto, K., Ishizaki, A. and Yoshino, S. (2004) High butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 in fed-batch culture with pH-stat continuous butyric acid and glucose feeding method. *Journal of Bioscience and Bioengineering.* 98 (**4**): 263-268.

United Nations Department of Economic and Social Affairs. World population prospects. *United Nations Department of Economic and Social Affairs.* Available at: https://esa.un.org/unpd/wpp/Publications/Files/WPP2017_KeyFindings.pdf. Published on 2017. Assessed on October 13, 2017.

Waight, A. B., Love, J. and Wang D. N. (2010) Structure and mechanism of pentameric formate channel. *Nature Structural and Molecular Biology.* 17(**1**): 1-20.

Wakasugi, T., Nagai, T., Kapoor, M, Sugita, M., Ito, M., Tsudzuki, J., Nakashima, K., Tsudzuki, T., Suzuki, Y., Hamada, A., Ohta, T., Inamura, A., Yoshinaga, K. and Sugiura, M. (1997) Complete nucleotide sequence of the chloroplast genome from the green alga *Chlorella vulgaris*: The existence of genes possibly involved in chloroplast division. *Proceedings of National Academy of Sciences of the United States of America.* 94: 5967-5972.

Wang, Y., Li, X., Mao, Y. and Blaschek, H. P. (2011) Single-nucleotide resolution analysis of the transcriptome structure of *Clostridium beijerinckii* NCIMB 8052 using RNA-Seq. *BMC Genomics.* 479 (**12**): 1-10.

Wang, Y., Guo, W. Q., Lom Y. C., Chang, J. S. and Ren, N. Q. (2014) Characterization and kinetics of bio-butanol production with *Clostridium acetobutylicum* ATCC824 using mixed sugar medium simulating

microalgae-based carbohydrates. *Biochemical Engineering Journal.* 91: 220-230.

Wang, Y., Guo, W., Cheng, C., Ho, S. H., Chang, J. S. and Ren, N. (2016) Enhancing bio-butanol production biomass of *Chlorella vulgaris* JSC-6 with sequential alkali pre-treated and acid hydrolysis. *Bioresource Technology.* 200: 557-564.

Wang, S., Dong, S. and Wang Y. (2017) Enhancement of solvent production by overexpressing key genes of the acetone-butanol-ethanol fermentation pathway in *Clostridium saccharoperbutylacetonicum* N1-4. *Bioresource Technology.* 245: 426-433.

Weizmann, C. and Rosenfeld, B. (1937) The activation of the butanol-acetone fermentation of carbohydrates by *Clostridium acetobutylicum* (Weizmann). *Biochemistry.* 85: 619-639.

Wu, X. (2014) Classic acetone-butanol-ethanol (ABE) fermentation with solventogenic clostridia. *Biofuel*. 139-144. Caister Academic Press.

Xiao, H, Gu, Y., Ning, Y., Yang, Y., Mitchell, W J., Jiang, W. and Yang, S. (2011) Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose and arabinose. *Applied Environmental Microbiology.* 77: 7886-7895.

Xin, F., Wang, C., Dong, W., Zhang, W., Wu, H., Ma, J. and Jiang, M. (2016) Comprehensive investigations of biobutanol production by a non-acetone and 1,3-propanediol generating Clostridium strain from glycerol and polysaccharides. *Biotechnology of Biofuels.* 9: 220.

Xin, F., Chen, T., Jiang, Y., Dong, W., Zhang, W., Zhang, M., Wu, H., Ma, J. and Jiang, M. (2017) Strategies for improved isopropanol– butanol production by a Clostridium strain from glucose and hemicellulose through consolidated bioprocessing. *Biotechnology for Biofuels.* 10: 118.

Xue, C., Zhao, X. Q., Liu, C. G., Chen, L. J. and Bai, F. W. (2013) Prospective and development of butanol as an advanced biofuel. *Biotechnology Advances.* 31: 1575-1584.

Yerushaimi, L. and Volesky, B. (1985) Importance of agitation in acetone-butanol fermentation. *Biotechnology and Bioengineering.* 27: 1297-1305

Yerushaimi, L., Volesky, B. and Szczesny, T. (1985) Effect of increased hydrogen partial pressure on the acetone-butanol fermentation by *Clostridium acetobutylicum. Applied Microbiology and Biotechnology.* 22: 103-107.

Zheng, Y. N., Li, L. Z., Xian, M., Ma, Y. J., Yang, J. M., Xi, X. and He, D. Z. (2009) Problems with the microbial production of butanol. *Journal of Industrial Microbiology and Biotechnology*. 36: 1127-1138.

Appendix

Appendix A. GCMS standard curves

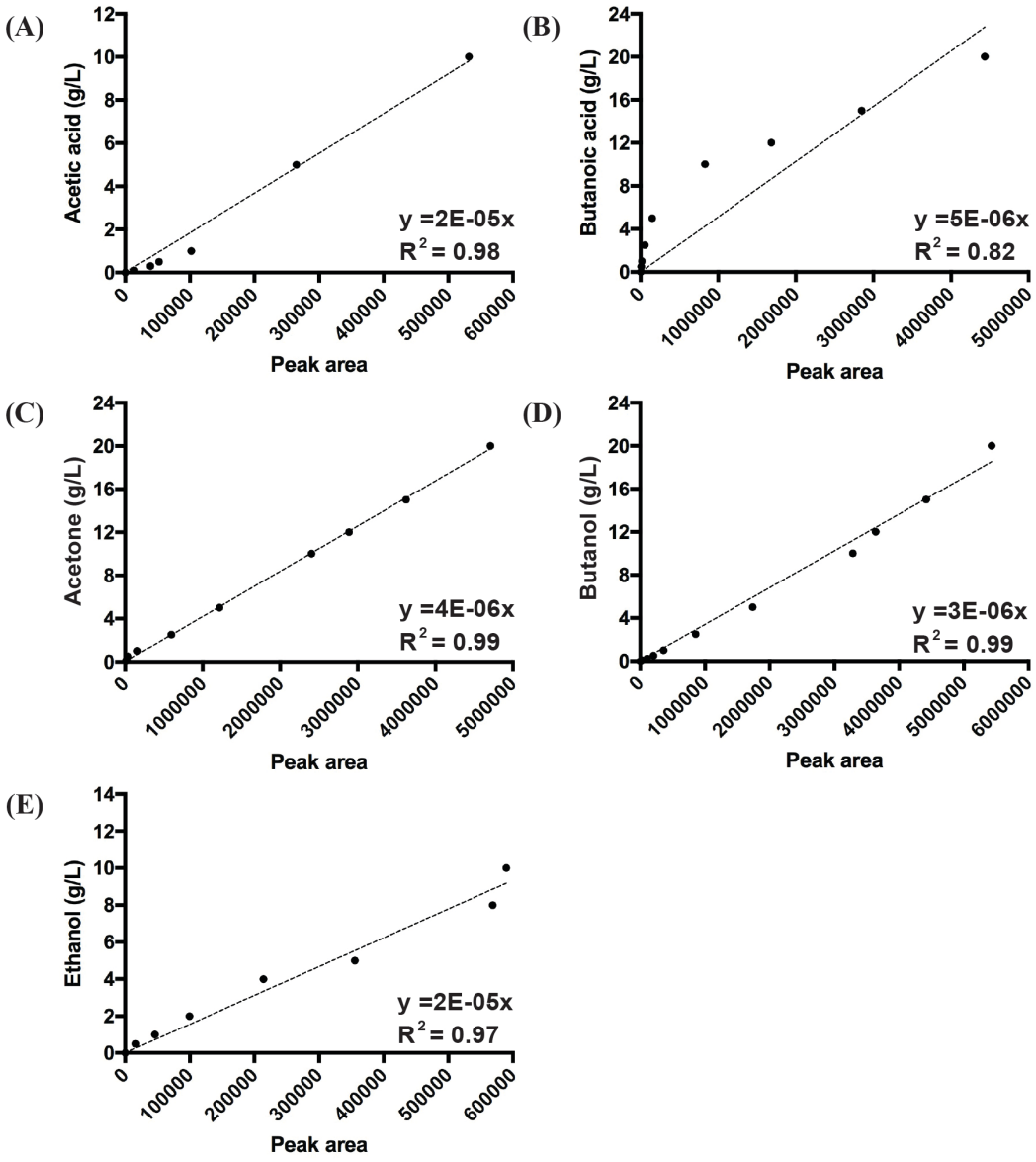

Figure A1. Solvents standard curve. (A) Acetic acid; (B) Butanoic acid; (C) Acetone; (D) Butanol; (E) Ethanol. Acetic acid and ethanol standard curves were generated over the concentration range of 0-10 g/L. Butanoic acid, acetone and butanol standard curve were generated over the concentration range of 0-20 g/L.

Appendix B. Full range of growth media in serum bottles

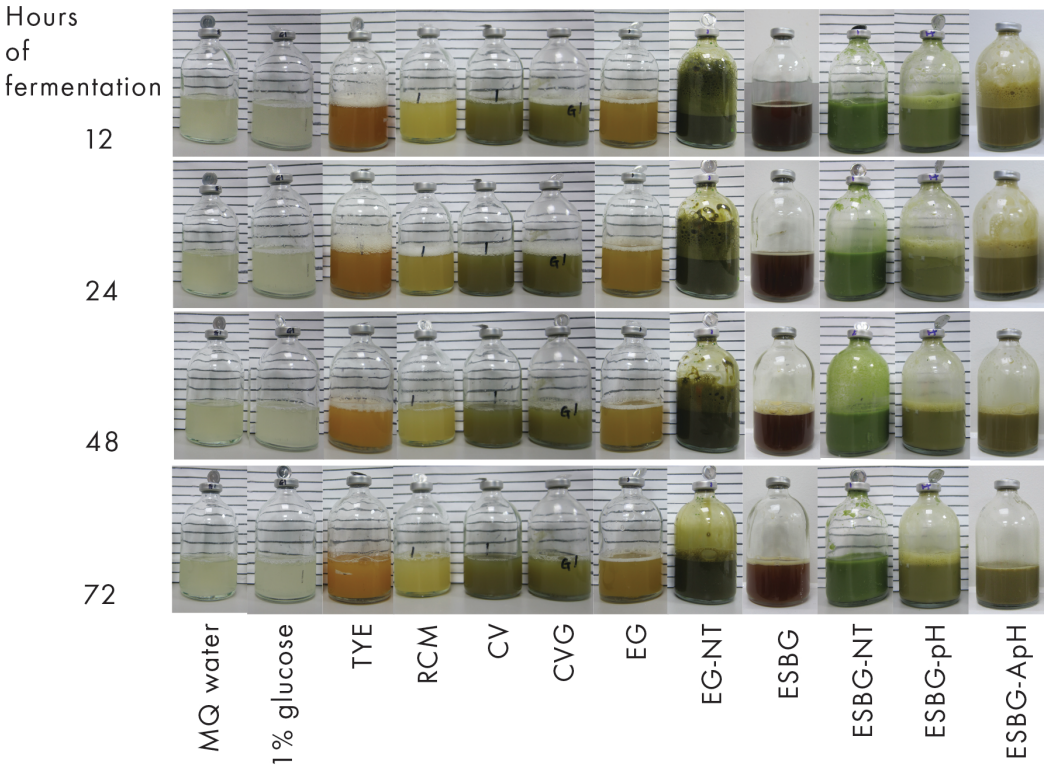

Figure B1. Full range of growth media in serum bottle. At 24 h of fermentation, a significant amount of foam was observed in TYE, RCM, CV, CVG, EG-NT, and ESBG-pH. The foam production subsided after 48 h of fermentation except for EG-NT and ESBG-pH. Positive controls: RCM and TYE. Negative controls: 1% glucose and MQ water. CV, CVG, EG and ESBG were centrifugated and autoclaved. EG-NT, ESBG-NT were not subjected to centrifugation and autoclaving. ESBG-pH was not subjected to centrifugation and autoclaving, the initial pH was adjusted to 6.0. ESBG-ApH was subjected to autoclaving and without centrifugation, the initial pH was adjusted to 6.0.

Appendix C. Microalgae

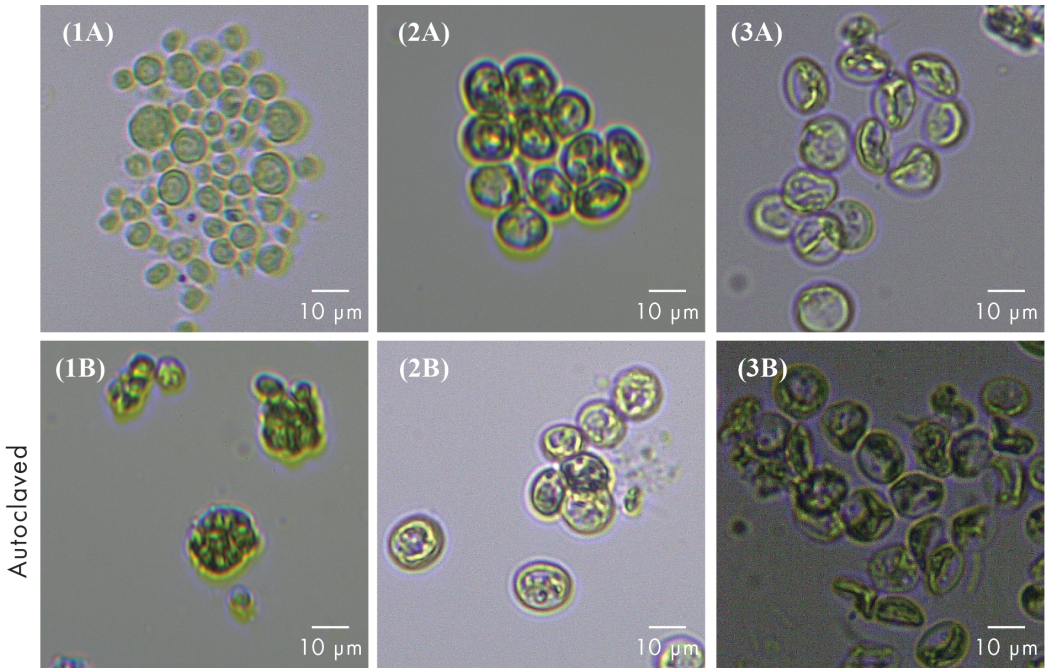

Figure C1. Morphology of microalgae using light microscopy. (1A). Broken cell wall *C. vulgaris* (1B). Autoclaved and centrifugated broken cell wall *C. vulgaris*; *C. vulgaris* was purchased from Seven Hills Wholefood, supplied as fragmented cells; (2A). Eustigmatophyceae whole cells; (2B). Autoclaved and centrifugated Eustigmatophyceae whole cells. The cell wall remained intact after centrifugation and autoclaving; (3A). Eustigmatophyceae spent biomass following oil extraction; (3B) Autoclaved Eustigmatophyceae spent biomass. The cell wall appears to be weakened furthered by autoclaving. The magnification was x400.

Appendix D. Microscope micrometer

Figure D1. Micrometer used in this study. Each division = 0.01mm = 10 µm. x400 magnification was used throughout this study.

Appendix E. List of equipment, reagents, accessories, consumable items

Table E1. List of equipment.

Equipment	Company	Model Number
Autoclave	Quirumed, Prestige Medical	2100 classic 9L without manometer
Weighing balance	Sartorius	CP2202 S
Oven	Gallenkamp	Economy incubator size 2
Capillary GC column	Zebron	ZA-WAX plus, Part No.7HG-G013-11
Centrifuges	Eppendorf MiniSpin®	Eppendorf AG 22331 Hamburg
Electrophoresis power supplies	Bio-Rad	PowerPac™ HC High-Current Power Supply
GCMS	Agilent Technologies	6890N GC system; 5973N Mass selective detector;7683 Series injector and auto-sampler; Enhanced ChemStation G1701 DA version D00.00.38
Gas cylinder – N_2	BOC	UN1002
Gel imager	GeneSys	G:BOX
High-speed centrifuge	Beckman Coulter	Avanti J-26 XP
Hotplate stirrer	Stuart	SB 126-3
Incubator	Heraeus	Function Line
Microscope	GT Vision	GXML 2800
Milli-Q® water system	Thermo- Scientific	Bernstead™ Easypure™ II
Microvolume Spectroscopy	NanoPhotometer® IMPLEN	N50
Orbital shakers	Stuart Scientific	mini shaker SO5
pH electrode	Mettler Toledo InLab® Semi-Micro-L	MT51343161
Pipet aid	Drummond	BC01108BR
Plasmid mapping software	SnapGene® Viewer Vector NTI Software	Version 4.2.1. Version 10.3.1
Precision weighting balance	A&D Company Limited	HR-100A
Redox probe	Mettler Toledo InLab® Redox Micro	MT51343203
Spectrophotometer	Agilent Technologies, USA	Cary 60 UV-Vis; Cary WinUV
PCR thermal cyclers	Applied Biosystems	Veriti 96 well Thermal Cycler
Transilluminator	Biostep	BIOView UV light

Ultra-low temperature freezer	New Brunswick Scientific	Premium U410
Water bath shaker	Innova®	3100

Table E1. (Continued)

Table E2. List of reagents.

Reagent	Company
1-butanol	Sigma-Aldrich
15% glycerol	Fisher Scientific
1 kb DNA ladder	Promega
10X CutSmart® buffer (Cat number: B7204S)	NEB
2X PCRBIO Taq Mix Red (cat number: PB10.11)	PCR Biosystems
Acetic acid glacial	Fisher Scientific
Acetone	Fisher Scientific
Agar technical	Oxoid
Agarose	Fisher Scientific
Ammonium sulphate (NH_2SO_4)	Acros Organics
Butyric acid	Sigma-Aldrich
Calcium chloride ($CaCl_2$)	Sigma-Aldrich
D-Glucose anhydrous	Fisher Scientific
Ethanol denatured (industrial methylated spirit)	Fisher Scientific
GenElute™ Bacterial Genomic Kit	Sigma-Aldrich
Gibson Assembly® Master mix (2X) (Cat number: E2611S)	NEB
Hydrochloric acid (HCl)	Fisher Scientific
Industrial methylated spirit; (IMS)	Fisher Scientific
Iron (II) sulphate 7 hydrate ($FeSO_4.7H_2O$)	AnalaR by BHD
Loading dye (6X)	Fisher Scientific
Magenesium Chloride ($MgCl_2$)	BDH ACS
Magenesium Sulfate ($MgSO_4$)	Sigma-Aldrich
MES, free acid	Merck Millipore
Potassium chloride (KCl)	Sigma-Aldrich
Restriction enzymes (NdeI and NheI)	NED
Sodium chloride (NaCl)	Fisher Scientific
Spectinomycin	Sigma-Aldrich
Q5® High-Fidelity 2X Master Mix (Cat number: M0492S)	NEB
QIAquick® PCR-purification kit	QIAGEN
QIAprep® Spin Miniprep Kit	QIAGEN
Reinforced clostridial medium (RCM)	Sigma-Aldrich
Tryptone	Oxoid
Yeast extract	Oxoid

Table E3. List of accessories.

Beaker (500 and 2000 mL)	Horizontal gel box
Chromacol vial and closure	Magnetic stirrer bar
Clamps	Masterflex tubing 6”
Crimping tool for center tears out septa	Metal spatula
Culture vessel (1000 mL)	Pipette
Duran bottle (500, 1000, 5000 mL)	Retaining clip
Flat flange lid (100 mm)	Rubber turn-over closure (Suba-seal®)
Gasket (PTFE Seal)	Serum vials (30 and 50 mL)
Gel comb	Shapes bin
Gel seal	Thermometer
	UV gel tray

Table E4. List of consumable items.

Aluminium foil	Needle (2 inches; 1.1 x 50 mm)
Autoclave tape	PCR tube (0.2 mL)
Butyl rubber septa (20 mm)	Peroxide-cured silicone tubing
Center tear out septa (20 mm)	Petri dish (10 cm × 1.5 cm)
Cryovial (2 mL)	Petroleum gel
Cuvette, semi-micro (Sarstedt)	Pipette tip (1000, 100 ul)
Examination glove	Powder-free nitrile glove
Minisart® filter (pore size: 0.2 μm)	Serological pipette (5 and 50 mL)
Microscope slide	Syringe (1, 2 and 5 mL)
Micro tube 2 mL	Weighing boats