



UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head Office: *Università degli Studi di Padova*
Department of Industrial Engineering

Ph.D. COURSE IN: *Industrial Engineering*
CURRICULUM: *Chemical and Environmental Engineering*
SERIES *XXXI*

Enhancement of hydrogen and methane production through anaerobic digestion using pre-treatments

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To the global peace.

Abstract

Dark fermentation of organic wastes is considered as a promising process in terms of sustainable waste management and simultaneous biofuel production. Pre-treatment technologies are known as essentials of dark fermentation to overcome obstacles responsible for low H₂ yield.

The effect of aerobic pre-treatment of food wastes with different compositions (carbohydrate-rich, protein-rich and lipid-rich) prior to two-stage anaerobic digestion, on H₂ and CH₄ productions was investigated. The results showed that pre-aeration of food waste did not constitute an effective treatment for the purpose of improving H₂ production potential during the first stage of the AD process. However, during the subsequent stage of AD, CH₄ yield for protein-rich substrate, increased by 45.6%, thus revealing that carbon conversion to CH₄ had an increase after pre-aeration.

In case of inoculum pre-treatment, a novel method using waste frying oil (WFO) was introduced. H₂ production from glucose was investigated for inoculum pre-treated with different concentrations of WFO. In the next step, a flux balance analysis model was developed to study the effect of inoculum pre-treatment on H₂ producing and H₂ consuming metabolic pathways. The results showed that H₂ consumption by hydrogenotrophic methanogens that was accounted for about 56% of the loss in the H₂ yield in untreated cultures, was negligible when the inoculum pre-treated with WFO.

Moreover, optimization of H₂ yield from food waste was performed in the next step of research, using a three-factor three-level Box-Behnken design method. Initial pH, pre-treatment duration and waste frying oil concentration were considered as the experimental factors. The results showed that combination of high WFO concentration, low initial pH and long pre-treatment could result in inhibition of methanogens.

Furthermore, two-stage anaerobic digestion of food waste was performed using the inoculum pre-treated with WFO and total energy yield was compared with three common pre-treatments (heat shock, aeration and alkaline pre-treatment) and untreated cultures. The results showed that inoculum pre-treatment with WFO resulted in higher H₂ and CH₄ productions compared to alkaline, aeration and heat shock pre-treatment.

Finally, microbial community of inoculum at different stages of dark fermentation (untreated, pre-treated with WFO, washed and fermented) was investigated to understand the effect of inoculum pre-treatment with WFO on H₂ producing and H₂ consuming microbial populations. The microbial diversity analysis showed that inoculum pre-treatment with WFO did not affect spore-forming H₂ producing bacteria. However, it resulted in increased relative abundances of non-spore forming H₂ producers which could be considered as an advantage in comparison with harsh pre-treatments such as heat shock.

Summary

Background and aim of the study

In the next generation bio-based refineries, hydrolysis and primary (or extractive) fermentations by mixed microbial cultures (MMC) are precursors of secondary bio-transformations, in which H₂, CO₂ and mixed carboxylates are used as substrate for achieving added-value target products. Dark fermentation is the simplest MMC-driven processes that include hydrolysis and primary fermentations to extract gaseous and soluble mixtures of compounds from raw biomass.

Pre-treatments technologies employed for dark fermentation are classified into inoculum and substrate pre-treatments. Inoculum pre-treatment technologies are aimed at selecting H₂ producing microorganisms and therefore increased H₂ production while the goal for substrate pre-treatment is the enhancement of H₂ yield through better hydrolysis of complex substrates and provide biodegradable nutrients for microbial growth and H₂ production. The present work aims at assessing inoculum and substrate pre-treatments on H₂ production. Moreover, the effect of pre-treatments has also investigated in case of integrated dark fermentation and anaerobic digestion. The research methodology include experiments, statistical analysis, metabolic modeling and microbial community analysis.

Pre-aeration of substrate prior to two-stage anaerobic digestion

Previous studies have shown that limited pre-aeration prior to anaerobic digestion could improve hydrolysis and methane production from different substrates [1–4]. However, the effect of composition variability of substrate in terms of carbohydrate, lipid and protein content, on pre-aeration effects and biogas production has not been addressed before. A part of this thesis is dedicated to study the aerobic pre-treatment effects on carbohydrate-rich, protein-rich and lipid-rich food waste prior to two-stage anaerobic digestion on both H₂ and CH₄ production.

Inoculum pre-treatment using waste frying oil

Anaerobic fermentation is mediated by complex microbial populations including H₂ producers, homoacetogens, methanogens, propionate producers and lactic acid bacteria. If the growth of H₂ consumers is not controlled, the H₂ produced by H₂ producing bacteria cannot be accumulated due the presence of H₂ consumers. The inhibitory effect of long chain fatty acids, on methane production by anaerobic digestion has been recognized since many years ago [5]. Long chain fatty acids could be adsorbed on the cell wall of some microbial species including methanogens, interfere metabolites transportation and subsequently hinder their growth [6]. This characteristic was used in the present study as an alternative pre-treatment using WFO (as a source long chain fatty acids) to suppress H₂ consumption by methanogens. Effect of waste frying oil concentrations on inhibition of methanogenic H₂ consumption and enhancement of H₂ accumulation were investigated using glucose as substrate. Moreover, a flux balance analysis model was developed and used to study the effect of pre-treatment on major microbial populations present in the mixed community. The findings of the present work showed that that low concentrations of WFO did not completely inhibited hydrogenotrophic methanogens. Flux balance analysis showed that H₂ consumption by hydrogenotrophic methanogens that was

accounted for about 56% of the loss in the H₂ yield in untreated cultures, was negligible when the inoculum pre-treated with WFO.

Microbial community analysis

Deciphering the microbial composition is one of the most important issues in dark fermentation studies in order to optimize H₂ production. In particular, changes in microbial composition after inoculum pre-treatment in comparison with the untreated inocula, reveals the efficiency of pre-treatment process. Microbial community at different stages of dark fermentation (untreated, pre-treated with WFO, washed and fermented) was investigated to understand the effect of inoculum pre-treatment with WFO on H₂ producing and H₂ consuming bacteria. Inoculum pre-treatment with WFO resulted in increased relative abundances of non-spore forming H₂ producers such as *Aeromonas* and *Citrobacter* spp. while it did not significantly affect spore-forming H₂ producers belonging to the *Clostridium* genus.

Optimization of hydrogen production from food waste using anaerobic mixed cultures pre-treated with waste frying oil

Optimization of H₂ yield from food waste, was performed using a three-factor three-level Box-Behnken design method. Initial pH, pre-treatment duration and waste frying oil concentration were considered as the experimental factors. Pre-treatment with waste frying oil decreased CH₄ productions significantly and in turn improved H₂ accumulation. The present study confirmed complete inhibition of methanogens with high WFO concentration, low initial pH and long pre-treatment.

Inoculum pre-treatment effects on two-stage anaerobic digestion of food waste

Two-stage anaerobic digestion of food waste was performed using four different inoculum pre-treatment methods to enrich H₂ producing bacteria from sludge. The pre-treatments used in this study include heat shock, alkaline treatment, aeration and novel pre-treatment using waste frying oil (WFO). The findings of the present study showed that alkaline pre-treatment and aeration did not completely inhibit methanogens in the first stage while no CH₄ was detected in the reactors cultivated either with heat shock or waste frying oil- pre-treated inocula. The highest H₂ and CH₄ yields were obtained using the inoculum pre-treated with waste frying oil. The highest total energy yield was obtained using inoculum pre-treatment with WFO. The total energy yield trend obtained using different pre-treatments was as follows: WFO>alkaline>heat>aeration> control.

Acknowledgement

I would like to thank my advisor Prof. Raffaello Cossu who provided me an opportunity to join his team and for the priceless experiences I gained during these three years of PhD.

I would like also to thank my co-supervisor, Prof. Alberto Pivato for his support and advices throughout this research. He was always available for my questions.

I acknowledge Prof. Maria Cristina Lavagnolo, for her precious support during three years of my PhD and providing me the unforgettable opportunities like the experience of teaching in Cameroon.

I would like to express my special appreciation to Dr. Annalisa Sandon, our energetic lab technician for her discipline during the research work and for her sense of humor that always make the atmosphere of LISA lab fresh.

I would like to thank Dr. Stefano Campanaro and Dr. Laura Treu for their precious help in microbial community analysis.

I would like to thank my lab mates Wei Peng, Mubasheer Saleem, Francesca Giroto, Valentina Grossule, Francesco Garbo, Giulia Cerminara and Giovanni Beggio for their helps throughout this research and for their friendship.

I would like to thank my parents for their love, dedication and support throughout my life. I also thank my brothers for their support, love and encouragement.

Finally, I would like to extend my special thanks to my husband, Siamak, whose love and support always keeps me going.

Publications

Journal papers:

- Rafieenia, Razieh, Francesca Girotto, Wei Peng, Raffaello Cossu, Alberto Pivato, Roberto Raga, and Maria Cristina Lavagnolo. "Effect of aerobic pre-treatment on hydrogen and methane production in a two-stage anaerobic digestion process using food waste with different compositions." *Waste management* 59 (2017): 194-199.
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- Rafieenia, Razieh, Alberto Pivato, Maria Christina Lavagnolo, and Raffaello Cossu. "Pre-treating anaerobic mixed microflora with waste frying oil: A novel method to inhibit hydrogen consumption." *Waste Management* 71 (2018): 129-136.
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- Rafieenia Razieh, Pivato Alberto, Treu Laura, Campanaro Stefano, Schievano Andrea, Lavagnolo, Maria Cristina. Study of microbial dynamics during optimization of hydrogen production from food waste by using LCFA-rich agent (under preparation)

Conference presentations and proceedings:

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- Rafieenia, Razieh, Girotto Francesca, Peng Wei, Cossu Raffaello. 6th International Symposium on Energy from Biomass and Waste

- Rafieenia, Razieh, Pivato Alberto, Lavagnolo Maria Cristina. Lab-scale tests for the investigation of a novel method to inhibit hydrogen consumption in anaerobic digestion. “16th international waste management and landfill symposium”. October 2017.
- Lavagnolo Maria Cristina, Rafieenia Razieh. Hydrogen production from biowaste-An overview. 3th Symposium of Urban Mining, 23-25 May 2016, Bergamo, Italy

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1. Dark fermentative hydrogen production from organic wastes

1.1. Dark fermentation process

Dark fermentative hydrogen production is considered as a sustainable process since it combines renewable energy production and waste reduction. During dark fermentation, anaerobic degradation of organic substances occurs by heterotrophic bacteria to obtain a variety of soluble metabolites as well as H_2 . H_2 production from a wide variety of industrial, agricultural and municipal wastes have already been investigated by different researchers [7–10]. Organic wastes are mainly composed of carbohydrate, protein and lipids; however, carbohydrate-rich substrates are favored by H_2 producing bacteria and support higher yields [11]. Figure 1-1 shows the schematic representation of dark fermentation.

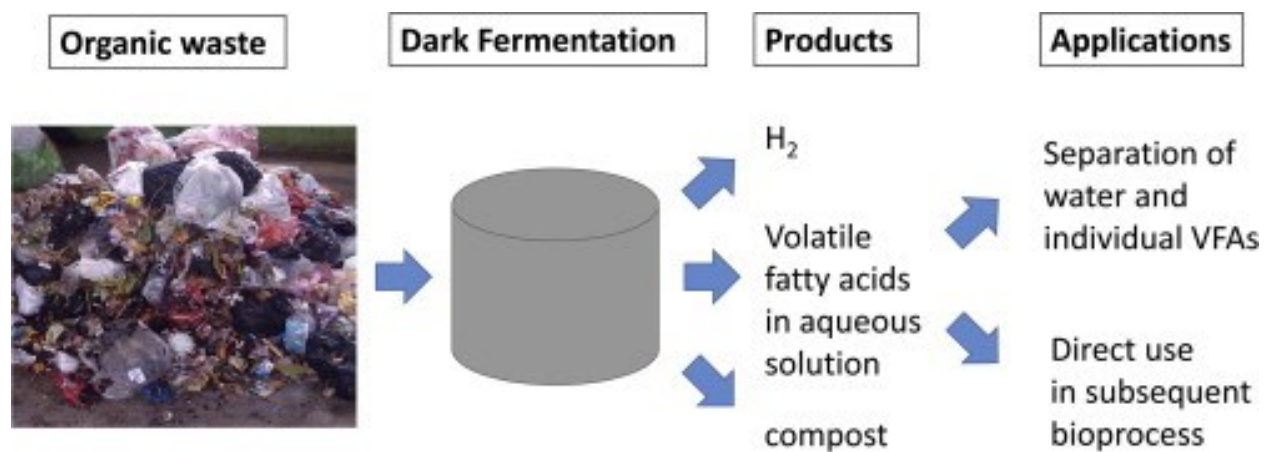


Figure 1-1 Schematic representation of dark fermentation of organic waste [12]

There are several studies performed using pure cultures and complex substrates such as cornstalk, sweet sorghum bagasse, rice straw, cheese whey, fruit and vegetable waste, waste paper and switchgrass [13–18].

In fact, H_2 is produced in some steps of anaerobic digestion (Figure 1-2). Dark fermentation is classified into three main categories based on the final products: butyrate-type, propionate-type and ethanol type [19]. Acetate and butyrate are the main soluble metabolites for butyrate-type fermentation and their metabolic pathways are accompanied with H_2 and CO_2 production (Eq. 1-2). Propionate-type fermentation mainly produces acetate and propionate without significant H_2 production. Ethanol-type fermentation which occurs in very low pH conditions (4-4.5) produces ethanol, acetate, H_2 and CO_2 (Eq. 1 and Eq. 4).

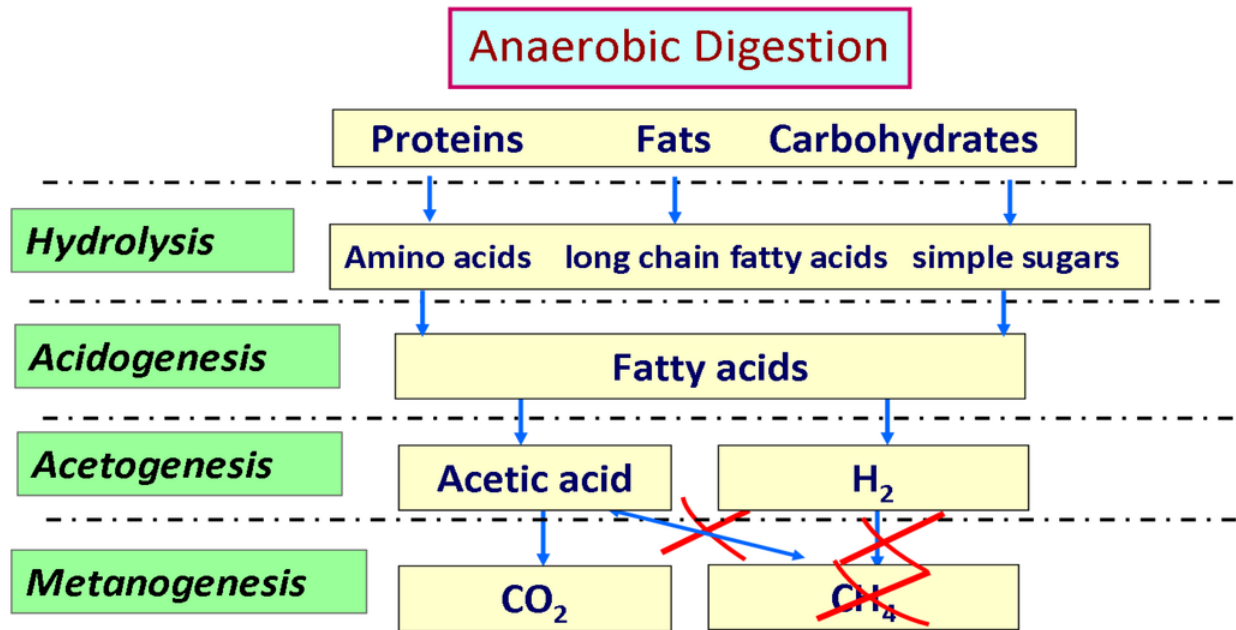
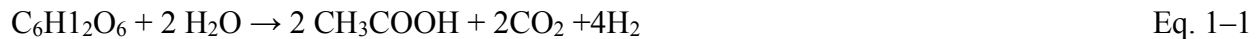


Figure 1-2 Overview of the metabolic pathways leading to hydrogen production in anaerobic digestion [20]



1.2. Factors affecting dark fermentation

Dark fermentation using mixed cultures is a complex process that is affected by many operational parameters. In addition, substrate and inoculum and their pre-treatments influence greatly the hydrogen production. These parameters are summarized below (Figure 1-3).

1.2.1. pH

pH is one of the most important parameters that influences on dark fermentative H₂ production as it affects hydrogenase activities and metabolic products. Operational pH conditions higher than 7 is favored by the production propionic acid while in very low pH conditions (lower than 5), ethanol- type fermentation may occur in which main soluble products are ethanol and acetic acid. The majority of dark fermentation studies have been performed in a pH range between 5 to 7 [11,21,22]. Acidic pH (lower than 6) inhibits hydrogenotrophic methanogens, a major group of H₂ consumers [23]. Selection of the operational pH is also strongly related to the substrate type and concentration which affect VFA production and subsequently pH. Optimal pH for lignocellulosic waste varies from 6.5-7 while the optimum pH for food waste varies from 4.5-7 [21].

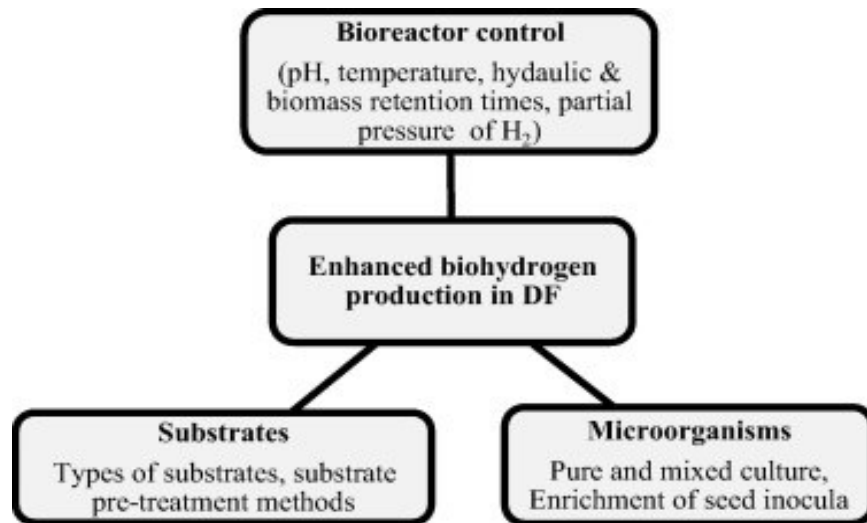


Figure 1-3 Factors affecting the biohydrogen production from organic waste [23]

1.2.2. Temperature

Temperature is another important parameter affecting the activities of H₂ producing bacteria. Temperature can significantly influence the substrate biodegradation rate, the activity of hydrogen-producing enzymes and the metabolism of H₂ producers [11,12]. The operational temperature should be chosen based on the inoculum type. Dark Fermentation can be performed at mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C) or hyperthermophilic (>80°C) conditions. Thermophilic conditions contribute in higher H₂ yields, inhibition of H₂ consumers and better stabilization of the digestate. On the other hand, due to the greater energy requirements, operational costs for thermophilic processes would be higher than mesophilic conditions. The majority of studies on dark fermentative H₂ production has been performed in temperatures between 35-37°C since mesophilic process is cost effective and easy to be controlled in large scale [13].

1.2.3. Substrate

Carbohydrate-rich substrates such as food waste are regarded as potential substrates for dark fermentative H₂ production due to its high carbohydrate content, being inexpensive and abundant [24–26].

Several studies reported that carbohydrate- rich substrates are favored for biohydrogen production due to their higher hydrolysis rates compared to lipids and proteins [11,27,28]. The main reason for this could be attributed to the short duration of dark fermentation which is not enough for the hydrolysis of proteins and lipids. Therefore, H₂ yield is proportional with the carbohydrate content of the substrate. Food waste could be regarded as one of the potential substrates for H₂ production due to its high carbohydrate content.

1.2.4. Inoculum

Using pure cultures has the benefit of exploitation of mutated strains. On the other hand, using mixed cultures for H₂ production reduces the operational costs compared to pure cultures since eliminates sterilization costs. Moreover, microbial diversity could enhance hydrolysis of different compounds present in organic wastes [29,30]. Many microbial species like *Enterococcus* sp., *Bacillus* sp., and *Clostridium* sp. are capable candidates for biohydrogen production. H₂ production studies from organic wastes using pure cultures have still not been widely mentioned in the literature. Recently, Shah et al., [31] could successfully produce H₂ from organic waste by pure cultures of *Bacillus* sp. for the first time. In another study, Srivastava et al., [32] produced 340 mL H₂/gCOD using pure cultures of *Bacillus licheniformis*. Kanchanasuta et al., [33] used pure cultures of *Clostridium butyricum* as the inoculum for H₂ production from organic waste. The mixed inoculum for dark fermentation can be found in environments such as soil, wastewater sludge and compost. The seed inoculum needs to be pre-treated in order to enrich H₂ producing bacteria and inhibit H₂ consuming ones.

1.2.5. Hydraulic retention time (HRT)

HRT can influence H₂ production by affecting hydrolysis, production of soluble products and controlling the growth rate of H₂ producing and H₂ consuming activities [23]. It has been reported that low HRT leads to wash out of methanogens and therefore is favoured during dark fermentation [34]; though, HRT alone cannot completely inhibit methanogens. Liu et al., [35] reported a combination of pH 5.5 and HRT of 3 days resulted in the optimum H₂ production from household waste. The optimum HRT for dark fermentation also depends on the biodegradability of substrate as it affects the hydrolysis rate.

1.2.6. H₂ partial pressure

The partial pressure of H₂ in the headspace of the bioreactor can influence dark fermentation as it affects mass transfer of H₂ from liquid to gas phase. High H₂ partial pressure leads to less oxidation of ferredoxin and subsequently less H₂ production and therefore is not favoured during dark fermentation [23]. In the presence of methanogens, the partial pressure of H₂ might be reduced due to the conversion H₂ and CO₂ to CH₄. Several strategies have been used to remove the H₂ from liquid phase, including sparging with N₂ and CO₂, strong mixing, using H₂-permeable membranes and reducing the pressure in the bioreactor using vacuum pump [36]. The most cost effective method to reduce the H₂ partial pressure could be avoiding the accumulation of H₂ inside the reactor by continuous removal of the produced H₂ from the reactor.

2. Inoculum pre-treatment prior to dark fermentation

Inhibiting H₂ consuming microorganisms such as hydrogenotropic methanogens, homoacetogens, lactic acid bacteria, propionate producing bacteria and sulphate reducers is one of the main steps for dark fermentative H₂ production when using mixed microbial communities [14]. Presence and growth of varying H₂ consumers depends on many factors and, therefore, may vary between different culture conditions. For instance, lactic acid and propionic acid bacteria

dominate in conditions such as high loading rates [15]. Nevertheless, hydrogenotropic methanogens have the biggest contribution for H₂ consumption among all H₂ consumers and their presence in mixed microflora reduces the H₂ yield significantly. Without inhibiting methanogens, H₂ will be consumed by them to produce methane. Considering this issue, the main goal of inoculum pre-treatment is enriching H₂ producing bacteria and suppresses H₂ consuming ones and mainly methanogens. The principle of inoculum pre-treatment technologies is that H₂ producers (mainly *Clostridium* spp. and *Bacillus* spp.) can sporulate when they are subjected to harsh environmental conditions of pH, temperature, irradiation, chemicals and etc [16–19]. Therefore, they can survive in such extreme conditions whilst non-spore-forming H₂ consumers that are not resistant to severe environmental conditions will be destroyed (Figure 2-1). Sporulating bacteria are able to be active again when the environmental conditions become suitable.

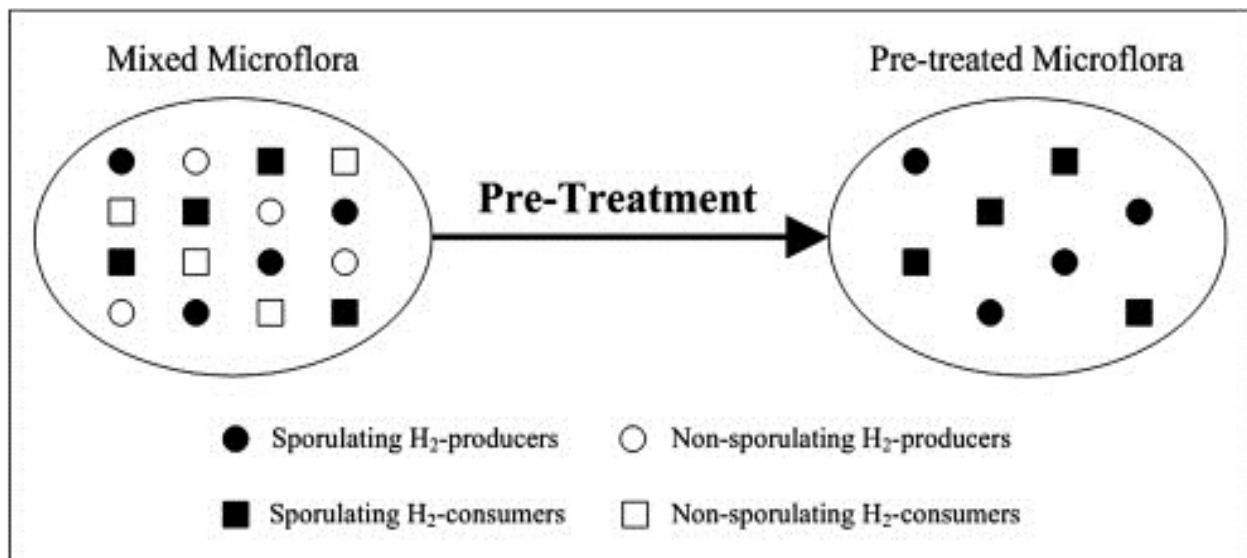


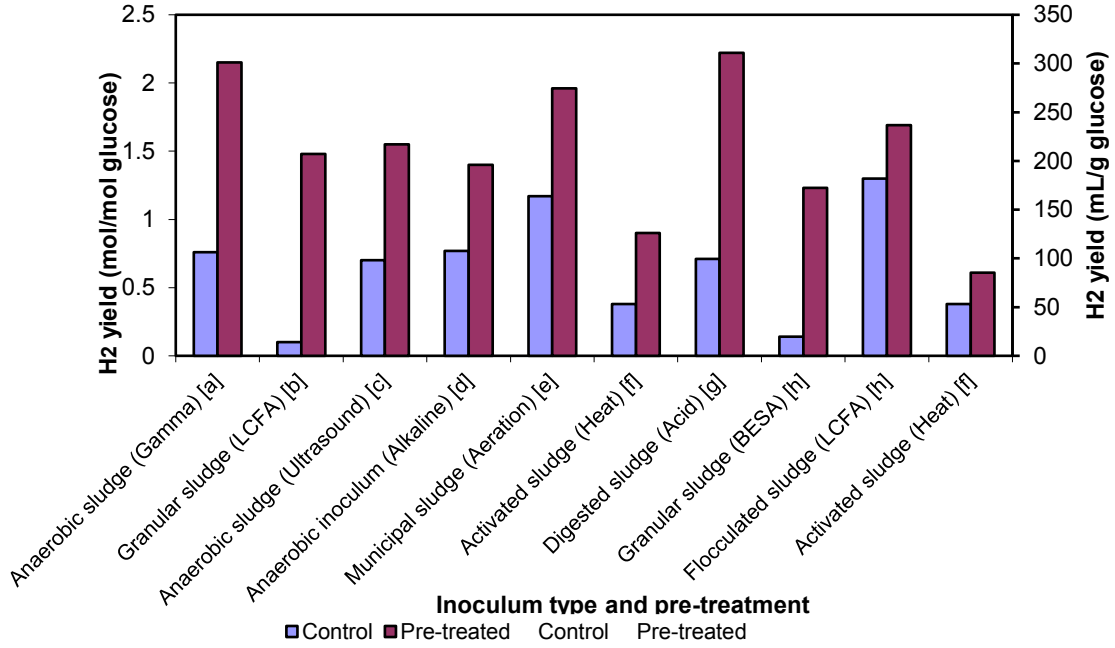
Figure 2-1 Effect of inoculum pre-treatment on H₂ producing and H₂ consuming microorganisms [22]

Many studies have been performed to investigate varying inoculum pre-treatment technologies and their impact on hydrogen production but most of them have used glucose as substrate. Instead, few works performed using complex substrates employed pre-treatments other than heat shock to select H₂ producer communities. The need to investigate different inoculum pre-treatment technologies using organic wastes as substrate is a crucial issue to design full scale plants. The most investigated inoculum pre-treatments either using glucose or organic wastes are discussed in the following section. Figure 2-2 and Figure 2- 3 show a summary of studies in the literature that used varying inoculum pre-treatments for dark fermentation when the substrates were glucose and organic waste respectively. Enhancement of H₂ yield has been considered as the main criteria for the efficiency of pre-treatments in most of the studies. However, there are additional criteria that should be considered to compare deeply the different pre-treatments.

2.1. Heat shock

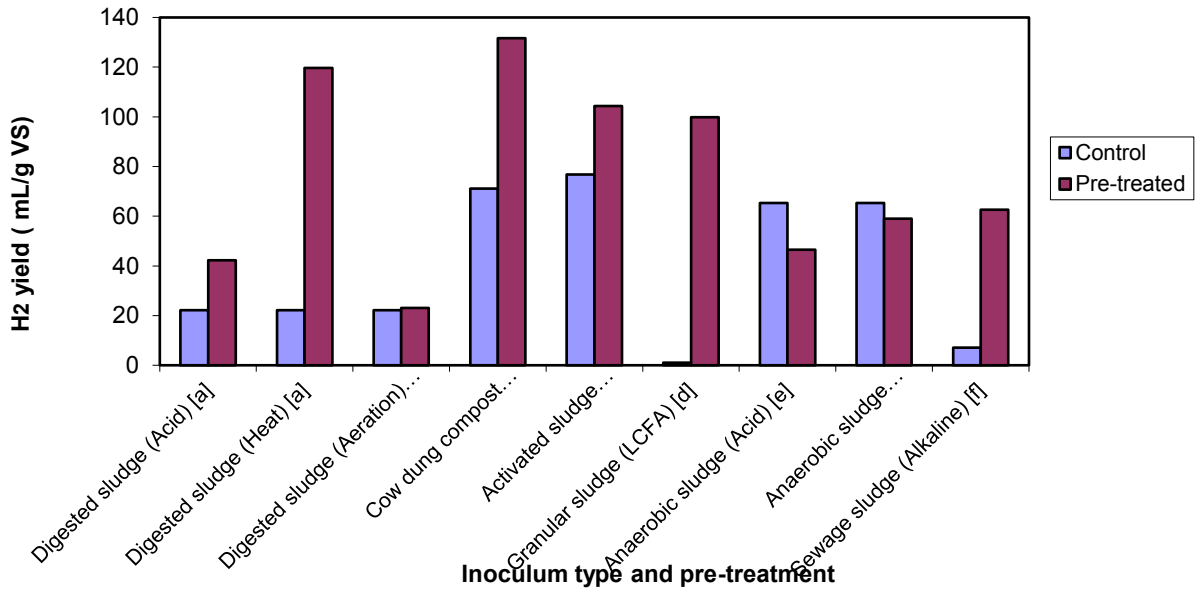
There are a lot of studies which used heat shock as an inoculum pre-treatment for dark fermentative H₂ production. They used varying temperatures and pre-treatment times and obtained different results. Wang and Wan, [37] obtained a H₂ yield of 215 ml.g⁻¹ glucose after pre-treating digested sludge in 100°C for 15 min which was 220% higher than untreated culture. They also reported heat shock pre-treatment led to the maximum H₂ yield compared to other inoculum pre-treatments (aeration, acid or base, chloroform). Applying the same pre-treatment conditions on another type of inoculum (anaerobic sludge), Yin et al., [38] reported a 100% increase in H₂ yield compared to the control. This difference between H₂ yields, which was obtained with the same pre-treatment conditions, is due to the varying microbial communities present in each inocula and therefore, their varying resistance to pre-treatment. Some studies reported a great enhancement of H₂ yield after heat treatment in low temperatures. As an instance, Baghchehsaraee et al., [39] obtained a H₂ yield of 1.6 mol.mol⁻¹ glucose for heat-treated activated sludge (65°C, 30 min) which was 530% higher than control. They observed that increasing the temperature to 80°C and 95°C led to decreased H₂ yields compared to the pre-treatment at 65°C. In contrast, Alibardi et al., [40] studied heat treatment of granular sludge at 100°C with varying pre-treatment times (0.5-4 h) and obtained the maximum H₂ yield of 2.14 mol.mol⁻¹ glucose for the sludge pre-treated for 4 h. Pendyala et al., [41] reported an increase of 542% in H₂ yield compared to untreated sludge after pre-heating granular sludge in 105°C for 45min.

As a step forward towards a more economically feasible process for H₂ production, several studies investigated effect of heat shock on microbial communities using different organic wastes. Liu et al., [35] used heat shock (pre-treatment of inoculum in 90°C for 1 h) for selecting H₂ producers in anaerobic sludge for H₂ production using household wastes and obtained a H₂ yield of 43 ml.g⁻¹ VS. Alibardi and Cossu, [11] showed that thermal pre-treatment of granular sludge in 80°C for 15 min was effective to collect H₂ producing bacteria. They obtained a H₂ yield of 188 ml.g⁻¹ VS using food waste as substrate. In contrast to the mentioned studies, Luo et al., [29] observed a decreased H₂ yield of 12% from cassava stillage as substrate after heating anaerobic sludge in 90°C for 1 h. It may be discussed that due to the intensive pre-treatment conditions, there is a possibility of partial suppression of hydrolyzing bacteria which their presence is vital when using organic wastes as substrate.



[a] [42], [b] [41], [c] [43], [d] [44], [e] [45], [f] [46], [g] [47]

Figure 2-2 Selected inoculum pre-treatment technologies for dark fermentative hydrogen production using glucose as substrate



[a] Switchgrass hydrolysate [48], [b] Food waste [49], [c] OFMWS [9], [d] Cassava stillage [29], [e] Cornstalk [50], [f] Apple pomace [51]

Figure 2-3 Selected inoculum pre-treatment technologies for dark fermentative hydrogen production using organic wastes as substrate

Improved H₂ yields after heat pre-treatment is also evident from changes in soluble metabolites production. For instance, higher H₂ production accompanied with lower lactic acid production is

a result of lactic acid bacteria inhibition by heat treatment [41]. Several studies have reported lower propionic acid and acetic acid concentrations with higher H₂ production after heat treatment which could be due to suppression of propionic acid and homoacetogenic bacteria respectively. Rossi et al., [52] observed lower acetic acid concentrations at the end of DF for heat-pre-treated sludge (obtained from an anaerobic sludge blanket reactor) relative to control. They heated the sludge for 2h in 105°C and used glycerol as substrate. Zhang et al., [53] reported lower propionic acid production for seed sludge subjected to thermal pre-treatment for 100°C for 1 h. Similarly, heat treatment of activated sludge in 95°C for 30 min led to the reduction in propionic acid concentrations compared to the control [22]. All the mentioned studies together show that heat pre-treatment may be inhibiting for different groups of H₂ consuming bacteria and therefore could be regarded as an effective pre-treatment during DF. However, long-term studies have shown that heat is not effective in long-term and therefore, it is required to repeat treatment during the process for permanent inactivation H₂ consuming populations. This makes the economics of the process doubtful [20,45].

2.2. Aeration

Aeration has been employed as an alternative inoculum pre-treatment method in order to enrich H₂ producing bacteria and inhibit H₂ consumers in DF. It is accepted that methanogens are strictly anaerobic microorganisms and are very sensitive to presence of oxygen. Aeration can inhibit all anaerobic H₂ consumers while the activity of spore forming H₂ producers can be recovered when the environment is changed to anaerobic conditions again [17]. Different duration and aeration intensities have been investigated for aerobic pre-treatment of mixed microflora prior to H₂ production. Zhu and Béland, [54] flushed air for 30 min into digested sludge obtained from a primary anaerobic digester. They performed DF using sucrose as substrate and observed that short time aeration, could not effectively suppress methanogenesis since H₂ yield was almost similar for pre-aerated sludge and non-pre-treated one. Wang and Wan, [37] pre-aerated digested sludge completely for 24 h and used the pre-treated inoculum for H₂ production from glucose. They obtained a higher H₂ yield (105 ml.g⁻¹ glucose) compared to control (75 ml.g⁻¹ glucose). Similar to other pre-treatments, the optimum condition for pre-treatment may vary among different inoculum types. Chang et al., [55] employed pre-aeration treatment on waste activated sludge for 24 h and achieved a cumulative H₂ of 73.53 ml. g⁻¹ glucose using glucose as substrate which was 125% higher than control (32.69 ml.g⁻¹ glucose). A more intensive aeration method was applied by Song et al., [56] who aerated the cow dung compost using an air pump for 72 h and obtained a H₂ yield of 247.6 ml.g⁻¹ glucose which is the highest yield reported for aeration pre-treatment. In order to optimize aeration pre-treatment, Giordano et al., [57] investigated aerobic pre-treatment of granular sludge for 2-14 days (aeration intensity of 100 LairL-1sludge h-1) prior to fermentation process. They observed that increased aeration time from 2-12 days led to an enhancement of H₂ yield with the highest H₂ yield of 160 ml.g⁻¹ glucose with 12 d pre-treatment.

There are quite few studies that studied aeration pre-treatment using organic wastes as substrates [29, 40, 47, 48]. Pre-aeration of anaerobic sludge for 2 h led to an increase of 4% in H₂ yield when a mixture of rice and lettuce powder was used as substrate [29]. Ghimire et al., [58] obtained a H₂ yield of 82.4 ml.g⁻¹ VS using the sludge subjected to aerobic pre-treatment (10 d,

air flow of 100 L air kg^{-1} sludge h $^{-1}$) and potato and pumpkin waste as substrate. The yield was 83% higher compared to heat-treated sludge.

Although the few studies performed on aerobic pre-treatment of mixed microflora reported enhanced H₂ yield compared to control, the H₂ yield is still lower if compared to other inoculum pre-treatment methods such as heat shock. In addition, this pre-treatment method is regarded quite time consuming if compared with the other pre-treatments and this would be problematic in long-term if repeating the aeration is needed. Moreover, in full scale, aeration would be energy intensive and therefore unfavorable from the economical point of view. With all aforementioned drawbacks, aeration is not considered as an appropriate technology for inoculum pre-treatment with the possibility of scale up and therefore, further investigations are discouraged.

2.3. Alkaline and acid pre-treatment

Acid and alkaline pre-treatments are widely used methods for enriching H₂ producing bacteria and inhibiting H₂ consumers with exploiting the sensitivity of non-spore forming H₂ consumers to pH changes. Similar to other inoculum pre-treatment technologies, the principle behind using acid or base is to make an extreme environment in which non-spore forming methanogens are suppressed due to cell wall disruption. Hydrogenotropic methanogens and some other non-spore forming H₂ consumers are not resistant to very low or very high pH conditions while the main H₂ producing bacteria (*Clostridium* spp and *Bacillus* spp) are able to sporulate and survive [19]. The most commonly employed acids are HCl, HClO₄, H₂SO₄ and HNO₃ and for alkaline pre-treatment NaOH and KOH have been employed widely. Acid and alkaline pre-treatments have been widely used to select H₂ producing cultures for DF. Chang et al., [55] pre-treated activated sludge for 24 h with 1M HCl and the pH was adjusted to 3 during treatment. Then the acid pre-treated inoculum was used for DF using glucose as substrate. An increased H₂ yield (almost 3 fold) was obtained with acid pre-treatment in comparison with control. Using the same pH conditions with corn stover hydrolysate as substrate a slight increase of 18% was observed compared to non-pre-treated inoculum [44]. Wang et al., [7] used HCl for acidic and NaOH for alkaline pre-treatment of anaerobic sludge. Alkaline and acidic pre-treatments (24 h) were performed in pH 12 and 3.5 respectively. H₂ yields of 55.4 and 41.5 ml/g VS obtained for acidic and alkaline pre-treatments respectively utilizing vinegar residues as substrate.

Pendyala et al., [41] added KOH 3M to granular sludge for 24 h (pH 12, 24 h). An enhanced H₂ yield of 0.83 mol.mol $^{-1}$ glucose than control (0.14 mol.mol $^{-1}$ glucose) revealed the effectiveness of alkaline pre-treatment. Chang et al., [55] pre-treated activated sludge with 1 M NaOH for 24 h, obtained an increase of approximately 2.5 fold relative to non-pre-treated culture using glucose as substrate. Mohammadi et al., [59] applied acid pre-treatment (6N HCl, pH 3, 24 h) on anaerobic sludge and observed a 166% increase in H₂ yield utilizing palm oil mill effluent as substrate. They also investigated alkaline pre-treatment (6N NaOH, pH 12, 24 h) using the same inoculum and obtained an increase of 208% in H₂ yield. Conversely, Chang et al., [43] reported that alkaline pre-treatment was more effective compared to acid pre-treatment to select H₂ producers from sewage sludge when glucose used as substrate. Similar to the other methods, the optimum conditions for pre-treatment may vary between different inocula due to characteristics of different microbial populations. In addition, when the low degradable substrates such as

lignocellulosic wastes are used as substrates, intensive pre-treatment may partially destroy hydrolytic bacteria that would be accompanied with a subsequent decreased H₂ yield. Using sewage sludge as inoculum, Hu and Chen, [60] reported a 28% decreased H₂ yield after acid pre-treatment (HCl, pH 3, 24 h) using glucose as substrate. Similarly, Ren et al., [61] observed 71% and 25% lower H₂ yields after acid and alkaline treatments respectively when glucose was used as substrate. The lower H₂ yields reported by Hu and Chen, [60] and Ren et al., [61] together with enhanced H₂ yields obtained by Chang et al., [55], Zhang et al., [53] and Wang et al., [7] after acid pre-treatment confirm that effectiveness of acid/alkaline pre-treatment strongly depends on the inoculum type and pre-treatment conditions. Nevertheless, the efficiency of either acid or alkaline pre-treatment is still lower than heat shock. Moreover, like other inoculum pre-treatment technologies, acid or alkaline pre-treatment have only short-term effects on H₂ consumers and repeated addition of acid or base is required for continuous H₂ production which impose more difficulties on the process [20]. Another drawback for using acid/alkaline in full scale would be the need for pH adjustment after treatment that seems to be challenging due to the significant difference between pre-treatment and fermentation pH conditions. Moreover, materials compatibility is another issue which should be taken into account for designing the reactors. With all aforementioned points, using acid/alkaline pre-treatment seems to be doubtful for commercialization.

2.4. Irradiation

Irradiation is considered as a novel technology to select H₂ producing bacteria from anaerobic mixed microflora. Different irradiation types that have been employed for inoculum pre-treatment in the past and they include microwave, ultrasound, gamma and infrared irradiation. The principle behind using all irradiation methods, the same as the previously mentioned technologies, is to create a harsh environment in which non-spore-forming microorganisms (which are mainly H₂ consumers) are not able to survive. However, the mechanism to achieve this goal is different among irradiation methods.

Ultrasound irradiation utilizes sound waves to create high temperature and pressure conditions in the medium. In such an extreme local conditions, reactive radicals may also be formed that finally disrupt the cell wall of sensitive organisms [19]. Elbeshbishy et al., [45] irradiated anaerobic sludge for 20 min at 20 kHz (62.5 W.g⁻¹ VS) and achieved an increase of 120% compared to the control using glucose as substrate. Dong et al., [9] sonicated anaerobic sludge (20 kHz, 20 min) and obtained an enhanced H₂ yield of 22.6 ml.g⁻¹ VS using lettuce powder compared to the 18.8 ml.g⁻¹ VS for non-pre-treated sludge.

Microwave irradiation corresponds to using electromagnetic waves with frequencies from 300 MHz to 300 GHz to generate friction and heat in polar liquids which subsequently leads to cell wall disruption [52]. Song et al., [50] pre-treated cow dung compost with microwave irradiation (0.5-2.5 min, 245 W.g⁻¹ TS) and used the resulted inoculum for H₂ production from corn stalk. They reported a maximum H₂ yield of 144.3 ml.g⁻¹ substrate with 1.5 min microwave irradiation. Veeravalli et al., [36] investigated the H₂ production from potato starch using an inoculum collected from a methanogenic reactor. They used microwave irradiation pre-treatment on the sludge (2 min, 25 W.ml⁻¹) and obtained a H₂ yield of 0.9 L.L⁻¹.d⁻¹ which was

comparable with the yield recorded for the heat treated sludge. In another study, [53] irradiated cow dung by microwave with varying powers (14.5-80 W.g-1TS) for 5 min and a frequency of 2450 MHz. H₂ production from *Benincasa hispida* waste using the irradiated inocula demonstrated that the optimum H₂ yield (14 mmol.mol⁻¹ sugar) obtained with a power of 22.66 W.g⁻¹TS.

Gamma irradiation is an emerging ionizing technology which is widely employed in the environmental engineering. Gamma irradiation changes the chemical, physical and biological properties of aqueous solutions. It creates free radicals react with DNA of non-spore-forming species and subsequently leads to cell wall disruption [25]. However, gamma irradiation is considered as a new technology for inoculum pre-treatment prior to dark fermentation and the studies performed using this method are scarce. Using gamma irradiation for pre-treatment of anaerobic sludge (20.8 Gy.min⁻¹), Yin et al., [46] obtained a H₂ yield of 267.7 ml.g⁻¹ glucose which was 194.3% higher than control. They concluded that besides inhibiting methanogens, gamma irradiation was effective in suppressing homoacetogens and propionic acid producers since propionic acid and acetic acid were not present among the soluble metabolites. In another study, [54] obtained a H₂ yield of 1.81 mol.mol⁻¹ glucose using the anaerobic sludge subjected to gamma irradiation (286 Gy.min⁻¹). In order to better understand gamma irradiation impact on microbial populations, [55] investigated the changes in microbial communities for gamma irradiated sludge and non-pre-treated sludge. They observed that many species were suppressed by gamma irradiation and H₂ producers including *Clostridium butyricum* were the predominant after gamma irradiation.

Infrared is another irradiation technique with the possible use as inoculum pre-treatment prior to dark fermentation. Infrared is an electromagnetic radiation with frequencies higher than microwaves that produces heat in the medium and breaks the cell wall of sensitive organisms. Efficiency of infrared to inhibit H₂ consuming species has been reported previously by Fan et al., [62] who baked cow dung for 2 h in an infrared oven and obtained a H₂ yield of 68.1 ml H₂.g-1 VS from acid treated wheat straw. Song et al., [56] employed infrared irradiation for 2 h on cow dung and obtained a H₂ yield of 290.8 ml.g⁻¹ glucose which was 6% and 17% higher than the yields achieved with heat shock and aeration respectively. Since both mentioned studies have used infrared irradiation on the similar inoculum, effectiveness of this method for other inoculum types is unknown.

Irradiation methods discussed above are considered as emerging technologies for inoculum pre-treatment prior to dark fermentation and the studies performed using them are quite scanty. More investigations are required using varying pre-treatment conditions and also utilizing complex wastes as substrate in order to obtain a comprehensive conclusion about possible utilization of these technologies for full scale applications. Also, cost and benefit analysis studies should be performed in order to calculate if the increased H₂ yield may compensate energy requirements for pre-treatment.

2.5. Chemical inhibition

Several chemicals have been shown to have inhibitory effects on methanogens and therefore employed for selecting H₂ producers from anaerobic mixed microflora to enhance H₂ yields. 2-

bromoethansulphonate (BES) or 2-bromoethansulphonate acid (BESA) is a widely used chemical to suppress methanogenic microorganisms. BES is an analogue of coenzyme-M (responsible to transfer a methyl group into methane in the final stage of methanogenesis) and its addition may hinder the completion of methanogenesis [18]. According to Valdez-Vazquez et al., [63] no methane was detected in the reactors fed with 25 mM BES pre-treated sludge. They used a mixture of food and paper waste as substrate. Using BES pre-treated digested sludge and grass as substrate, Kosse et al., [64] reduced methane concentration in the biogas to 1.84% relative to 33.58% for non-pre-treated sludge. Pendyala et al., [41] added 50 mM BES to granular sludge and observed more than 7 fold increase in H₂ yield with sucrose as substrate. Other studies have used BES or BESA in a range of 50-500 mM, reported 23-700% increased H₂ yields [20,21,23,45]. Kumar et al., [65] reported that BESA treatment did not significantly improved H₂ yield, However, the H₂ production rate was almost doubled (210 mL.L⁻¹.d⁻¹) compared to the control (106 mL.L⁻¹.d⁻¹). Despite several studies obtained higher H₂ yields after BES pre-treatment, Pendyala et al., [41] observed survival of some methanogens and homoacetogens. Also, increased production of propionic acid after BES pre-treatment was reported by Chang et al., [55]. Higher propionic acid production is regarded as an indicator for inefficacy of BES on inhibiting propionic acid bacteria, another group of H₂ consumers. Shanmugam et al., [42] studied long term impact of BES pre-treatment methods on inhibiting H₂ consumers. Although they obtained higher H₂ yields with granular sludge pre-treated with 50 mM BES relative to the control, it was the lowest yield among all other pre-treatment methods (heat shock, acid and alkali, long chain fatty acids).

Chloroform is another compound which has been employed for its inhibitory effect on methanogens during dark fermentation to select an inoculum rich in H₂ producing bacteria. Similar to BES, the mechanism of chloroform function relies on preventing the final step of methanogenesis to be completed. It suppresses methyl-CoM reductase and therefore reduction of methyl group of methyl-CoM to CH₄ [51]. The inhibitory effect of chloroform (0.05-5% v/v) on of methanogens present in granular sludge was studied by Hu and Chen, [60]. They obtained the highest H₂ yield of 135 ml.g⁻¹ glucose with 0.05% chloroform which was significantly higher than control (0.42 ml.g⁻¹ glucose). When they used another type of inoculum (sewage sludge) the maximum increase in H₂ yield was 16%. Adding chloroform with a concentration of 1% v/v to activated sludge increased H₂ yield from 3.59 mmol.g⁻¹glucose to 5.76 mmol.g⁻¹glucose [22]. Similarly, Mohammadi et al., [59] reported a H₂ yield of 0.23 mmol.g⁻¹COD with chloroform pre-treated sludge which was 97% higher than control. In contrast to the mentioned studies that reported enhanced H₂ yields after chloroform treatment, there are some reports who observed a decreased H₂ production. Wang and Wan, [37] observed a lower H₂ yield from glucose (19% lower than control) following addition of 2% chloroform to digested sludge relative to control. They concluded that higher concentrations of chloroform not only inhibit methanogens, but also may reduce H₂ producing bacterial activities. In another study, Luo et al., [29] reported a 49% decreased H₂ yield from cassava stillage after pre-treatment of inoculum (anaerobic sludge) with 0.2% chloroform. They contributed the lower H₂ production to partial inhibition of hydrolytic bacteria by chloroform.

Acetylene is another stressing agent that has been used as an inhibitor for methanogenesis during dark fermentation. Its action mechanism is disruption of H^+ gradient in cell membrane and decrease energy for methanogenesis by lowering ATP synthesis [60]. Acetylene is considered a cheap compound for inoculum pre-treatment and there are promising results on methanogenic inhibition and improving H_2 yields after using it. Zhao et al., [66] used acetylene as an inhibitor for CH_4 production in landfills. Valdez-Vazquez et al., [63] found out acetylene 1% (V/V) has an inhibitory effect on methanogens which was comparable to inhibition by BES. They used anaerobic sludge as inoculum and organic fraction of municipal solid waste as substrate. Another study done by Valdez-Vazquez et al., [67] showed that pre-treatment of inoculum with acetylene led to enhanced H_2 yields when the fermentation process was conducted in mesophilic conditions ($37^\circ C$) in comparison with thermophilic ($55^\circ C$) conditions.

Chemical inhibition seems to be an economically feasible pre-treatment for full scale since it does not need high energy requirements or capital costs. Nevertheless, this method has not received much attention in the recent years. This could be mainly due to the presence of these toxic chemicals in the effluents that may lead to serious ecological problems. Other drawbacks include being flammable (acetylene), major cost implications (BES) and failure on inhibiting all the H_2 consumers (chloroform) [19,21,30].

2.6. Long chain fatty acids pre-treatment

The inhibitory of long chain fatty acids (LCFAs) on gram positive bacteria have been proven since last decades. LCFAs have the ability to be absorbed on the cell wall of methanogens (which are similar to gram positive bacterial cell wall), reduce permeability and limit the transport of soluble substrates [63]. Absorption of LCFAs on the cell wall can also disrupt the membrane by acidification and changing the pH [64]. Most of the enzymatic reactions within the cell cannot be preceded in pH condition lower than 7 and therefore incomplete metabolism results in cell death.

LCFAs naturally may exist in lipid rich wastewaters and are inexpensive when compared with chemical inhibitors. Moreover, in contrast to chemical inhibitors, they are non-toxic for the environment. The inhibitory effect of LCFAs has been utilized recently to suppress methanogenesis and subsequently enhance H_2 production. Linoleic acid (LA), palmitic acid (PA), stearic acid (SA) and Lauric acid (LUA) have been shown to have inhibitory effects on methanogens [65–69]. Chaganti et al., [68] added 2000 mg L^{-1} LA to mixed anaerobic cultures fed with xylose and obtained a H_2 yield of 1.94 mol.mol^{-1} xylose. Shanmugam et al., [69] observed that adding LUA and LA to granular sludge cultures fed with glucose decreased H_2 consumption by 86% and 65% respectively compared to the control. Recently, Shanmugam et al., [42] reported a H_2 yield of 2.58 mol.mol^{-1} glucose for thermophilic dark fermentation using LA addition that was the highest yield obtained among all pre-treatment methods (heat shock, BES, acid and alkali). Veeravalli et al., [48] pre-treated granular sludge with 1750 mg.L^{-1} LA and obtained a H_2 yield of 99.86 ml.g^{-1} VS from switchgrass hydrolysate. Inhibition of H_2 consumers with LCFAs is a relatively new technology in the field of dark fermentation. Inhibition with LCFAs could be regarded as an inexpensive option with the possibility of using in full scale. In comparison to other methods such as heat shock or irradiation, it is less energy

intensive and more economically feasible. Moreover, in case of need, repeating the pre-treatment during fermentation seems to be practical. However, since few studies have investigated effects of LCFAs on dark fermentative H₂ production, it would be interesting to investigate their effect on H₂ production from organic wastes since using low-value substrates is a prerequisite for sustainable H₂ production.

2.7. Influence of pre-treatments on fermentation products

Employing inoculum pre-treatments may affect different microbial populations and subsequently distribution of soluble products. For a better understanding about this issue, Ren et al., [61] investigated the fermentation products after four different pre-treatments on (repeated aeration, acid, alkaline and heat shock) using glucose as substrate. They observed that different treatments were accompanied with varying soluble products at the end of the fermentation. For heat shock and alkaline pre-treatment, butyric acid and acetic acid (46.1% and 45.4% respectively) were the final products. In contrast, the main products after acid pre-treatment were acetic acid (45.4%) and propionic acid (34.2%). Ethanol production decreased for all the pre-treatments compared to control except that repeated aeration that changed the fermentation type from mixed acid to ethanol type. The increased levels of ethanol could be somehow attributed to proliferation of *Ethanoligenens harbinensis* due to repeated aeration, which was not detected after the other treatments. It should be emphasised that both inoculum type and pre-treatment method have a significant effect on microbial communities and therefore fermentation products. For instance, Enterobacter species (which are facultative bacteria) have been detected in inocula such as composts [46,71,72] or cultures pre-treated with aeration (Jeong et al., [70]; Ren et al., [61]; Wang et al., [71]). The impact of pre-treatments on growth of different populations may be demonstrated by electron flow towards different products. Shanmugam et al., [42] reported that in control cultures, 32.4% of electron flow of substrate was diverted towards CH₄ production, while in all treated cultures no detectable CH₄ was found. They observed significant activities of acetoclastic methanogens and homoacetogens in control cultures that were responsible for lower H₂ yields. In another study performed by Yin et al., [46], mixed acid fermentation was observed for control and alkali treated cultures while acetic acid was the only VFA detected in heat shock and acid treated cultures. Likewise, Chaganti et al., [47] reported different fermentation types after varying pre-treatments on granular sludge. Cultures pre-treated with LA or heat, showed acetic acid, butyric acid and ethanol were dominant while controls and alkali treated cultures showed mixed acid fermentation. However, there are also some reports on similarity of the fermentation products after various pre-treatments only with changes in concentrations [16,21,44,45,73,74]. These different observations could be due to inoculum source, substrate type or operational conditions.

2.8. Comparison of different inoculum pre-treatments in long-term

The majority of studies mentioned above, investigated the pre-treatment technologies only for a single batch while the long-term effect of pre-treatments are neglected. The effectiveness of a pre-treatment on inhibiting H₂ consumers may vary over time due to different behaviors of each microbial population that are present in anaerobic mixed microflora. Although enrichment in H₂ producing species including Enterobacter and Clostridium is the main target of inoculum pre-

treatment, other microbial populations such as hydrolysers, granulation amplifiers and those removing toxic oxygen, that support long-term stabilization of fermentation process, should not be neglected [75]. In other words, pre-treatment conditions should be optimized in such a way that does not lead to major loss of mentioned populations. Despite its importance, studies on comparison long-term effect of different technologies on H₂ yields and changes in microbial diversity are quite scanty. Zhu and Béland, [54] pre-treated digested sludge with varying methods (heat shock, acid, alkaline, BES and aeration) and studied H₂ production from sucrose in mesophilic conditions. They observed that aeration and BES pre-treated sludge, produced less H₂ in the second batch compared to the first one whilst H₂ yield using acid, base and heat pre-treatment increased in the second batch. In the first batch the highest H₂ yield (5.28 mol.mol⁻¹ sucrose) was obtained using BES treated sludge while for the second batch, base pre-treated sludge produced the highest H₂ (6.12 mol.mol⁻¹ sucrose). O-Thong et al., [72] pre-treated anaerobic sludge with four different methods (acid, base, BES and heat shock) and compared the corresponding H₂ yields from sucrose. For all the pre-treatments higher H₂ yield was obtained in the second batch compared to the first one. Heat shock was the best pre-treatment method at the end of the second batch with a H₂ yield of 1.16 mol.mol⁻¹ hexose which was 286% higher than control. Shanmugam et al., [42] investigated long-term effects of different inoculum pre-treatments (acid, alkaline, heat shock, BES and LA) on granular sludge and studied H₂ production from glucose in mesophilic (37°C) and thermophilic (55°C) conditions. For mesophilic conditions, the H₂ yield decreased after 5th batch for alkaline, BES and heat pre-treated cultures while for acid and LA pre-treated sludge, higher H₂ yields was achieved in the 5th batch compared to the first one. At the end of the 5th batch, the highest H₂ yield was obtained for LA pre-treated culture (2.1 mol.mol⁻¹ glucose) which was almost 40% higher than the yield with LA pre-treated culture in the first batch. Conversely, H₂ production decreased for all the pre-treated cultures in the 5th batch compared to the first one when the incubation temperature was set to 55°C. For thermophilic fermentation, LA pre-treated sludge produced the highest H₂ yield (1.48 mol.mol⁻¹ glucose) among all other methods after the 5th batch. Luo et al., [73] studied long-term effect of heat shock and acid pre-treatment on H₂ production from glucose using digested sludge as inoculum. They observed that H₂ yields for both heat-treated and acid-treated inocula were lower compared to the untreated sludge after 5th batch cultivation. In another study, Luo et al., [29] studied thermophilic H₂ production using different inoculum pre-treatments including chloroform, acid, base and heat shock with cassava stillage as substrate. They observed that effectiveness of pre-treatments varied for batch and continuous fermentations indicating the short-term impacts of pre-treatments on H₂ yield. H₂ production by non-pre-treated granular sludge was higher than pre-treated sludge in batch tests. Conversely, in continuous experiments, no significant difference was observed between all pre-treatment methods and control culture.

It is difficult to make a conclusion about the best inoculum pre-treatment method for long-term only based on the very few studies mentioned. Nevertheless, it is obvious that effectiveness of a pre-treatment in long-term depends on inoculum, substrate and also temperature of incubation. According to Shanmugam et al., [42], LA pre-treatment seems to be a potential technology for long-term inhibition of H₂ consumers. However, more investigations are required to be performed on LA pre-treated cultures using organic wastes as substrate instead of glucose.

Likewise, effect of other pre-treatments needs to be studied in long-term using organic substrates.

3. Substrate pre-treatment

After selecting a culture rich in H₂ producing bacteria, the availability of easily degradable substrates is crucial for microbial growth and H₂ production. Lignocellulosic wastes are the most abundant biomass residues (220 billion tons per year) that may be regarded as potential substrates for hydrogen production [74]. Lignocellulosic compounds that may be found in biofuel production residues, agricultural and forestry wastes, food industry residues or even household wastes, contain low degradable polymeric compounds such as cellulose, hemicellulose and lignin. These compounds are not biodegradable due to their crystalline structure which makes them inaccessible for microorganisms. In order to achieve high H₂ yields, cellulose and hemicellulose content of these wastes should be hydrolysed into biodegradable carbohydrates which are regarded as the preferred substrate for dark fermentation (Figure 3- 1).

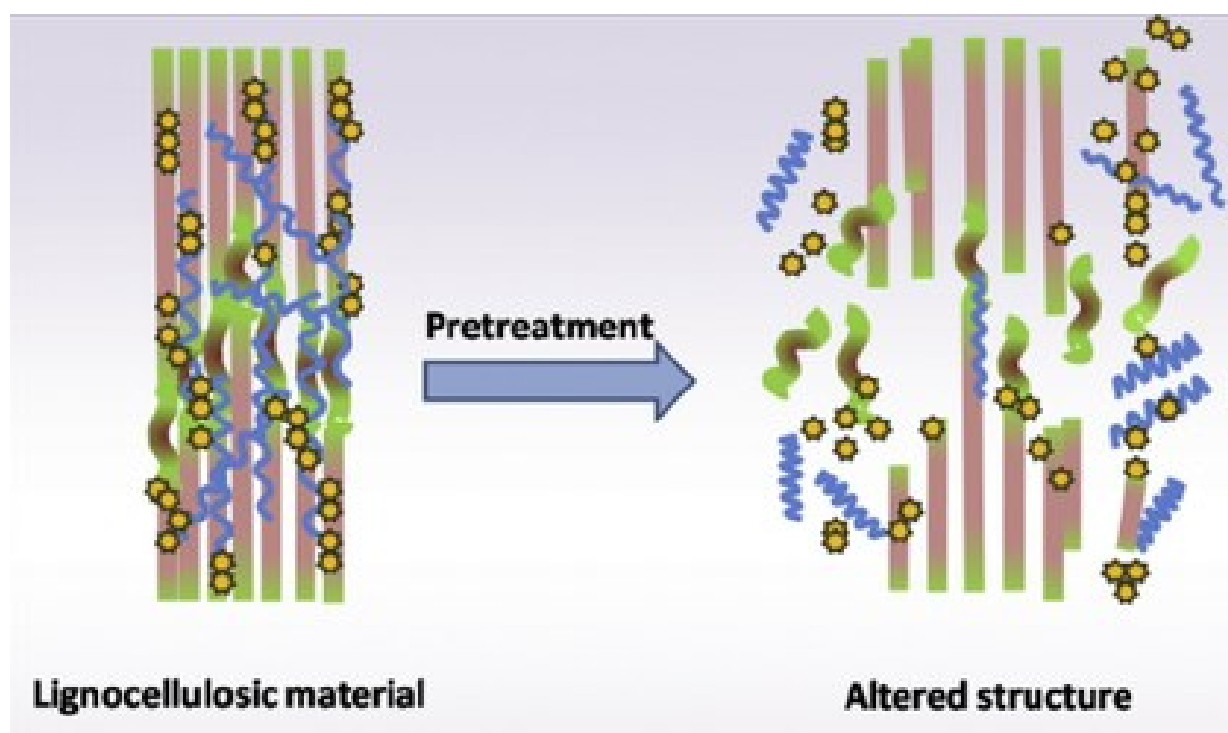


Figure 3- 1 Schematic representation of substrate pre-treatment [75]

Substrate pre-treatment technologies are aimed at breaking the complex structure of low degradable organics, improve solubilisation and subsequently enhance product yield. Impact of varying pre-treatments on H₂ yields and effluents characteristics depends on substrate and pre-treatment method. Hence, the best pre-treatment for each substrate should be identified based on the H₂ yield, costs, energy requirements and sustainability of the process. The most commonly investigated pre-treatment technologies that have been employed on varying substrates are discussed in the next section. A summary of the pre-treatment conditions and corresponding H₂ yields are shown in Table 3-1.

Table 3-1 Pre-treatment technologies for organic wastes investigated for dark fermentative hydrogen

Substrate	Pretreatment method	H ₂ yield	References
Food waste	Thermal pre-treatment (134°C, 20min)	Control: 5 mL.g ⁻¹ VS Pre-treated: 23 mL.g ⁻¹ VS	[76]
Food waste	Sonication (500W, 79kJ/g TS,)	Control: 42 mL.g ⁻¹ VS Pre-treated: 97 mL.g ⁻¹ VS	[77]
Food waste	Acid (HCl, pH 3, 24 h)	Control: 42 mL.g ⁻¹ VS Pre-treated: 55 mL.g ⁻¹ VS	[77]
Food waste	Heat (70°C, 30 min)	Control: 42 mL.g ⁻¹ VS Pre-treated: 70 mL.g ⁻¹ VS	[77]
Food waste	Alkaline (NaOH, pH 11, 24 h)	Control: 42 mL.g ⁻¹ VS Pre-treated: 46 mL.g ⁻¹ VS	[77]
Sewage sludge	Thermal pre-treatment (121°C, 30 min)	Control: 0.3 mL.g ⁻¹ VS Pre-treated: 1.2 mL.g ⁻¹ VS	[78]
Sugar beet pulp	Alkaline (2M NaOH, pH 12, 30min)	Control: 90.1 mL.g ⁻¹ VS Pre-treated: 115.6 mL.g ⁻¹ VS	[79]
Activated sludge	Acid (HCL 0.5w/v, 24h)	Control: 9 mL.g ⁻¹ VS Pre-treated: 41 mL.g ⁻¹ VS	[80]
Slaughterhouse sludge	Microwave (850W, 3min)	Control: 0.18 mL.g ⁻¹ COD Pre-treated: 12.77 mL.g ⁻¹ COD	[81]
Grass	Acid-heat (1 g grass with 20 ml HCl 4%w/v, boiled for 30 min)	Control: 4.38 mL.g ⁻¹ dry grass Pre-treated: 72.2 mL.g ⁻¹ dry grass	[82]
Sewage sludge	Thermal pre-treatment (121°C, 30 min)	Control: 1.21 mL.g ⁻¹ VS Pre-treated: 8.62 mL.g ⁻¹ VS	[83]
Sewage sludge	Sonication (20 KHz, 30 min, 0.8 W.ml ⁻¹ sludge)	Control: 1.21 mL.g ⁻¹ VS Pre-treated: 3.83 mL.g ⁻¹ VS	[83]
Sewage sludge	Acid (HCl, pH 2, 5 min)	Control: 1.21 mL.g ⁻¹ VS Pre-treated: 3.25 mL.g ⁻¹ VS	[83]
Sewage sludge	Alkaline (NaOH, pH 12, 5 min)	Control: 1.21 mL.g ⁻¹ VS Pre-treated: 1.46 mL.g ⁻¹ VS	[83]
Algal biomass	Acid (HCl 200 mL.L-1 , 121°C, 20 min)	Control: 1.42 mL.g ⁻¹ VS Pre-treated: 95 mL.g ⁻¹ VS	[84]
Vinegar residues	Acid (HCl, pH 1, 10ml.g ⁻¹ TS, 99°C 30 min)	Control: 23.8 mL.g ⁻¹ VS Pre-treated: 53.2 mL.g ⁻¹ VS	[7]
Vinegar residues	Alkaline (NaOH, pH 12, 24 h)	Control: 23.8 mL.g ⁻¹ VS Pre-treated: 55.4mL.g ⁻¹ VS	[7]
Vinegar residues	Heat (boling, 30 min)	Control: 23.8 mL.g ⁻¹ VS Pre-treated: 47.3mL.g ⁻¹ VS	[7]
Rice straw	Alkaline (NaOH 8%, 24 h,55°C)	Control: 0.3 mL.g ⁻¹ VS Pre-treated: 15.4 mL.g ⁻¹ VS	[85]
Waste sorghum leaves	Acid (HCL, 24 h)	Control: 47.3 mL.g ⁻¹ sugars Pre-treated: 213.4 mL.g ⁻¹ sugars	[86]
Cornstalk	Biological treatment (15 days)	Control: 20 mL.g ⁻¹ VS Pre-treated: 176 mL.g ⁻¹ VS	[87]
Potato	Enzyme (α -amylase, glucoamylase)	Control: 200.4 mL.g ⁻¹ VS Pre-treated: 217.5 mL.g ⁻¹ VS	[88]
Cornstalk	Biological pretreatment (fungi, 6 days)	Control: 18.65 mL.g ⁻¹ VS Pre-treated: 54.1 mL.g ⁻¹ VS	[89]
Olive pomace + olive mill wastewater	Sonication (1.8 kW, 30 min)	Control: 54 ml.g ⁻¹ VS Pre-treated: 81 ml.g ⁻¹ VS	[90]
Cassava wastewater	Ultrasound (50 kHz, 45 min)	Control: 80.4 ml.g ⁻¹ COD Pre-treated: 200.8 ml.g ⁻¹ COD	[91]
Food waste	Ultrasound (20 kHz, 100 W.g ⁻¹ VS, 45 min)	Control: 87.5 ml.g ⁻¹ VS Pre-treated: 299 ml.g ⁻¹ VS	[92]
Food waste	Heat (130°C, 50 min)	Control: 87.5 ml.g ⁻¹ VS Pre-treated: 0 ml.g ⁻¹ VS	[92]

3.1. Thermal pre-treatment

In addition to use as a method for selecting H₂ producing bacteria and inhibit H₂ consuming populations, thermal pre-treatment has also been used as a substrate pre-treatment technology for complex substrates mainly for lignocellulosic wastes. Thermal pre-treatment can be used to breakdown the bonds between molecules, increased solubilisation and subsequently enhanced hydrolysis [39,73,93]. Also, heat pre-treatment may help to pathogen removal which is a vital characteristic for process sustainability. Temperatures above 160°C may lead to solubilisation of lignin and hemicellulose. However, heat pre-treatment in very high temperatures (about 400°C) may lead to possible formation of undesirable byproducts such as phenolic compounds, furfural and hydroxufurfural which are regarded as inhibitory compounds for the hydrolyzing and fermentative microorganisms [94,95]. According to Carrère et al., [93] in temperatures higher than 170°C the so called Mallaird reaction may occurs between amino acids and carbohydrates resulting in the formation of melanoidins which are very difficult to be degraded. Most of the studies on thermal pre-treatment have been performed with temperatures ranged between 50°C to 250°C [97]. Temperature and duration of pre-treatment are two key factors affecting process efficiency. However, the composition of substrate and inoculum type may affect the results. Kim et al., [94] heated food waste at 90°C for 20 min and reported a significant enhancement in H₂ yield (96.9 ml.g⁻¹ VS) compared to 4.4 ml.g⁻¹ VS for non-pre-treated substrate. Elbeshbishy et al., [95] reported an increase in H₂ yield from food waste by 67% using heat pre-treatment for 30 min at 70°C. Pagliaccia et al., [76] pre-heated food waste at a higher temperature (134°C) with duration of 20 min and observed only 30% increase in H₂ production. They attributed the improvement in H₂ yield to better solubilisation of carbohydrates in food waste due to thermal treatment. Applying thermal pre-treatment on corn starch as substrate (100°C, 20 min), Bao et al., [96] obtained a cumulative H₂ of 1186 ml which was 40% higher than control (838 ml). Xiao and Liu, [83] pre-heated sewage sludge (a protein and carbohydrate rich substrate) at 121°C for 30 min and reported a H₂ yield of 8.62 ml.g⁻¹ VS relative to the non-pre-treated substrate (1.21 ml.g⁻¹ VS). In spite of the promising results reported using thermal pre-treatment, energy requirements to create high temperatures is regarded as a drawback for this pre-treatment. Energy balance analysis should therefore be performed in order to evaluate if the consumed energy for heating may be compensated by excess H₂ production.

3.2. Acid or alkaline pre-treatment

Acids or alkaline pre-treatment have been utilized not only for selecting rich H₂ producing cultures, but also as an established method to improve substrate solubilisation. The mechanism of pre-treatment is to destroy the polymeric bonds, enhanced availability of substrate and therefore increased biodegradability [94]. Acid pre-treatment may enhance solubilisation of hemicellulose but it is not effective on delignification [94]. Alkaline solutions saponify the ester bands in lignocellulosic substrates, breakdown their crystalline structure and subsequently enhance hydrolysis. HCl and H₂SO₄ are the most commonly used acids and NaOH is widely used for alkaline pre-treatment. Rorke and Kana, [86] reported a 77% hemicellulose solubilisation using combined heat/acid pre-treatment (5.95% HCL, 100°C, 176 min). Fangkum and Reungsang [97] used different concentrations of H₂SO₄ (0.25-5% v/v) for pre-treating sugarcane bagasse and reported an optimum pre-treatment condition using H₂SO₄ 1% (v/v) with

a maximum total sugar (glucose, xylose and arabinose) yield from sugarcane bagasse. Pan et al., [98] investigated the acid pre-treatment of cornstalk with H₂SO₄ 1.5% v/v, 21°C, 60 min) and reported a maximum H₂ yield of 103.3 ml. g⁻¹ VS with an increase of 368% relative to the untreated substrate (22.1 ml. g⁻¹ VS). Assawamongkholsiri et al., [80] used acid pre-treatment (HCl and H₂SO₄) for H₂ production from activated sludge. The solubilisation of proteins and carbohydrates with HCL was higher in comparison with H₂SO₄. A maximum H₂ yield of 41 (5% w/v, 6h, pH 1.0) was obtained with HCL pre-treated substrate (5% w/v, 6h, pH 1.0) which was 240% higher than control (12 (5% w/v, 6 h, pH 1.0).

Kim et al., [94] obtained an increase of 1056% in H₂ yields with alkaline pre-treatment of food waste (24 h, pH 13). H₂ yield with pre-treated and untreated food waste were 50.9 ml. g⁻¹ VS and 4.4 50.9 ml. g⁻¹ VS respectively. Ruggeri and Tommasi, [99] reported an increase of 1272% and 1130% in H₂ yield with NaOH pre-treated (24 h, pH 12.5) and HCL pre-treated (24 h, pH 3) vegetable wastes respectively relative to the control (6.6 ml. g⁻¹ VS). Xiao and Liu, [83], pre-treated sewage sludge with NaOH (pH 12, 5 min) and reported a H₂ yield of 11.68 ml. g⁻¹ VS which was 54% higher than control (7.57 ml. g⁻¹ VS).

The use of acids for pre-treatment of complex substrates is regarded as an inexpensive and efficient method. However, it may results corrosion in reactors. Another disadvantage for either alkaline or acid pre-treatment is possible formation of inhibitory compounds which may suppress the fermentation process. Moreover, pH adjustment after pre-treatment increases the complicacy of the process and also operational costs.

3.3. Microwave Irradiation

Microwave irradiation generates high temperatures in the medium which subsequently leads to cell wall disruption and increased solubilisation similar to heat pre-treatment. There are also non-thermal effects for microwave irradiation when dipoles in polar liquids are aligned and realigned continuously and generate frictional heat. When the cell wall is broken, the solubilisation and therefore degradability will be enhanced [52]. Microwave irradiation is an established method to improve solubilisation and subsequently biodegradability of complex substrates [102–105]. However, there are not many studies on dark fermentative H₂ production using microwave pre-treatment alone for substrate whilst most of the studies have combined microwave irradiation with acid or alkaline pre-treatment [106–108]. Serrano et al., [100] applied microwave pre-treatment (30 W.g⁻¹ TS, 66 Sec) on sewage sludge and reported an increase of 39% in soluble compounds compared to the untreated sludge. Guo et al., [101] heated wastewater sludge with microwave for 2 min at a power of 2.6 W.ml⁻¹ sludge and obtained a H₂ yield of 11.44 ml. g⁻¹ COD. They observed enhanced protein (570%) and carbohydrate (3100%) contents of sludge after microwave pre-treatment compared to untreated substrate. Thungklin and Sittijunda, [81] pre-treated sludge of poultry slaughterhouse wastewater with microwave (3 min, 42.5 W.g⁻¹ TS) and reported a H₂ yield of 12.77 ml. g⁻¹ COD while the H₂ yield for untreated sludge was 6994% higher than untreated substrate. Conversely, Bundhoo, [102] reported a decreased H₂ yield with microwave irradiated mixture of food and yard waste using varying pre-treatment time (0-30 min) and power (0-3.85 W.g⁻¹ TS). With applying a specific energy of 0.99 W.g⁻¹ TS, they obtained a H₂ yield of 7.03 ml. g⁻¹ VS which was significantly lower than control (21.27 ml. g⁻¹

VS). They concluded that high concentrations of soluble compounds such as ethanol and propionic acid after microwave pre-treatment could be a possible cause for lower H₂ yields.

As mentioned above, there are only a very few studies on using microwave pre-treatment alone as a substrate pre-treatment prior to dark fermentative H₂ production with controversial results. It should be noticed that microwave pre-treatment has high energy requirements which should be taken into account when performing economic analysis. Moreover, similar to thermal pre-treatment, the possible formation of inhibitory compounds is inevitable due to high temperatures. Hence, very high intensity powers should be avoided during pre-treatment in order to inhibit very high temperatures and subsequent formation of inhibitory compounds.

3.4. Sonication

Sonication means irradiation of ultrasound waves with frequencies between 20 KHz to 10 MHz to release high acoustic energy to the medium and subsequently produce local conditions of high temperature and pressure. In this method, high shearing forces, thermal effects and also formation of radicals can destroy the cell wall and therefore improve solubilisation by increased accessibility of intracellular compounds for hydrolysis [112]. Several studies showed the positive effect of sonication on enhanced biodegradability of substrate and therefore H₂ yield. Gadhe et al., [103] studied ultrasonic irradiation pre-treatment of food waste and determined total solids, pre-treatment time and specific energy as three factors affecting solubilisation and H₂ yield. They observed a 75% enhanced H₂ yield (149 ml. g⁻¹ VS) with optimum pre-treatment condition (8% TS, 12 min, 18.75 W.g⁻¹ TS). Similarly, Elbeshbishy et al., [104] sonicated food waste (500 W, 79 J.g⁻¹ TS) and observed a 63% increase in H₂ yield compared to the untreated food waste. Yang et al., [105] obtained a H₂ yield of 7.24 ml.g⁻¹ TS applying sonication pre-treatment (82.35 W.g⁻¹ TS, 60 Sec) on waste activated sludge which was compared 513% more than control (1.18 ml.g⁻¹ TS). Xiao and Liu, [83] pre-treated sewage sludge, a carbohydrate and protein rich substrate with ultrasound irradiation (88.8 W.g⁻¹ TS, 30 min). After pre-treatment, they observed 21 and 4.58 fold increase in protein and carbohydrate content of sludge respectively. A H₂ yield of 3.83 ml.g⁻¹ VS was obtained for the sonicated food waste which was 216% higher than untreated substrate. Battista et al., [90] used ultrasonic pre-treatment (32.7 W.g⁻¹ TS, 30 min) on a mixture of olive pomace and olive mill wastewater (a lignin and cellulose rich waste) used as substrate for ethanol-type fermentation. They obtained an increased H₂ yield by 50% and also a 23% decrease in polyphenols level (which are regarded as toxic compounds) after sonication. Bundhoo, [102] investigated varying ultrasound intensities (0-6946 J.g⁻¹ TS) for hydrogen production from a mixture of food and yard waste. Sonication enhanced solubilisation of organic matter; however, in contrast to the other studies mentioned, he observed a lower H₂ yield for sonicated substrate (6946 J.g⁻¹ TS) compared to the control (47% decrease). He attributed the lower H₂ yield to the high concentrations of ethanol and propionic acid and also formation of toxic compounds which are considered as inhibitors for dark fermentation. Likewise, Wongthanate et al., [106] reported lower H₂ yields after sonication of food waste (20 min) compared to untreated substrate. Unfortunately they did not mention the pre-treatment conditions in their manuscript and therefore, it is not possible to judge if the lower H₂ products would be due to intensive sonication. Ultrasonic irradiation has been shown to be beneficial for releasing the multiple nutrients entrapped in waste activated sludge. Sonication of activated sludge for 40

min led to increased levels of proteins and polysaccharides to 3700 and 800 mg.L⁻¹ respectively while the corresponding concentrations of mentioned nutrients were below 200 mg.L⁻¹ before pre-treatment [116].

Despite the fact that majority of studies have shown positive results, using sonication for full-scale applications is still doubtful because of the mentioned negative results. In this paper we tried to normalized the pre-treatment intensities for sonication as W.g⁻¹ TS whenever enough data was available in original articles since some of the studies presented the intensity of sonication by different units or even do not report the details of pre-treatment. Energy requirements and feasibility of using in full scale for ultrasound pre-treatment should be considered when performing economic studies in order to determine if the excess H₂ yield may compensate energy consumed by sonication. Moreover, possible formation of inhibitory compounds such as furans and phenolic compounds is another drawback which is inevitable using some substrates [111].

3.5. Biological Treatment

Biological pre-treatments technologies are also utilized to destroy cross-linking structure of lignocellulosic wastes and therefore enhance hydrolysis rate. There are different pre-treatment methods which are classified as biological technologies including fungal treatment, enzymatic hydrolysis and aeration. Fungal treatment corresponds to use of specific microorganisms such as white-rot and soft-rot fungi (to degrade lignin and cellulose) and brown-rot fungi (to degrade hemicellulose) prior to dark fermentation [117]. These specific groups of fungi produce extracellular enzymes that convert cellulosic compounds to reducing sugars and subsequently increase hydrolysis rate and H₂ production. With the aid of different types of fungi for biological pre-treatment, Hatakka, [107] increased the biodegradation of wheat straw by 35% in five weeks. Cheng and Liu, [89] reported an increase of 209% in cumulative H₂ production (194.9 ml) compared to untreated substrate (93.4 ml) using fungal pre-treatment of cornstalk (fungus *Trichoderma reesei* Rut C-30). Likewise, Zhao et al., [108] studied fungal pre-treatment of cornstalk with *Phanerochaete chrysosporium* using varying pre-treatment time ranged from 3 to 15 days and observed that enzymatic saccharification increased with pre-treatment time. They obtained a H₂ yield of 80.3 ml. g⁻¹ VS with fungal pre-treatment for 15 days.

Besides fungal pre-treatment, biological treatment also corresponds to direct use of enzymes required for hydrolysis. Several studies performed enzymatic hydrolysis as a substrate pre-treatment technology for enhancement of H₂ production. Cui et al., [109] pre-treated poplar leaves (a by-product of forestry) with a mixture of cellulose, hemicellulase, arabanase, xylansae and β-glucanase. They obtained an optimum H₂ yield of 44.92 ml. g⁻¹ dry poplar leaves which was 300% higher than raw substrate. Leañó and Babel, [91] reported a H₂ yield of 5.02 ml.g⁻¹ COD after pre-treating cassava wastewater with 0.2% α-amylase which was 50% higher than control. Contreras-Dávila et al., [110] investigated the effect of enzymatic hydrolysis on continuous H₂ production from agave baggase collected from a tequila distillery. Using Celluclast 1.5 L® for pre-treating agave baggase, they obtained a H₂ yield of 1.35 mol.mol⁻¹ substrate.

Another pre-treatment technology which has been studied recently by a few studies is termed pre-aeration or micro-aerobic pre-treatment. The principle behind this method is the fact that hydrolysis rates are higher in presence of oxygen [122]. Rafieenia et al., [111] applied aerobic pre-treatment (24 h, air flow rate 5 L/h) on food waste with varying composition prior to dark fermentation. They observed decreased H₂ yields by 19%, 33% and 24% for carbohydrate-rich, protein-rich and lipid-rich food waste respectively. They attribute lower H₂ yields with pre-aeration to carbon loss during pre-treatment. Li et al., [112] studied micro-aerobic fermentation for H₂ production from corn straw. They obtained a H₂ yield of 41.6 ml. g⁻¹ VS with an optimal oxygen addition equal to 0.28 ml/(g TS.day) which was 43% higher than control (with no oxygen addition). They observed presence of facultative anaerobes *Citrobacter* sp. and *Escherchia* sp. (which are H₂ producers) only with micro-aerobic fermentation but not in strictly anaerobic conditions.

In contrast to negative effects of pre-aeration on H₂ production from food waste, micro-aerobic fermentation with controlled oxygen addition was shown to be effective to enhance H₂ production from corn straw. However, further investigations should be carried out using shorter pre-treatment times and aeration intensities using varying substrates before making a conclusion on effectiveness of aerobic pre-treatment on H₂ production.

Biological treatment technologies regarded inexpensive compared to other pre-treatment due to less energy requirements. However, the disadvantage of long pre-treatment times should be considered for commercialized implementation.

3.6. Comparison of different substrate pre-treatments

Comparative analysis between different studies in order to determine the best pre-treatment method for the same substrate are not reliable due to varying inoculum and operational conditions applied among different studies. However, there are few reports about using varying pre-treatments on the same substrate in one study. Hence, it is not easy to provide somehow conclusive results about the most effective pre-treatments for that single substrate. A summary of the pre-treatment conditions and corresponding H₂ yields are shown in Table 3-2. Wang et al., [7] applied varying pre-treatments (heat, acid and alkaline) on vinegar residues prior to dark fermentation and reported alkaline pre-treatment as the most effective technology among others. The H₂ yield obtained by alkaline pre-treatment was 33% and 17% higher than acid and heat treatments respectively. Battista et al., [90] compared sonication and alkaline pre-treatment effects on H₂ yield from olive oil waste. A H₂ yield of 198 ml.g⁻¹ VS was obtained with alkaline pre-treatment which was 144% and 266% more than sonicated and raw substrates respectively. Bundhoo, [102] studied microwave and ultrasound irradiation with varying intensities (6946 kJ/kg TS) for pre-treating a mixture of food and yard wastes. He reported enhanced solubilisations of 175% and 259% with the highest specific energies for microwave and ultrasound pre-treatments respectively. However, none of the methods improved H₂ yields from food and yard waste compared to untreated substrate. The main reason for lower H₂ yields with pre-treated substrates was attributed to increased levels of propionic acid and ethanol that are considered as inhibitors for dark fermentation. Menon et al., [92] investigated effect of substrate pre-treatment (heat, base and ultrasound) on food waste solubilisation and H₂ production and

reported an increase of 400% in H₂ yield for alkaline pre-treated food waste compared to the control. H₂ yield obtained with ultrasonic irradiation was 48% lower than alkali pre-treated food waste while no hydrogen was detected for thermally treated food waste possibly due to formation of inhibitory compounds.

According to Table 3-2, the majority of studies that have been conducted using the same substrate, reported alkaline pre-treatment as most effective technology in terms of improving H₂ yield. However, the results are controversial in some cases as it can be seen for Elbeshbishy et al., [95] and Menon et al., [92] who reported different technologies as the best option for pre-treating food waste. Altogether, it may be deduced that alkaline pre-treatment is a potential technology for full scale applications. Nevertheless, more comprehensive studies must be conducted using varying pre-treatment conditions on a single substrate.

Table 3-2 Comparison between substrate pre-treatment methods

Substrate	Pre-treatment methods	Best pre-treatment method	Maximum H ₂ yield	References
Vinegar residues	Acid, alkaline, heat	Alkaline (NaOH, pH 12, 24 h)	55.4 ml.g ⁻¹ VS	[7]
Olive oil waste	Ultrasound, alkaline	Alkaline (NaOH, pH 12, 24 h)	198 ml.g ⁻¹ VS	[90]
Poplar leaves	Acid, alkaline, enzyme	Enzyme (Viscozyme L, 2%, 5 h)	44.92 ml.g ⁻¹ dry poplar leaves	[109]
Sewage sludge	Acid, alkaline, heat, ultrasonic	Alkaline (NaOH, pH 12, 5 min)	11.68 ml.g ⁻¹ VS	[83]
Sewage sludge	Acid, alkaline, heat, ultrasonic	Alkaline (NaOH, pH 12, 30 min)	2.2 ml.g ⁻¹ VS	[78]
Cassava wastewater	Enzyme, ultrasound	Enzyme (α -amylase, 0.2%)	113.6 ml.g ⁻¹ COD	[91]
Food waste	Acid, alkaline, heat, ultrasonic	Ultrasonic Sonication (500W, 79kJ/g TS,)	97 ml.g ⁻¹ VS	[77]
Food waste	Alkaline, ultrasonic, heat	Alkaline (NaOH 3M, pH 9, 12 h)	350 ml.g ⁻¹ VS	[92]

4. Integrated systems for bioenergy and materials recovery from organic waste

Energy conversion efficiency of the substrate in dark fermentation is low due to the high COD conversion to organic acids and alcohols. Combining the dark fermentation with other processes would be a sustainable biorefinery option to improve the overall performance of the system, enabling the recovery of a wide variety of chemicals as well as biofuels. Integrated biorefineries can use biomass and produce any combination of biofuels, power, heat and high-value chemicals. Some of the most promising combined process using organic waste are summarized in the next sections. Schematics of integrated systems are shown in Figure 4-1.

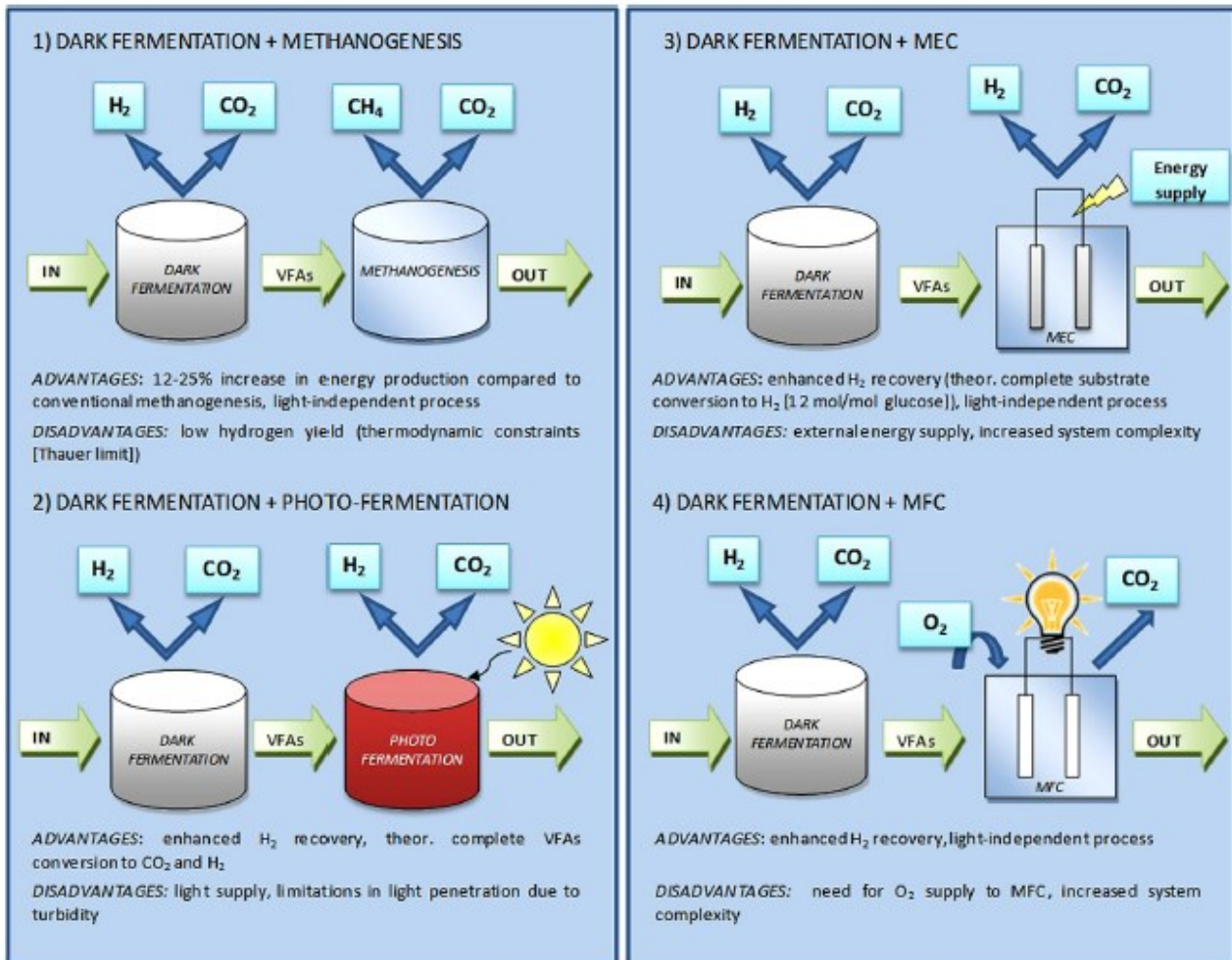


Figure 4-1 Schematization of integrated process [26]

4.1. Two-stage AD for H₂ and CH₄ recovery

Besides the possibility of H₂ recovery, two-stage AD could improve CH₄ yields compared to one-stage AD, due to the enhanced hydrolysis in the first stage [34]. Moreover, methanogens have been shown to be more tolerant towards high organic loading rates in a two-stage AD when compared to traditional one-stage AD process [113,114]. In fact, the first AD stage might be considered also as a pre-treatment for the second stage. In a two-stage AD, organic compounds are first hydrolysed and then utilized by acidogenic bacteria to produce H₂ and volatile fatty acids (VFAs). Then in the second stage, VFAs are converted to CH₄ by acetoclastic methanogens. H₂ produced in the first stage could be either utilized separately as an energy carrier or mixed together with CH₄ to obtain hythane. Enrichment of CH₄ with H₂ (5-25%) reduces the greenhouse gas emission due to the decreased C/H ratio, increases the flammability of the fuel as well as burning speed, and enhanced heat efficiency [115,116]. The bioconversion of organic wastes into H₂ and CH₄ through two-stage AD has been reported to yield as high as 40-160 mL H₂/gVS and 300-500 mL CH₄/gVS [36,73,117–119]. Although coupling DF and AD might improve the energy recovery as well as substrate degradation efficiencies, the investment and maintenance costs associated with using two separate reactors should be taken into account. Moreover, neutralization of VFA-rich DF effluents before starting the methanogenesis phase stage is an additional challenge.

4.2. Integrated processes for H₂ and biopolymers production

Another integrated biorefinery could be combining energy recovery together with biopolymers production from the acidogenic effluents.

Polyhydroxyalkanoates (PHAs), completely biodegradable polymers naturally produced by a wide variety of bacteria, have been successfully tested as substitutes for conventional petroleum based plastics. PHAs have gained much attention due to their biodegradability as well as their thermoplastic properties [120]. PHA is synthesized and accumulated as granules in the bacterial cytoplasm for carbon and energy storage when the limitation in nutrients leads to the lower growth rates. In order to stimulate PHA accumulation, the process is mainly operated under feast and famine conditions [121]. Many species have been studied for PHAs synthesis including *Alcaligenes* spp, *Bacillus* spp, *Nocardia* spp *Pseudomonas* spp and *Azotobacter* spp. The production cost for PHAs is largely influenced by the price of the substrate and therefore, using low-value substrates could reduce the price of final products. On the other hand, VFAs-rich effluents from dark fermentation might be considered as abundant and inexpensive substrates for PHAs production. Integrating dark fermentation with PHA production has the benefit of reducing environmental pollution while obtaining value added chemicals.

Integration of H₂ and PHA production has been investigated using a variety of biowastes. Giroto et al., [122] reported that optimizing the process conditions (pH and food to microorganism ratio) for H₂ production from OFMSW, also led to enhanced recovery of volatile fatty acids such as butyrate and acetate (precursors for PHA production). Applying the optimum operational conditions, they obtained a H₂ yield of 90.6 mL/gVS together with 34 mg/gVS total VFA after 48 h incubation. Recently, Luongo et al., [123] used *Rhodobacter sphaeroides* to produce

polyhydroxybutyrate from effluents of dark fermentation. They reported a yield of 155 mg PHB/gCOD together with a H₂ yield of 105 mL/gVS in the first stage.

4.3. Integrated dark and photo fermentative H₂ production

Coupling dark and photo fermentative H₂ production is another strategy for improving energy recovery from organic wastes. In the presence of light, organic acids resulted from dark fermentation can be used as substrates for H₂ and CO₂ production by purple non-sulphur bacteria (Eq. 4-1):



Several studies investigated integration of dark and photo H₂ production from a variety of substrates including sugar beet juice, olive mill wastewater, cornstalk and cheese whey [124–127]. Luongo et al., [123] obtained H₂ yields of 105.0 mL /gVS from organic waste in dark fermentation and then studied the possibility of using effluents for photo fermentative H₂ production. They obtained a yield of 233.8 mL H₂/gCOD using an enriched mixed culture of purple non-sulphur bacteria. The main drawbacks associated with coupling dark and photo fermentation are light dependency of photo bioreactors and complicated design of large scale reactor processes for efficient light penetration. Due to the high turbidity of effluents resulted from DF of organic waste, an additional step needs to be added prior to photo fermentation in order to dilute the DF effluents and enhance light penetration Bundhoo, [128].

4.4. Integration of dark fermentation and bioelectrical systems

Integration of dark fermentation and electricity production is another combined technology for converting low value organic compounds to electricity. Dark fermentation effluents that are rich in organic acids are potential substrates for electricity production by bioelectrical systems (BES). BES can be classified into two groups: Microbial fuel cells (MFC) and microbial electrolysis cells (MEC). In MFC the organic acids produced during dark fermentation are oxidized by anaerobic microbial cultures in the anode part generating electrons, proton and CO₂. Electron transfer from anode to cathode via an external circuit generates electric current while protons migrate to the cathode where they react with oxygen and produce water [129]. Integration of dark fermentation and MFC is considered as a novel method to improve the bio energy recovery from organic rich effluents. Furthermore, MFC are also beneficial for odour removal from VFA-rich effluents. Integrated MFC and hydrogen production has been conducted using a few types of substrate including cellulose [130], rice bran [131], crude glycerol [132], sucrose [133], and liquid fraction of pressed municipal solid waste [134].

MEC, another type of BES, is a systems used to produce hydrogen from organic substances with the aid of an external electric current. Similar to MFC, organic rich effluents from dark fermentation, are potential substrates for MEC to enhance total hydrogen production from organic waste. In contrast to MFC, no oxygen is needed in the cathode and therefore no water is produced. In spite of the additional electricity required for hydrogen production by MEC, it is considered as a promising technology for hydrogen production from a wide variety of organic wastes. The theoretical voltage needed to produce hydrogen at neutral pH at the cathode is 0.11 V, basically lower than the potential required for hydrogen production from electrolysis of water

(1.8-2.0 V) [135]. Chookaew et al., [132] combined dark fermentation of crude glycerol and MEC (with an external voltage of 1.0 V) and reported a hydrogen yield of 0.55 mole/ mole glycerol that was equal to an energy yield of 142 kJ/mole glycerol. Other substrates that have been investigated for two-stage hydrogen production by coupling dark fermentation and MEC are waste peach pulp cellulose [136,137], waste paper [138], sugar beet juice [139], cornstalk [140], and a wide variety of agro industrial wastewater [141].

5. Effect of aerobic pre-treatment on hydrogen and methane production in a two-stage anaerobic digestion process using food waste with different compositions

5.1. Introduction

Hydrogen has been indicated as one of the most promising fuels for the future [26,142]. However, subsequent to anaerobic hydrogen production, substrate conversion remains incomplete, with the majority remaining as a residue after the process. A promising system is represented by a two-stage AD process combining H₂ and CH₄ productions. During the first stage, organic compounds are hydrolysed and utilized by hydrogen producing bacteria to produce H₂ and volatile fatty acids (VFAs), whilst in the second stage, VFAs are used as substrates for CH₄ production by methanogens. Two-stage AD provides a positive energy yield (40-90% available energy), thus underlining the highly important process sustainability [143]. Several studies have demonstrated the ability of two-stage AD to improve CH₄ yields during the second stage, likely due to better hydrolysis [34,35]. Moreover, compared to one-stage AD, process control would be simpler and stability would be improved [114,144].

During hydrolysis, the rate limiting step of anaerobic digestion, organic compounds including proteins, carbohydrates and lipids are broken down by hydrolytic bacteria into amino-acids, sugars and long chain fatty acids, respectively. Substrate pre-treatment methods are aimed at promoting and improving hydrolysis of high molecular weight compounds to readily-biodegradable constituents, and subsequently increasing the AD process product yields.

Hydrolysis occurs under both aerobic and anaerobic conditions; however, hydrolysis rates are significantly higher under aerobic conditions, likely due to the higher production of enzymes [145]. In addition, pre-aeration reduces accumulation of VFAs, resulting in a drop of pH during the process, thus improving the start-up stability of food waste anaerobic digestion. Limited pre-aeration prior to anaerobic digestion has been shown to improve hydrolysis and biogas production [1–4].

Composition of organic wastes varies according to the source from which the wastes are collected. Slaughterhouse wastes may be rich in proteins and lipids, while food wastes and organic fraction of municipal solid wastes are rich in carbohydrates. An in-depth understanding of effective pre-treatment methods for each kind of waste is fundamental in improving biogas production.

To the best of the Authors' knowledge, no scientific reports have been published to date on the effects of aerobic pre-treatment on food waste with different compositions for either H₂ and/or CH₄ production in a two-stage AD process. Moreover, the effect of carbohydrate, lipid and protein content of food waste on pre-aeration efficiencies has not been addressed before. Therefore, the present work aims to study the aerobic pre-treatment effect of carbohydrate-rich (C), protein-rich (P) and lipid-rich (L) food waste prior to two-stage anaerobic digestion on both H₂ and CH₄ production.

5.2. Materials and Methods

5.2.1. Organic waste samples

Synthetic food waste samples were prepared in order to simulate industrial or municipal food waste with different compositions as indicated in a previous study [11].

Three different substrates were prepared and classified as C (carbohydrate-rich), P (protein-rich), and L (lipid-rich) substrates. The composition of samples is shown in Table 5-1. The percentages are based on wet weight.

Food waste samples were shredded after preparation and characterized (Table 5-2) in order to have more detailed information for each substrate category.

Table 5-1 Composition of synthetic food wastes (%W/W)

Ingredients	C	L	P
Tuna (%)	6.7	7.5	31.1
Butter (%)	5.5	22.3	5.5
Apple (%)	27.8	27	7.85
Banana (%)	27.8	27	7.85
Chicken breast (%)	6.7	7.5	31.1
Bread (%)	5.4	1.5	3.2
Pasta (%)	5.4	1.5	3.2
Minestrone soup (%)	14.7	5.5	10.2

Table 5-2 Average Characteristics of food wastes with different compositions.

Parameters	C	L	P
TS(%)	28.56	30.72	43.2
VS(%TS)	95.4	96.1	97.3
TOC(%TS)	58.7	65.9	66.3
TKN(%TS)	3.34	3.05	7.98
Lipid (%TS)	16.1	41	17.3
Protein(%TS)	19.8	18.1	47.3
Glucose(%TS)	4.2	1.54	3.11
Fructose (%TS)	12.36	5.29	2.75
Sucrose (%TS)	15.56	7.42	2.78

5.2.2. Aerobic pre-treatment of substrate

In order to compare the two-stage AD process with and without pre-aeration on the prepared substrates featuring different compositions, half the waste samples from each category were air injected using an aquarium pump (EIN WELTWEIT-Elite799) connected to a porous stone for better air diffusion. The air flow rate was fixed at 5 l/h using a flow meter (BROOKS SHORATE 1355). After 24h, aeration was stopped. The inoculum was then added to each bottle with and without pre-treatment.

5.2.3. Two-stage AD – Hydrogen production

Laboratory scale tests were performed to evaluate Biochemical Hydrogen Potential (BHP) of the examined substrates. Batch tests were carried out using 1-litre glass bottles which were subsequently sealed with silicon plug. Substrate concentration and food to microorganism ratio (F/M) were 5gVS/l and 0.3 gVS/gVS, respectively. Granular sludge was used as inoculum for BHP and was collected from a full-scale Upflow Anaerobic Sludge Blanket (UASB) digester of a brewery factory located in Padova, Italy.

Heat treatment was carried out on granular sludge in a rotary water-bath incubator at a fixed temperature of 80°C for 15 minutes in order to suppress methanogenic bacteria [11]. pH was set at 6.0 using phosphate buffer before the start of tests. The bottles were flushed with N₂ gas for 3 minutes to ensure anaerobic conditions and incubated at a temperature of 35±1°C. All tests were performed in duplicate.

5.2.4. Two-stage AD – Methane production

After completing the H₂ production phase, the bottles were opened and pH, dissolved organic carbon (DOC) and VFAs were measured (Table 3 and Table 4). Non-pre-treated granular sludge (at the same amount as the first stage) was then added to each bottle and all were sealed again, flushed with N₂ gas for 3 min, and incubated at the same initial mesophilic conditions of 35±1°C.

5.2.5. Analytical Methods

TS, VS and TKN were analysed according to standard methods (APHA, 1999). Total organic carbon (TOC) values were calculated on the basis of the difference between total carbon and inorganic carbon present in the samples. Concentrations of carbohydrates, proteins, lipids and free sugars were obtained according to official methods (AOAC, 2003). The volume of biogas produced during the anaerobic digestion process was measured by means of the water displacement method. The produced gas composition in terms of H₂ first, and then CH₄, was analyzed using a micro-GC (Varian 490-GC) equipped with an MS5A column to measure H₂ and CH₄, and a PPU column for CO₂ and two Thermal Conductivity Detectors. Argon was used as the carrier gas at a pressure of 60 kPa. Temperatures of column and injector were set to 80°C.

VFAs concentrations were measured using a gas chromatograph (Varian 3900) equipped with a CP-WAX 58 WCOT fused silica column and a Flame Ionization Detector. Nitrogen was used as carrier gas with a flow of 4 ml/min in column. The oven temperature was set at 80°C for the first

minute and then increased at a rate of 10°C/min to 180°C for two minutes. Column and injector temperatures were set to 250°C.

5.2.6. Hydrogen and methane production calculations

Hydrogen, methane and carbon dioxide volumes produced during the first and second stages of AD were calculated according to Eq. 5-1 [146].

$$V_{c,t} = C_{c,t} * V_{b,t} + V_H * (C_{c,t} - C_{c,t-1}) \quad \text{Eq. 5-1}$$

In which:

$V_{c,t}$ = Volume of H₂, CH₄ or CO₂ produced between intervals of t and t-1

$V_{b,t}$ = Volume of total biogas produced between intervals of t and t-1

V_H = Volume of headspace of bottles

$C_{c,t}$ = Concentrations of H₂, CH₄ or CO₂ in headspace in time of t

$C_{c,t-1}$ = Concentrations of H₂, CH₄ or CO₂ in headspace in time of t-1

5.3. Results and discussion

5.3.1. Effect of aeration pre-treatment on the first stage of AD

5.3.1.1. Hydrogen production

Three main H₂-producing enzymes are used by anaerobic microorganisms: [Fe/Fe]/hydrogenases, [Ni/Fe]/hydrogenases and nitrogenases. These H₂-producing enzymes are generally all highly oxygen-sensitive and presence of oxygen may reduce their activities [147]. Accordingly, H₂ production should be carried out under strictly anaerobic conditions and following aerobic pre-treatment, N₂ should be flushed in order to ensure anaerobic conditions.

The results obtained for hydrogen production potential from three different food waste samples are shown in Figure 5-1. Data obtained through GC analysis revealed a lack of methane in the emitted gas, due to efficiency of the thermal pre-treatment of inoculum. In the first stage of AD, substrate C without aeration produced considerably more hydrogen (55.31 ml/g VS) compared to L (27.93 ml/g VS) and P (7.96 ml/g VS) substrates. This finding is in agreement with Alibardi and Cossu [11], who concluded that carbohydrate rich food waste is capable of producing much higher quantities of H₂ compared to lipid or protein rich substrates. This could be attributed to faster hydrolysis rate of carbohydrates (almost 20 times faster) compared to lipids and proteins [25]. Since the duration of H₂ production is short (around 3 days) it is not enough for the hydrolysis of proteins and lipids. In addition, conversion of long chain fatty acids from hydrolysis of lipids to H₂ is feasible only at very low hydrogen partial pressure [27]. Degradation of some amino acids from hydrolysis of protein is H₂ consuming. According to Hallenbeck [27],

readily-biodegradable carbohydrates are the preferred substrates by anaerobic microorganisms during dark fermentative H₂ production. Similarly, Chu et al., [28] showed that H₂ yield is strongly dependent on the carbohydrate content of organic wastes.

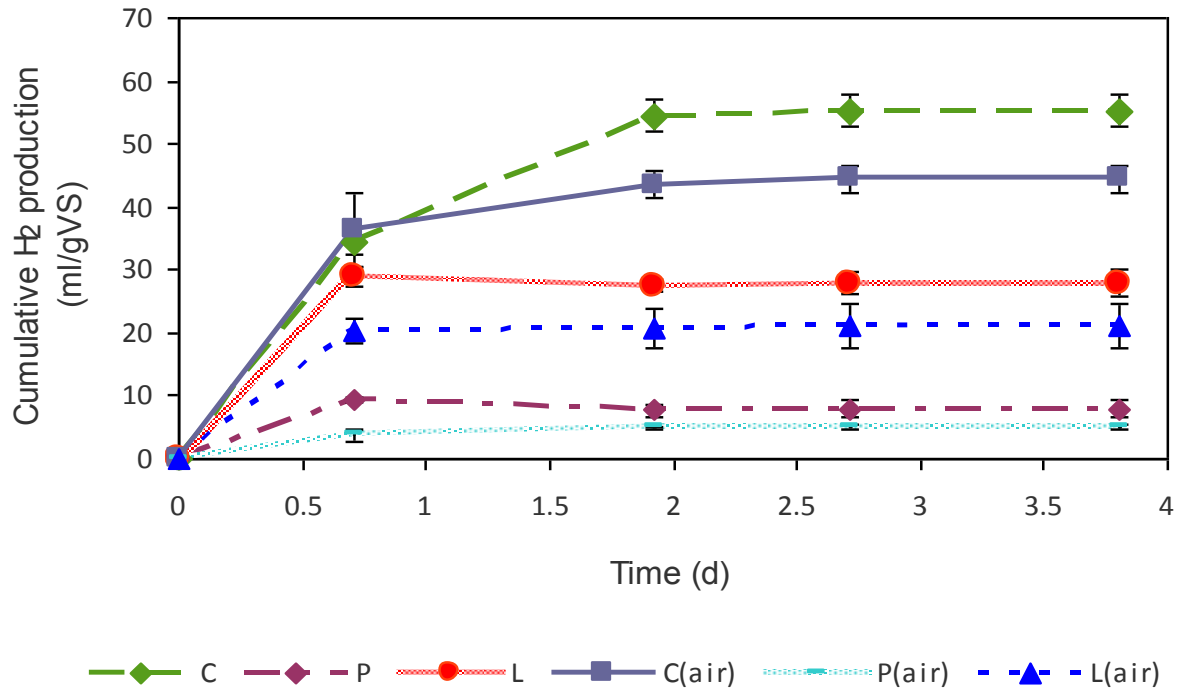


Figure 5-1 Hydrogen production potential for the three different substrates, namely C, P, and L, with and without aerobic pre-treatment. C-rich, P-rich and L-rich are carbohydrate, protein and lipid rich substrates without aerobic pre-treatment, respectively. C (air), P (air) and L (air) are carbohydrate, protein and lipid rich substrates with aerobic pre-treatment, respectively.

For samples subjected to aerobic pre-treatment, substrate C achieved the highest H₂ yield (44.4 ml/g VS), followed by substrate L (21 ml/g VS) and P (5.27 ml/g VS). Aerobic pre-treatment lowered average H₂ production for C (19%), L (24%) and P (33%) substrates. Although aerobic pre-treatment has been indicated as a strategy to increase hydrolysis and CH₄ production from sludge [2,3], it proved ineffective in achieving an increase in H₂ production from food waste. The latter could convincingly be explained by a low solid retention time (SRT) for H₂ (3 days) compared to CH₄ production (15 days or more). In addition, during aeration, part of the available readily-biodegradable carbon (mainly free sugars) is converted to CO₂ or consumed for cell growth instead of in product formation [145]. In the present study, the carbon loss after aeration was proved by 37%, 6%, and 12% decreased TOC content for C, P, and L substrates, respectively. Although this drawback may also be present in pre-aeration studies on CH₄ production, it may be compensated by a longer SRT, which enhances carbon hydrolysis with lower degradability, subsequently leading to higher product yields.

5.3.1.2. Composition after the hydrogen-producing phase

At the end of the first stage of the AD process, liquid samples were collected to investigate the effect of aeration on VFA composition and on pH (Table 5-3 and Table 5-4). The major VFA components for all food waste samples were acetic acid (AC) and butyric acid (BU), while propionic acid (PA) was detected only in P-rich samples. Aerobic pre-treatment slightly increased PA production for P-rich substrate. Presence of PA at the end of the first stage is not favorable since unlike AC and BU it is produced by a metabolic pathway which consumes substrate without producing H₂ [148]. The average concentrations of VFAs at the end of the first stage of AD are reported in Table 5-3. When compared to non-aerated samples, AC yield in samples P decreased slightly with aeration (4%) while in samples C and L, AC decrease was much more significant, 33% and 25% respectively. Similarly, aeration lowered BU production for sample C by 43%. On the contrary, in samples P and L, BU concentration increased by 34% and 10%, respectively. PA concentration in pre-aerated P samples was slightly higher (5%) than in non-pre-treated P ones.

P-rich samples, both with and without aeration, displayed the lowest BU/AC ratios amongst all substrate types. For non-aerated samples, correlation between BU/AC ratio and H₂ production is in agreement with previous studies which suggested that BU/AC ratio is directly proportional to H₂ yield [151]. Conversely, other studies reported the absence of a correlation between BU/AC ratio and H₂ yield [15,152]. Indeed, Table 5-3 highlights how for aerated P and L samples the BU/AC ratios increased in comparison to non-aerated samples, although lacking any positive effect on H₂ production.

Table 5-3 Average volatile fatty acid (VFA) production for the three different substrates, namely C, P, and L, with and without pre-treatment

Specific VFA(mg/l)	C	C (air)	P	P (air)	L	L (air)
Acetic acid	593±66	392±28	490±63	473±51	510±43	381±29
Butyric acid	413±50	236±23	139±35	187±32	220±38	243±43
Propionic acid	0	0	88.9±11	94.2±21	0	0
BU/AC ratio (mmol/mmol)	0.47	0.41	0.19	0.26	0.29	0.43
Total VFAs (mg/l)	1006	628	717.9	754.2	730	624

pH, DOC concentrations and cumulative CO₂ production values obtained following the first stage for samples with and without aerobic pre-treatment are shown in Table 5-4. Pre-aeration was not found to have significantly affected cumulative CO₂ production at the end of the first stage. However, DOC values were lower for all pre-aerated samples in comparison to samples without pre-aeration. This could be mainly due to lower amount of easily degradable carbon in pre-aerated samples as a result of partial loss of carbon during aeration.

pH values were measured at the end of the first stage for all samples. Substrate P (both with and without pre-aeration) showed higher values compared to other substrates. Generally, substrates with high nitrogen content (such as protein rich wastes) inhibit excessive acidification due to their buffering capacity [149].

Table 5-4 Average CO₂ and DOC concentration and pH at the end of first and second stages of AD process for the three different substrates, namely C-rich, P-rich, and L-rich with and without aerobic pre-treatment

	Parameter	C	C (air)	P	P (air)	L	L (air)
First stage	CO ₂ (ml/g VS)	82.42±2.11	72.5±1.41	42.81±2.02	39.41±2.11	65.64±3.87	65.41±2.51
	DOC (mg/l)	1003.5±23.11	626.5±31.33	734.5±86.08	690.5±18.12	790.25±28.14	678.5±16.2
	pH	4.56±0.01	4.88±0.02	5.51±0.1	5.31±0.03	4.56±0.1	4.56±0.1
Second stage	CO ₂ (ml/g VS)	169.76±13.2	159.18±12.65	150.16±21.37	214.62±12.8	151.94±12.12	151.95±31.19
	DOC (mg/l)	<15	<15	<30	<25	<20	<30
	pH	7.49±0.03	7.39±0.1	7.69±0.02	7.56±0.02	7.3±0.07	7.14±0.1

In terms of pH, during the first stage, aeration led to a slightly higher pH in substrate C as a consequence of the lower VFA concentration (see Table 5-3). Instead, aeration led to a lower pH in substrate P compared to non-aerated samples. For substrate L, with and without aerobic pre-treatment, pH values at the end of the first stage of the AD process were substantially similar.

5.3.2. Effect of pre-aeration on the second stage of AD

5.3.2.1. Methane production

Cumulative methane productions for the three investigated food waste substrates, with and without aeration, are shown in Figure 5-2. Long lag phase of almost one week (except for sample C without aeration with about 3 weeks) was observed for all substrate types. The most probable reason was a low pH following completion of the first AD stage. However, for P-rich substrate without aeration the lag phase lasted only 3 days.

For substrate C, aerobic pre-treatment ensured a better acclimatising of the bacteria and increased cumulative CH₄ production by 6% at the end of second stage of AD. Cumulative CH₄ production for the aerated substrate C was 600% higher than the non-aerated substrate C until day 14. Subsequently, CH₄ produced until day 20 for aerated C was approximately equal to cumulative CH₄ until day 34 for non-aerated substrate. Similarly, Charles et al., [4] observed an accelerated CH₄ production after aerating OFMSW, a carbohydrate rich substrate.

For substrate P with aeration, cumulative CH₄ production was lower compared to non-aerated samples until day 50. After this time, CH₄ production remained virtually constant for non-aerated samples, whilst it increased significantly (45.6%) for samples with pre-aeration. Cumulative CH₄ production for P-rich with and without aerobic pre-treatment was 351.69 and 241.52 ml/g VS, respectively.

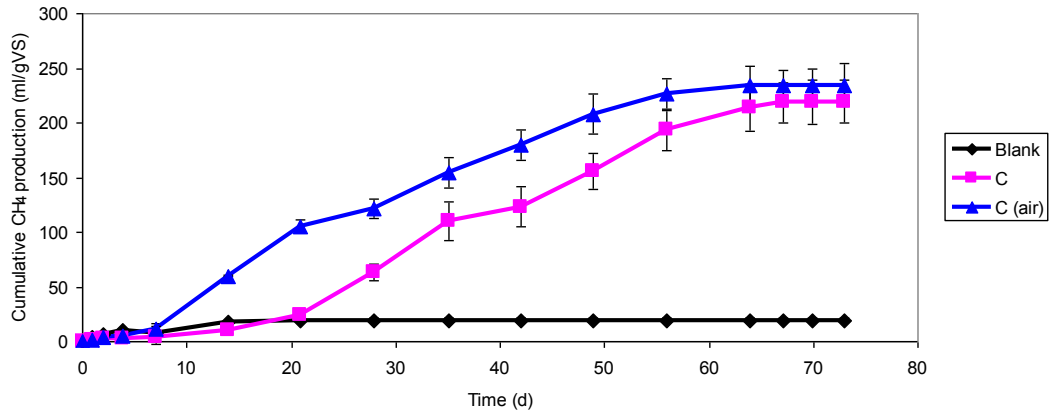
Cumulative CH₄ productions for substrate L were around 263 and 240 ml/gVS for non-aerated and pre aerated samples, respectively. L-rich was the only substrate that produced less CH₄ with pre-aeration. However, when taking into consideration non-aerated substrates, L was the best substrate type for CH₄ production in two-stage AD. Similarly, Johansen and Bakke, [150] reported that micro aeration led to higher hydrolysis of carbohydrates and proteins of primary sludge, while lipids hydrolysis failed to increase without the addition of inoculum.

Alibardi and Cossu, [151], reported that proteins and lipids produce higher quantities of CH₄ than carbohydrates. These Authors separated different fractions of municipal solid waste to produce CH₄ in single stage AD from each single fraction. They observed that the highest CH₄ production was achieved using the fraction containing meat, cheese and fish, and the lowest was produced using a fraction containing bread and pasta alone. This finding is in agreement with the present study in which substrates P and L produced more CH₄ compared to substrate C.

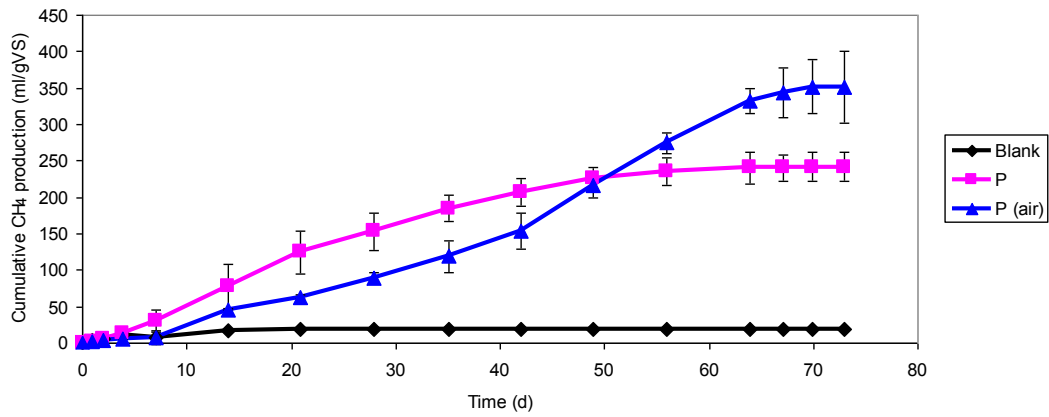
Several researchers have observed higher CH₄ production in single stage AD process following aerobic pre-treatment. Lim and Wang [144] showed that aerobic pre-treatment of a mixture of brown water and food waste improved AD treatment performance with a 10% increase in CH₄ production. Pre-aeration of sewage sludge and primary sludge increased cumulative CH₄ production by 25% [2], and 14% [3], respectively. According to Botheju et al. [145], pre-aeration may increase substrate conversion efficiency due to enhanced hydrolysis.

Jang et al., [152] applied aerobic thermophilic pre-treatment prior to mesophilic AD for sludge digestion. They obtained higher CH₄ production and higher carbon conversion efficiencies with aeration. The study was reported as using sludge with a higher protein compared to carbohydrate content.

a)



b)



c)

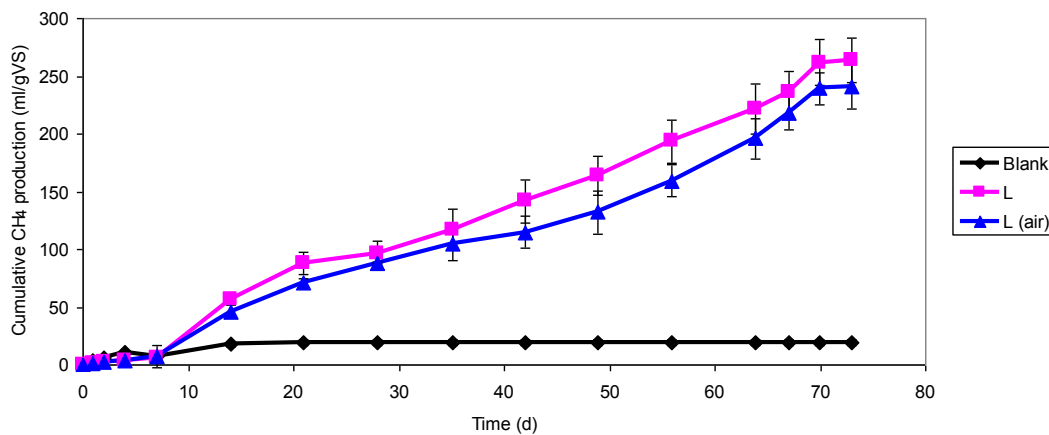


Figure 5-2 Methane production potential of the three different substrates, namely C (a), P, (b) and L (c), with and without aerobic pre-treatment, after hydrogen production phase. C (air), P (air) and L (air) are carbohydrate, protein and lipid rich substrates with aerobic pre-treatment, respectively. C, P, L are carbohydrate, protein and lipid rich substrates without aerobic pre-treatment. B is blank.

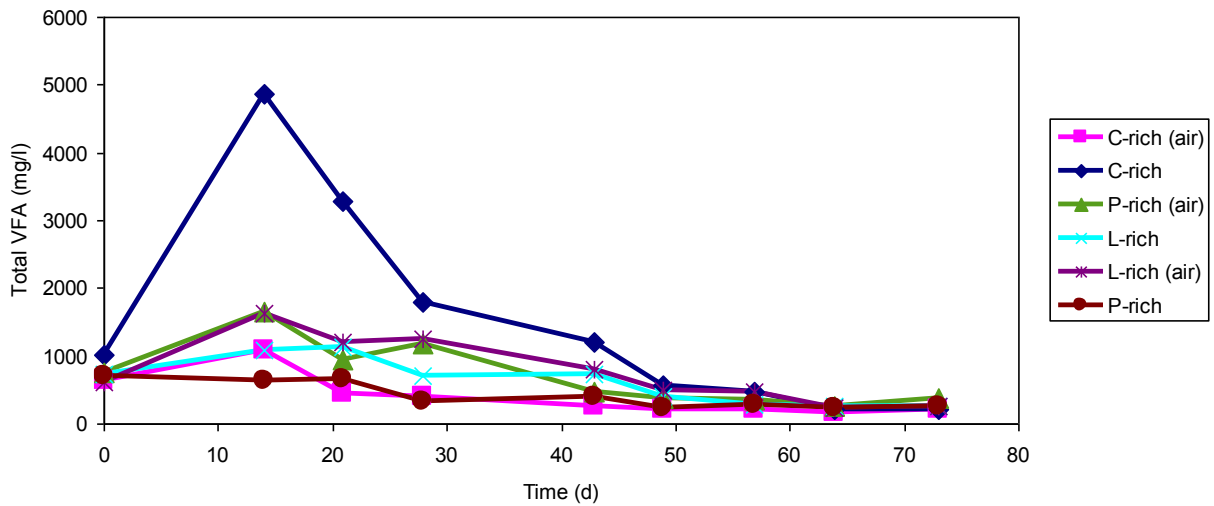


Figure 5-3 VFA accumulation during the second stage of the AD process

In anaerobic digesters VFA accumulation, and consequent drop in pH, is a major issue during CH₄ production. Limited aeration may remove excess degradable carbon, thus delaying the onset of acidification. Several studies have reported lower VFA accumulation during anaerobic digestion following aerobic pre-treatment [153,154]. The present study likewise showed positive effects of pre-aeration on VFA accumulation during the second stage of AD (Figure 5-3). A high VFA accumulation was observed until day 15 for C-rich substrate without aerobic pre-treatment. However, for the same substrate with pre-aeration, VFA accumulation was controlled, resulting in lower accumulation and higher CH₄ production (Figure 5-2.a).

Table 4 illustrates pH, CO₂ and DOC values after the second stage of AD. At the end of the second stage, pH values for all samples ranged from 7.1 to 7.6. Similar to the first stage, pH was slightly lower in pre-aerated samples, possibly due to higher carbon conversion to VFAs. The most significant difference between cumulative CO₂ production for pre-aerated and non-aerated samples was observed for P-rich substrate (214.62 and 150.16 ml/g VS, respectively). This sample also featured the highest CH₄ production.

5.3.3. Total Energy yields from H₂ and CH₄ in the two-stage AD

H₂ and CH₄ productions obtained with each substrate revealed that more H₂ were accompanied by a lower production of CH₄ and vice versa. In order to define the efficiency of the two-stage process in terms of total energy generation, total energies from CH₄ and H₂ have been calculated and presented in Table 5-5. It is evident that P-rich substrate with pre-aeration was the best in terms of total energy generation when both H₂ and CH₄ production are considered. When taking into account non-aerated samples, the total energy production from L-rich substrate was higher in comparison with C-rich and P-rich substrates.

Table 5-5 Total energy generation in the process in terms of H₂ and CH₄

Substrate	Energy from H ₂ (kJ/gVS)	Energy from CH ₄ (kJ/gVS)	Total Energy (kJ/gVS)
C	0.23	5.98	6.21
P	0.038	6.58	6.61
L	0.14	7.22	7.36
C (air)	0.2	6.4	6.6
P (air)	0.017	9.63	9.64
L (air)	0.14	6.58	6.72

5.4. Conclusion

The efficiencies of a two-stage AD treatment using organic wastes with different compositions in both the presence and absence of aeration as a treatment were compared by evaluating the H₂ and CH₄ production. This study suggested that pre-aeration of organic waste did not constitute an effective treatment for the purpose of improving H₂ production potential during the first stage of the AD process. However, during the subsequent stage of AD, CH₄ yield for substrate P, increased by 45.6%, thus revealing that carbon conversion to CH₄ had an increase after pre-aeration.

In the two-stage AD process, the best CH₄ production yield was obtained from substrate P with pre-aeration, whilst among non-aerated substrates, L produced the highest CH₄ yield and featured the best total energy generation considering both H₂ and CH₄. Further studies should however be undertaken using shorter and therefore less expensive, pre-aeration times in order to assess whether this may result in a positive effect on both H₂ and CH₄ productions. From the very beginning of the food waste treatment up to digestate management and disposal, the totality of the two-stage AD processes, with and without aeration, should be investigated and compared in terms of a complete Life Cycle Assessment.

6. Pre-treating anaerobic mixed microflora with waste frying oil: A novel method to inhibit hydrogen consumption

6.1. Introduction

Using mixed microflora for dark fermentation is less expensive and more practical compared to pure cultures due to the elimination of sterilization costs, improved substrate degradation, and easier process control. Therefore, using mixed microbial communities is economically and technically feasible for simultaneous waste reduction and clean energy production. A major problem for H₂ production by anaerobic mixed communities would be the presence of H₂ consuming microorganisms such as hydrogenotrophic methanogens, homoacetogens and propionate producers in the raw inoculum, which convert H₂ to CH₄, acetic acid, and propionic acid respectively. Among the H₂ consumers, hydrogenotrophic methanogens are recognized with the major contribution for H₂ consumption [155,156].

Many investigations have been performed to suppress methanogenic H₂ consumption and enrich H₂ producing bacteria and various pre-treatment methods have been employed to fulfil this aim. However, intermittent treatment would be an indispensable issue in full scale due to the subsequent proliferation of anaerobic and facultative H₂ consumers which are present in non-sterile feedstocks. Regarding the pre-treatments, using acid or alkali needs periodical pH adjustment, heating and irradiation are energy intensive and chemical inhibitors are discouraged due to their toxicity. Therefore, none of the mentioned methods are regarded as a perfect solution for full-scale application when the pre-treatment is repeated.

The inhibitory effect of long chain fatty acids (LCFAs) on anaerobic digestion has been recognized since many years ago [127,128]. Several studies have used pure LCFAs such as palmitic and stearic acid [65], oleic acid [131], lauric acid [69], and linoleic acid ([42,157]) to limit growth of hydrogenotrophic methanogens. Therefore, lipid rich wastes which contain mixtures of LCFAs may be utilized as inhibitors for H₂ consumption. A potential option could be waste frying oil (WFO) which is generated by restaurants, households, canteens, and food processing industries worldwide. This study is aimed at evaluating the simultaneous effect of initial pH and WFO concentration on inhibition of H₂ consumption and subsequently enhancement of H₂ yield. In order to better analyze the experimental results, a quadratic model also was developed to predict the simultaneous effect of initial pH and WFO concentration on H₂ production. In the next step, H₂ production from a synthetic food waste was also investigated to confirm the impact of WFO on H₂ production performances using complex substrates. The significance of this study would be introducing an inexpensive and practical method to inhibit methanogenic H₂ consumption during dark fermentation of mixed microbial cultures.

6.2. Materials and methods

6.2.1. Inoculum pre-treatment using waste frying oil

Granular sludge was collected from a full-scale Up-flow Anaerobic Sludge Blanket (UASB) digester of a brewery factory located in Padova, Italy. WFO (sunflower oil) was chosen in this study as it is widely used as cooking oil in Italy and collected from a local restaurant in Padova,

Northern Italy. In order to solubilize WFO, 100 g of the oil was added to 14g NaOH (98%) and mixed rigorously at 55°C. Fatty acid composition of WFO was analyzed using gas chromatography and the main fatty acids detected were as follows: linoleic acid (52%), oleic acid (30.24%), palmitic acid (7.04%) and stearic acid (3.22%). For the fermentation tests performed using glucose, different concentrations of saponified WFO solution (0-20 g WFO/l) were added to the reactors contained 10 gVS/l of granular sludge. After 24 h, 5g/L glucose was added to granular sludge plus WFO and control cultures. Untreated cultures were selected as controls. For the tests performed using synthetic food waste, granular sludge cultures were pre-treated with 10 g/l WFO with different durations (0, 24 and 48 h).

6.2.2. Hydrogen production studies

The first series of experiments were designed to assess the effect of varying concentrations of WFO on H₂ production from glucose. Since this study is the first report on inoculum pre-treatment with WFO, glucose was chosen as substrate in order to confirm reproducibility of the results which would be impossible using complex substrates due to the composition variability of organic wastes. Laboratory scale tests were performed using 1-liter glass reactors with a working volume of 500 mL. The reactors were nitrogen injected after substrate addition for 3 min to ensure anaerobic conditions and then incubated at 37±1°C. Different initial pH conditions were applied (5.5, 6.5 and 7.5) before incubation using NaOH (3M) and HCl (3M). The start of the process was considered as the time of glucose addition. The tests were done in triplicate. H₂ and CH₄ volumes produced during the dark fermentation were calculated according to Vanginkle et al., [146]. In order to better demonstrate if inhibitory effect of WFO on hydrogenotrophic methanogens can be remained even after removing the WFO, an additional test was also performed. Granular sludge cultures were pre-treated with 10 g/L WFO for 48 h. Then, the cultures were washed twice in order to remove the WFO before adding 5 g/L glucose as substrate and incubation with pH 5.5.

The second series of the experiments were performed to study H₂ production from food waste in order to investigate the impact of WFO on H₂ production performances using complex substrates. A synthetic food waste was prepared with the aim of reproducibility of the results. The synthetic food waste was mainly composed of vegetables (14.7 %), meat (13 %), fruits (54 %), cheese (5.5 %), bread and pasta (10.8 %) to simulate the food waste composition in Italy. After preparation, the samples were shredded in a kitchen mill to make a homogeneous mixture and analyzed. The characteristics of the synthetic food waste based on wet weight were as follows: Total solids (30.10 %), Volatile solids (28.59%), Total Organic Carbon (14.11 %) and Total Kjeldahl Nitrogen (0.99 %). H₂ and CH₄ productions were studied for four conditions: untreated cultures (U), cultures received WFO and substrate at the same time (A), cultures pre-treated with WFO for 24 h before substrate addition (B) cultures pre-treated with WFO for 48 h before substrate addition (C). The initial pH for food waste fed cultures was adjusted at 5.5.

6.2.3. Data analysis

A quadratic model (Eq. 6-1) was fitted in this study to analyse the effect of concentration of WFO and pH on cumulative H₂ production. Curve fitting was performed using Matlab (The Mathworks Inc., version 2016a).

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{11}(X_1)^2 + a_{22}(X_2)^2 + a_{12}X_1X_2$$

Eq. 6-1

Where X_1 and X_2 are input variables (WFO concentration and initial pH respectively) which influence Y (H_2 production), a_0 is the offset term, a_1 and a_2 linear coefficients and a_{11} and a_{22} quadratic coefficients and a_{12} interaction coefficient. Minitab 17 statistical software (Minitab Inc., State College, PA, 2010) was used to obtain main effect plots for experimental factors.

6.3. Results and discussion

6.3.1. Effect of WFO pre-treatment on dark fermentation using glucose as substrate

H_2 yields obtained for the cultures pre-treated with varying concentrations of WFO and initial pH conditions are shown in Figure 6-1. Generally, for all the initial pH conditions tested, H_2 production increased with WFO concentration and in turn, CH_4 decreased. H_2 productions in untreated cultures for all the initial pH conditions tested were significantly lower when compared to those pre-treated with high concentrations of WFO. CH_4 productions in untreated controls were significantly higher compared to the pre-treated cultures for all initial pH conditions. A maximum CH_4 production equal to 408 mL/g glucose added was observed for the untreated culture with initial pH 6.5 which was accompanied with the lowest H_2 yield (27.07 mL/g glucose added). CH_4 yields equal to 233.14 and 133.66 mL/g glucose added were recorded for untreated cultures with initial pH 7.5 and 5.5 respectively. A maximum H_2 yield of 209.26 mL/g glucose added was obtained for the cultures pre-treated with 20 g/L WFO and incubated with initial pH of 5.5. This yield was 514% higher than untreated cultures incubated with the same initial pH conditions.

CH_4 productions during dark fermentative H_2 production could be a representative for the presence of either hydrogenotrophic or acetoclastic methanogens. However, decreased CH_4 production together with enhanced H_2 yields might be regarded as indicators for inhibition of hydrogenotrophic methanogens to convert H_2 to CH_4 . According to the literature, pH levels in the range of 6.5-7.2 are favoured by methanogens [68]; therefore, maximum methanogenic activities for the initial pH of 6.5 compared to the other pH conditions would be expectable. Similarly, Ray et al., [158] reported an increase of 70% in H_2 yields for culture pre-treated with 2 g/L linoleic when the initial pH of decreased from 7.6 to 5.5.

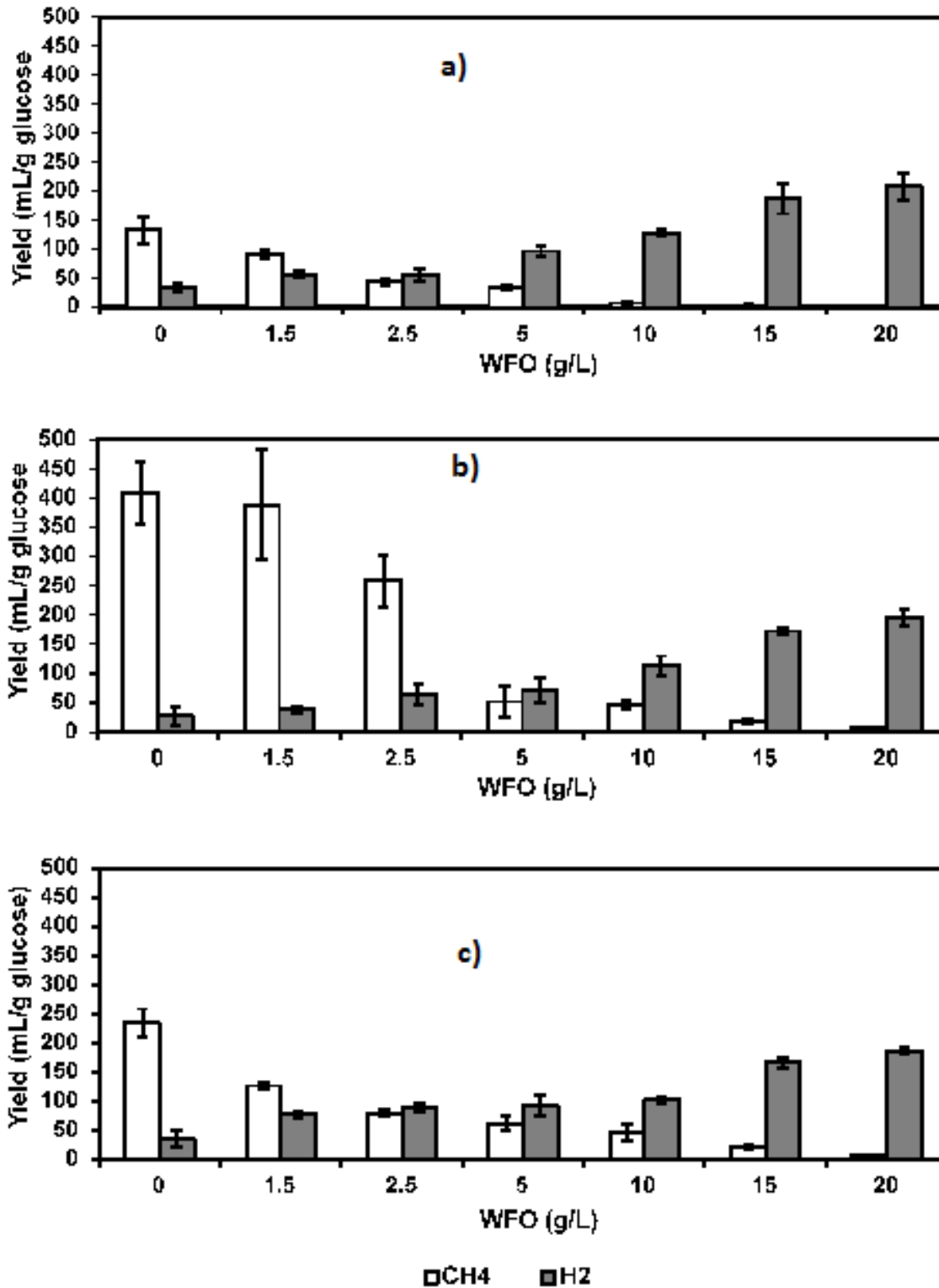


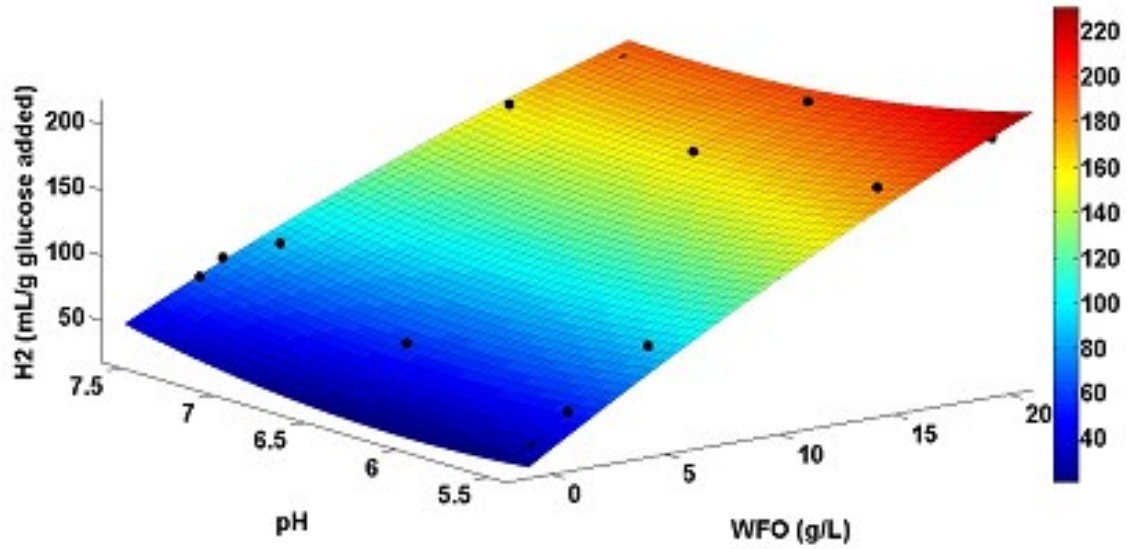
Figure 6-1 Effect of different concentrations of WFO on H₂ and CH₄ yields after 72 h incubation with initial pH a) 5.5 b) 6.5 and c) 7.5

LCFAs degradation by anaerobic bacteria occurs in a slow process called β -oxidation in which, they are converted to acetate and H_2 and subsequently to CH_4 by acetoclastic and hydrogenotrophic methanogens respectively [135,136]. However, LCFAs can be degraded to H_2 and acetate only at extremely low H_2 partial pressures that is impossible to be maintained without methanogenic H_2 consumption [159,160]. In the present study, in order to ensure that increased H_2 yields were mainly due to suppression of H_2 consuming microorganisms, WFO control cultures were prepared without glucose addition and negligible H_2 productions were recorded in the absence of glucose. Considering the aforementioned points, improved H_2 production using WFO could be attributed to suppression of hydrogenotrophic methanogens.

A quadratic model was used to describe simultaneous effect of waste frying oil concentration and initial pH on cumulative H_2 production. R^2 values (0.9672) revealed that quadratic model explained more than 90% of the variation in cumulative H_2 production with initial pH and WFO concentration. 3-D and 2-D plots for cumulative H_2 production are developed and presented in Figure 6-2.

The inhibitory effect of pure LCFAs on H_2 consumption using mixed cultures have been demonstrated by several studies [20,65,69,131]. Shanmugam et al., [69] reported a 10 fold decreased CH_4 production after pre-treatment of anaerobic sludge with 2 g/L linoleic acid. Differences in cell structures may influence the resistance of microbial species to changes in the surrounding environment. Therefore, the response of a mixed microbial culture to an inhibitor might be different depending on the microbial populations exist in it. Dasa et al., [6], observed that concentration of 4.5 g/L oleic acid resulted in 50% decreased CH_4 production. A recent study performed by Silva et al., [161] investigated the effect of palmitate and oleate addition to pure cultures of acetoclastic and hydrogenotrophic methanogens. They observed that threshold levels of these LCFAs inhibited CH_4 production significantly either from acetate or H_2 ; however, inhibitory concentrations for hydrogenotrophic methanogens were higher, suggesting higher sensitivity of acetoclastic methanogens to LCFAs. LCFAs adsorption on the cell wall of microorganisms might interfere with mass transfer. Since hydrogen is a smaller molecule than acetate, mass transfer in acetoclastic methanogens is more affected by LCFAs [139].

a)



b)

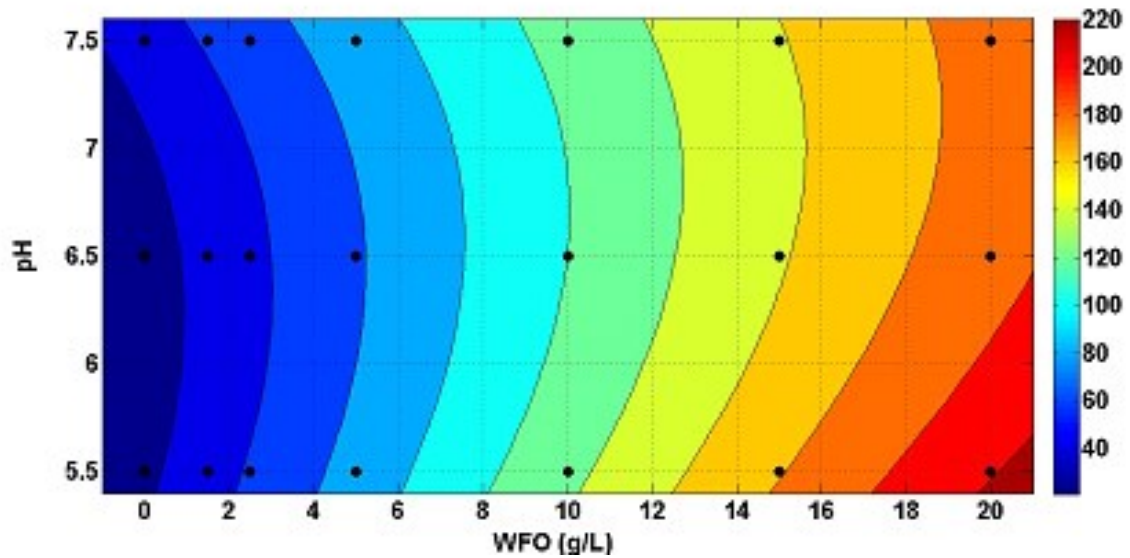


Figure 6-2 3D and b) 2D plots for cumulative H₂ production under varying initial pH and WFO concentrations

The effects of pH and WFO concentration on H₂ production were also investigated using the main effect plot (Figure 6-3). The large vertical displacement for WFO suggests that WFO concentration had a stronger effect on H₂ production compared to initial pH.

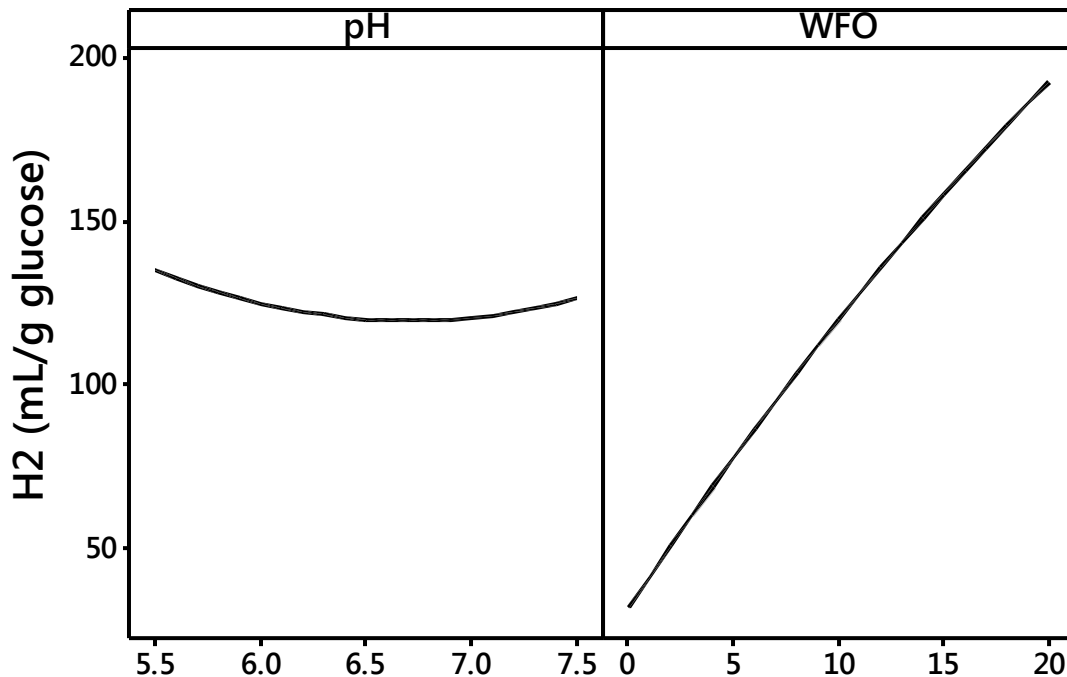


Figure 6-3 Main effect plot of the experimental variables (WFO concentration and initial pH) for response variable (H₂ production)

For the cultures pre-treated with 10 g/L WFO for 48 h and then washed, no CH₄ was detected during 72 h fermentation while a H₂ yield of 117.2 mL/g glucose added was obtained. Washing was performed in order to confirm that methanogens were inhibited during pre-treatment with WFO. This study revealed that H₂ producing bacteria can tolerate higher concentrations of LCFAs compared to methanogens. This characteristic might be exploited to develop an inexpensive and applicable method to enrich H₂ producing cultures.

The present study was performed using an inoculum concentration of 10 gVS/L. Reducing the inoculum to LCFAs ratios that is equal to higher bioavailable LCFAs concentrations, might reinforce their inhibitory effect [131]. Therefore, more studies should be undertaken using varying inoculum to WFO ratios before making any conclusion about the optimum pre-treatment conditions.

6.3.2. Volatile fatty acids production

Volatile fatty acids (VFA) production could be used to study the performance of dark fermentation. Figure 6-4 shows the VFA concentrations at the end of dark fermentation with varying initial pH and WFO concentrations. Acetate and butyrate, which are produced in H₂ producing pathways, were the major VFAs identified in all the cultures.

Elevated levels of acetate and butyrate were detected in the cultures pre-treated with high concentrations of WFO. VFA analysis together with H₂ production might be useful to evaluate hydrogenotrophic and acetoclastic methanogens inhibition. Acetate accumulation together with decreased CH₄ production in pre-treated cultures could be regarded as a possible indicator for lower activities of acetoclastic methanogens. In addition, decreased CH₄ production together with enhanced H₂ accumulation, could suggest the inhibition of hydrogenotrophic methanogens.

LCFAs have been proven not only to have inhibitory effects on acetoclastic methanogens, but also on acetogens which convert VFAs to acetate [129,135,140]. Average butyrate concentrations in the control cultures were 732, 255 and 732 mg/L at initial pH of 5.5, 6.5 and 7.5 respectively. Butyrate concentrations reached 1031, 1258 and 1552 mg/L at initial pH of 5.5, 6.5 and 7.5 respectively when the cultures pre-treated with 20 g/L WFO. Similarly, acetate concentrations in the cultures pre-treated with 20 g/L WFO increased by 423, 2530 and 184% at initial pH of 5.5, 6.5 and 7.5 respectively compared to the corresponding controls. In the present study, increased accumulation of VFAs for cultures pre-treated with WFO could be either due enhanced H₂ production through acetate or butyrate metabolic pathways or reduced activities of acetogenic bacteria to convert VFAs to acetate.

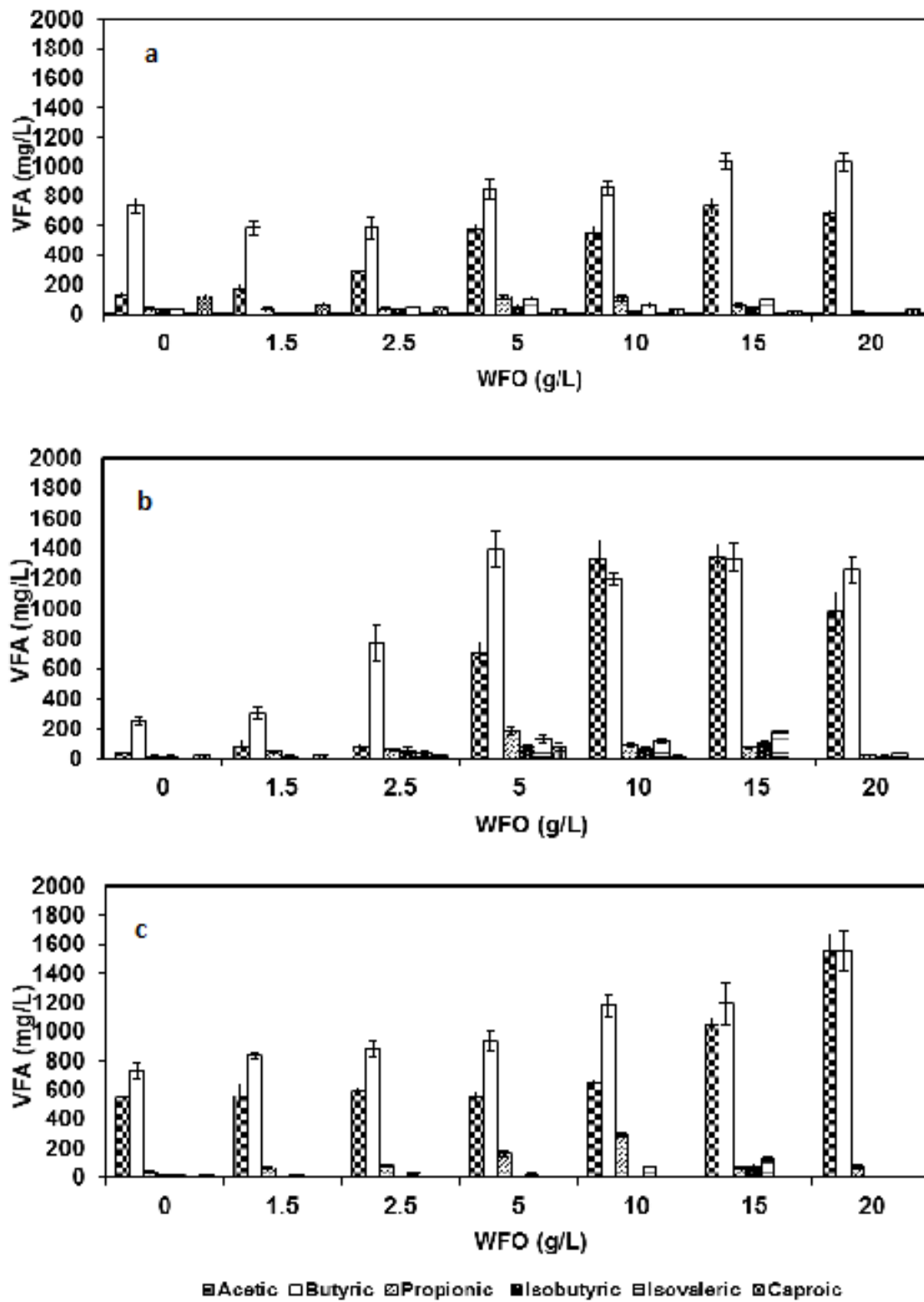


Figure 6-4 Volatile fatty acids production from glucose for the cultures pre-treated with varying concentrations of waste frying oil with initial pH a) 5.5 b) 6.5 and c) 7.5.

6.3.3. Effect of WFO pre-treatment on hydrogen production from food waste

A prerequisite to develop economically sustainable approaches for H₂ production is utilizing low value substrates such as municipal, industrial or agricultural wastes. Food waste is the major component in municipal solid waste and regarded as a suitable substrate for dark fermentative H₂ production due to the high carbohydrate content, balanced carbon to nitrogen ratio and abundance [5,13,123]. Therefore, H₂ productions from food waste with applying inoculum pre-treatment using WFO was also studied.

Cumulative productions of H₂ and CH₄ for cultures pre-treated with 10 g/L WFO with different durations are shown in Figure 6-5.

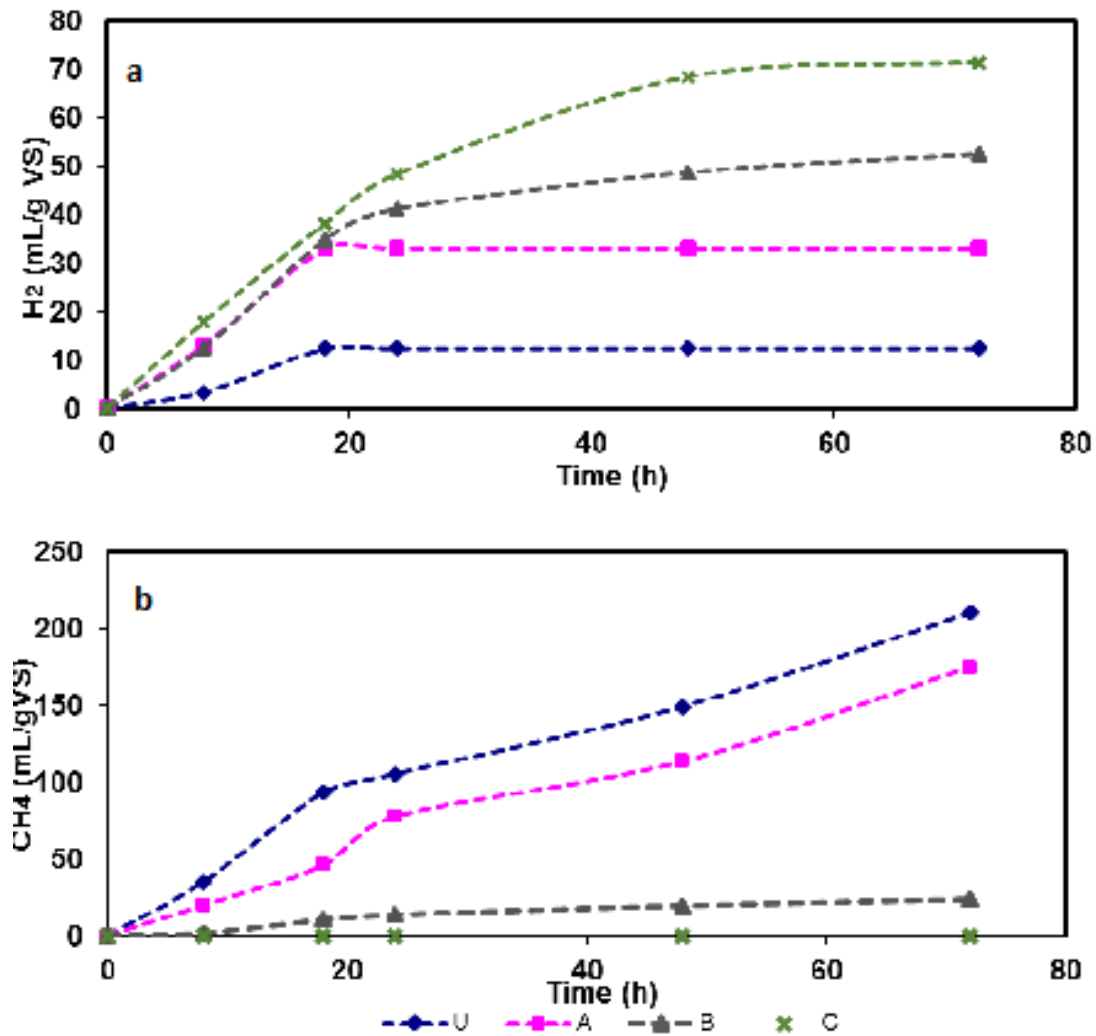


Figure 6-5 H₂ and CH₄ productions from food waste for untreated cultures (U), cultures received WFO and substrate at the same time (A), cultures pre-treated with WFO for 24 h before substrate addition (B) and cultures pre-treated with WFO for 48 h before substrate addition (C)

Inhibition of methanogens and subsequently H₂ production were affected by exposure time. A CH₄ yield of 210.73 mL/gVS was recorded for the untreated cultures while CH₄ yield of 174.66

mL/gVS was recorded for the cultures received food waste and WFO simultaneously. Interestingly, increased exposure time led to significant decrease in CH₄ and in turn improved H₂ production. Average H₂ yields 52.48 and 71.46 mL/g VS were obtained by the cultures pre-treated for 24 and 48 h before substrate addition respectively. CH₄ yield for the former was 23.5 mL/g VS while no CH₄ was detected when pre-treatment time increased to 48 h. Therefore, it could be deduced that the combination effects of WFO concentration and exposure time together had a more strong effect on methanogenic inhibition compared to individual effect of each factor.

Figure 6-6 shows the VFA analysis at the end of the fermentation for different pre-treatment conditions. Increased VFAs recovery with exposure time could support the inhibition of methanogens and acetogens. Butyrate was the major VFA found in the condition A in which food waste and WFO were added at the same time to the cultures. Conversely, in the cultures pre-treated either for 24 or 48 h, acetate was the predominant VFA. VFA accumulation in untreated cultures were quite low in comparison to pre-treated cultures, suggesting the high activities of acetogens and acetoclastic methanogens.

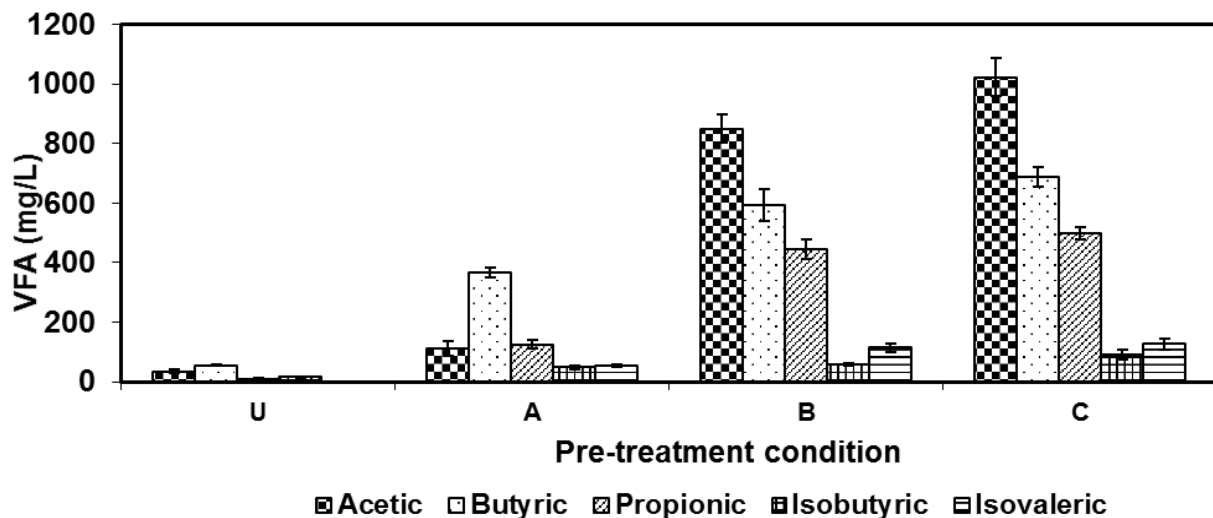


Figure 6-6 Volatile fatty acids production from food waste for untreated cultures (U), cultures received WFO and substrate at the same time (A), cultures pre-treated with WFO for 24 h before substrate addition (B) and cultures pre-treated with WFO for 48 h before substrate addition (C).

Sousa et al., [162], investigated the impact of oleic acid on pure cultures of *Methanospirillum hungatei* and *Methanobacterium formicicum*, two predominant hydrogenotrophs in anaerobic digesters. They studied cell disintegration and observed that the number of damaged cells was proportional to exposure time. Rodríguez-Méndez et al., [163], investigated CH₄ production from slaughterhouse waste (a lipid rich substrate) and observed that high concentrations of LCFAs inhibited methanogenesis and acetogenesis but hydrolysis and acidogenesis were not affected. These reports are in agreement with the present study as no inhibition on H₂ production was observed in presence of WFO, while complete inhibition of methanogens achieved.

Although there are recent reports on using various inoculum pre-treatment, all of these studies have been performed using sugars as substrate. The majority of the fermentation studies using complex substrates have used heat shock for enriching H₂ producing bacteria. A summary of the studies carried out using various organic substrates are shown in Table 6-1.

Table 6-1 Comparison of hydrogen yields from different substrates using different inoculum pre-treatment methods

Substrate	Inoculum	Inoculum pretreatment method	pH	Temperature (°C)	H ₂ yield (mL/g VS)	Reference
Food waste	Anaerobic sludge	Heat (100°C, 30 min)	6.0	35	Control:34.7 Pre-treated: 43.0	[164]
Potato and pumpkin waste	Anaerobic sludge	Chemical (BESA, 25mM)	7.4	35	Control:- Pre-treated: 171.1	[58]
OFMSW	Anaerobic sludge	Aeration (air pump, 2 h)	5.5	37	Control:22.1 Pre-treated: 23.0	[9]
Food waste	Anaerobic sludge	Ultrasound irradiation (79 kJ/gTS)	5.0-6.0	37	Control:- Pre-treated: 180.0	[104]
Food waste	Anaerobic sludge	Alkaline (KOH, pH 12.5, 24 h)	5.3	35	Control:7.1 Pre-treated: 62.6	[49]
Food waste	Anaerobic sludge	Heat shock (105°C, 90 min)	4.5	55	Control: - Pre-treated: 60.6	[85]
Vinegar residues	Anaerobic sludge	Alkaline (HCl, pH 3.5, 24 h)	6.0	37	Control: - Pre-treated: 55.4	[7]
Vinegar residues	Anaerobic sludge	Alkaline (NaOH, pH 12, 24 h)	6.0	37	Control: - Pre-treated: 41.5	[7]
Glucose	Activated sludge	Chloroform (1% V/V, 24 h)	7.0	37	Control: 0.361* Pre-treated: 0.68*	[43]
Glucose	Granular sludge	Heat shock (105°C, 45 min)	6.0	37	Control: 0.14* Pre-treated: 0.9*	[41]
Glucose	Granular sludge	Alkaline (KOH, pH 12, 24 h)	6.0	37	Control: 0.14* Pre-treated: 0.83*	[41]
Glucose	Cow dung	Acid (HCl, pH 3, 24 h)	6.5	37	Control: 1.07* Pre-treated: 1.70*	[165]
Glucose	Granular sludge	WFO (10 g/L, 48 h and then washed)	5.5	37	Control: 0.24* Pre-treated: 0.83*	This study
Glucose	Granular sludge	WFO (20 g/L, 24 h)	5.5	37	Control: 0.24* Pre-treated: 1.48*	This study
Food waste	Granular sludge	WFO (10 g/L, 48 h)	5.5	37	Control: 12.97 Pre-treated: 71.3	This study

6.4. Conclusion

Waste frying oil was successfully used as an inhibitor for H₂ consumption during dark fermentation of mixed cultures. Increased WFO up to 20 g/L did not show inhibitory effects on H₂ production while CH₄ production decreased significantly with 5 g/L WFO, suggesting the higher sensitivity of methanogens compared to hydrogen producing bacteria. H₂ production from pre-treated cultures was also studied using a synthetic food waste and a H₂ yield of 71.46 mL/g VS was obtained for cultures pre-treated with 10 g/L WFO for 48 h that was corresponded to 100% methanogenic inhibition. The present study suggests that inoculum pre-treatment with waste frying oil might be considered as a promising approach to enhance H₂ production from food waste. However, more studies should be performed to investigate the long-term effects of waste frying oil on hydrogen consumers before confirming the economic viability of the process.

7. Optimization of hydrogen production from food waste using anaerobic mixed cultures pre-treated with waste frying oil

7.1. Introduction

The activity of H₂ consuming species is affected by several factors including pH, inoculum type and presence of inhibitors. The microbial activities of both H₂ producing and H₂ consuming microorganisms are pH dependent since the flow of electron towards metabolic pathways is regulated by pH [61,175]. When pre-treating the inoculum with microbial inhibitors, duration of pre-treatment and concentration of inhibitor are two key variables which should be optimised [176]. Previous experiments have proven that inoculum pre-treatment with WFO could be a promising technology to improve H₂ production [177]. This study is aimed at optimizing H₂ production from food waste with a combination of operative variables represented by concentration of frying oil (WFO), pre-treatment duration and value of initial pH at mesophilic conditions.

7.2. Materials and methods

7.2.1. Experimental set-up

Granular sludge was collected from a full-scale Up-flow Anaerobic Sludge Blanket (UASB) digester of a brewery factory located in Padova, Italy. WFO was collected from a local restaurant in Padova, Italy. In order to solubilise WFO, 100 g of the oil was added to 14 g NaOH and mixed rigorously at 55°C. Fatty acid composition of WFO obtained by gas chromatography was as follows (w/w%): linoleic acid (52%), oleic acid (30.24%), palmitic acid (7.04%) and stearic acid (3.22%). A synthetic FW was prepared at lab; it was mainly composed of meat, cheese, pasta, bread, fruits and vegetables, in order to simulate the food waste composition in Italy (Table 7-1). Laboratory scale batch tests were carried out for 72 h using 1-litre glass reactors with a working volume of 500 ml. The bottles were filled with granular sludge with concentrations of 10 gVS/L. pH adjustment were made using NaOH (3M) and HCL (3M) in order to provide different initial pH conditions (5.5, 6.5 and 7.5). Subsequently, varying concentrations of WFO saponified solution (0, 2, 6 and 10 g/l) were added to the bottles at different times (0, 24 and 48 h) before substrate addition. After pre-treatment, the bottles were opened and FW (5 gVS/L) was added to the bottles. In order to ensure anaerobic conditions, all the bottles were nitrogen injected for 3 min and then incubated at mesophilic conditions (37±1°C). Control cultures were prepared with granular sludge (10 gVS/L) and FW (5 gVS/L) without WFO addition with varying initial pH conditions. All the tests were performed in triplicate.

7.2.2. Experiment optimization

The Design of Experiment approach (DOE) has the benefits of reduced experiments and therefore overall costs compared to the conventional optimization methods. Response surface methodology (RSM) is a useful statistical modelling approach to examine the effect of multiple

parameters on a response variable. In this study, Box-Behnken design (BBD), a widely used RSM method was chosen for optimization studies [178]. RSM approach has been already successfully employed for optimization of H₂ production by pure and mixed cultures [179–182]. The factors investigated in this study include initial pH, duration of pre-treatment and waste frying oil concentration.

Table 7-1 Characteristics of inoculum and substrate (Food waste) used in the present study

Parameter	Food waste	Granular sludge
TS (%)	30.1	15.0
VS (%TS)	95.4	53.0
TOC (%TS)	47.4	29.6
TKN (%TS)	3.3	43.0

Table 7-2 Experimental design with three independent variables and the corresponding responses. Three controls codified as C1, C2 and C3 were performed at pH 5.5, 6.5 and 7.5 respectively

Exp. no.	Duration of pre-treatment (h)		WFO (g/L)		pH		H ₂ (mL/gVS)	CH ₄ (mL/gVS)
	Actual	Coded	Actual	Coded	Actual	Coded		
C1	0		0		5.5		12.97 ± 0.77	210.73 ± 8.00
C2	0		0		6.5		7.89 ± 0.32	328.86 ± 5.06
C3	0		0		7.5		5.92 ± 0.14	273.45 ± 4.20
1	0	-1	2	-1	6.5	0	1.74 ± 0.63	387.07 ± 18.15
2	0	-1	10	1	6.5	0	14.78 ± 3.08	259.41 ± 29.02
3	48	1	2	-1	6.5	0	1.93 ± 0.11	315.62 ± 17.55
4	48	1	10	1	6.5	0	25.60 ± 3.07	41.21 ± 7.25
5	0	-1	6	0	5.5	-1	29.48 ± 2.65	266.71 ± 31.81
6	0	-1	6	0	7.5	1	19.40 ± 1.21	370.23 ± 22.35
7	48	1	6	0	5.5	-1	68.20 ± 2.66	16.77 ± 1.29
8	48	1	6	0	7.5	1	13.20 ± 0.47	54.68 ± 2.34
9	24	0	2	-1	5.5	-1	22.40 ± 1.33	194.41 ± 55.33
10	24	0	2	-1	7.5	1	1.07 ± 0.51	287.66 ± 18.52
11	24	0	10	1	5.5	-1	52.48 ± 7.68	23.53 ± 3.55
12	24	0	10	1	7.5	1	17.3 ± 1.11	94.04 ± 17.77
13	24	0	6	0	6.5	0	15.20 ± 0.74	168.68 ± 25.37

DOE was performed in order to investigate combination effects of three individual parameters on dark fermentation. The factors chosen in this study include initial pH (5.5, 6.5 and 7.5), WFO concentration (2, 6, and 10 g/l) and duration of pre-treatment (0, 24 and 48 h). The range for the factors selected based on a previous study [177] and the corresponding experimental responses (H₂ and CH₄) are shown in Table 7-2. Minitab 17 statistical software (Minitab Inc., State College, PA) was used to optimize the response variables, generating the contour plots and analysis of ANOVA. The quadratic polynomial Eq. 7-1 and Eq. 7-2 were fitted to explain the H₂

and CH₄ variations with factors mentioned above. Curve fitting was performed using Matlab (The Mathworks Inc., version 2016a).

$$H_2 \text{ (ml/g VS)} = a_0 + a_1 * (\text{Time}) + a_2 * (\text{WFO}) + a_3 * (\text{pH}) + a_4 (\text{Time})^2 + a_5 (\text{WFO})^2 + a_6 (\text{pH})^2 + a_7 (\text{Time}) * (\text{WFO}) + a_8 (\text{Time}) * (\text{pH}) + a_9 (\text{WFO}) * (\text{pH}) \quad \text{Eq. 7-1}$$

$$CH_4 \text{ (ml/g VS)} = b_0 + b_1 * (\text{Time}) + b_2 * (\text{WFO}) + b_3 * (\text{pH}) + b_4 (\text{Time})^2 + b_5 (\text{WFO})^2 + b_6 (\text{pH})^2 + b_7 (\text{Time}) * (\text{WFO}) + b_8 (\text{Time}) * (\text{pH}) + b_9 (\text{WFO}) * (\text{pH}) \quad \text{Eq. 7-2}$$

7.3. Results and discussion

7.3.1. Effects of experimental variables (duration of pre-treatment, WFO concentration and initial pH) on response variables (H₂ and CH₄ yields)

The reactors were operated for 72 h, though the H₂ production for most of the experimental conditions was not observed after 48 h (data not shown). CH₄ productions during dark fermentation represents the presence of either hydrogenotrophic or acetoclastic methanogens. The experimental H₂ and CH₄ yields obtained with varying levels of selected parameters are shown in Table 7-2. Average yields of H₂ and CH₄ varied from 1.07 to 68.20 mL/gVS and from 16.77 to 387.07 mL/gVS respectively. Increased H₂ and decreased CH₄ production from FW with elevated levels of WFO were observed for cultures pre-treated either for 24 or 48 h. Figure 7-1 shows the contour plots for H₂ and CH₄ productions with different experimental variables. Correlation between increased levels of pure LCFAs and H₂ yields from sugars has been previously reported [48,157,179]. However, to the best of the authors, there is no study in the literature to investigate simultaneous effects of LCFAs concentrations and duration of pre-treatment on inhibition of methanogens and enhancement of H₂ production using response surface models.

Inhibition of methanogens and subsequently enhanced H₂ accumulation were affected by both WFO concentration and exposure time. CH₄ and H₂ yield of 328.86 and 7.89 mL/gVS were recorded respectively for the untreated cultures with an initial pH of 6.5. CH₄ yield decreased by 259.41 mL/gVS when the cultures received food waste and 10 g/L WFO simultaneously and incubated with the same pH. Interestingly, increased exposure time to 48 h led to significant decrease in CH₄ (41.21 mL/gVS) and in turn improved H₂ yield (25.60 mL/gVS). Therefore, it could be deduced that the combination effects of WFO concentration and exposure time together had a stronger effect on methanogenic inhibition compared to individual effect of each factor. At a pH 5.5, average H₂ and CH₄ yields of 68.20 and 16.77 mL/gVS were obtained respectively by the cultures pre-treated for 48 h (6 g/L WFO) before substrate addition respectively. pH had also a strong effect on H₂ and CH₄ production from food waste. This is evident when comparing the yields obtained for the tests 7 and 8. Tests 7 and 8 were conducted with similar conditions of WFO concentration and exposure time (6 g/L, 48 h), but initial pH conditions were different (5.5 and 7.5 respectively). Higher H₂ and lower CH₄ yields obtained for test 7 (68.20 and 16.77 mL/gVS respectively) in comparison with test 8 (13.20 and 54.78 and mL/gVS respectively)

indicate the reduced activities of methanogens when the pH is low. According to the previous studies, the optimum pH for H₂ producing bacteria is in the range of 5.0-6.0 [42,68,168]. Several studies have reported the effect of pH on H₂ production from sugars in presence of pure LCFAs (linoleic or oleic acid) [48,157,158]. There are also a few works about simultaneous effects of pH and linoleic or oleic acid concentration on H₂ production from glucose or xylose [48,179]. However, it is of great importance to investigate effect of LCFAs mixtures on H₂ production using complex substrates when there is a more diverse microbial population due to the presence of different nutrients.

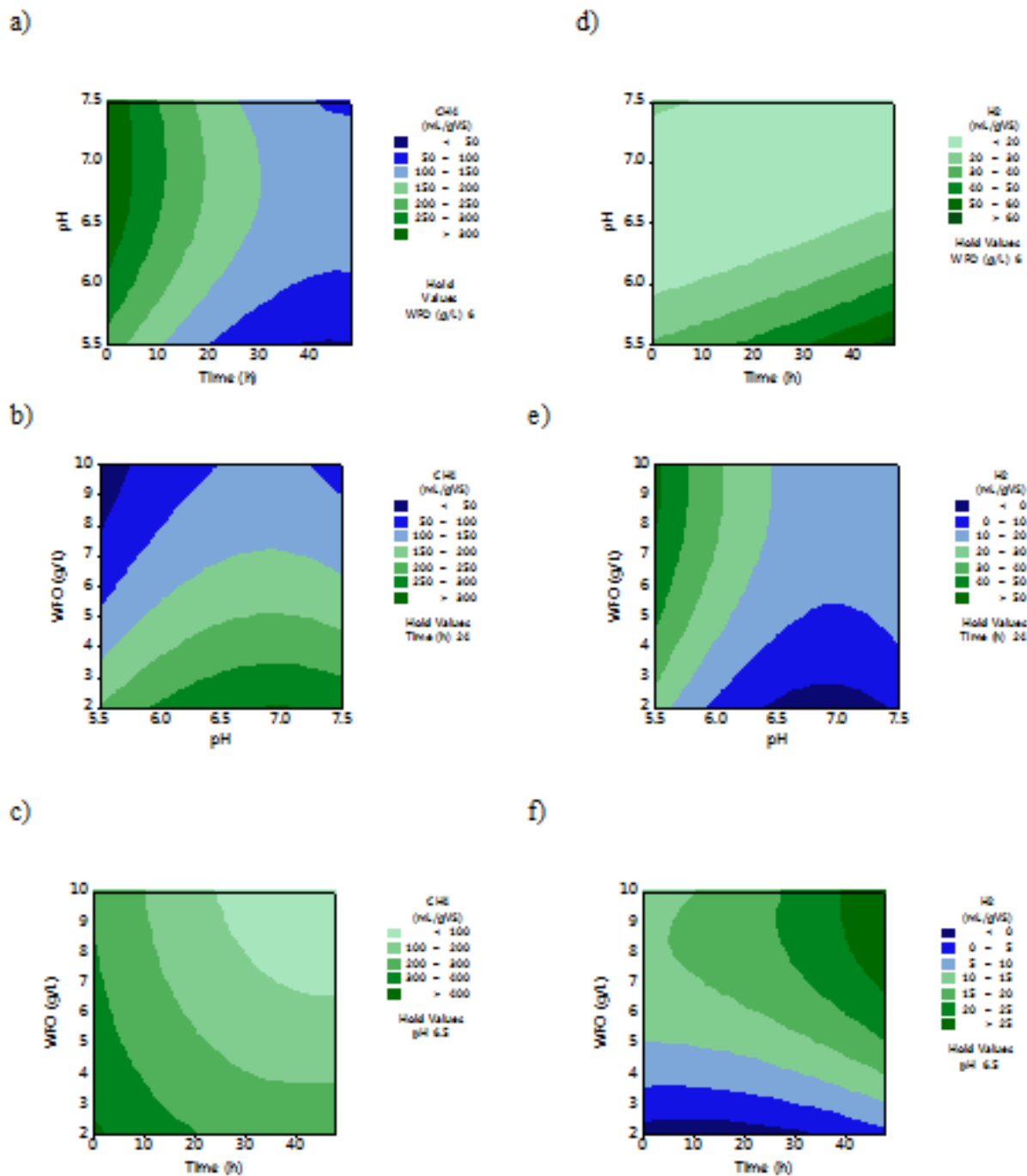


Figure 7-1 Contour plots for CH₄ (a,b,c) and H₂ production (d,e,f).

CH₄ productions by control cultures were not always lower than those pre-treated with WFO. For instance, CH₄ productions by control cultures with initial pH of 6.5 (328.86 mL/gVS) was lower than the cultures incubated with the same pH with simultaneous addition of FW and 2 g/L WFO (387.07 mL/gVS). This is in agreement with other studies that have reported addition of pure LCFAs such as oleic acid, palmitic acid, or linoleic acid with low concentrations could improve CH₄ production [6,163,173].

The inhibitory effect of LCFAs on microorganisms underlies different mechanisms. They can be adsorbed on the cell wall of some species including methanogens, reduce the permeability of the membranes and limit nutrients transport into the cell [183]. In continuous systems, the surrounding lipid layer increases floatation and wash out of biomass [173]. In addition, when LCFAs are entered into the cell, they can dissociate, cause acidification and subsequently cell disruption [174]. When the concentrations of LCFAs in a system are low, they are not able to accumulate around the cell. Instead, LCFAs could be used as energy sources when they are needed.

Besides concentration of LCFAs, inhibition of methanogens and subsequently H₂ production was also affected by exposure time. Sousa et al., [162] investigated cell disintegration in pure cultures of hydrogenotrophic methanogens in presence of oleic acid and reported that increased exposure time led to greater number of damaged cells. Rodríguez-Méndez et al., [163] studied anaerobic digestion using lipid rich substrates and observed that increased levels of LCFAs were inhibitory for methanogenesis and acetogenesis while hydrolytic and acidogenic bacteria were not inhibited. LCFAs have been shown to have inhibitory effect on either acetoclastic or hydrogenotrophic methanogens; however, the latter are more sensitive and inhibitory levels for them might be lower than hydrogenotrophic methanogens [161].

The effects of experimental variables (pH, WFO concentration and duration of pre-treatment) on H₂ and CH₄ productions are also shown using three-factor main effect plots and interaction plots (Figure 7-2 and Figure 7-3). According to main effect plots, the largest effect on H₂ production was due to pH while for CH₄ minimization effect of WFO concentration and duration of pre-treatment was greater than pH. Increasing the levels of WFO concentration or duration of pre-treatment resulted in low levels of CH₄ production. It should be considered that main effect plots show the average response for each experimental factor without taking into account the effects of other experimental factors. Optimum condition predicted by the model for the complete inhibition of methanogens were initial pH 5.5, WFO 7.74 g/L, and a duration of pre-treatment equal to 42.67 h.

7.3.2. Model fit using ANOVA

3.2. Model fit using ANOVA

In order to evaluate the fitted models for H₂ and CH₄, analysis of ANOVA was performed (Table 7-3). The significance of each term in the model was determined by p-values (terms with p-values < 0.05 were considered significant). Considering equations 1 and 2, ANOVA revealed that linear terms (duration of pre-treatment and WFO) were significant for CH₄ production while quadratic and interaction terms were insignificant. For H₂ production, linear terms (pH, duration of pre-treatment and WFO), quadratic terms (pH and WFO) and interaction term (Time*pH) were the most significant.

Table 7-3 ANOVA outputs for the fitted models

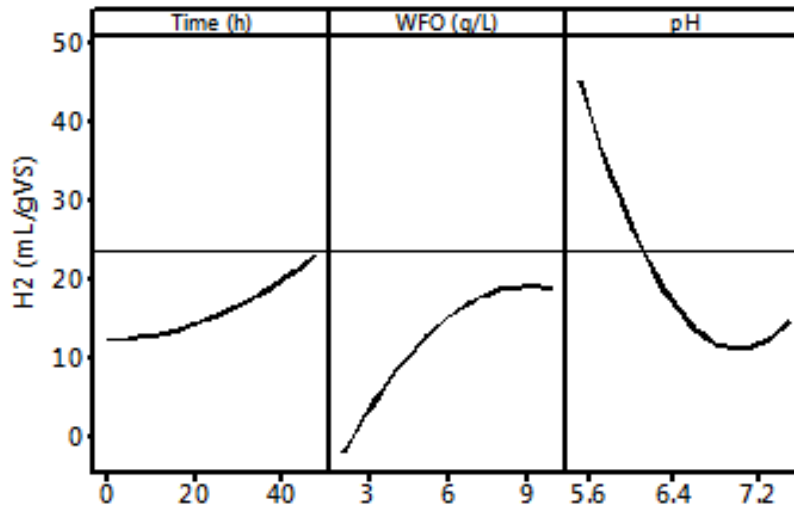
Source	DF	Adj SS	Adj MS	F-Value	P-Value
H₂					
Model	9	4584.84	509.43	32.53	0.001
Linear	3	2945.63	981.88	62.70	0.000
Time	1	237.73	237.73	15.18	0.011
WFO	1	859.88	859.88	54.91	0.001
pH	1	1848.02	1848.02	118.01	0.000
Square	3	1058.13	352.71	22.52	0.002
Time*Time	1	23.54	23.54	1.50	0.275
WFO*WFO	1	167.36	167.36	10.69	0.022
pH*pH	1	813.69	813.69	51.96	0.001
2-Way Interaction	3	581.08	193.69	12.37	0.009
Time*WFO	1	28.68	28.68	1.83	0.234
Time*pH	1	504.45	504.45	32.21	0.002
WFO*pH	1	47.96	47.96	3.06	0.141
Error	5	78.30	15.66		
Lack-of-Fit	3	78.30	26.10	*	*
Pure Error	2	0.00	0.00		
Total	14	4663.14			
CH₄					
Model	9	206123	22902.6	11.85	0.007
Linear	3	176326	58775.4	30.42	0.001
Time	1	91380	91380.3	47.29	0.001
WFO	1	73228	73227.7	37.89	0.002
pH	1	11718	11718.3	6.06	0.057
Square	3	23250	7749.9	4.01	0.085
Time*Time	1	11021	11021.0	5.70	0.063
WFO*WFO	1	2760	2759.7	1.43	0.286
pH*pH	1	7990	7990.1	4.13	0.098
2-Way Interaction	3	6547	2182.3	1.13	0.421
Time*WFO	1	5343	5342.9	2.76	0.157
Time*pH	1	1083	1083.1	0.56	0.488
WFO*pH	1	121	121.0	0.06	0.812
Error	5	9662	1932.4		
Lack-of-Fit	3	9662	3220.7	*	*
Pure Error	2	0	0.0		
Total	14	215785			

DF: degree of freedom

Adj SS: adjusted sum of squares

Adj MS: adjusted mean squares

a)



b)

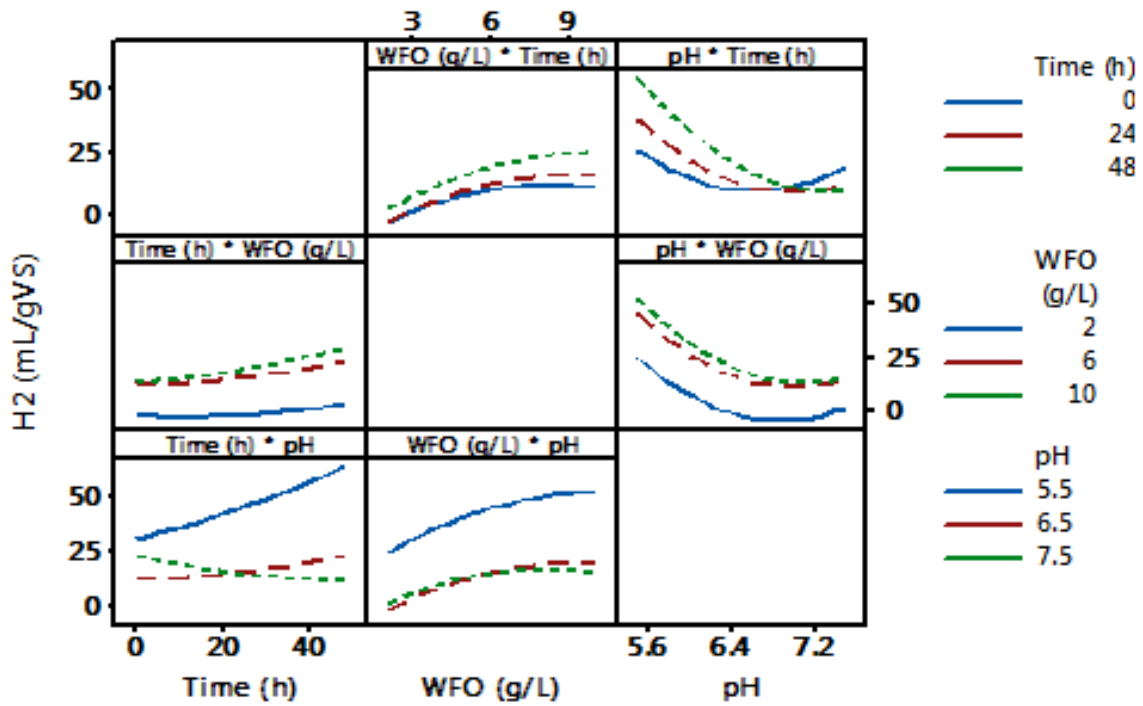
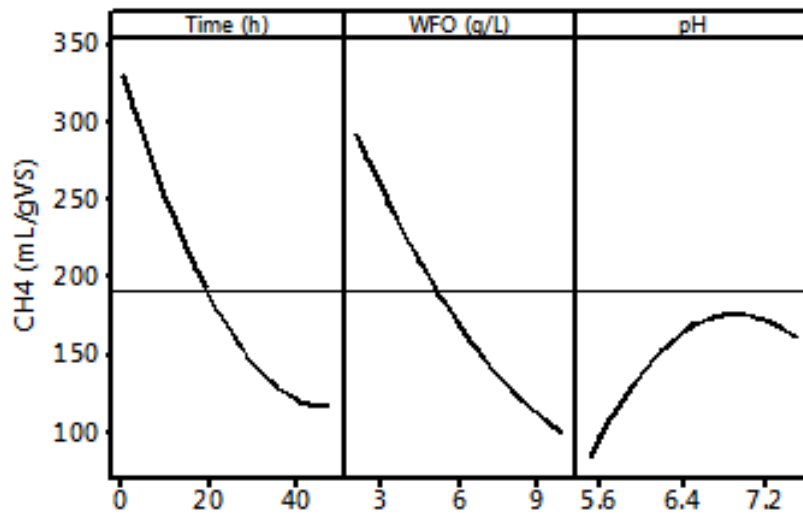


Figure 7-2 a) Main effects plot and b) interaction plots for H₂ production. Dashed lines in the first graph indicate the mean response values

a)



b)

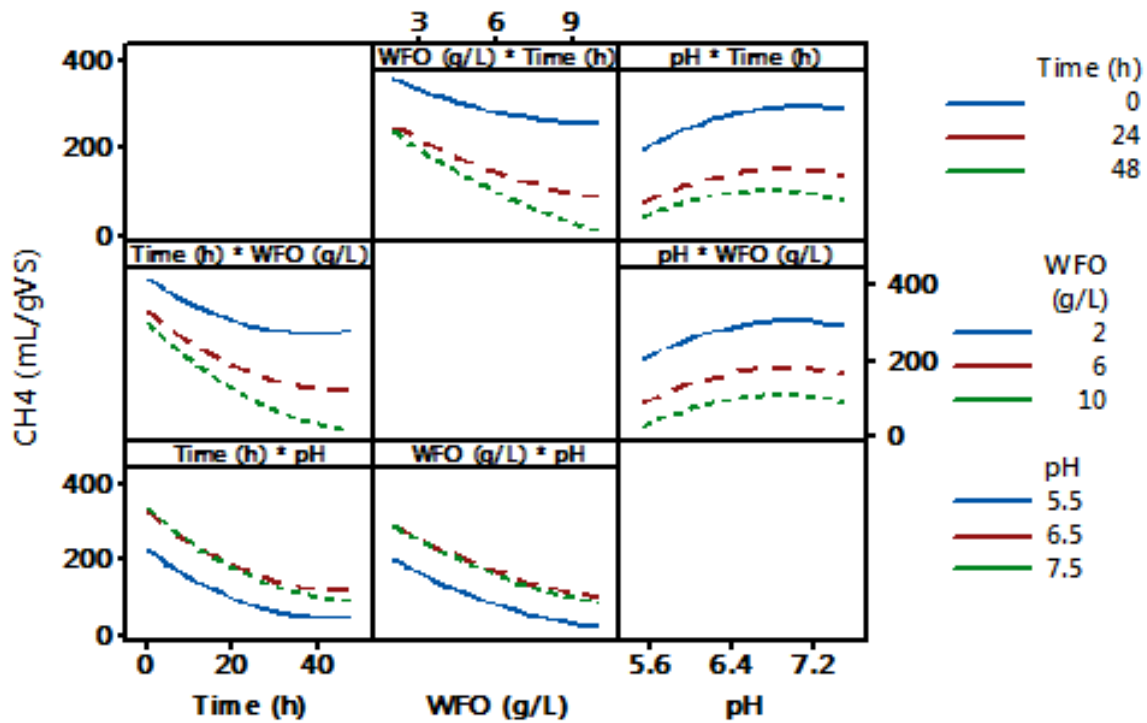
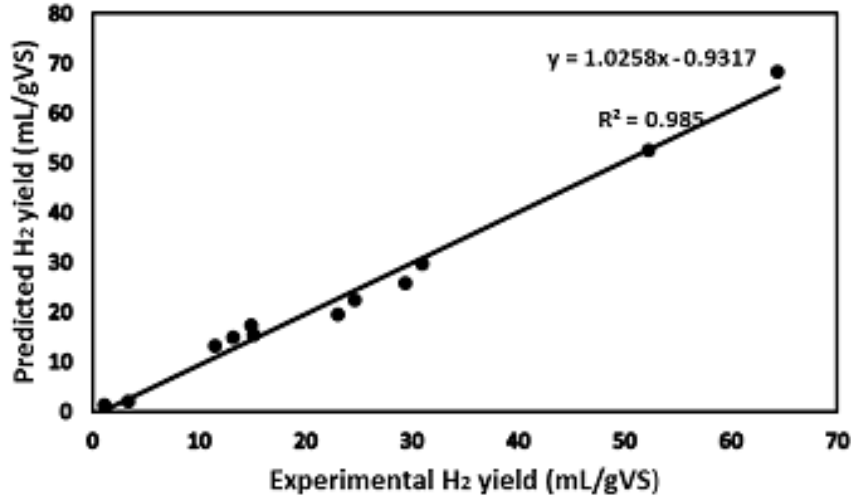


Figure 7-3 a) Main effects plot and b) interaction plots for CH₄ production. Dashed lines in the first graph indicate the mean response values

7.3.3. Model verification and validation

The response variables (H₂ and CH₄) predicted by the quadratic models fitted reasonably well with the experimental data. The R² values were 0.985 and 0.954 for H₂ and CH₄ models respectively (Figure 7-4).

a)



b)

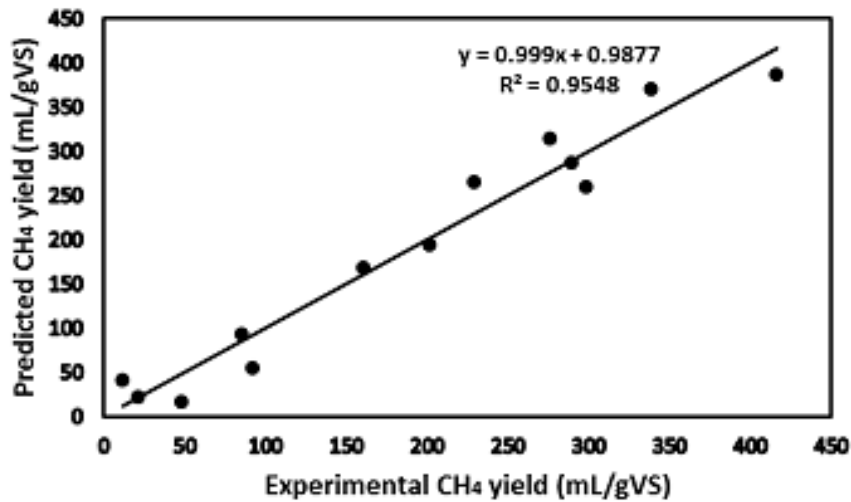


Figure 7-4 Predicted values versus experimental data for a) H₂ and b) CH₄

A validation study was performed based on the optimum conditions predicted by the model. The predicted conditions for the complete inhibition of methanogens were 7.74 g/L, pH 5.5 and 42.67 h. Further experiments were performed in order to compare the experimental responses with the

predicted values under optimum conditions. A CH₄ yield of 2.30 mL/gVS with a H₂ yield of 71.34 mL/gVS was obtained for the conditions predicted by the model for complete inhibition of methanogens.

7.3.4. VFA production and fate of organic carbon

Analysis of soluble metabolites production at the end of fermentation could be useful to evaluate metabolic pathways for H₂ production from food waste as well as H₂ consuming pathways. Fermentation types in mixed cultures are classified into three main categories: 1) butyrate-type, 2) propionate-type and 3) ethanol type [19]. In butyrate-type fermentation, acetate and butyrate are the main soluble compounds produced together with H₂ and CO₂. In propionate-type fermentation, acetate and propionate are the main products with small amounts of iso valerate and without significant production of H₂. Ethanol-type fermentation which occurs in very low pH conditions (4-4.5) produces ethanol, acetate, H₂ and CO₂. In this study, acetate, butyrate, propionate and small quantities of iso valerate and iso butyrate, were the main soluble metabolites. Figure 7-5 shows the VFA concentrations at the end of fermentation using different experimental conditions. For all the experimental conditions tested, the low concentrations of acetic acid were corresponded with high CH₄ production, suggesting the increased acetic acid consumption for CH₄ production by acetoclastic methanogens due to absence of WFO.

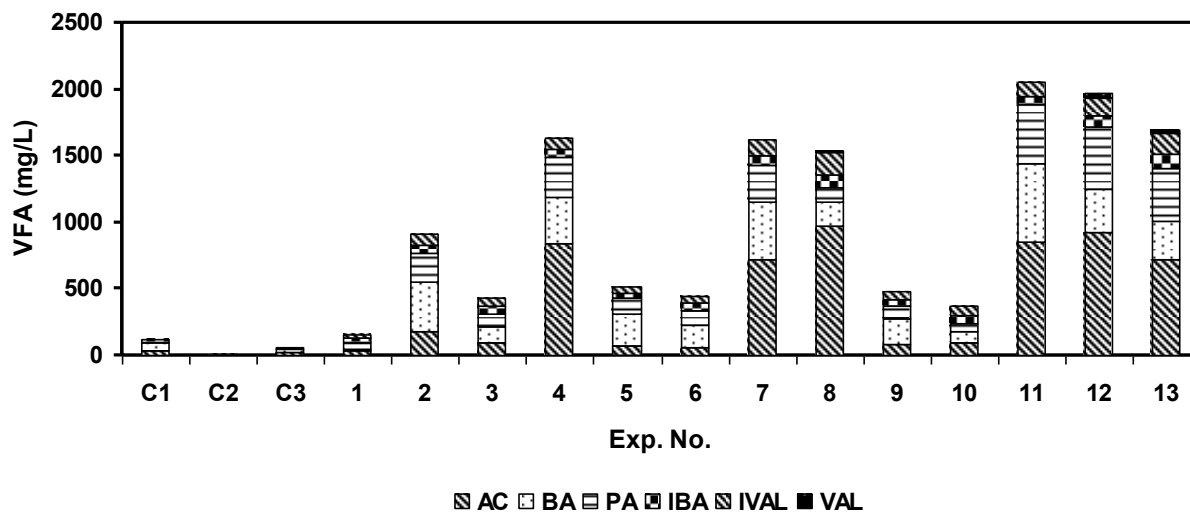


Figure 7-5 Average VFA productions at the end of fermentation using different experimental conditions. Ac:Acetic, BA: Butyric, PA: Propionic, IBA: Iso butyric, IVAL: Iso valeric, VAL: Valeric

VFA concentrations for all the untreated controls were very low compared to the pre-treated cultures, indicating the higher activities of acetogens (to convert propionate and butyrate to acetate) and acetoclastic methanogens (to convert acetate to CH₄). Shin et al., [184] have already reported the inhibitory effects of oleate, linoleate, stearate and palmitate on degradation of propionate to acetate. Similarly, Lalman and Bagley, [5] observed lower degradation of butyrate in presence of linoleic acid. Other studies have also reported higher accumulation of propionate and butyrate in presence of linoleic acid compared to the control cultures [42,69]. VFA concentrations in the cultures pre-treated with 2 g/L WFO either for 24 or 48 h, were

significantly lower compared to the cultures pre-treated with 6 or 10 g/L WFO. Also, acetate concentrations in cultures pre-treated with 2 g/L WFO were much lower compared to those pre-treated with higher concentrations of WFO. This might suggest that pre-treatment with low concentrations of WFO could not inhibit the conversion of acetate to CH₄ by acetoclastic methanogens.

Initial organic carbon transformed to DOC, or emitted as CO₂ and CH₄ in each experimental condition were calculated to better analyze the impact of experimental factor on the fate of the carbon (Table 7-4). Carbon hydrolyzed to DOC varied between 4.6% and 55.98% of the initial organic carbon. For the experimental conditions with high H₂ accumulation, the majority of the initial carbon remained in the liquid phase. Carbon percentages emitted as CH₄ were very low for experiments 7 and 11 (0.73% and 0.71% respectively) in which high H₂ productions were detected.

Table 7-4 Average percentages of initial organic carbon (TOC) converted to dissolve organic carbon (DOC) or emitted as CO₂ and CH₄ at the end of fermentation from different experimental conditions. All the numbers are presented as %.

Exp. no.	Hydrolysed to DOC	Emitted as CO ₂	Emitted as CH ₄
C1	12.22 ± 2.1	32.13 ± 3.1	22.01 ± 2.5
C2	4.87 ± 0.6	28.17 ± 4.3	43.30 ± 5.1
C3	8.45 ± 1.4	28.65 ± 3.2	36.01 ± 3.4
1	9.29 ± 0.8	24.03 ± 3.6	26.96 ± 2.8
2	31.77 ± 2.8	10.49 ± 0.6	10.76 ± 1.3
3	20.05 ± 3.4	21.98 ± 2.5	22.67 ± 2.2
4	39.58 ± 5.3	6.85 ± 1.1	1.75 ± 0.1
5	10.10 ± 2.1	14.06 ± 1.9	13.12 ± 1.7
6	14.78 ± 2.9	14.76 ± 3.1	18.08 ± 2.2
7	55.98 ± 6.9	8.69 ± 0.9	0.73 ± 0.0
8	36.26 ± 4.7	10.62 ± 1.1	2.54 ± 0.4
9	21.84 ± 4.4	23.91 ± 3.1	19.11 ± 1.5
10	31.97 ± 3.6	22.96 ± 3.2	20.16 ± 3.3
11	33.59 ± 4.3	6.69 ± 0.9	0.71 ± 0.0
12	30.81 ± 3.9	7.75 ± 0.8	4.05 ± 0.6
13	22.18 ± 3.3	13.18 ± 1.1	10.90 ± 1.2

7.4. Conclusion

WFO was used as an inhibitor for methanogenic H₂ consumption during anaerobic conversion of food waste to H₂ using mixed cultures. Inoculum pre-treatment conditions with WFO were optimized to inhibit methanogenic H₂ consumption and improve H₂ yields from food waste. BBD approach was used to develop response surface models to evaluate effect of inoculum pre-treatment with WFO on H₂ and CH₄ production from food waste. Duration of pre-treatment, WFO concentration and initial pH were the three experimental factors tested in this study. Combination of high WFO (7.74 g/L), low initial pH (5.5) and long pre-treatment (42.67 h) led to complete inhibition of methanogens.

8. Effect of inoculum pre-treatment on mesophilic hydrogen and methane production from food waste using two-stage anaerobic digestion

8.1. Introduction

Selection of the best inoculum pre-treatment for H₂ production depends on substrate and inoculum type. There are several studies that investigated different inoculum pre-treatments for H₂ production from glucose [41,42,165]. Also, there are few reports that compared different inoculum pre-treatment methods for H₂ production from complex wastes including potato and pumpkin waste [58], corn stover [53], waste ground wheat [185], and stale corn [186]. However, it is not reported the effects of inoculum pre-treatment methods on two-stage AD for H₂ and CH₄ production from food waste (FW). Most of the two-stage AD studies have been performed using heat shock as the inoculum pre-treatment [187]. Therefore, in order to fill this knowledge gap, this study aims to:

- 1) Investigate the possibility of using anaerobic mixed cultures pre-treated with WFO in two-stage AD for H₂ and CH₄ production.
- 2) Compare the two-stage AD of FW using WFO pre-treated cultures with three common inoculum pre-treatment methods (aeration, heat shock and alkaline pre-treatment).
- 3) Evaluate the overall performance of two-stage AD in terms of energy yield and substrate degradation.

8.2. Materials and methods

8.2.1. Seed sludge pre-treatments

Four different pre-treatments were used to enrich H₂ producing bacteria from granular sludge:

- Heat shock: Granular sludge was boiled at 90°C for 30 min [122].
- Aeration: The granular sludge was aerated for 24 h using an aquarium pump with an air flow rate of 3 L/min [43].
- Alkaline pre-treatment: The pH of the granular sludge was adjusted to 12.0 ± 0.1 with 3 N NaOH and maintained for 24 h [7].
- Pre-treatment with WFO: a saponified WFO solution was prepared according to the method described by Rafieenia et al., [177]. Fifteen g/L of WFO was added to the granular sludge cultures (33 gVS sludge/L saponified WFO solution) and maintained for 24 h. After the treatment, the pre-treated cultures were washed three times with tap water. In order to wash the granular sludge, the pre-treated cultures were remained stagnant for 30 min to precipitate the granular sludge. After which, the supernatant containing saponified WFO solution was removed using a syringe. The washing was repeated two additional times by adding tap water and removing the supernatant.

Control cultures were also prepared without any form of pre-treatment.

It should be mentioned that pre-treatment conditions for each method might vary from one study to another and optimal conditions depend on both the inoculum and substrate. Therefore, the pre-treatment conditions applied in the present study were chosen from the studies that used similar inoculum or substrate.

8.2.2. Two-stage anaerobic digestion tests

Synthetic FW samples with the composition reported in previous sections was used as substrate. Two-stage batch AD tests were performed using 500 mL glass reactors with a working volume of 250 mL. In the first stage (H_2 production), pre-treated cultures as well as the control received 5 gVS/L FW. Followed by a pH adjustment to 5.5 using NaOH (3M) and HCl (3M). Subsequently, the bottles were sealed with silicone rubber stoppers, purged with nitrogen for 3 min to ensure anaerobic conditions were achieved, and incubated in a water bath at 35°C. After 96 h (when the biogas production ceased), the reactors were opened and samples (5 mL) were taken from each reactor and stored in a freezer (-20°C) for further analysis. Before storage in the freezer, the samples were filtered using membrane filters with a pore size of 0.45 μm to be ready for dissolved organic carbon (DOC) and volatile fatty acid (VFA) analysis. In order to start the second stage (CH_4 production), 5 gVS/L of untreated granular sludge was added to the reactors [111]. The pH of all the reactors was adjusted at 7.0 since it is the optimal pH for methanogens [168] and incubated at 35°C. The second stage lasted for 32 days. All of the tests were performed in triplicate. The schematic of the different steps are shown in Figure 8-1.

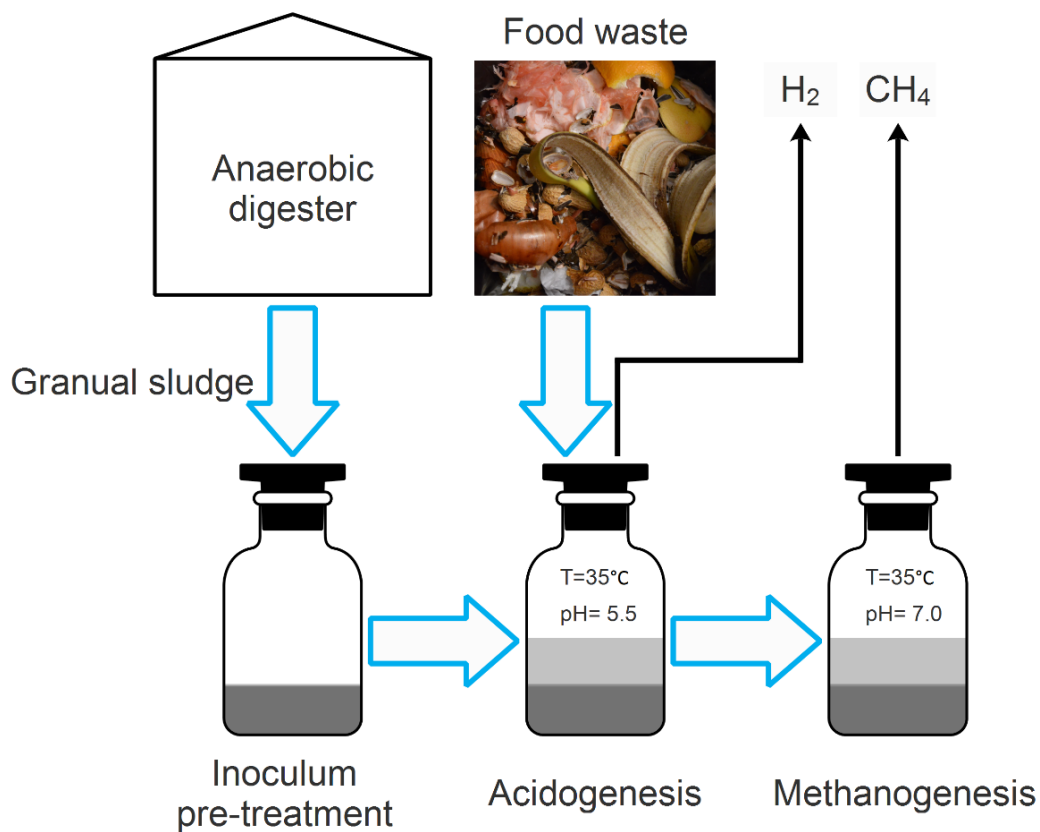


Figure 8-1 Schematic of the different steps of two-stage anaer

A modified Gompertz equation was used to model H₂ and CH₄ production and quantification of kinetic parameters (Eq. 8-1). The modified Gompertz model is the most widely used method to analyse biogas production performance and therefore it was ideal to evaluate the H₂ and CH₄ productions using different inoculum pre-treatment methods.

$$H(t) = P * \exp \left\{ - \exp \left[\frac{R_m * e}{P} \right] (\lambda - t) + 1 \right\} \quad \text{Eq. 8-1}$$

Where:

H is the cumulative hydrogen production potential (ml)

P the H₂ and CH₄ production potentials (ml)

R_m is the maximum H₂ and CH₄ production rates (ml/h)

λ is the lag phase (h)

t is incubation time.

P, λ and R_m were estimated by using a curve fitting tool in Matlab (The Mathworks Inc., version 2016a).

8.3. Results and discussion

8.3.1. Effect of inoculum pre-treatment methods on the first stage of anaerobic digestion

8.3.1.1. Hydrogen production

In the first stage of AD, no detectable CH₄ was observed in the reactors pre-treated either with WFO or heat shock, indicating the efficiency of the mentioned pre-treatments on the inhibition of methanogens. For alkali treated and pre-aerated inoculum, small amounts of CH₄ were detected in the produced biogas (4.4 and 3.2%, respectively). Cumulative H₂ production obtained in the first stage of AD using various inoculum pre-treatment methods are shown in Figure 8-2. H₂ produced by the control cultures was less than that of all pre-treatments (27.6 mL/gVS). The highest H₂ yield was obtained for WFO pre-treated cultures (76.1 mL/gVS) followed by heat shock (53.8 mL/gVS). H₂ yields of 42.8 mL /gVS and 35.3 mL/gVS were obtained for alkali pre-treated and pre-aerated inoculum, respectively. Higher H₂ yields obtained by WFO-pre-treated inoculum compared to heat shock suggest that inoculum pre-treatment with WFO could provide a less severe condition for H₂ producing bacteria to be enriched. The lower H₂ yields obtained by aeration and alkaline pre-treatment could be attributed to the incapability of the mentioned methods to suppress H₂ producing bacteria.

Kinetic parameters calculated using the modified Gompertz equation are shown in Table 8-1. The results showed that H₂ production increased for all of the pre-treatment methods when compared to the control. Inoculum pre-treatment with WFO and heat shock increased the

cumulative H₂ production potential (P) by 182% and 109%, respectively, compared to the untreated culture. For alkaline and aeration pre-treatments cumulative H₂ productions were 56.5% and 31.2% higher than the control, respectively. Regarding the lag time (λ), the shortest lag time of 2.4 h was obtained for untreated inoculum while the longest lag time of 12.4 h was obtained for heat shock. The literature also reported that the longest lag time was observed after heat shock pre-treatment [46,188]. The highest lag time for heat shock pre-treated cultures could be presumably due to the inhibition of some H₂ producing bacteria during the heat shock pre-treatment. Therefore, additional time is needed for H₂ producing bacteria to proliferate and produce H₂.

Table 8-1 Modified Gompertz equation coefficients for different inoculum pre-treatments

Inoculum pre-treatment	R _m (mL H ₂ /h)	λ (h)	P (ml)	R ²
First stage				
Waste frying oil	4.0	5.8	110.6	0.9864
Alkaline	4.1	3.0	61.2	0.9991
Aeration	2.5	3.2	51.3	0.9985
Heat shock	3.3	12.4	81.9	0.9853
Control	3.7	2.4	39.1	0.9996
Second stage				
	R _m (mL CH ₄ /d)	λ (d)	P (ml)	R ²
Waste frying oil	50.0	3.9	903.0	0.9972
Alkaline	41.6	0.9	608.2	0.9955
Aeration	41.9	0.6	534.1	0.9953
Heat shock	51.2	0.7	640.0	0.9945
Control	33.4	0.3	438.8	0.9861

Based on these results, the best inoculum pre-treatment method to enrich H₂ producing bacteria reported by a specific study might differ from other studies. This inconsistency could be due to the strong effect of substrate biodegradability on efficiency of each inoculum pre-treatment method. This means that the best inoculum pre-treatment using a specific substrate is not necessarily as effective using other substrates.

Many studies have used different substrate pre-treatment methods prior to DF in order to increase the hydrolysis rate and nutrient conversion efficiencies [77,189,190]. However, the techno-economic feasibility of the combined processes needs to be evaluated for industrial application [191]. Moreover, composition variability of the inoculum is another issue that affects the specific inoculum pre-treatments and therefore results in contradiction between other studies. Microbial populations present in the initial inocula show different tolerability towards the changes in the environmental conditions and act differently to the severe conditions imposed by pre-treatments.

The majority of the comparison studies on the inoculum pre-treatment methods have been performed using glucose as substrates. Comparison studies on H₂ production from complex

substrates using various inoculum pre-treatment methods are rare in the literature. Yin et al., [46] investigated H₂ production from glucose using four inoculum pre-treatment methods namely heat shock, gamma irradiation, acid, and alkaline pre-treatment and reported the highest H₂ yields for gamma-irradiated cultures. Using the same inoculum, a significant variation was observed in the H₂ production performances when glucose was replaced by raw grass as the lowest H₂ yield was obtained by gamma-irradiated cultures [45]. Ghimire et al., [58] evaluated H₂ production from potato and pumpkin waste using aeration, heat shock, and bromoethane sulphonic acid (BESA) addition and achieved the highest H₂ yield using BESA treatment. Dong et al., [9] reported that heat shock was the best inoculum pre-treatment to enhance H₂ yields from the organic fraction of municipal waste. Similarly, heat shock has been reported as the best inoculum pre-treatment using palm oil mill effluent [59], waste ground wheat [185], brewery wastewater [192], and corn stover hydrolysate [53].

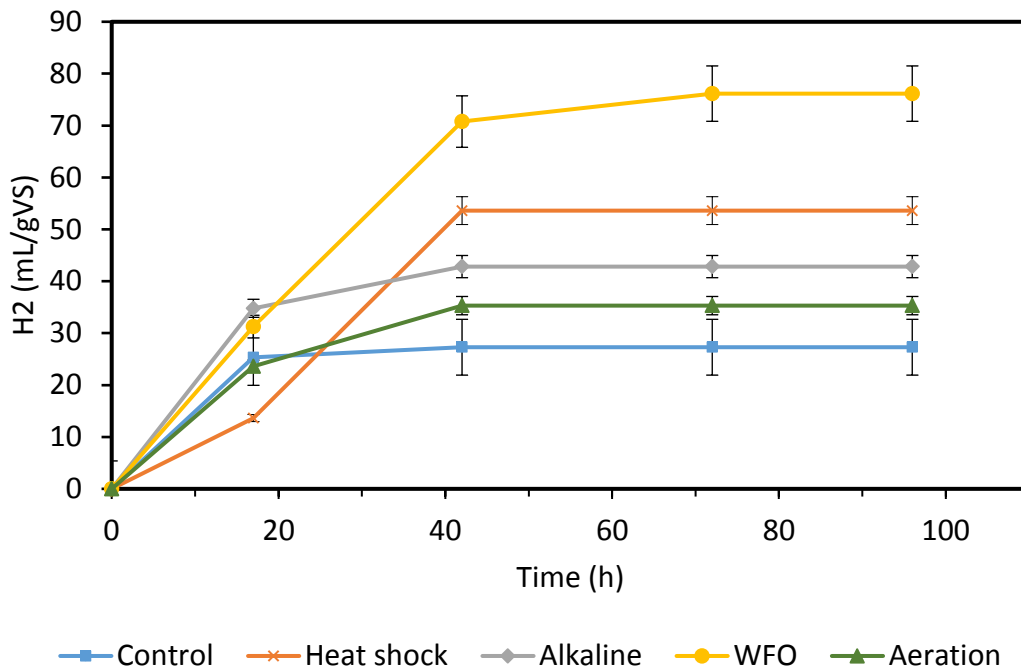


Figure 8-2 Cumulative H₂ production using different inoculum pre-treatment methods in the first stage of AD

8.3.1.2. Volatile fatty acids production

H₂ production through DF is accompanied with the production of soluble metabolites and mainly VFAs. VFAs produced in the first stage of AD using different inoculum pre-treatment methods are shown in Figure 8-3. Acetic acid and butyric acid were the main VFAs detected by Gas Chromatography for all the pre-treatments. Lower acetic acid concentrations in the untreated culture could be attributed to the possible conversion of acetic acid to CH₄ by acetoclastic methanogens. The highest concentration of butyric acid was observed for WFO pre-treated inoculums (385.33 mg/L) while the alkali treated cultures produced the highest amount of acetic acid (258.66 mg/L). Several studies observed that H₂ yield was directly proportional to the

butyrate/acetate ratio [166,167] while other studies reported no correlation between the butyrate/acetate ratio and H₂ yields [111,165]. Moreover, there are several studies reporting that H₂ yields were inversely proportional to the butyrate/acetate ratio [46,61,72]. A possible explanation for these contradictory results could be related to acetic acid and butyric acid producing pathways. Theoretically, 4 and 2 mol H₂/mol glucose can be obtained when H₂ is produced through acetic acid and butyric acid production pathways, respectively.

However, higher acetic acid productions can also be caused by H₂ consumption to acetic acid production by homoacetogens. Therefore, higher acetate concentrations during DF may not always be accompanied by higher H₂ yields.

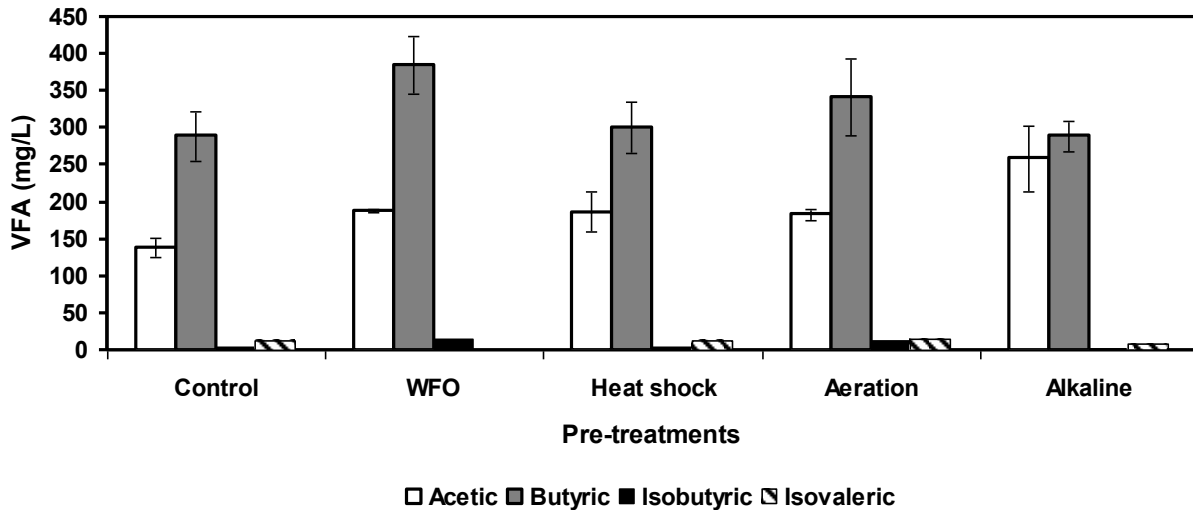


Figure 8-3 VFA concentrations at the end of the first stage of AD

VFAs generation resulted in a pH drop at the end of the first stage for all treatments. Table 8-2 shows the pH values at the end of the first stage for the different pre-treatments, ranging from 4.23 (WFO) to 4.69 (alkaline). The lowest pH value for WFO pre-treated inoculum is in agreement with the highest production of VFAs.

8.3.1.3. Substrate degradation

The percentages of the initial TOC hydrolyzed to DOC have been shown in Table 8-3. TOC percentages hydrolyzed to DOC in the first stage were in the range of 35-38% for all the pre-treatments methods while the hydrolysis performance in the untreated culture was the lowest (28%). The hydrolysis yields reported during DF of FW are in the range of 35-45% [193,194]. During the first stage, no CH₄ was detected in the WFO and heat shock treated cultures while for the aeration and alkali treated cultures, 1.4% and 0.9% of the initial organic carbon was converted to CH₄, respectively. TOC conversion efficiencies for heat shock and WFO were similar (43.4%). TOC conversion for control cultures was less than all treatments (40.58%). The highest TOC conversion during the first stage was obtained for the alkaline pre-treatment (48.6%).

Table 8-2 pH values at the end of the first and second stage for different pre-treatment methods

	Control	Heat shock	WFO	Alkaline	Aeration
First stage	4.56 ± 0.19	4.42 ± 0.04	4.23 ± 0.15	4.69 ± 0.08	4.30 ± 0.13
Second stage	7.15 ± 0.06	7.21 ± 0.05	7.06 ± 0.02	7.17 ± 0.07	7.14 ± 0.01

8.3.2. Effect of inoculum pre-treatment methods on the second stage of anaerobic digestion

8.3.2.1. Methane production

Figure 8-4 shows the cumulative CH₄ production during the second stage of AD using the effluents of the DF subjected to the various inoculum pre-treatments. The CH₄ and CO₂ content in the produced biogas varied between 60-76% and 20-27%, respectively. The highest CH₄ yield of 598.2 mL/gVS was obtained for the WFO pre-treatment. CH₄ yields of 422.91, 432.2 and 381.6 were obtained for heat shock, alkaline, and aeration pre-treatment, respectively. Similar to the first stage, the lowest CH₄ yield was obtained for the untreated cultures (321.5 mL CH₄/gVS). The significant differences in the CH₄ yields for the cultures undergoing different pre-treatments in the first stage (while they were received the same amount of raw granular sludge for the second stage) suggests that the first stage can also play an important role on the efficiency of the second stage. Firstly, the inoculum pre-treatment used in the first stage affects the amount of VFAs produced which are then converted to CH₄ by acetoclastic methanogens in the second stage. Second, the inoculum pre-treatment not only has an influence on methanogens but also on many other species present in the mixed culture. This effect may change the behavior of the granular sludge in terms of many characteristics including hydrolysis.

The modified Gompertz model was used to evaluate CH₄ production performance in the second stage of AD. Kinetic parameters calculated using the modified Gompertz equation for the second stage of AD are shown in Table 8-1. Regarding the cumulative CH₄ production potential (P), the highest value was obtained for the inoculum pre-treated with WFO (903 mL) which was 106% higher than the untreated cultures (438.8 mL). The lowest CH₄ production potential among the four pre-treatment methods was recorded for aeration pre-treated inoculum (534.1 mL) but was still higher compared to the untreated inoculum.

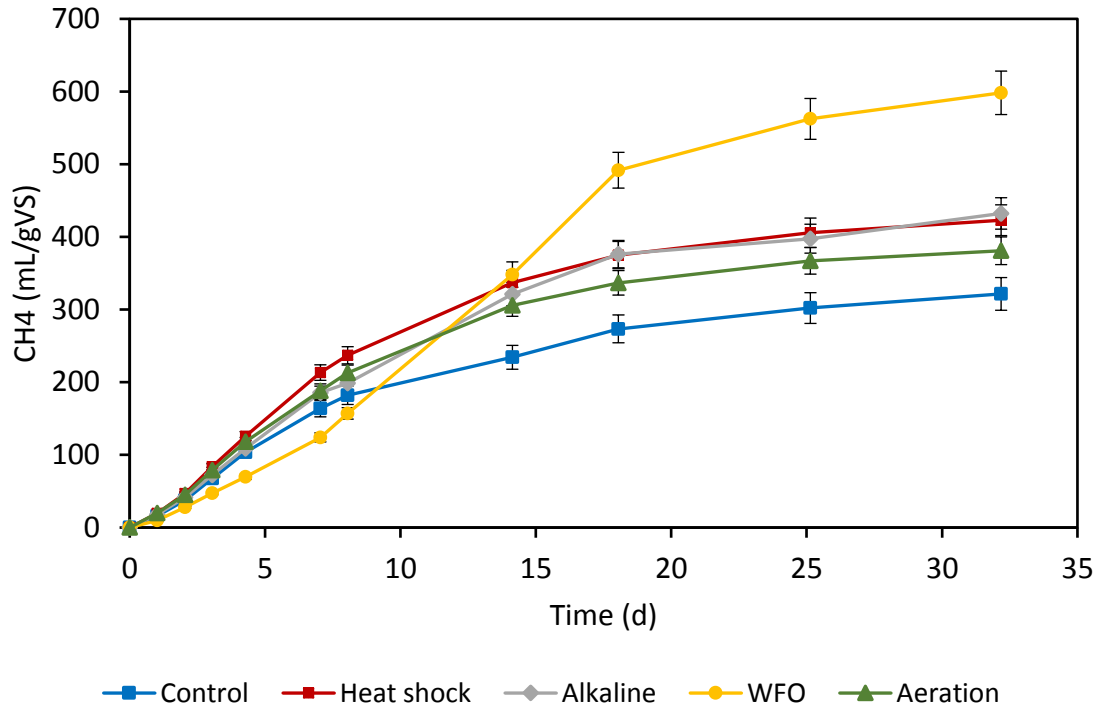


Figure 8-4 Cumulative CH₄ production for various inoculum pre-treatment methods

As for the lag time (λ), the longest value of 3.9 d was obtained for the WFO pre-treatment. A possible explanation for this observation could be the partial loss of acetogens (which convert VFAs to acetate) during the first stage of AD. The inhibitory effect of LCFAs on acetogens has been proven by several studies [6,195]. The highest CH₄ production rates (R_m) of 51.2 and 50.2 mL CH₄/d were obtained for heat shock and WFO, respectively. CH₄ production rates of 41.9, 41.6, and 33.4 mL CH₄/d were calculated for aeration, alkaline, and control pre-treatments, respectively.

The pH values at the end of the second stage were in the range of 7.06 (WFO) to 7.21 (heat shock), which are close to the optimum pH for methanogens [196] (Table 8-2).

8.3.3. TOC removal

During DF, a major fraction of the initial TOC remains in the liquid phase in the form of organic acids that can be further converted to CH₄. Coupling DF and AD could enhance the substrate conversion and subsequently result in a more stabilized digestate that can be used as a fertilizer [197,198].

As shown in Table 8-3, during the first stage of AD, carbon removal was in the range of 8-11% of the initial TOC (in the form of CO₂ or CH₄). This removal means that approximately 90% of the initial TOC remained in the solid or liquid phases at the beginning of the second stage of AD. The lowest carbon removal during the second stage was observed for the untreated cultures (51.17% of the initial TOC). The lowest carbon conversion to CH₄ for the untreated cultures could be attributed to the lower conversion of substrate to organic acids in the first stage. The

highest carbon removal was obtained by WFO with 87.36% of the initial TOC removal during the second stage. The rank of TOC removal relative to other inoculum pre-treatments was alkaline > heat > aeration.

Table 8-3 Average percentages of initial organic carbon (TOC) degraded during two-stage anaerobic digestion.

Inoculum Pre-treatment	First stage				Second stage				Overall TOC removal (%)
	Hydrolysed to DOC (%)	Emitted as CO ₂ (%)	as CH ₄ (%)	Emitted as CH ₄ (%)	Emitted as CO ₂ (%)	as CH ₄ (%)	Emitted as CH ₄ (%)		
Heat shock	35.22 ± 3.34	8.25 ± 0.96	0.00 ± 0.00	± 0.00	14.56 ± 0.78	51.64 ± 1.45	74.45		
Waste frying oil	35.04 ± 3.55	8.41 ± 0.52	0.00 ± 0.00	± 0.00	23.15 ± 1.32	64.21 ± 1.61	95.77		
Aeration	36.23 ± 4.47	8.87 ± 0.87	1.40 ± 0.03	± 0.03	18.03 ± 0.96	42.76 ± 1.21	71.06		
Alkaline	37.98 ± 0.77	9.81 ± 0.59	0.90 ± 0.01	± 0.01	19.08 ± 1.35	48.52 ± 1.11	78.31		
Control	28.85 ± 2.77	9.73 ± 0.04	2.01 ± 0.04	± 0.04	15.03 ± 0.61	36.14 ± 2.92	62.91		

8.3.4. Overall performance of the two-stage process

Energy conversion efficiency of the substrate in DF is low due to the major conversion of substrate to soluble metabolites. Combining DF with AD could improve the overall energy yield of the system and enhance the substrate degradation. In order to analyze the overall performance of the two-stage AD, carbon removal and total energy yield from H₂ and CH₄ were evaluated for all of the inoculum pre-treatments as well as untreated cultures.

The overall energy yield from two-stage AD was calculated as the energy obtained from H₂ in the first stage together with energy yield from CH₄ in the second stage of AD. Figure 8-5.a shows the overall energy yield of the two-stage process using different inoculum pre-treatments. The energy yields obtained from H₂ and CH₄ were calculated based on their corresponding calorific values (142 and 55 kJ/g, respectively) [194]. Two-stage AD using inoculum pre-treated with WFO resulted in the highest total energy yield of 21.69 kJ/gVS, which was significantly higher than the untreated cultures (11.52 kJ/gVS). Total energy yields of 15.34, 15.55, and 13.68 kJ/gVS were obtained for heat shock, alkaline, and aeration, respectively. Moreover, the conversion rates of H₂ and CH₄ for all of the pre-treatments were higher relative to the control (Figure 8-5b).

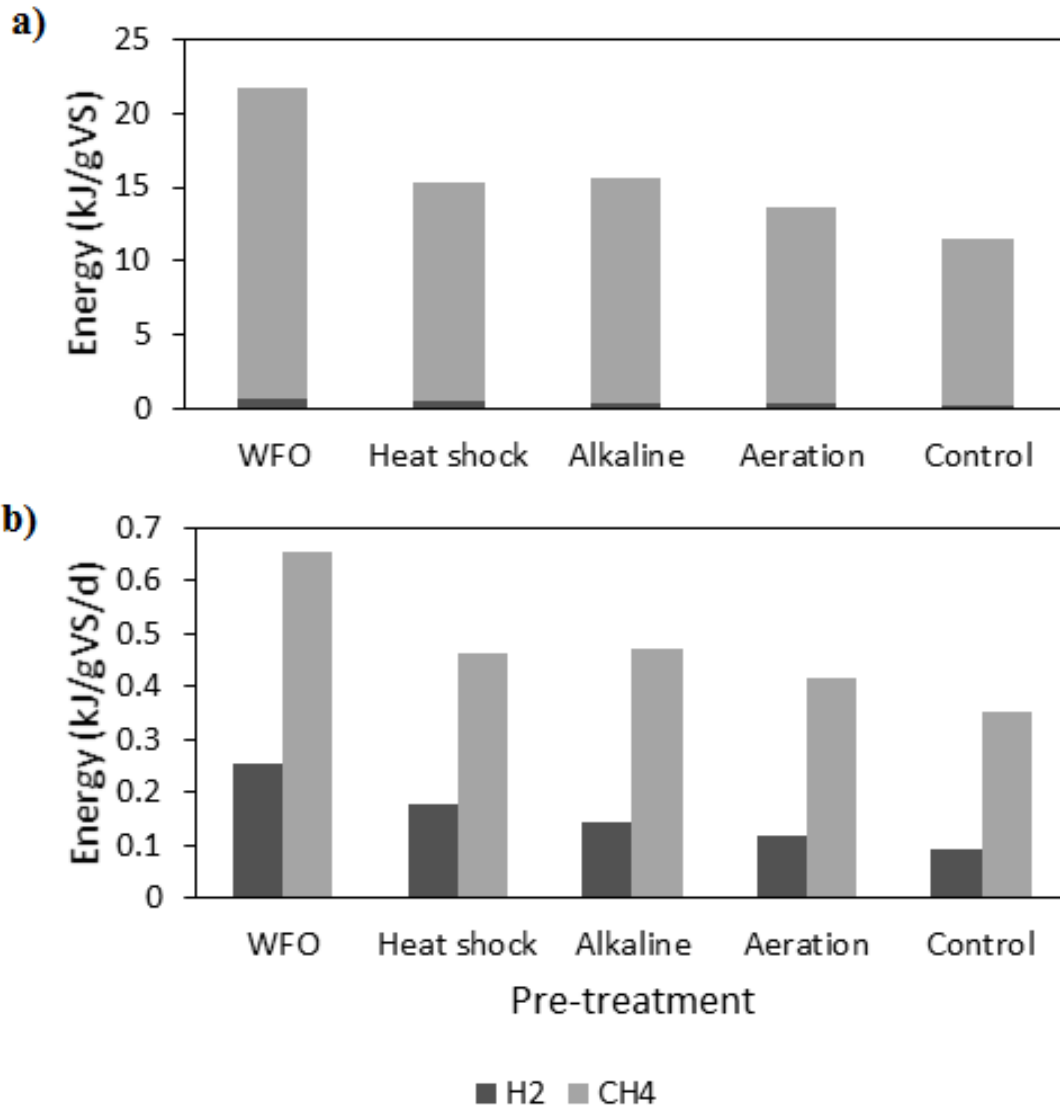


Figure 8-5 a) Total energy recovery from H₂ and CH₄ and b) Conversion rates for H₂ and CH₄ using different inoculum pre-treatment methods

Total TOC removal was calculated as the sum of TOC emitted as CO₂ and CH₄ during two-stage AD. The two-stage process resulted in an acceptable carbon removal (95.77% initial TOC) for WFO pre-treatment. The range for the other inoculum pre-treatments was between 71.06% and 78.31%. The lowest carbon removal for the untreated cultures (62.91%) might indicate that there was a positive effect of inoculum pre-treatment on substrate degradation. Rodríguez-Méndez et al., [163] reported that elevated levels of LCFAs are not inhibitory for hydrolysing and acidogenic bacteria. This means that LCFAs could be used to selectively enrich H₂ producing bacteria without reducing the hydrolysis efficiencies.

Table 8-4 Two-stage AD of organic wastes using various inoculum pre-treatment methods

Substrate	Inoculum pre-treatment method	H₂ yield (mL/gVS)	CH₄ yield (mL/gVS)	Total energy yield (kJ/gVS)	Reference
Household waste	Heat shock (100°C, 1 h)	43.0	511.0	18.31	[35]
Vinegar residues	Alkaline (NaOH, pH 12, 24 h)	53.2	192.0	7.25	[7]
Potato waste	Heat shock (70°C, 2 h)	85.0	364.0	13.59	[28]
Food waste	Heat shock (90°C, 30 min)	55.0	94.0	3.84	[199]
Food waste	Heat shock (105°C, 4 h)	104.1	99.0	4.50	[200]
Food waste	Heat shock (100°C, 30 min)	43.0	511.6	18.32	[164]
Food waste	Heat shock (120°C, 20 min)	11.8	391.2	14.10	[194]
Food waste	WFO (24 h, 15 g/L and then washed)	76.1	598.2	21.69	This study
Food waste	Heat shock (90°C, 30 min)	53.8	422.9	15.34	This study

Table 8-4 shows the H₂ and CH₄ yields obtained from two-stage AD of organic substrates in the literature. Additionally, the total energy yields obtained by two-stage processes have been shown to be compared with the results of the present study. As previously mentioned, the majority of the two-stage AD studies have used heat shock to enrich H₂ producing bacteria in the first stage. The total energy recovery in terms of H₂ and CH₄ obtained in the present study is among the highest values found in the literature. However, it should be mentioned that the composition variability of the FW as well as the composition of the inoculum can affect the H₂ and CH₄ yields. Therefore, any direct comparisons should be made with caution. In the present study, which was performed with similar substrates and inoculum for all of the pre-treatments, the total energy yield obtained by WFO pre-treatment was higher than the other pre-treatments studied.

8.4. Conclusion

Two-stage AD tests were performed to measure the H₂ and CH₄ recovery from FW. The effect of the four inoculum pre-treatment methods namely heat shock, aeration, and alkaline, pre-treatment with WFO to enrich H₂ producing bacteria in the first stage was studied. Moreover, the possible effect of the inoculum pre-treatment method on the second stage was also evaluated. This study revealed that inoculum pre-treatment not only could increase H₂ yields but it also has an impact on the second stage of AD and CH₄ production. Inoculum pre-treatment with WFO resulted in the highest H₂ and CH₄ yield in the first and second stages, respectively. The highest total energy yield and substrate degradation efficiencies were obtained using inoculum pre-treatment with WFO. In order to better analyze the effect of inoculum pre-treatment methods on enhanced H₂ and CH₄ yields, further studies should be performed to investigate the microbial community changes during the first and second stages of AD. Energetic costs due to inoculum pre-treatment would need also to be considered in order to calculate the net energetic production.

9. Study of microbial dynamics during optimization of hydrogen production from food waste by using LCFA-rich agent

9.1. Introduction

Deciphering the microbial composition is one of the most important issues in dark fermentation studies in order to optimize H₂ production. In particular, changes in microbial composition after inoculum pre-treatment in comparison with the untreated inocula, reveals the efficiency of pre-treatment process. Selection of the inoculum enrichment method is highly dependent on the inoculum type and its microbial composition, since different species respond in a different way to the applied pre-treatment microbial communities might contain variable populations. Several studies have reported significant variations in the microbial community composition after applying different inoculum pre-treatment methods [41,61,201]. Moreover, substrate has a great effect on the efficiency of enrichment method. The objective of the present study is to investigate microbial community changes after inoculum pre-treatment with WFO. Moreover, changes in microbial diversities abundance after fermentation using food waste as substrate was studied by means of high-throughput 16S rRNA amplicon sequencing.

9.2. Materials and methods

9.2.1. Seed sludge and inoculum pre-treatment

The anaerobic sludge, used as the inoculum, was obtained from a full-scale mesophilic sludge digester treating the excess sludge of a municipal wastewater treatment plant located in Padova. Anaerobic sludge was characterized by a Total Solids (TS) concentration of 12.13% and Volatile Solids (VS) concentration of 6.93% TS. The sludge was pre-treated with 20 g/L WFO as previously described [177] and a fraction was stored at -20 °C for microbial analysis. In order to better investigate the inhibitory effect of WFO, a fraction of pre-treated sludge was washed and stored at -20 °C to investigate if this treatment washing may lead to any change in the microbial composition structures.

9.2.2. Hydrogen production studies

Dark fermentation studies were performed using 500 mL glass bottles sealed with silicone rubber stoppers. The composition of FW samples (W/W) was as follows (W/W): meat (13.4%), cheese (5.5%), bread (5.4%), pasta (5.4%), fruits (54.1%), and vegetables (16.2%). The synthetic FW samples were characterized by a TS concentration of 28.1% and a VS concentration of 94.6% TS. Each bottle contained pre-treated inoculum (5 gVS/L) and synthetic food waste (5 gVS/L) and the working volume was reached to 250 mL by addition of tap water. Subsequently, the pH adjusted to 5.5 using NaOH (3M) and HCl (3M). The bottles were purged with nitrogen gas for 3 min to ensure anaerobic conditions and incubated at 35°C. H₂ productions during fermentation were calculated according to Vanginkel et al., [146].

9.2.3. Analytical Methods

Biogas composition was analysed using a gas-chromatograph (Varian 490-GC) equipped with a 10-meter MS5A column to measure H₂ concentrations and a 10-meter PPU column to analyse carbon dioxide (CO₂) concentration and two thermal conductivity detectors. Argon was used as the carrier gas with a pressure of 60 kPa in the columns. Temperatures of the column and injector were set to 80°C. The volume of biogas produced during the two-stage AD tests was measured by means of the water displacement method.

TS and VS were analysed according to standard methods. VFA concentrations were analysed at the end of fermentation. To do this, the liquid phase was first filtered using membrane filters with a pore size of 0.45 µm. Then, the VFA concentrations were analysed using a gas chromatograph (Varian 3900) equipped with a CP-WAX 58 WCOT fused silica column and a Flame Ionization Detector. Nitrogen was used as the carrier gas with a flow rate of 4 mL/min in the column. The temperature of oven was set at 80°C for one minute and then increased at a rate of 10°C/min up to 180°C and kept constant (remained for two minutes) at a rate of 10°C/min. The temperatures of the column and injector were held at 250°C.

9.2.4. Microbial analysis

9.2.4.1. DNA sampling collection and extraction

Liquid samples from each inoculum were collected during steady state condition. Genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO laboratories Inc., Carlsbad, CA USA) and following manufacturer's instructions, except from the addition of an initial purification step using 2 mL of Phe:Chl:IAA pH 8 (Sigma-Aldrich, DK). DNA quantity and quality were determined using NanoDrop (ThermoFisher Scientific, Waltham, MA) and Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA).

9.2.4.2. 16S rRNA amplicon sequencing, data analysis

Microbial community composition was determined considering the 16S rRNA gene V4 hypervariable region which was amplified with universal primers (515F/806R). Sequencing was performed using Illumina MiSeq platform at the Ramaciotti Centre for Genomics (Sydney, Australia). Raw sequencing data processing was performed with the CLC Workbench software (V.8.0.2) using the Microbial genomics module plug in (QIAGEN Bioinformatics, Germany). Standard quality filtering was used to remove low-quality reads, and a pipeline was used to determine OTUs (operative taxonomical units) clustering, taxonomical assignment (Greengenes v13_5 database), Alpha and Beta diversity. In order to confirm the taxonomical assignment of the OTUs' consensus sequences belonging to the most abundant microbes, a BLAST search was performed considering the 16S ribosomal RNA database of NCBI. Heat maps and hierarchical clustering analyses obtained considering only the high abundant OTUs (i.e. relative abundance > 0.5%) were visualized with the Multi experiment viewer software (MeV 4.9.0) and a complete linkage Pearson correlation was calculated using the same software. Two groups comparison t-test (equal variance) was used to calculate the significance of changes in abundance determined between samples (p-value < 0.05). Both the statistical analysis and the graphics were obtained using STAMP software

9.3. Results and discussion

9.3.1. Microbial diversity at different stages

In order to observe in detail the changes affecting the microbial community, relative abundance and fold change of relative abundance at different stages were examined and results are reported shown in Figure 9-1.

The microbial community in the untreated inoculum (C), was dominated by *Bacteroidetes* (16.44%) and *Proteobacteria* (12.89%), while after inoculum pre-treatment with WFO (P), there was a dramatic change in the microbial community dominated by *Proteobacteria* became the dominant taxonomic group (33.17%). The washing procedure applied on the pre-treated inoculum (W) did not affect the microbial composition significantly since *Proteobacteria* were again the dominant phylum in the washed inoculum with 34.98% abundance. *Bacteroidetes* were the second most abundant phylum both in pre-treated and washed inoculum (7.61 and 8.39% respectively). The other abundant phyla contributions in diversity were less than 3%. *Proteobacteria* was also the most abundant phylum at the end of fermentation (F) with 48.2% and *Firmicutes* was the second most abundant major phylum (34.83%). The microbial composition after inoculum pre-treatment was more diverse compared to other studies used different inoculum pre-treatment methods. *Clostridium* genus was highly abundant at the end of fermentation with three species of *Clostridium butyricum* (a well-known H₂ producer) identified. Many studies previously performed using mixed cultures, reported a relationship between high H₂ production and the presence of *Clostridium* genus as a dominant taxonomic group [70,202]. In the untreated inoculum, an Operational Taxonomic Unit (OTU) sequence belonged to *Marinilabiliaceae* family (*Marinilabiliaceae* sp.4) was the most abundant with 91% identity to *Mangroviflexus xiamenensis* and *Alkaliflexus imshenetski*, both are known as propionic acid producers. Propionic acid production is unfavorable in dark fermentation as it is produced through a H₂ -consuming pathway [156]. After inoculum pre-treatment with WFO, the genus *Acinetobacter* underwent a dramatic increase and it became was the most abundant OTU with a by 802- fold increase in relative abundance (from 0.01% in sample “C” to 8.02% in sample “P”). Other two dominant most abundant genera after pre-treatment were *Thauera* (5.13%) and *Dechloromonas* (6.83%) with 4.31 and 3.21- fold increase in relative abundance compared to the untreated inoculum. An increased relative abundance from 0.69% to 3.46% was observed for *Thauera* sp. 5 (100% identity to *Tauera aminoaromatica* and *Tauera phenylacetica*, two denitrifying bacteria) after pre-treatment. Relative abundances of four *Acinetbacter* spp. increased between 458 to 889- fold after inoculum pre-treatment.

Two unclassified *Bacteroidetes* (*Bacteriodetes* sp. 27 and *Bacteriodetes* sp.73) that were among the most abundant OTUs in the raw inoculum, showed significant decrease in relative abundances after pre-treatment with WFO (138.3 and 70.1- fold respectively). Other major OTUs showing a decreased relative abundances after pre-treatment were *Lewinellaceae* sp. 62 (12.5- fold), *Chitinophagaceae* sp. 66 (52.7 fold), *Sterolibacterium denitrificans* 56, a denitrifying member of *Proteobacteria* (13.8 fold).

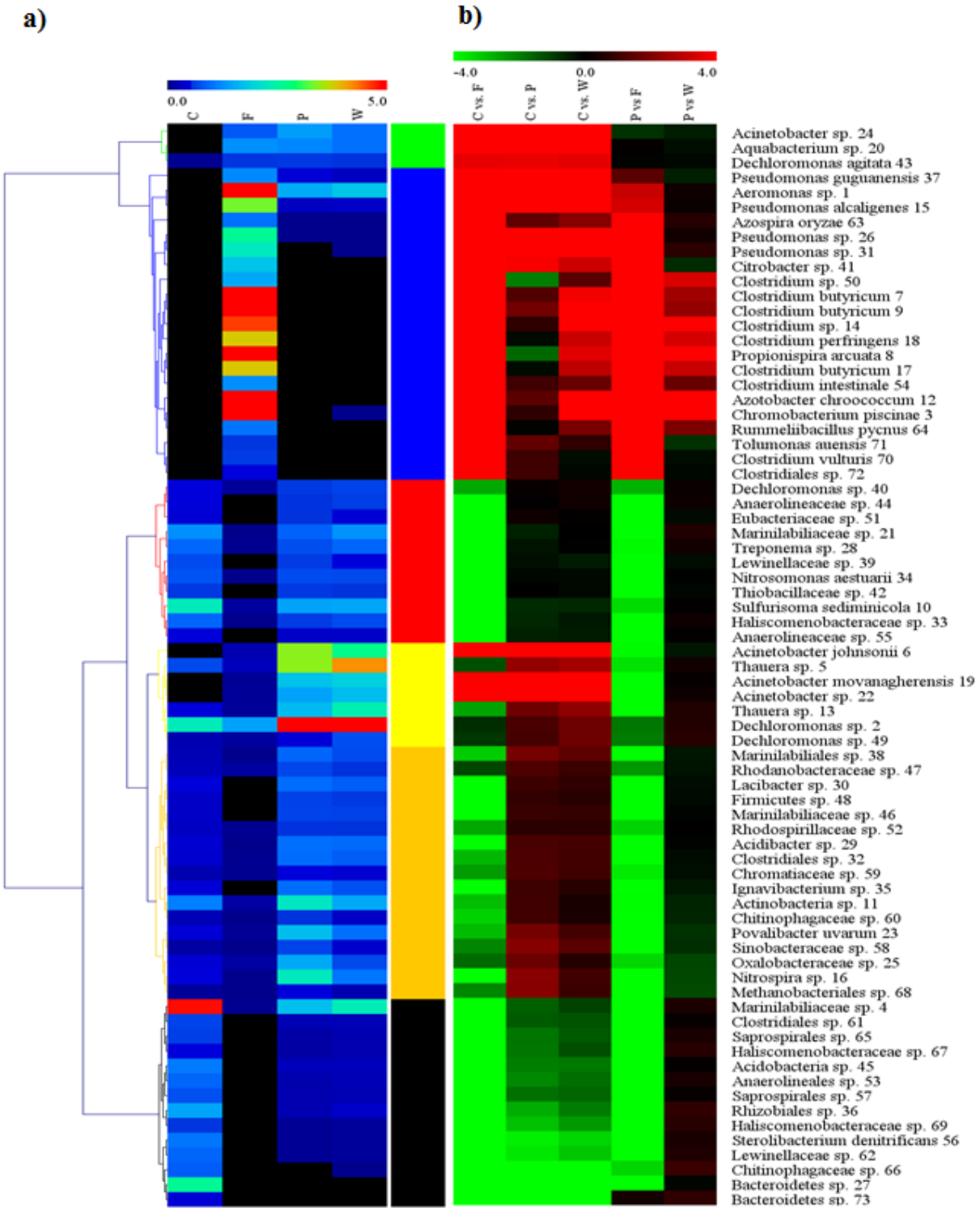


Figure 9-1 Microbial composition in anaerobic sludge a) Relative abundance (%) and b) Fold change is reported only for of the most abundant OTUs . C (untreated), P (pre-treated), W (pre-treated and then washed), F (after fermentation).

Although heat shock pre-treatment has been extensively used by many researchers, the risk of an excessive reduction of the diversity in the less diverse microbial community may affect the H₂ yield obtained from complex wastes. For example, heat shock pre-treatment provides an extreme environment in which non-spore forming H₂ consumers and producers are inhibited or suppressed. Therefore, in many of the studies used heat shock pre-treatment, the spore-forming H₂ producers (such as *Clostridium* spp. and *Bacillus* spp.) are the most abundant at the end of dark fermentation [56,72,201]. An interesting issue observed in the present study was associated to the increased abundances of non-spore forming fermentative bacteria (including *Aeromonas* spp.) after inoculum pre-treatment with WFO (*Aeromonas* sp.1 and *Citrobacter* sp.41; 973 and 15- folds respectively). In contrast, the majority of *Clostridium* spp. did not experienced a significant change in relative abundances after pre-treatment. However, increased relative abundances of 2.5 and 3.5- folds were observed for *Clostridium butyricum* 7 and *Clostridium butyricum* 9, respectively, after pre-treatment. A *Clostridium* spp. (*Clostridium* sp.50) was identified with 99% identity to *Clostridium acetobutylicum*, a well-known homoacetogenic bacteria.

Besides adsorption on the cell wall and hinder microbial growth, the surrounding lipid layer may increase the floatation and removal of microbial populations [203]. According to this, the inoculum pre-treated with WFO was washed after pre-treatment (W) and its microbial community was investigated in comparison with WFO-pre-treated inoculum before washing (P). This was done to better investigate if washing the inoculum has any effect on relative abundances of H₂-producing or H₂-consuming populations. As can be seen from Figure 9-1 a), washing the inoculum after pre-treatment did not affect significantly the relative abundances of many genera. However, relative abundances of *Oxalobacteraceae* sp. 25, *Nitrospira* sp. 16 and *Methanobacteriales* sp. 68 decreased by 2-fold after washing the inoculum. Interestingly, the same treatment determined an increased relative abundance (between 3 to 10 fold) for the majority of *Clostridium* spp.

9.4. Conclusions

Microbial community of sludge at different stages of dark fermentation (untreated, pre-treated with WFO, washed and fermented) was investigated to understand the effect of inoculum pre-treatment with WFO on H₂ producing and H₂ consuming bacteria. Inoculum pre-treatment with WFO resulted in increased relative abundances of non-spore forming H₂ producers such as *Aeromonas* and *Citrobacter* spp. while it did not significantly affect significantly spore-forming H₂ producers from belonging to the *Clostridium* genus. The predominant genera at the end of dark fermentation were *Clostridium*, *Aeromonas* and *Chromobacterium*.

10. Elucidating the effect of inoculum pre-treatment on H₂ producing and H₂ consuming metabolic pathways using flux balance analysis

10.1. Introduction

Mixed microbial cultures should be pre-treated by different means to suppress hydrogen consuming species and enrich H₂ producing bacteria. In order to better investigate the effect of inoculum pre-treatment on the H₂ yield, contribution of H₂ producing and H₂ consuming pathways before and after pre-treatment should be quantified. Hydrogenotrophic methanogens that convert H₂ and CO₂ to CH₄ are considered as the main group of H₂ consumers in anaerobic mixed cultures (Eq. 10-1).



CH₄ could also be produced from acetate degradation by acetoclastic methanogens (Eq. 10-2). However, quantification of CH₄ production from H₂ or acetate is quite difficult in the lab.

During dark fermentation of carbohydrate-rich substrates, acetic acid and butyric acid constitute the highest proportion of the produced soluble metabolites. Although the theoretical H₂ yield of glucose for acetate production is twice of that for butyrate, several studies reported that there was no correlation between higher acetate production and increased H₂ yield [111,166,167]. This could be because of possible acetate production from H₂ by homoacetogenic bacteria (Eq. 10-3)



Therefore, quantification of the acetate produced by H₂ producing bacteria and that produced by H₂ consumers is of great importance to analyze the activities of the two mentioned microbial populations and optimizing the inoculum pre-treatment conditions. This issue cannot be addressed easily using lab experiments while using metabolic network models it is possible to estimate this ratio. Several studies have been used metabolic network models for H₂ production using pure microbial cultures [145–148]. Metabolic network model construction for a mixed culture is more challenging compared to a pure culture since the syntrophic relationships between different microbial populations should be considered. To the best of our knowledge, there are only two studies on metabolic network modeling of hydrogen production for mixed cultures [68,149]. Among these two studies, the latter did not include methanogenic and homoacetogenic H₂ consumption since they used a heat pre-treated mixed culture. Chaganti et al., [168] used a simplified model without considering pentose-phosphate (PP) and tricarboxylic

acid (TCA) pathways. Moreover, CO₂ flux was not included in the models presented by Chaganti et al., [168] and Gonzalez-Garcia et al., [169] while it is well understood that a major part of the initial carbon is emitted as CO₂ and therefore should be considered for carbon mass balance. Considering the lack of information about the mentioned issues, developing a comprehensive model which addresses the missing information, seems to be an important issue.

Metabolic network models can be beneficial in dark fermentation studies of mixed cultures to give a more comprehensive understanding of metabolic pathways involved in H₂ production and consumption. Flux balance analysis (FBA) is an interesting approach that can be utilized to investigate how any changes in substrate or operational parameters can change the metabolic flux distribution towards different metabolic pathways and different products. FBA approach can increase our understanding of the complex metabolic reactions occur in a mixed culture and define the contribution of a substrate to products by quantification of intracellular fluxes that is quite difficult with experimental methods.

The objectives of the present study are 1) to develop a metabolic network model for anaerobic mixed cultures and provide a comprehensive insight into the H₂ producing and H₂ consuming pathways and 2) to investigate the effect of inoculum pre-treatment with WFO on flux distribution towards different metabolic pathways compared to the untreated culture. The main focus will be on the estimation of H₂ consumption by hydrogenotrophic methanogens and homoacetogens, two major H₂ consuming populations.

10.2. Materials and methods

10.2.1. Culture conditions

Batch fermentation tests were performed using 1-liter glass bottles received 5 g/L glucose as substrates. In order to pre-treat the sludge with WFO, 10 gVS/L of granular sludge was mixed with varying concentrations of WFO (0, 5, 10 and 20 g/L) for 24 h before glucose addition. After glucose addition, tap water was added to the bottles to reach the working volume to 500 mL. All the bottles were sealed with silicone rubber and incubated in a water bath at a temperature of 35°C for 72 h. The initial pH for untreated and pre-treated cultures was set at 5.5 using NaOH (3M) and HCl (3M) after glucose addition. All the tests were done in triplicate.

10.2.2. Theory of FBA

FBA is a constraint-based technique to compute the flux distribution from a substrate to products in microorganisms, assuming pseudo-steady state of intermediate metabolites. In order to develop an FBA model, a biochemical network composed of the metabolic reactions involved in degradation of substrate and formation of products should be defined. Biochemical reactions could be collected from bioinformatics databases and experimental works. In the next step, mass balance is carried out for all the metabolites involved in the metabolic network and a matrix of stoichiometric coefficients is written as:

$$S \cdot V = 0$$

Eq. 10-4

Where S is a stoichiometric matrix with a dimension of $m \times n$ (m and n are the number of metabolites and reactions respectively) and V is the flux vector with a dimension of $n \times 1$. Equation 6 could be solved using linear optimization. Since the number of metabolites usually is higher than the number of reactions, the system would be under-determined. FBA method calculates all the unknown metabolic fluxes for the under-determined system by restricting the solution space using stoichiometric constraints and experimentally measured fluxes. In order to use FBA method to calculate the in-vivo metabolic fluxes, an objective function which is based on the system variables should be chosen. The optimized flux distribution could be achieved with minimization or maximization of this objective function. The methodology of FBA is shown in Figure 10-1.

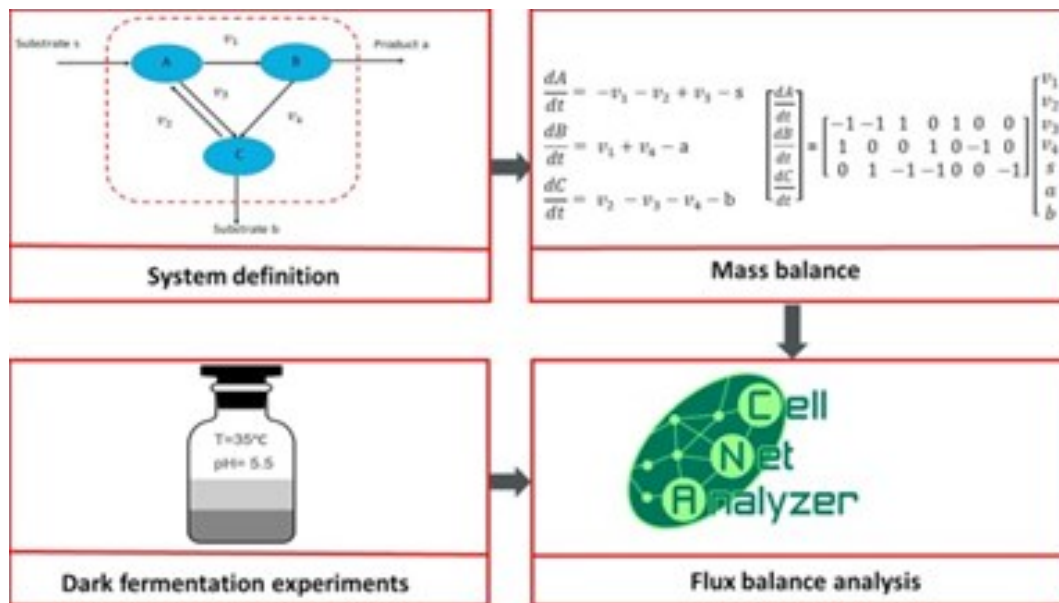


Figure 10-1 Research methodology performed in the present study

10.2.3. Metabolic network model development and FBA analysis

The basis of developing a metabolic network model for a mixed microbial culture is the same for pure and mixed cultures as it is based on the stoichiometry of metabolic reactions and solving the system of linear equations. However, due to the presence a wide variety of microorganism types, the possible fermentation products are more versatile. Moreover, in mixed microbial cultures, the syntrophic relationships between different strains affect final products distribution pattern. Hence, unlike pure cultures, reactions for products consumption by specific types of bacterial strains should be considered in the model.

In order to construct a metabolic network model for a mixed microbial culture, it is assumed as a universal bacterium which produces all the possible products of single types of the bacteria present in the culture. This concept has already been used by several metabolic flux analysis studies using mixed cultures [68,149,151,152]. The in silico metabolic network model for the mixed culture was constructed from bioinformatics databases including KEGG (www.kegg.jp) and BioCyc (www.biocyc.org) and previous studies [168–172].

To the best of our knowledge, the model proposed by Chaganti et al., [168], is the only metabolic network model to predict H₂ production by anaerobic mixed cultures considering methanogenic and homoacetogenic H₂ consuming pathways. In our work, significant changes were introduced compared to the work of Chaganti et al., [168] since they did not consider a complete TCA cycle and pentose phosphate pathway for the universal bacterium. Moreover, CO₂ flux was also considered in the model while it was not presented by Chaganti et al., [168] and Gonzalez-Garcia et al., [169]. According to the previous experimental studies, all the possible products from the dark fermentation of glucose and their metabolic routes were considered in the model. In this regard, the main products included in the metabolic network model are acetate, butyrate, lactate, propionate, valerate, caproate, ethanol, hydrogen, carbon dioxide and methane. The experimentally measured fluxes used as constraints for the metabolic model are shown in Table 10-1. The biomass formation equation included in the model was assumed to be similar to the equation used by Gonzalez-Garcia et al., [169].

All the metabolites (both external and internal) which affect flux distribution are involved in the metabolic network model. The lists of all reactions and metabolites which used in the metabolic network model have been shown in Appendix A and Appendix B respectively. In this study, the biomass growth was chosen as the objective function which should be maximized, since it is believed that microorganisms optimize their metabolic network in a way that leads to their maximum growth [147,153,154]. FBA analysis was performed using CellNetAnalyzer [155]. Figure 10-2 shows the proposed metabolic network model.

Table 10-1 Experimental metabolite rates used in the FBA model

Metabolite		WFO (g/L)			
		0	5	10	20
Substrate uptake rate (mmol/L/h)	Glucose	1.137	1.273	1.388	1.175
	Acetate	0.113	0.545	0.483	0.596
	Propionate	0.026	0.083	0.074	0.009
Production rate (mmol/L/h)	Valerate	0.000	0.000	0.000	0.000
	Caproate	0.053	0.016	0.013	0.008
	Butyrate	0.146	0.445	0.509	0.616
	CO ₂	1.743	1.278	2.355	1.630
	CH ₄	0.899	0.181	0.054	0.000
	H ₂	0.254	0.450	0.770	1.334

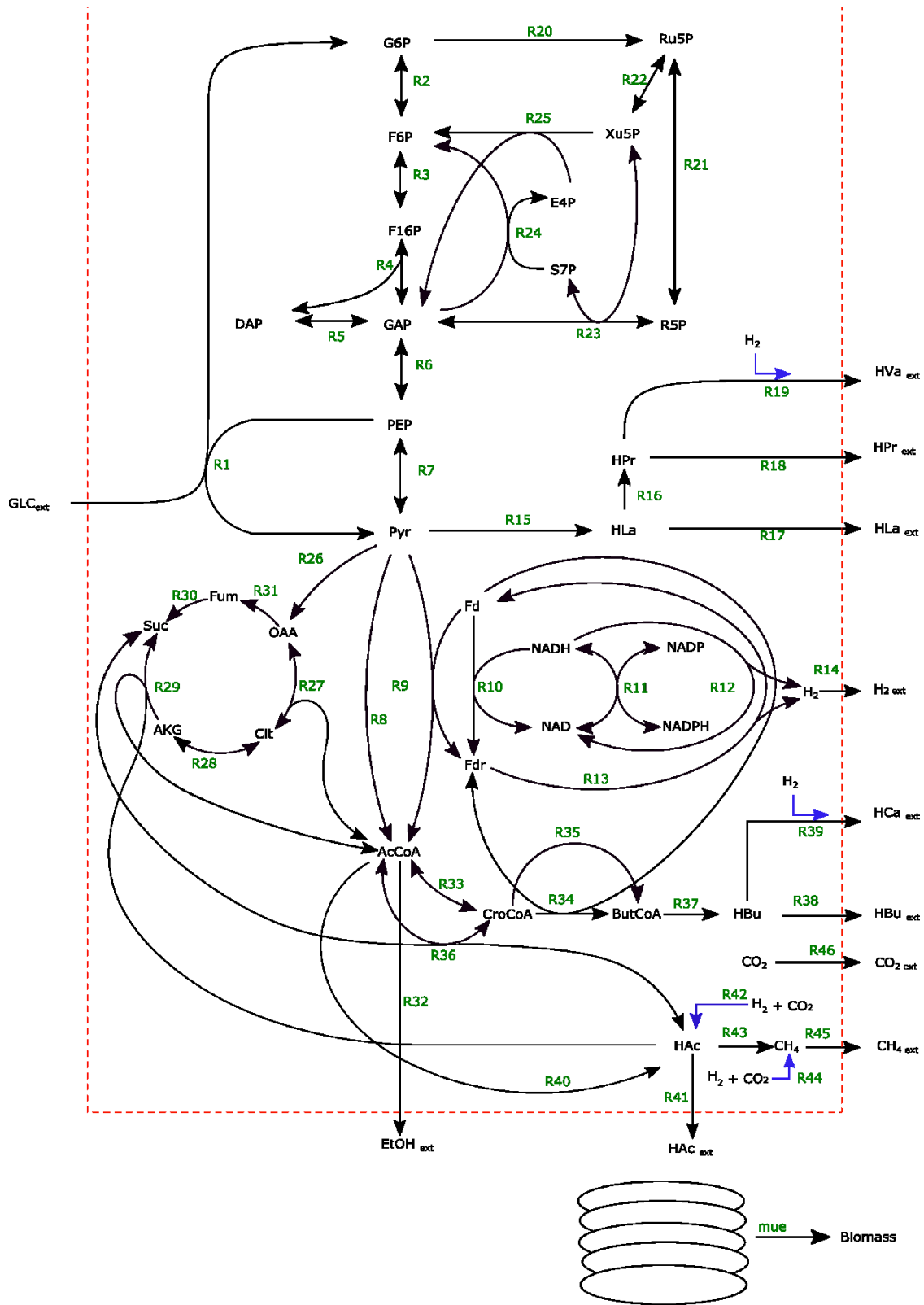


Figure 10-2 Proposed metabolic network model for anaerobic mixed culture

10.3. Results and discussion

10.3.1. Effect of inoculum pre-treatment on flux distribution

Metabolic flux distribution through different pathways computed by FBA for untreated and three different pre-treated cultures are shown in Figure 10-3. In order to validate the FBA model, experimental yields for all the external metabolites except butyrate were used as constraints of the model and the calculated butyrate yields were compared to the experimental ones. On average, FBA resulted in 11% error in predicting the butyrate yields. The proposed model can be used to study H₂ production by any other mixed microbial culture with similar byproducts. During dark fermentation, glucose is consumed by anaerobic bacteria resulting H₂, CO₂ and a variety of soluble carbon by-products. Glucose is converted to pyruvate and NADH through EM pathway and subsequent oxidation of pyruvate to acetyl-CoA generates reduced ferredoxin (Fdr) and CO₂. H₂ can be produced either from NADH or oxidation of reduced ferredoxin. In the present study, more than 70% of the H₂ produced either by untreated or pre-treated cultures, was generated through Fdr (R13).

Glucose-6-phosphate (G6P) is the branch point between glycolysis and PP pathway. The majority of the flux from G6P was directed to Embden-Meyerhof (EM) pathway (88-90%) except for the cultures pre-treated with 10 g/L WFO. PP pathway is an important part of microbial metabolism as it produces essential precursors for cell growth. Higher fluxes through PP pathway leads to lower TCA fluxes and higher conversion of NADPH to NADH that is favored for H₂ production [149,156]. The fluxes towards different soluble products could affect H₂ production due to their impact on balances of ATP, NADH, and Fdr. NADH is an important cofactor for H₂ production which is produced through different metabolic pathways. NADH could be used either for H₂ production through R12 or Fdr which its subsequent oxidation generates H₂ (R13). Table 10-2 shows the balance for NADH and NADPH cofactors for untreated and pre-treated cultures. Lactate production (R15) is an NADH consuming pathway and results in lower NADH availability for H₂ production. Moreover, redirection of the flux from pyruvate to lactate production results in the lower formation of acetyl-CoA from pyruvate which is coupled with NADH formation. Productions of other soluble metabolites such as butyrate, ethanol, and propionate are regarded as NADH sinks.

ATP production through EM pathway was reduced by the increase in WFO concentration; however, higher WFO concentrations led to enhanced ATP generation through butyrate producing pathway (Table 10-3). ATP produced through EM pathway was mainly consumed by biomass growth or by TCA cycle. Since the lowest TCA fluxes were obtained for the inoculum pre-treated with 10 g/L WFO, less ATP consumption by TCA led to the redirection of the more available ATP to biomass growth. The biomass yield from glucose in anaerobic mixed cultures has been reported to be relatively low [68,146,149]. In the present study, considering a biomass formula equal to CH_{1.976}O_{0.629}N_{0.149} as previously reported by Gonzalez-Garcia et al., [169], biomass yield ranged from 3.2 to 4.2 mol.mol⁻¹ of glucose, that is comparable with previous studies [146,148,149].

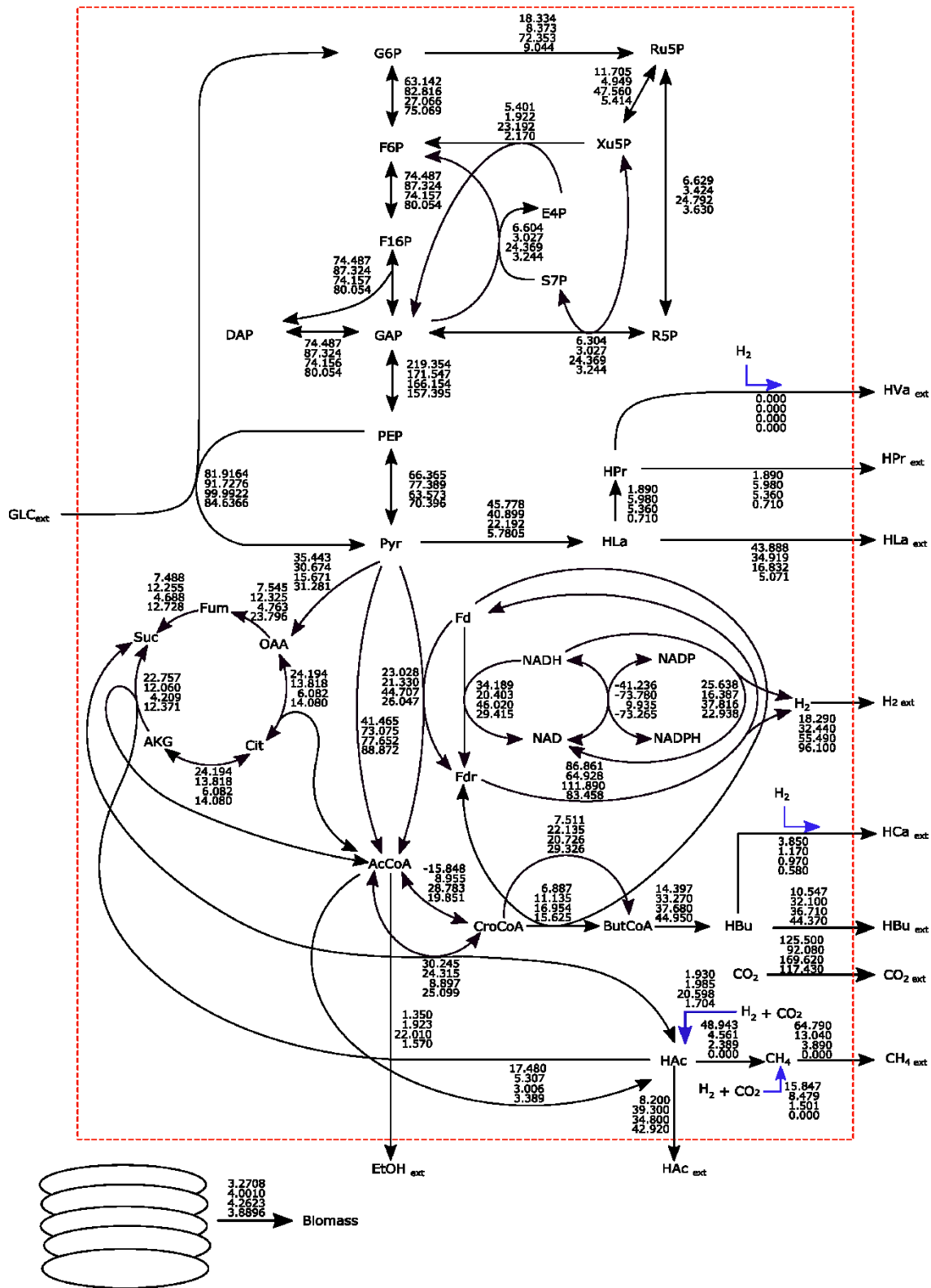


Figure 10-3 Metabolic fluxes for cultures pre-treated with different concentration of WFO (0, 5, 10 and 20 g/L). The FBA analysis was based on 100 mol of glucose.

Table 10-2 Balance for NADH and NADPH cofactors for untreated and pre-treated cultures

Reaction number	Pathway	WFO (g/L)			
		0	5	10	20
NADH balance					
R6	GAP \rightleftharpoons ATP + NADH + PEP	150.267	171.546	166.153	157.394
R8	Pyr \Rightarrow CO ₂ + AcCoA + NADH	41.465	73.075	77.651	88.872
R11	NADPH \rightleftharpoons NADH	41.236	73.780	9.934	73.265
R10	2 Fd + NADH \Rightarrow 2 Fdr	-34.189	-20.403	-46.020	-29.415
R12	NADH \Rightarrow H ₂	-25.638	-16.3872	-37.8157	-22.938
R15	NADH + Pyr \Rightarrow Lactate	-45.7784	-40.899	-22.1918	-5.780
R16	Lactate + NADH \Rightarrow HPr	-1.890	-5.980	-5.360	-0.710
R32	AcCoA + 2 NADH \Rightarrow EtOH	-2.700	-3.847	-44.020	-3.141
R33	2 AcCoA + NADH \rightleftharpoons CroCoA	-15.847	-8.955	-28.783	-19.850
R34	CroCoA + 2 Fd + 2 NADH \Rightarrow ButCoA + 2 Fdr	-13.773	-22.140	-33.91	-31.250
R35	CroCoA + NADH \Rightarrow ButCoA	-7.510	-22.135	-20.7261	-29.325
R36	2 AcCoA + NADPH + NADH + Suc \rightleftharpoons 2 Acetate + CroCoA	-30.244	-24.314	-8.8967	-25.099
mue	0.1804 AcCoA + 1.62 ATP + 0.01056 E4P + 0.00421 F6P + 0.00067 Fum + 0.00515 G6P + 0.048 GAP + 0.4499 NADPH + 0.054 NADH + 0.0433 OAA + 0.02322 PEP + 0.03 Pyr + 0.0038 R5P + 0.0168 AKG \Rightarrow	-4.6193	-5.650	-6.019	-5.493
NADPH balance					
R20	G6P \Rightarrow CO ₂ + NADPH + Ru5P	18.333	8.372	72.352	9.044
R28	Cit \rightleftharpoons CO ₂ + NADPH + AKG	24.194	13.817	6.081	14.080
R11	NADPH \rightleftharpoons NADH	-41.236	-73.780	-9.934	-73.265
R30	Fum + NADPH \Rightarrow Suc	-7.488	-12.254	-4.688	-12.727
R31	NADPH + OAA \Rightarrow Fum	-7.545	-12.324	-4.762	-12.796
R36	2 AcCoA + NADPH + NADH + Suc \rightleftharpoons 2 Acetate + CroCoA	-30.244	-24.314	-8.896	-25.099
mue	0.1804 AcCoA + 1.62 ATP + 0.01056 E4P + 0.00421 F6P + 0.00067 Fum + 0.00515 G6P + 0.048 GAP + 0.4499 NADPH + 0.054 NADH + 0.0433 OAA + 0.02322 PEP + 0.03 Pyr + 0.0038 R5P + 0.0168 AKG \Rightarrow	-38.485	-47.077	-50.151	-45.766

Table 10-3 ATP balance for untreated and pre-treated cultures

Reaction number	Pathway	WFO (g/L)			
		0	5	10	20
ATP balance		0	5	10	20
R6	GAP \rightleftharpoons ATP + NADH+ PEP	150.267	171.546	166.153	157.394
R7	PEP \rightleftharpoons ATP + Pyr	66.365	77.389	63.573	70.396
R37	ButCoA \Rightarrow Butyrate + ATP	14.397	33.270	37.680	44.950
R40	AcCoA \Rightarrow Acetate + ATP	17.479	5.307	3.006	3.389
R3	ATP + F6P \rightleftharpoons F16P	-74.486	-87.323	-74.156	-80.054
R26	CO ₂ + ATP + Pyr \Rightarrow OAA	-35.443	-30.673	-15.670	-31.281
mue	0.1804 AcCoA + 1.62 ATP + 0.01056 E4P + 0.00421 F6P + 0.00067 Fum + 0.00515 G6P + 0.048 GAP + 0.4499 NADPH + 0.054 NADH + 0.0433 OAA + 0.02322 PEP + 0.03 Pyr + 0.0038 R5P + 0.0168 AKG \Rightarrow	-138.580	-169.515	-180.586	-164.795

10.3.2. Fluxes through H₂ producing and H₂ consuming pathways

Figure 10-4 shows the H₂ fluxes produced or consumed through different metabolic pathways for pre-treated cultures and untreated inoculum. It can be seen that WFO increased R14, the net H₂ production flux. For the untreated culture, the majority of H₂ produced by hydrogenase activity was converted to CH₄ by hydrogenotrophic methanogens (R44).

It has been reported that higher fluxes through PP pathway could increase the H₂ production [149,157]. Redirecting the flux through PP pathway generates additional NADPH that will be subsequently converted to NADH which is available for H₂ production. Moreover, high PP fluxes lead to lower TCA fluxes which are favorable for H₂ production. This is in agreement with the present study in which the highest fluxes through PP pathway computed for cultures pre-treated with 10 g/L WFO, was accompanied with the highest H₂ production through hydrogenase activity (R12 and R13). Oh et al., (2008) reported that if the glucose is metabolized through PP pathway, a H₂ yield of 8.7 mol.mol⁻¹ glucose can be obtained for *Citrobacter amalonaticus* Y19. In the present study, the highest H₂ producing fluxes (R12 and R13) was obtained for the cultures pre-treated with 10 g/L WFO; though, the lower net H₂ yield compared to the cultures pre-treated with 20 g/L WFO could be attributed to the higher H₂ consumption by homoacetogenic (R42) and hydrogenotrophic methanogens (R44).

Three main H₂ consuming pathways were identified by the FBA model include homoacetogens, hydrogenotrophic methanogenesis, and caproic acid production. The activity of all these pathways was affected by the concentration of WFO. Hydrogenotrophic methanogens consume 4 mole H₂ to produce 1 mole CH₄ (R44). The model predicted about 56% of the H₂ produced from hydrogenase activity was consumed by hydrogenotrophic methanogens. H₂ consumption through caproic acid production and homoacetogens were 20.0 and 7.7% respectively in the untreated

culture. H₂ consumption by hydrogenotrophic methanogens is thermodynamically favoured over homoacetogenesis and therefore, homoacetogens are normally outcompeted by hydrogenotrophic methanogens. Homoacetogenesis could be significant in the absence of hydrogenotrophic methanogens resulting large amounts of acetate [20,158]. In the present study, homoacetogenesis was significant only for the cultures pre-treated with 10 g/L (57% loss in the H₂ produced through R12 and R13 pathways) for which a low activity of hydrogenotrophic methanogens was calculated (4% H₂ loss). H₂ consumption through caproic acid production decreased with increasing the WFO concentration. For the cultures pre-treated with 20 g/L WFO, only 3.2 % of the H₂ generated through hydrogenase was consumed for caproic acid production.

An increase of WFO concentration from 10 to 20 g/L, led to lower H₂ producing and H₂ consuming fluxes. The FBA results revealed that H₂ generation through Fdr and NADH decreased by 39 and 25% respectively in comparison with the cultures pre-treated with 10 g/L WFO. However, due to the complete inhibition of hydrogenotrophic methanogens as well as lower homoacetogens and caproic acid fluxes, only 9.5% of the H₂ produced by the hydrogenases were consumed through the mentioned pathways. This led to an increase of 73% in the net H₂ production compared to the cultures received 10 g/L WFO.

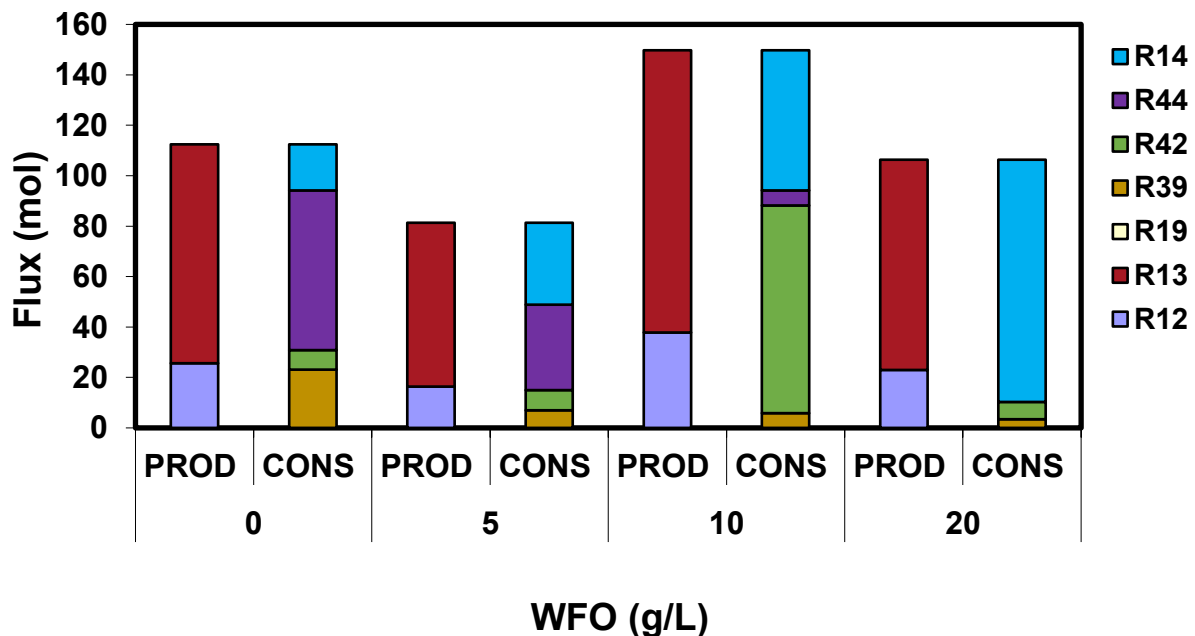


Figure 10-4 Fluxes through hydrogen producing and hydrogen consuming pathways

10.3.3. Inhibition of acetoclastic and hydrogenotrophic methanogens

FBA can be used to estimate the amount of CH₄ produced from acetoclastic or hydrogenotrophic methanogens in different conditions without performing complicated lab experiments. Many studies have reported the inhibition of anaerobic digestion and CH₄ production in presence of high concentrations of long chain fatty acids [139,159,160]. The inhibitory effect of long chain

fatty acids underlies their adhesion to the cell wall, limits mass transfer and leads to biomass washout [173,174].

Rodríguez-Méndez et al., [163] investigated anaerobic digestion using lipid-rich substrates and reported that elevated levels of long chain fatty acids did not suppress acidogenic bacteria while the significant lower CH₄ production suggests the inhibition of methanogens. However, it was not clear if the lower CH₄ production could be due to the inhibition of both groups of either acetoclastic or hydrogenotrophic methanogens.

In the present study, FBA predicted that hydrogenotrophic methanogens were responsible for about 25% of the total CH₄ production in untreated culture (Figure 10-5). When the inoculum pre-treated with 5 g/L WFO, the activity of acetoclastic and hydrogenotrophic methanogens decreased by 90 and 46% respectively compared to the untreated inoculum. This could suggest that hydrogenotrophic methanogens were less sensitive to WFO in comparison with acetoclastic methanogens. This issue was also reported previously by Sousa et al., [162] and [136] who studied the effect of oleate concentrations on pure cultures of acetoclastic and hydrogenotrophic methanogens. When the concentration of WFO increased by 10 g/L, CH₄ productions by acetoclastic and hydrogenotrophic methanogens decreased from 48.94 to 2.38 and from 15.84 to 1.50 respectively compared to the untreated inoculum. FBA results suggested that inhibitory levels of WFO for acetoclastic methanogens were lower than hydrogenotrophic methanogens.

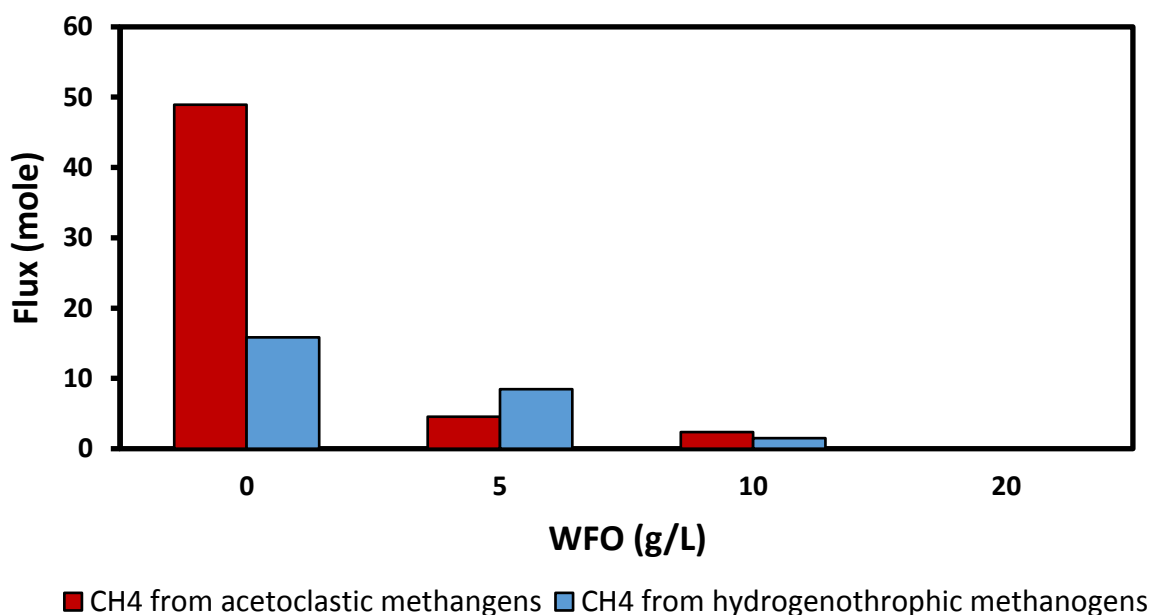


Figure 10-5 Contribution of hydrogenotrophic and acetoclastic methanogens in CH₄ production

10.3.4. Fate of the initial carbon

Inoculum pre-treatment with WFO not only affected H₂ producing and consuming fluxes but also affected flux distribution towards different byproducts. Table 10-4 and Table 10-5 show the product yields and the fate of the initial carbon respectively. Initial carbon metabolized by the mixed culture was emitted as CH₄ or CO₂ or converted to the soluble products. For the untreated culture, a CH₄ yield of 0.79 mol.mol⁻¹ glucose that was equal to 13.18% of the initial carbon resulted in lower fluxes towards different products. A major part of the initial carbon either in untreated or pre-treated inoculum was emitted as CO₂ (16.73-28.27%).

Higher butyrate/acetate ratio for the untreated cultures in comparison with pre-treated cultures could be attributed to the conversion of acetate to CH₄. Regarding the effect of butyrate/acetate ratio on H₂ yield, there are contradictory reports in the literature. Some studies reported that this ratio was proportional with H₂ yield [166] while on the contrary, the others observed lower H₂ yields with increased butyrate/acetate ratio [40,164]. This controversy could be explained by the metabolic pathways of butyrate and acetate production as well as the activity of acetate producing (homoacetogens) and acetate consumers (hydrogenotrophic methanogens). Theoretically, H₂ generation through acetate and butyrate producing pathways results in 4 and 2 mol.mol⁻¹ glucose. Therefore, higher production of acetate could be in favor of higher H₂ yields. Nevertheless, acetate production by homoacetogens or its consumption by hydrogenotrophic methanogens could affect this ratio, resulting in an incorrect conclusion.

Table 10-4 Product yields under different pre-treatment conditions

WFO (g/L)	Product yields (mol/mol glucose)									
	Acetate	Butyrate	Caproate	Propionate	Lactate	Ethanol	H ₂	CO ₂	CH ₄	Butyrate/Acetate
0	0.10	0.12	0.04	0.02	0.53	0.01	0.22	1.53	0.79	1.20
5	0.42	0.34	0.01	0.06	0.38	0.01	0.35	1.00	0.14	0.80
10	0.34	0.36	0.00	0.05	0.16	0.22	0.55	1.69	0.03	1.05
20	0.50	0.52	0.00	0.00	0.05	0.01	1.13	1.38	0.00	1.04

Table 10-5 Carbon conversion for untreated and pre-treated cultures

WFO (g/L)	% Initial carbon converted									
	Acetate	Butyrate	Ethanol	Propionate	Lactate	Caproate	CH ₄	CO ₂	Biomass	
0	3.37	8.58	0.54	1.15	26.78	4.70	13.18	25.53	0.66	
5	14.28	23.33	0.69	3.26	19.03	1.27	3.26	16.73	0.72	
10	11.60	24.47	7.33	2.68	8.41	0.97	2.68	28.27	0.71	
20	16.90	34.94	0.61	0.41	2.99	0.68	0	23.12	0.76	

10.3.5. Maximizing the hydrogen yield

In order to further assess the effect of flux distribution on H₂ yield, two theoretical conditions were analyzed by the model: 1) acetate is the only soluble product and maximization of acetate was chosen as the objective function and 2) butyrate is the only soluble product and maximization of butyrate was chosen as the objective function. In both cases, H₂ consumption either by homoacetogens or hydrogenotrophic methanogens was assumed to be zero. Flux distributions computed by FBA method for the mentioned conditions are shown in Figure 10-6. A H₂ yield of 2.65 mol.mol⁻¹ glucose was estimated by the model if acetate is the only soluble product of dark fermentation. The H₂ yield obtained by applying the second condition (if butyrate is the only soluble product) was equal to 1.44 mol.mol⁻¹ glucose. As mentioned before, the theoretical H₂ yield through acetate producing pathway is higher than that for butyrate. Butyrate-producing pathway consumes more NADH compared to acetate producing pathway and therefore increase in butyrate production could reduce the available NADH for H₂ production [145,148,165]. In order to redirect the flux through H₂ producing pathways, experimental conditions could be controlled to provide favored conditions for maximizing acetate production. FBA can be used to estimate the intracellular acetate production and analyze the effect of different operational parameters on intracellular acetate producing pathways.

10.4. Conclusion

Developing a metabolic network model for dark fermentative H₂ production using anaerobic mixed cultures is very helpful for interpreting the H₂ production in such a complex system. In the present study and FBA model was developed and used to investigate the effect of inoculum pre-treatment with WFO on metabolic flux distribution through H₂ producing and H₂ consuming pathways. Methanogenic H₂ consumption accounted for about 56% of the loss in the H₂ yield in untreated cultures. Although the maximum H₂ production from hydrogenases was computed for 10 g/L WFO, the lower net H₂ yield compared to that for 20 g/L WFO could be attributed to the lower H₂ flux through H₂ consuming pathways.

The proposed FBA model can be used to investigate the effect of different operational parameters or other inoculum pre-treatment methods on inhibition of hydrogenotrophic methanogens and homoacetogens. FBA could provide valuable information to establish strategies to reduce H₂ consumption by anaerobic mixed cultures and optimization of dark fermentation.

11. General conclusions and future works

11.1. Effect of aerobic pre-treatment of food waste on two-stage anaerobic digestion

We studied pre-aeration effect of food waste on two-stage anaerobic digestion. The results showed that aerobic pre-treatment of food waste did not constitute an effective treatment for the purpose of improving H₂ production during the first stage of the anaerobic digestion process. However, during the subsequent stage of AD, CH₄ yield for substrate P, increased by 45.6%, thus revealing that carbon conversion to CH₄ had an increase after pre-aeration. In the present study, duration of pre-aeration and its intensity was constant. Therefore, further research on pre-aeration of food waste with different pre-treatment times and aeration intensities could be the next step of this part of the work.

11.2. Inoculum pre-treatment with WFO

The present study suggests that inoculum pre-treatment with waste frying oil, might be considered as an alternative pre-treatment method to suppress H₂ consumption and subsequently enhance H₂ yield from food waste. In the present study, the effect of WFO concentration on inhibition of H₂ consumption was studied without considering the concentration of inoculum. Therefore, the next step of this part of the work would be to investigate the effect of WFO availability for microorganisms considering the inoculum and WFO concentrations (g sludge/ml WFO).

11.3. Flux balance analysis to study strategies for H₂ consumers inhibition

Flux balance analysis of H₂ production by mixed communities showed that H₂ consumption by hydrogenotrophic methanogens that was accounted for about half of the loss in the H₂ yield in untreated cultures was negligible when the inoculum pre-treated with WFO. The proposed model could be used to optimize H₂ production by any other mixed microbial culture with similar byproducts. Moreover, the model would be used to study the effect of other inoculum pre-treatment methods on H₂ producing and H₂ consuming pathways.

11.4. Microbial community analysis

The microbial diversity analysis showed that inoculum pre-treatment with WFO did not affect spore-forming H₂ producing bacteria. However, it resulted in increased relative abundances of non-spore forming H₂ producers which could be considered as an advantage in comparison with harsh pre-treatments such as heat shock. The next step could be investigation of microbial community during the first and also second stage of anaerobic digestion.

An issue which can help to understand better the anaerobic digestion process could be microbial community analysis during the process. In the present study, microbial communities were analyzed only at the end of the process. However, it would be interesting if the changes during fermentation are studied. Moreover, microbial communities during the second stage (methane production) could be another important issue when studying the inoculum or substrate pre-treatment.

11.5. Effect of inoculum pre-treatment on two-stage anaerobic digestion of food waste

Inoculum pre-treatment with WFO resulted in higher H₂ and CH₄ productions compared to alkaline, aeration and heat shock pre-treatment. In order to better analyze the effect of inoculum pre-treatment with WFO on energy recovery through anaerobic digestion, energetic costs due to inoculum pre-treatment would need also to be considered in order to calculate the net energetic production.

11.6. Future works

The present research was aimed at investigating inoculum and substrate pre-treatment on H₂ and CH₄ production through anaerobic digestion.

We suggested an alternative method for enriching H₂ producing bacteria using WFO to enhance H₂ production from organic waste. The experimental results together with FBA metabolic modelling and microbial analysis were used to optimize H₂ production using the novel pre-treatment. FBA showed that inoculum pre-treatment with high concentrations of WFO inhibited hydrogenotrophic methanogens, the main H₂ consuming microorganisms in anaerobic digestion.

As confirmed by microbial analysis, this technology has an advantage of enriching both spore-forming and non-spore forming H₂ producers, the latter is absent for common methods such as heat shock. This method has an advantage of less energy consumption compared to heat shock that is an important issue for full scale implementation.

In order to implement the technique for organic waste management, some issues should be considered. For a more sustainable process, the possibility of reusing the WFO after pre-treatment should be investigated. A possible option in case of two-stage anaerobic digestion could be the partial addition of used WFO to the methanogenic reactor as it is confirmed that low concentrations of LCFAs could increase the methane production.

In the present research, the effect of aeration as a substrate pre-treatment was also studied in two-stage anaerobic digestion using organic wastes with different compositions. The results showed that although aerobic pre-treatment (24 h) enhanced methane production for protein-rich substrate (46%), it did not increase the total energy recovery of the two-stage process as the energy obtained by higher methane production did not compensate the energy consumption for preaeration. However, before testing different intensities of aeration and durations, it could not be judged if this method could be implemented. The most important finding of this research was that aerobic pre-treatment affects proteins degradation more than carbohydrates and lipids. Therefore, in case of anaerobic digestion of protein rich substrates, it could be considered as a potential pre-treatment method.

From the very beginning of the food waste treatment up to digestate management and disposal, the totality of the two-stage AD processes, with and without pre-treatment, should be investigated and compared in terms of a complete Life Cycle Assessment.

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Index A. List of the biochemical reactions included in the metabolic model

- R1 $\text{GLC} + \text{PEP} \implies \text{G6P} + \text{Pyr}$
- R2 $\text{G6P} \iff \text{F6P}$
- R3 $\text{ATP} + \text{F6P} \iff \text{F16P} + \text{ADP}$
- R4 $\text{F16P} \iff \text{DAP} + \text{GAP}$
- R5 $\text{DAP} \iff \text{GAP}$
- R6 $\text{ADP} + \text{iP} + \text{GAP} + \text{NAD} \iff \text{ATP} + \text{H}_2\text{O} + \text{NADH} + \text{PEP}$
- R7 $\text{ADP} + \text{PEP} \iff \text{ATP} + \text{Pyr}$
- R8 $\text{Pyr} + \text{CoA} + \text{NAD} \implies \text{CO}_2 + \text{AcCoA} + \text{NADH}$
- R9 $2 \text{Fd} + \text{Pyr} + \text{CoA} \implies \text{CO}_2 + \text{AcCoA} + 2 \text{Fdr}$
- R10 $2 \text{Fd} + \text{NADH} \iff 2 \text{Fdr} + \text{NAD}$
- R11 $\text{NADPH} + \text{NAD} \iff \text{NADH} + \text{NADP}$
- R12 $\text{NADH} \implies \text{H}_2 + \text{NAD}$
- R13 $2 \text{Fdr} \implies \text{H}_2 + 2 \text{Fd}$
- R14 $\text{H}_2 \implies \text{H}_2(\text{ext})$
- R15 $\text{NADH} + \text{Pyr} \implies \text{HLa} + \text{NAD}$
- R16 $\text{HLa} + \text{NADH} \implies \text{HPr}$
- R17 $\text{HLa} \implies \text{HLa}(\text{ext})$
- R18 $\text{HPr} \implies \text{HPr}(\text{ext})$
- R19 $6 \text{H}_2 + \text{HPr} \implies \text{HVa}(\text{ext})$
- R20 $\text{H}_2\text{O} + \text{G6P} + \text{NADP} \implies \text{CO}_2 + \text{NADPH} + \text{Ru5P}$
- R21 $\text{Ru5P} \iff \text{R5P}$
- R22 $\text{Ru5P} \iff \text{Xu5P}$
- R23 $\text{R5P} + \text{Xu5P} \iff \text{GAP} + \text{S7P}$
- R24 $\text{GAP} + \text{S7P} \implies \text{E4P} + \text{F6P}$
- R25 $\text{E4P} + \text{Xu5P} \implies \text{F6P} + \text{GAP}$

R26 $\text{CO}_2 + \text{ATP} + \text{Pyr} + \text{H}_2\text{O} \implies \text{OAA} + \text{ADP} + \text{iP}$
R27 $\text{AcCoA} + \text{OAA} + \text{H}_2\text{O} \implies \text{Cit} + \text{CoA}$
R28 $\text{Cit} + \text{NADP} \implies \text{CO}_2 + \text{NADPH} + \text{AKG}$
R29 $\text{HAc} + 2 \text{Fd} + \text{AKG} + \text{CoA} \implies \text{CO}_2 + \text{AcCoA} + 2 \text{Fdr} + \text{Suc}$
R30 $\text{Fum} + \text{NADPH} \implies \text{Suc} + \text{NADP}$
R31 $\text{NADPH} + \text{OAA} \implies \text{Fum} + \text{H}_2\text{O} + \text{NADP}$
R32 $\text{AcCoA} + 2 \text{NADH} \implies \text{EtOH}(\text{ext}) + \text{CoA} + 2 \text{NAD}$
R33 $2 \text{AcCoA} + \text{NADH} \iff \text{CoA} + \text{H}_2\text{O} + \text{CroCoA} + \text{NAD}$
R34 $\text{CroCoA} + 2 \text{Fd} + 2 \text{NADH} \implies \text{ButCoA} + 2 \text{Fdr} + \text{NAD}$
R35 $\text{CroCoA} + \text{NADH} \implies \text{ButCoA} + \text{NAD}$
R36 $2 \text{AcCoA} + \text{NADPH} + \text{NADH} + \text{Suc} \implies 2 \text{HAc} + \text{CroCoA} + \text{CoA} + \text{H}_2\text{O} + \text{NAD} + \text{NADP}$
R37 $\text{ButCoA} + \text{ADP} + \text{iP} \implies \text{Butyrate} + \text{ATP} + \text{CoA}$
R38 $\text{HBu} \implies \text{HBu}(\text{ext})$
R39 $\text{HBu} + 6 \text{H}_2 \implies \text{HCa}(\text{ext})$
R40 $\text{AcCoA} + \text{ADP} + \text{iP} \implies \text{HAc} + \text{ATP} + \text{CoA}$
R41 $\text{HAc} \implies \text{HAc}(\text{ext})$
R42 $\text{CO}_2 + 4 \text{H}_2 \implies \text{HAc}$
R43 $\text{HAc} \implies \text{CO}_2 + \text{CH}_4$
R44 $\text{CO}_2 + 4 \text{H}_2 \implies \text{CH}_4$
R45 $\text{CH}_4 \implies \text{CH}_4(\text{ext})$
R46 $\text{CO}_2 \implies \text{CO}_2(\text{ext})$

mue $0.1804 \text{ AcCoA} + 1.62 \text{ ATP} + 0.01056 \text{ E4P} + 0.00421 \text{ F6P} + 0.00067 \text{ Fum} + 0.00515 \text{ G6P} + 0.048 \text{ GAP} + 0.4499 \text{ NADPH} + 1.14499 \text{ H}_2\text{O} + 0.003 \text{ H}_2\text{S} + 0.05408 \text{ NADH} + 0.0433 \text{ OAA} + 0.02322 \text{ PEP} + 0.03 \text{ Pyr} + 0.382 \text{ R5P} + 1.62179 \text{ iP} + 0.0168 \text{ AKG} \implies \text{Biomass} + 0.18043 \text{ CoA} + 0.04205 \text{ CO}_2 + 1.62179 \text{ iP} + 0.05408 \text{ NAD} + 0.44993 \text{ NADP}$

Appendix B. List of metabolites and abbreviations

Metabolites

Not balanced

Acetate (ext) HAc (ext)

Biomass Biomass

Butyrate (ext) HBu(ext)

Caproic acid (ext) HCa (ext)

Carbon dioxide(ext) CO2(ext)

Ethanol EtOH (ext)

Glucose GLC (ext)

Hydrogen (ext) H2(ext)

Lactate (ext) HLa(ext)

Methane (ext) CH4(ext)

Propionic acid (ext) HPr(ext)

Valeric acid (ext) HVa (ext)

Balanced

Acetate HAc

Butyrate HBu

Carbon dioxide CO2

Hydrogen H2

LactateHLa

Methane CH4

Propionic acid HPr

Acetyl-Coenzyme-A AcCoA

Adenosine triphosphateATP

Butyryl-Coenzyme-A ButCoA

Citrate Cit
 Crotonyl-Coenzyme.A CroCoA
 Dihydroxyacetone-phosphate DAP
 Erythrose-4-phosphate E4P
 Ferredoxin Fd
 Ferredoxin reduced Fdr
 Fructose-1,6-bi-phosphate F16P
 Fructose-phosphate F6P
 Fumarate Fum
 Glucose-6-phosphate G6P
 Glyceraldehyde-phosphate GAP
 Nicotinamide adenine dinucleotide phosphate reduced NADPH
 Nicotinamide adenine dinucleotide reduced NADH
 Oxalacetate OAA
 Phosphoenolpyruvate PEP
 Pyruvate Pyr
 Ribose-6-phosphate R5P
 Ribulose-6-phosphate Ru5P
 Sedoheptulose-7-phosphate S7P
 Succinate Suc
 Xylulose-5-phosphate Xu5P
 α -Ketoglutarate AKG
 Nicotinamide adenine dinucleotide NAD
 Nicotinamide adenine dinucleotide phosphate NADP
 Inorganic phosphate iP
 Water H2O
 Hydrogen sulfide H2S

ACoenzyme-A CoA

Adenosine diphosphate ADP