UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

Development and evaluation of an acidogenic biorefinery for food waste valorisation

Joana Resende Ortigueira

Supervisor: Doctor Carla Alexandra Monteiro da Silva

Co-Supervisors: Doctor Patrícia Maria Brito Madeira da Silva Moura

Doctor Maria do Rosário Sintra de Almeida Partidário

Thesis approved in public session to obtain the PhD Degree in Environmental Engineering

Jury final classification: Pass with Distinction and Honour

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Abstract

Food waste (FW) is an organic, highly degradable residue which amounts for approximately 32% of the worldwide food production. Current strategies for FW processing focus on treatment and disposal in detriment of valorisation. This research explores the valorisation of non-avoidable FW and agro-industrial residues/byproducts through acidogenic fermentation for hydrogen production, strongly adapted to the Portuguese biomass availability and characteristics. The experimental stage focused on the selection of residues according to two typologies: agro-industrial residues or byproducts (AG) and catering industry waste (CIW). The agro-industrial residues and/or byproducts were selected from a process of cross-referencing between Portuguese data on waste production and the identification/characterisation of substrates appropriate for acidogenic fermentation. The fermentability of the agro-industrial residues was enhanced through optimised pretreatments for production of sugar-rich solutions. Small-scale assays were performed with the use of a well-known microbial H_2 producer, *Clostridium butyricum*, for the selection of the most appropriate AG carbon source. The highest H_2 production yield coupled with the simpler procedure for sugar solubilisation supported the selection of carob pulp (CP). CP and CIW were successfully tested in bench-scale pH-controlled batch fermentation under sterile conditions. CIW was used as a model substrate for the development of a simplified non-sterile conversion process with CO_2 sequestration. The optimised settings of the batch system were used as the basis for the operation of a continuous FW conversion bioreactor (CSTR) during which continuous stable H_2 and acid production were registered. Produced H_2 was converted directly into electricity through the integration of a proton-exchange fuel cell. The quantification of valuable streams and the production efficiencies of the CSTR were used for the simulated scale-up and global warming impact evaluation, which culminated with the comparison between the new food waste biorefinery and the conventional food waste treatment and disposal pathway.

Keywords: dark fermentation, electricity, food waste, fermentative hydrogen, organic acids.

Resumo

Desperdício alimentar (*FW*) é definido como qualquer alimento retirado da cadeia alimentar que não tenha um uso secundário definido, sendo geralmente constituído por material orgânico degradável. A nível mundial estima-se que a quantidade produzida corresponda aproximadamente a 32% da produção total alimentar. O estudo apresentado explora a valorização de *FW* por fermentação acidogénica para a produção de hidrogénio (H_2), ácidos orgânicos e fertilizante, segundo o conceito de biorrefinaria. A fase experimental foi iniciada com a seleção de duas tipologias de *FW*: resíduos/subprodutos da indústria agro-alimentar (AG) e resíduos de hotéis, restaurantes e cantinas (HORECA ou na designação inglesa, *CIW*). Os AG foram selecionados por referenciação cruzada entre dados de identificação e caracterização físico-química e informação disponível relativa a quantidades geradas no território Português. Os resíduos selecionados foram processados para a obtenção de soluções fermentáveis e testados em ensaios preliminares de fermentação com uma bactéria produtora de H_2, *Clostridium butyricum* DSMZ 10702. Polpa de alfarroba foi selecionada como substrato modelo de AG devido à facilidade do processo de extração de açúcares solúveis, bem como ao elevado rendimento em H_2. CP e CIW foram testados de forma bem-sucedida em condições estéreis em bioreactor de bancada (modo *batch*) mas declinando substancialmente em performance após remoção da etapa de esterilização. Um processo de controlo de contaminação do substrato por aplicação de micro-ondas foi instituído de forma a contrariar este efeito. Os parâmetros operacionais de fermentação de CIW em *batch* foram transcritos para operação em contínuo (RPA). O biogás produzido durante a fermentação foi alimentado a uma célula de combustível de membrana de permuta protónica (PEMFC) para conversão em eletricidade. Os resultados obtidos serviram de base para o desenvolvimento de um modelo de aumento de escala teórico para a valorização de FW, para comparação com um sistema de tratamento de referência por digestão anaeróbia.

Palavras-chave: ácidos orgânicos, desperdício alimentar, electricidade, fermentação escura, hidrogénio fermentativo.

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List of Acronyms and Abbreviations

AC – Aerobic composting

Ac – Acid pretreatment

AD – Anaerobic digestion

AG – Agro-industrial waste

APA – *Agência Portuguesa para o Ambiente*, Portuguese Environmental Agency

BSG – Brewers' spent grain

CC – Corn cob

CIW – Catering industry waste

CO_{2eq} – Carbon dioxide equivalent

CP – Carob pulp

DF – Dark fermentation

EU – European Union

FAO - Food and Agriculture Organization of the United Nations

FW – Food waste

FLI – Food loss index

GHG – Greenhouse gas

GR – Green residues

GWP – Global warming potential

MSW – Municipal solid wastes

MW – Microwave pretreatment

OSD – Objective for sustainable development

OECD – Organization for economic cooperation and development

PEC – *Pacote de economia circular* (Circular Economy Package)

PERDA – *Projeto de estudo e reflexão sobre o desperdício alimentar* (Project on the study and reflection about food waste)

UN – United nations

VAT – Value added tax

WRI – World Resources Institute

WS – Wheat straw

List of Units

°C	Degree Celsius
g	gram
gvs	gram volatile solids
GJ	Giga Joule
ha	hectare
k	kelvin
kg	kilogram
km	kilometre
kWh	kilowatt- hour
L	litre
m^3	cubic metre
MJ	Mega Joule
MWh	Mega Watt-hour

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1. Introduction

1. Introduction

1.1. Motivation and problem statement

Food waste (FW) is considered to be one of the most problematic types of organic waste worldwide, representing an average of 16-65% of the municipal solid waste residues (Yun et al., 2018). According to recent alterations in the European directive 2008/98/EC, FW has now been defined as food lost "along the entire food supply chain from production until consumption. Food also includes inedible parts were those were not separated from the edible parts when the food was produced […], hence, food waste can comprise items which include parts of food intended to be ingested and parts of food not intended to be digested." Furthermore, this definition does not consider as waste, losses performed at stages of the food supply chain where products have yet to become food and by-products from food production with a defined use (European Union, 2019). The main causes appointed for FW production can be summarized as: over and faulty production, inadequate marketing rules and strategies, and deficient stock management (Lipinski et al., 2013), i.e., is a direct result of the inefficiency of food production and transformation systems and consumption. The European Union (EU) estimates that 88 Mton of FW are generated annually by the member states, a number equivalent to almost 50% of the EU total food production (Lipinski et al., 2013). At a worldwide level, FW is produced at an estimated value of 1.3 billion tonnes per year. Both values are expected to rise due to a predicted increase in worldwide population, accompanied by dietary changes, decrease in food production caused by climate change, land-use change derived from increase in biofuel production and soil erosion and degradation (Vittuari et al., 2016). Furthermore, according to the Food and Agriculture Organization of the United Nations, the FW discarded in the 28 countries of the European Union in 2013 represents up to 186 Mt CO_{2eq} of additional carbon emissions (Scherhaufer et al. 2018). The challenge behind this problematic can be summarised into two pertinent questions: can the produced food waste be reduced or eliminated and, if such is not possible, what should be done with the referred waste to diminish its impact upon the environment and economy of the territory.

Recent EU legislation defends that FW decrease is highly dependent of producer daily practices (waste prevention), efficient use of acquired food (loss minimization), and recycling practices that diverge from landfill (waste as resource) (European union, 2018; Hecht et al., 2009). However, it not possible to eliminate all produced FW, as no

production system is 100% efficient. The non-preventable fraction of FW would still be significant and require proper treatment and disposal. Currently, FW is either disposed through landfilling or valorised, particularly through composting and anaerobic digestion (AD). Landfill is a method which is quickly becoming obsolete, as recent EU legislation restricts the biological material deposition in its facilities. Anaerobic digestion and composting are examples of biological conversion processes, capable of converting organic matter into methane and digestate (AD) and compost (Composting), respectively. However, while a definite improvement over landfilling, both valorisation systems are not ideal solutions (Bryant, 1979). It is, perhaps, a mistake to see FW as a residue solely, something to be disposed of as securely and as efficiently as possible. From a biochemical point of view, FW is largely composed by functionalized molecules and fermentable compounds, such as carbohydrates, proteins, fat, etc. It is an incredibly varied substrate with undeniable potential for biological valorisation. This is the starting point for the design and evaluation of a possible biological valorisation process which permits the recovery of value while diminishing the environmental burden caused by an unpreventable type of residue.

1.2. Objectives and research questions

The main objective of this research is to design and test a biorefinery process at lab-scale which would permit the processing of food waste (FW) into hydrogen, bulk chemicals and sludge, through biological conversion with CO_2 sequestration. After proper continuous operation achievement at lab-scale, the process will be theoretically scaled-up and evaluated by energy and global warming potential indicators.

The proposed urban biorefinery sought to expound the benefits of the conversion of FW through dark fermentation, integrating the decentralized participation of consumers' in self-waste handling to reinforce FW prevention, proposed the generation of a highly valorisation vector from non-preventable FW while taking advantage of the existing infrastructures for biowaste treatment and included an additional upgrade of the bioconversion co-metabolites. Finally, the suggested biorefinery benefits and drawbacks would be compared with the current conventional food waste treatment (composting/anaerobic digestion, biogas conversion to electricity). The main steps followed for the targeted objective can be summarised as follows.

- **Target 1. Evaluation of the feedstock availability**

This target relates to the in-depth characterisation of the production profiles of FW in the Portuguese territory and, when not possible, ascertain their relation to the profile of production of municipal solid wastes on the territory. Causes, consequences, loss prevention practices and current and future legislation for FW minimisation will be researched and taken into consideration. The identification of the stages of greater loss and waste in the Portuguese food supply line are also of outmost importance.

Research question 1: *Is there a significant food waste potential in the Portuguese territory?*

- **Target 2. Feedstock selection and characterization**

Two case-studies were selected of the catering industry and industrial/agricultural waste typologies considering their importance at a national level, in accordance with chemical and physical characteristics adequate to acidogenic fermentation.

Research question 2: *How does Clostridium butyricum perform in terms of hydrogen and organic acid production when using food waste as carbon and energy source*?

- **Target 3. Biorefinery concept and optimisation of operational parameters**

The presented target will focus on the design of a biorefinery concept which will include a lab-scale prototype for conditioning and pre-processing and a lab-scale prototype for conversion of the biomass through dark fermentation. Testing of the following operational parameters will be performed focusing on the sterilisation, supplementation, medium optimization and minimisation, contamination control and mode of operation of the fermentation system. The optimised conditions will be used as basis for the development of a continuous production process.

Research question 3: *Is it technologically possible to set up a lab-scale biorefinery in continuous mode?*

- **Target 4. Biohydrogen conversion**

The fermentative H_2 will be converted through a proton-exchange fuel cell under variation of several operation parameters (temperature, H_2 flow, substrate recirculation). Analysis of the viability of the use of bioH_2 versus commercial H_2 will be performed as well as the influence of temperature in the conversion.

Research question 4: *How does the biohydrogen produced at ambient temperature and pressure, with moisture, affect a proton exchange fuel cell performance*?

- **Target 5. Optimisation of the culture medium**

This stage will consist on the optimisation of the culture conditions in order to obtain acid-rich fermentates with appropriate characteristics for biological conversion into polyhydroxyalkanoates (bioplastics).

Research question 5: *Will the fermentation byproducts be suitable for bioplastic production*?

- **Target 6. Global warming potential evaluation**

The viability, advantages and disadvantages of the proposed biorefinery will be assessed and evaluated.

Research question 6: *How does the virtual food waste biorefinery energy demand and global warming potential compares with conventional food waste treatment*?

1.3. Document organization

The figure bellow depicts the structure of the present document as well as the connection between the referred targets and the different chapters.

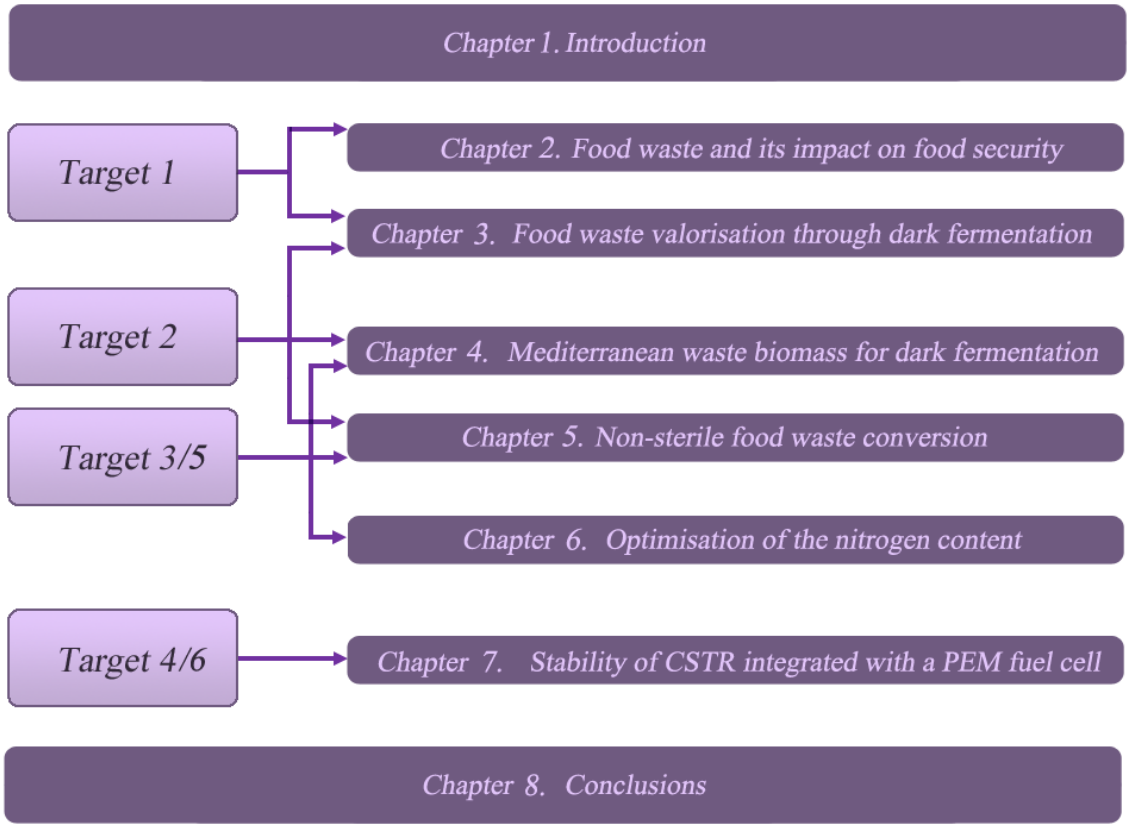

Fig 1. Schematic representation of the structure of the document.

2. Food waste and its impact on food security

2 Food waste and its impact on food security

2.1 Food security thematic

Food security is defined by the Food and Agriculture Organization of the United Nations (FAO) as "*a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life*" (Lipinski et al., 2013). Food security varies greatly from community to community, being a measure of the wellbeing and safety of its populations as well as characteristic of its culture, environment and social context. Population growth, urbanization and rise in per capita incomes are key factors for variations denoted in food security (Grafton et al., 2015). In fact, up to 2050, the worldwide population is expected to reach a total of 9 billion people due to the sudden industrial development of countries, such as India or China (Lutz et al., 2001). This growth implies an associated need to increase the supply of food, water, mineral and land resources, among others. By 2030, it is predicted that the food, energy and fresh water societal requirements will increase 50%, 50% and 30%, respectively. By 2050, predictions are even direr. According to FAO data, for the worldwide food necessities to be completely satisfied, the current crop production needs to be increased by 70% until 2050, which corresponds to a predicted growth of approximately 6500 trillion kcal per year (Lipinski et al., 2013). The most immediate and easiest measure to counterbalance such a situation would be to intensify production up to a point where it matches demand. However, the expansion of intensive agricultural production has severe associated ecological impacts including, aggravated erosion, decrease in organic matter levels, increase of soil compaction, impoverished micro fauna and flora, pollution and leachates from high fertiliser and pesticide use, among others (Stoate et al., 2001). Conversely, the expansion of arable land would encroach upon carbon sinks, soil covered with species which contribute greatly to atmospheric carbon sequestration and that cannot and should not be encroached upon without severe environmental consequences (Bruinsma, 2017). The increase of food availability as described must be done in such a manner which permits its sustainable supply while not hindering neighbouring ecosystems, human communities or even the capacity to continue food production in the future (Grafton et al., 2015).

According to FAO, part of the worldwide food production does not actually reach the consumer, being wasted or lost well before the consumption stage. Food loss and waste are defined as the edible parts of plants and animals that are produced or harvested for human consumption but that are not ultimately consumed (Lipinski et al., 2013). FAO further differentiates both concepts. According to recent reports, food loss refers to "*a decrease in mass or nutritional value of food which was originally intended for human consumption*", i.e., the loss of food which occurs due to inefficiency of the food production and distribution systems up until it reaches the final consumer. Food waste, on the other hand, is considered to be food appropriate for human consumption but which was discarded through a conscious decision, either due to expiration or spoilage (Lipinski et al., 2013). For the effect of this work, food waste and loss will be referred to in conjunction as *Food Waste* (FW). This term was defined as the food intended for human consumption, edible or inedible, which is removed from the food production and supply chain to be disposed of, including that which is obtained from the stages of primary production (agriculture, animal production, etc.), transformation, production, transportation, storage, distribution and consumption, excepting loss in primary production (European Union, 2018). The referred legislation excludes from this concept: animal feed, live animals unless prepared for insertion into the food market for human consumption, plants prior to harvest, medicine, tobacco, drugs and residues, contaminants and by-products from food production with a definite destination (European Union, 2019).

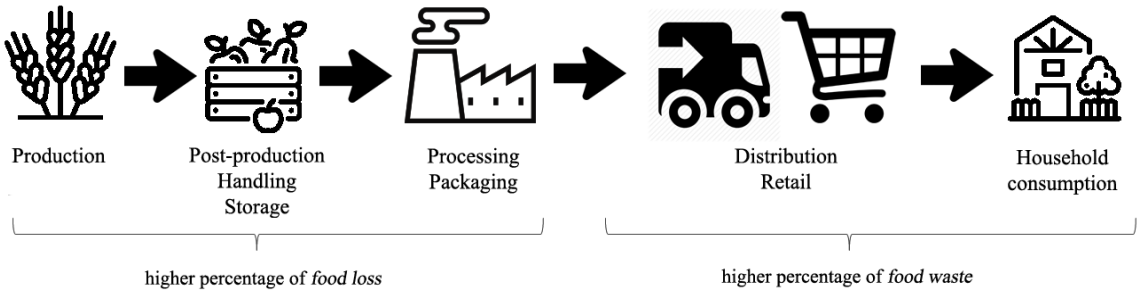

Fig 1. Schematic representation of the stages of the food production and supply line chain and identification of stages associated with food waste and loss.

The disparity between the produced food and the food which is actually consumed can be caused by several reasons, ranging from aesthetic (due to high market appearance standards) to biological (spoiling, for example), and can happen from the primary points of process production, such as the harvest, all up to the final consumption stage. Common

examples of FW are the loss of grain or fruit due to deficient harvesting and/or transportation equipment, loss of material during processing or packaging and rejection of food prior to distribution due to aesthetic preferences. The main causes appointed for FW are overproduction, faulty production, inadequate marketing rules and strategies, and deficient stock management and its production directly affects food security and availability, forcing the food industry to increase the production to compensate increasing demand. FAO estimates that approximately 1.3 billion tonnes of food produced worldwide were wasted in 2012, a value equivalent to a total waste of 24% of all produced food (Lipinski et al., 2013). It represents not only an immense economical loss but also an increment in greenhouse gas emissions (GHG), wasted resources and land. For the reported year, 3,300-5,600 million metric tons of GHG were registered as being caused by FW production, as well as 173 billion cubic meters of water and 28 million tons of fertilizer.

Strategies for FW reduction must be applied at different stages of the production process and must be tailored to the type of commodity. The facilitation of food redistribution or donation is paramount, as is the betterment of food storage, packaging and transportation, the lowering of appearance standards by both retailers and consumers, the education of the communities about food supply, distribution and waste problems, etc. These measures can be summarized as waste prevention (waste control on the producer and consumer daily practices), loss minimization (efficient use of the acquired food) and use of waste as resource (recycling practices which diverge from landfill) (Gustavsson et al., 2011). The referred measures are in agreement with EU legislation (European Union, 2018). Additional strategies can be summarised as follows:

- Development of FW quantification protocols
- Definition of targets for FW reduction and maximum production
- Investment to increase the efficiency on the production, post-harvesting, transportation and distribution stages, especially in developing countries
- Creation of regulatory entities in developed countries
- Acceleration and support of collaborative initiatives for FW reduction

According to the recently shared Objectives for Sustainable Development (OSD), one of the major community goals related to the reduction of FW recognizes that the FW per capita at a worldwide level should be halved by 2030 (United Nations, 2015). The

OSD also underlines that the reduction of residue must be undertaken through prevention, reduction, recycling and reuse.

FAO is already acting upon the referred resolution, focussing on natural resource management, such as soil, energy and water, and associated GHG emissions. This organization also aims to establish a "Food Loss Index" (FLI), which would allow the quantification and worldwide characterisation of the FW by country. Furthermore, in 2010, the Organisation for Economic Co-operation and Development (OECD) founded the "Food Chain Analysis Network", a platform based on the inherent necessity to understand the operation and sustainability of the food production and supply chain and productivity data. Only after clear analysis of the evolution of the FW generation trends can appropriate measures for FW reduction be employed.

It is estimated that a reduction in half of the current levels of worldwide FW production would lead to savings of 1,314 trillion kcal. This value is equivalent to, approximately, 22% of the amount of food required worldwide by 2050 (Lipinski et al., 2013). It is important to underline, however, that even a 50% reduction in global FW seems a faraway goal. The food market is highly dynamic and a reduction of food loss at the production level, for example, might lead to higher product availability and a consequent lowering of the selling price to the consumer. This effect, in turn, can cause an increase in consumption which might influence producers to revaluate their production pattern. Every strategy undertaken must be custom tailored for a given territory and dynamic enough in order to counteract market fluctuations. Taking this into consideration, it is impossible to deny that FW reduction will reduce pressure on the ecosystems, climate and water and provide positive influence for poverty reduction.

2.2 Food waste quantification

As stated in the previous section, legislative measures for FW reduction depend heavily, as already stated, on the economic, cultural and social development of a given territory. The majority of the FW produced in developed countries, for example, tends to occur at the consumption level (*closer to fork)*, while in developing countries the FW levels are higher at the production, processing and handling stages (*closer to farm*). Therefore, it can be inferred that the latter is heavily caused by inefficiency of the

production systems rather than the cultural/economic factors in the former (Lipinski et al., 2013).

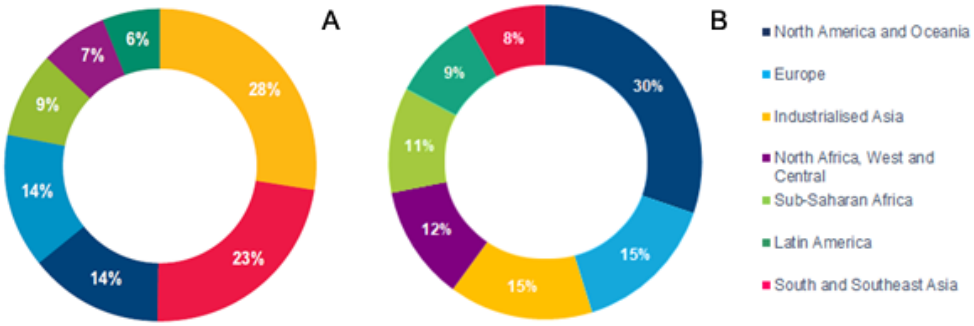

Fig 2. A) Share of global FW amounts generated by region and B) Share of global FW per capita by region (adapted from Lipinski et al., 2013).

According to the World Resources Institute (WRI, Washington DC, USA), the greater share of FW produced worldwide originates from Asia, both the industrialised and South/Southeast areas (Lipinski et al., 2013). This share reflects the higher population numbers of the territory (Fig 2. A). However, when the FW values are evaluated per capita, the results are staggeringly different. North America and Oceania waste approximately 1520 Kcal capita^{-1} day^{-1}. This value is approximately twice the amount calculated for Europe and Industrialised Asia (748 and 746 Kcal capita^{-1} day^{-1}, respectively). The total share of FW lost per region per capita ranges from 15-25% of all available regions except for, once more, in the North America and Oceania where this percentage reaches a maximum of 30%. However, these estimates are complicated by the associated difficulty in quantifying FW. The inefficient separation of FW from the general waste by the population, inefficient organic waste sorting in the treatment facilities. and the lack of information from both food producers and distributors are contributing factors. The absence of a general procedure to obtain quality FW data still produces large disparities in the information, even among the countries of the European Union (Fig 3).

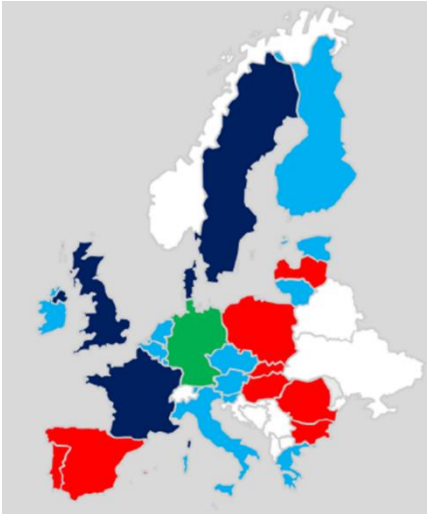

Fig 3. A) Representation of the quality of FW collection data at the EU level: not sufficient quality (red), sufficient quality (light blue), good quality (dark blue) and high quality (green) (adapted from Stenmarck et al., 2016).

The present EU FW estimates are based on 2012 information collected under the seventh framework program of the European Committee. The project "FUSIONS: Reducing food waste through social innovation" divided the food production supply line into 5 stages in an effort to normalise data collection: primary production, processing (food manufacturing), wholesale and logistics (distribution), food service and household (consumption) (Stenmarck et al., 2016). The estimates of waste production for each stage were obtained through the collection of waste treatment data from the different countries and selected studies. The data collected points to a yearly generation of approximately 88 million tonnes in both edible and inedible food, a value equivalent to 173 Kg of waste per person. This amount corresponds to 20% of the total of food produced in the EU (865 Kg per capita^{-1}, 2012 data) and amounts to a 143 billion euros loss.

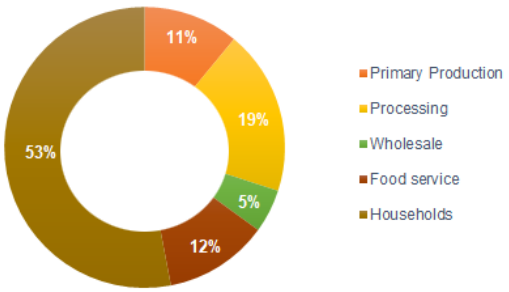

Fig 4. Share of FW by stage of the food production supply line estimated for the EU-28 (2012 data, adapted from Lipinski et al., 2013).

According to this estimative, it was also possible to identify the most wasteful and inefficient stages of the EU food production supply line: households (47 million tonnes) and food processing (17 million tonnes). The first, especially, is characteristic of more developed societies where FW is mostly generated at the consumption level and not during production and distribution. In fact, the fraction of FW obtained at the household is composed by a larger fraction of wasted edible food (Lipinski et al., 2013).

2.3 Food waste prevention strategies

Chapter 2.1 and 2.2 defined that the FW production is an incredibly all-encompassing and encroaching issue, impacting negatively several areas of society. As such, the EU has agreed that FW prevention is necessary for the establishment of a sustainable and fairer society. The establishment of the "EU food losses platform", the definition of the Sustainable development goals directly connected to sustainable food production and consumption and the project FUSIONS itself serve to underline the clear interest shown by the European Union to reduce FW within its borders.

Towards this goal, a non-compulsory amendment to the Directive 2008/98/EC on waste has been designed, solely devoted to the establishment of a methodology for FW quantification and its associated quality requirements which will be applied to the whole of the EU territory (European Union, 2019). The basis of this document is the obligation of each member-state to quantify national production of FW according to the guidelines expounded upon the document, particularly the requirement to estimate yearly FW amounts and to undertake in-depth quantification every 4 years. Included in the document is also the obligation to include FW prevention measures into national waste prevention programs and the assessment of the impact of already implemented measures.

The "EU platform on Food losses and food waste" (European Union, 2016) and the EU project FUSIONS (Stenmarck et al., 2016) have both suggested preventative measures for several points of the food production supply line. The "EU platform on Food losses and food waste" focuses on two main areas of action: food donation and FW quantification (table 1).

Table 1. Definition of the plan of action devised by the EU platform on food losses and food waste (European Union, 2016).

Food donation	FW quantification
Preparation of legislation for food donation: ➢ Definition of food business operators and the clear distinction between these and charity and food donation organisations. **Correct labelling:** ➢ Food donation organisations should be defined as establishments of food service and follow proper EU legislation referent to consumer information. ➢ Differentiation between the labelling use-by and best before. **Oversight:** ➢ Tax deductions ➢ Implementation of good practices for food donation in all the EU-members. ➢ Implementation of a pilot-project for evaluation of good practices for food donation.	➢ Establishment of a delegation for the evaluation and communication among the EU-member states of FW definitions and what it represents. This event is in accordance to a proposal for alteration of a residue-related EU-legislation and will operate in close cooperation with FAO. ➢ Development of FW indicators for monitorization of minimization policies. ➢ Identification of data, practices for data recovery and associated experience existent in the EU-member states. ➢ Evaluation of a possible monitorisation of FW production or food resource flow throughout the food production systems in the EU. ➢ Continuation of the project FUSIONS.

The prevention methods contained in the referred package focus strongly in the later stages of the food production supply line, particularly at the distribution level (retail of food goods) and restauration/household level. It implies that the FW is either close to spoiling or already cooked and attempts to prevent the waste of food which is still in an edible stage. It also considers extremely relevant the correct quantification of FW and the identification of different points of FW production. In fact, it is a characteristic strongly shared with the project FUSIONS (Vittuari et al., 2016). In their own recommendations package, FUSIONS underlines that, not only is FW quantification of paramount importance, as the definition of FW itself needs to be clear, concise and shared between different countries. This project further proposes a common framework for data collection and characterization and suggest the possible introduction of practical information for in-field procedures. Food donation should be encouraged through the creation of adequate

EU legislation and an associated national legislative incentive package towards social innovation promotion related to food donation. It is necessary, the document claims, to encourage food business operators to donate unsold edible food to charities instead of defaulting to disposal systems. This can only be properly performed when there are proper guidelines in place, supported by national legislation. A final suggestion for the improvement of food donation is associated with the Value added tax (VAT). This value is defined as "*the purchase price at the moment of the donation adjusted to the state of those goods at the time when the donation takes place*" and, according to EU Council directive 2006/112/EC (European Union, 2006), food donation is not exempt of VAT. FUSIONS underlines that this stature should not be changed. The adoption of VAT for food donation of zero or near zero can nullify other policy options, such as deduction of corporate tax credit (Vittuari et al., 2016).

Social innovation is defined as "*a novel solution to a social problem that is more effective, efficient, sustainable, or just than existing solutions and for which the value created accrues primarily to society as a whole rather than private individuals*" (Phills et al., 2008). Social innovation enterprises are the basis for a successful FW prevention and, as with food donation organizations, guidelines and policy interventions should be defined and proper financing established for their correct development. The good examples undertaken, while possibly not implementable in different realities (urban *versus* rural, for example), should be adapted and the information gained from their performance should be shared. In all the referred suggestions, both local and national governmental organizations have the strongest role. The sharing of information, the design of awareness campaigns and common framework for evaluation of policies should be under their purview. Final recommendations involve the necessity of the evaluation of the impact of FW production, its drivers and impacts.

2.4 Case-study: the particular case of Portugal

2.4.1 Food waste production in the Portuguese territory

Project PERDA (*Projecto de Estudo e Reflexão sobre o Desperdício Alimentar* – project on the study and reflection about food waste) (Baptista et al., 2012) aimed to quantify and analyse the amount and type of FW produced in the Portuguese territory. PERDA identified four stages of the food production supply line instead of the 5

recognised by FUSIONS: production, processing, distribution and consumption. According to statistical data, 17% of the food processed or produced for human consumption in the Portuguese territory is wasted. This value is equivalent to a total amount of, approximately, 1 million tonnes per year and relates to the amount of food wasted in the 4 stages of the food production supply line. Processing is the most efficient stage while both production and consumption slightly more wasteful (Baptista et al., 2012). The design of FW prevention measures can only be performed after an in-detail analysis of the causes of FW in each stage of the food production supply line.

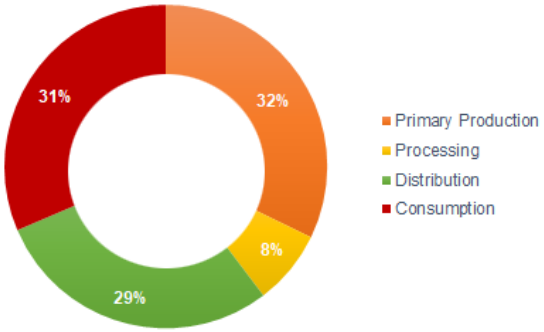

Fig 5. Share of FW by stage of food production supply line estimated for Portugal (2012 data, adapted from Baptista et al., 2012).

The Portuguese analysis identified shortcomings in the agricultural production stage, particularly during harvest, related to the mechanical damage caused by harvesting machines, inappropriate storage and post-harvest processing. Sickness, plagues and animal attacks cause heavy losses, not only in agricultural products but also in animal production. Transportation from production areas to slaughterhouses is also an important point of loss for animal production. Fishing FW can be derived from a common practice in the community which promotes the return to the sea of species which are either not adequate for human consumption or are not of the appropriate size. Additional loss is promoted when species without commercial value are not supplied for human consumption but instead forwarded for animal ration manufacture. Processing is a heavily automatized phase of the food production supply line. The associated losses are usually related to mechanical damage during packing or inherent to the processing method itself (example, the loss of tomato skin during tomato pulp production). Further loss can be caused by procedures of the factories such as machinery start-up, end of production, cleaning stages or new product testing. The third identified stage of the food production supply line, according to this study, is food distribution. FW attained in this stage is often related to the lack of/bad storage and handling, inept stock management and accumulation

of unsold material (Baptista et al., 2012). Furthermore, an additional detail should be taken into consideration. In developed countries, there is a clear distinction between the areas of production and the areas of consumption, the first being generally associated to rural areas while the latter is focused mostly in the large littoral-based urban centres (Lipinski et al., 2013). This fact, associated with the low self-provision capability of the country, i.e., "*the capacity to produce the necessary amount of food for the population existent within its boundaries*", leads to an enlengthening of the food distribution chain. Therefore, the number of elements in the distribution stage and the associated FW is significantly increased. Finally, the last stage to be considered is the consumption stage. The authors recognised this period as all food consumption, either at a household level or in large-scale food service. FW at this level relates to, again, inadequate storage and handling as well as unsuitable stock management but the human component has the strongest influence. FW loss prior to food preparation due to ignorance of the 'use-by' or 'best before' nomenclatures, during food preparation or after service are all too common.

2.4.2 Food waste reduction policies in the Portuguese territory

The Portuguese government used the information acquired by PERDA and FUSIONS in the elaboration of plan of action which was included in the '*Pacote de Economia Circular*' (Circular economy package) (Portuguese government, 2018). This package contains guidelines which aim to improve the competitiveness of the Portuguese economy while taking into consideration the environmental issues inherent to economic growth (European Union, 2013). Examples of the targets related to FW reduction are:

- Adequate and careful management of raw materials and their efficient use
- Prevention of residues through increase of durability
- Improvement of the donation and share of food in replacement of disposal
- Promotion of legislation which guarantees the quality of products which use byproducts as raw materials in their composition
- Reduce FW in all stages of the food production and supply chain, including households
- Monitorisation and report of FW levels

Several FW reduction measures can be transversal, applying strongly to more than one stage of the production supply line. Those defined by the Portuguese government in the PEC involve revision of guidelines for food safety and promotion of information campaigns for consumer awareness (at a national level and targeted to schoolchildren, in a manner similar to recycling incentive programs), specific for food producers and at an EU level. Furthermore, statistics of FW production should be obtained consistently and shared with the public as well as examples of good practices, exemplary social innovation systems and, especially when successful. The food donation should be facilitated, particularly since it tends to clash with the current food safety legislation, and the establishment of specific areas for sale of edible food goods close to waste conditions has been suggested. The definition of this problem shows that while the reduction is possible and necessary, the elimination of FW remains elusive, for the moment. Therefore, it is important to identify the final destination of FW and how this residue can be appropriately disposed of, preferably with an associated creation of value. Fig 6 depicts the most common destinations of FW in the Portuguese territory.

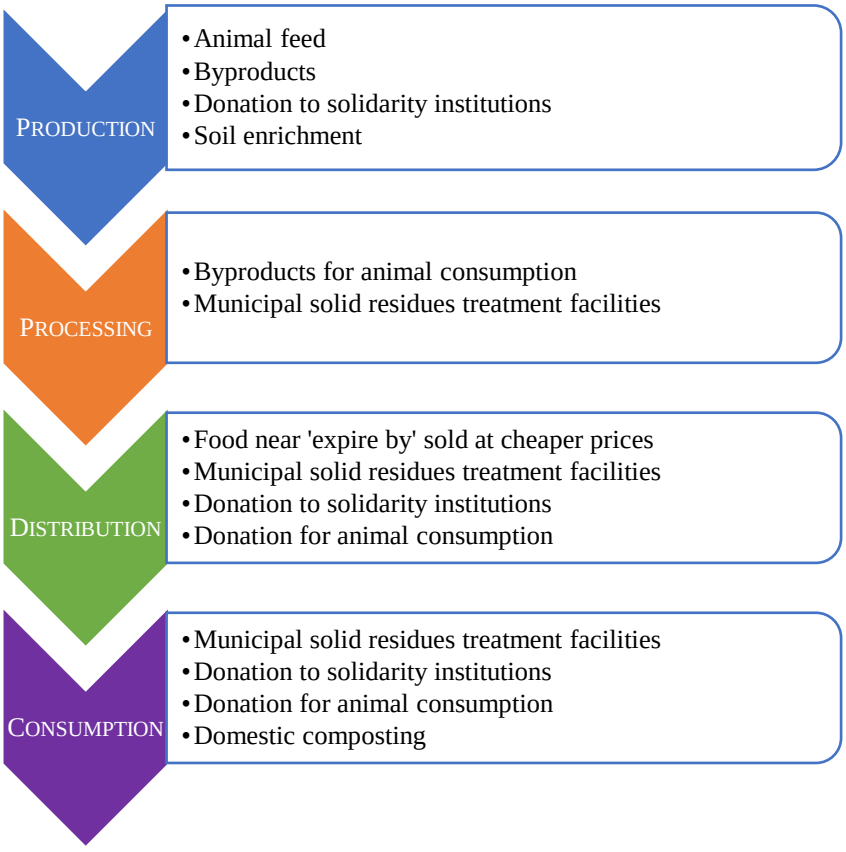

Fig 6. Common destinations of the FW produced in the Portuguese territory for stage of the food production supply line (adapted from Baptista et al., 2012).

The production and processing stages tend to the direct FW towards a secondary use. A common example can be seen during harvest of wheat or corn. This activity is performed with large-scale harvesting machines which break down the plants and recover the product. However, while doing so, they can cause high mechanical damage to the cultures. Broken fibres and product are commonly abandoned in the field and used as enrichment to the fields or, if recovered, as animal feed. FW which are not donated, sold at cheaper prices or used as byproducts are disposed of through the municipal solid waste (MSW) treatment facilities.

2.4.3 Municipal solid wastes: quantification and disposal

The report of the MSW generated in the Portuguese territory is of the responsibility of "*Agência Portuguesa para o Ambiente"* (APA, Portuguese Agency for the Environment). This type of waste is defined as the sum of the selective and non-selective waste recovered by the different waste disposal companies acting in the territory (Marçal et al., 2015). The organic fraction of MSW is composed primarily by FW and green residues. As per national legislation, GR are treated independently from the MSW, generally managed by the local government institutions. The food-derived waste obtained from the various points of the food production supply line are mainly produced by private households, school canteens, restaurants or supermarkets, among other large-scale food retailers.

The Portuguese MSW management companies employ four methods of waste valorisation and disposal: energy recovery (through incineration), organic valorisation (methanogenic fermentation and composting) and disposal through landfill. Disposal through landfill, while on a decline due to recent legislation, remains the primary method of waste management (Silva et al., 2014). Over 60% of the MSW produced in the Portuguese territory in the year of 2005 were deposited in landfill. This number was reduced from 2005-2014 in almost 20% as landfill was slowly replaced by organic valorisation or coupled with mechanic and biological treatment systems. In the timeframe of a decade, energy production through biological means has increased from a mere 7% to over 20%, an increase which represents an incredibly effort by the legislating authorities. The application of both energetic valorisations did not vary excessively throughout the decade. The majority of the waste treatment facilities does not perform selective separation of the waste received into organic and inorganic fractions. Therefore,

the material channelled into landfill has a similar organic matter fraction to the one registered in the original untreated waste. The physical characterisation of the MSW has been performed yearly since 2008. Fig 7 depicts the variation of the municipal solid wastes deposited in landfill as quantified by APA from 2009 up to 2017 and their respective organic fraction.

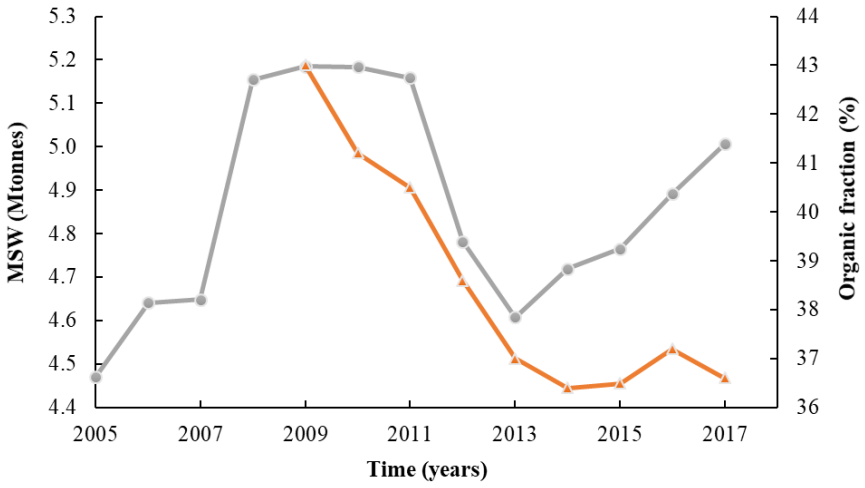

Fig 7. Variation of the amount of MSW produced in the Portuguese territory (●) and corresponding organic fraction (▲) (Marçal & Teixeira, 2017).

In the year of 2008, 43% of the total MSW was composed by organic material. This value gradually lowered in the following years to a minimum of 36.4% registered in 2014 before increasing slightly for the three following years. An active organic material and waste production decrease may be indicative of a concerted effort by the communities to diminish their loss of income or by the application of measures of waste prevention such as specific food sharing programs established in later years ("*Desperdício zero*", Zero waste program) (Marçal et al., 2015). The connection between waste production and the economic status of the country appears to be corroborated with the results registered after 2015. The increasing stability of the country coupled with the governmental deficit reduction and diminution of the financial weight of the Portuguese families appear to have a direct implication in waste production. For the year of 2017, of the 5 Mtonnes produced of MSW. 1.8 Mtonnes of the produced MSW were identified as organic material and 0.98 Mtonnes of this material were ultimately disposed of in landfill without appropriate valorisation (Marçal & Teixeira, 2017). Considering the sheer amount of organic material which is, in all accordance, retaking the increasing trend registered prior to 2008, it is

important to analyse the disposal and valorisation methods in use by the solid waste treatment facilities.

2.5 Organic waste valorisation

The term organic valorisation can be defined as the process of "*valorisation of organic materials through the conversion of materials in end-of-life situation into a product of interest*" (Arancon et al., 2013). This product can be a fuel, an energy vector, compost or an added-value product, such as organic acids, surfactants or pigments (Science and Technology Select Committee H of L, 2014). In this study, the term organic valorisation is defined as the biological conversion of organic waste materials, i.e., the generation of products of interest through the biological activity of microbial populations, using organic waste as carbon source for their development.

The principal methods currently in operation for organic matter valorisation are composting (aerobic and vermicomposting) and anaerobic digestion (biogas production and biomethanation). The introduction of a biological treatment prior to disposal allows control of the natural process which would, otherwise, take place in the landfill stage causing several of the issues already referred. Lou et al. (2009) registered that the major emissions of CO_2 originated in landfills are due to their organic content. The reduction or, if possible, the removal of this organic fraction would decrease greatly the amount of methane and CO_2 detected from landfill (Lou et al., 2009). Organic valorisation also permits the shift of the value from the waste management chain back into the market in a manner much the same as the recycling of inorganic materials (metal, plastic, glass) already in use (Sharholy et al., 2008). The following section expands further on the characteristics of each organic valorisation process.

2.5.1 Aerobic composting

The process of aerobic composting consists in the degradation of organic matter by its own bacterial community in the presence of oxygen (Sharholy et al., 2008; Sharma et al., 1997). This type of valorisation allows for a reduction in waste volume of over half

(50-85%), producing a highly nutritious material as principal product (compost) which is primarily used as fertilizer for agriculture (Sharma et al., 1997).

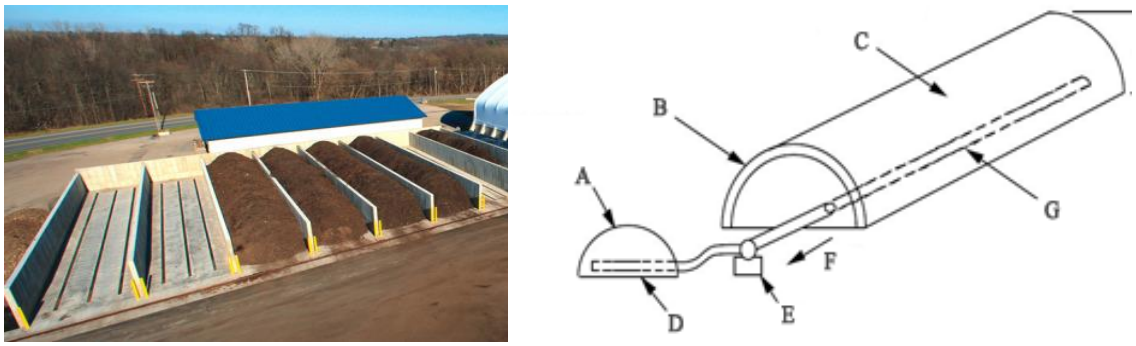

Fig 8. Representation of the Amboy Compost Facility (New York) and the aerobic composting system (adapted from JI network, 2017): A) Finished compost; B) Blanket of finished compost; C) Yard trimmings, sourced separated organics or mixed MSW; D) Odour filter; E) Blower; F) Air flow; G) Perforated aeration tube.

The process can be divided into four stages: mesophilic, thermophilic, cooling and compost maturation. In the first stage, the degradation of readily fermentable materials is performed by a mixture of bacteria, actinomycetes and fungi, generally functioning at temperatures between 15-40 ºC. This event is conjoined with the formation of water, CO_2 and heat before finalizing with partial auto-oxidation of the cellular material. The thermophilic stage occurs at temperatures over 40 ºC, incentivising the growth of actinomycetes and thermophilic bacteria. The conversion of monomeric carbon sources leads to a gradual decrease in temperature – the cooling stage – where the degradation of polymeric recalcitrant materials is generally performed. The process is finalized with the maturing stage, performed at lower temperatures (25 ºC) with reduced oxygen uptake rates, whereas further degradation of the more recalcitrant materials is undertaken (Maqbool and Rehman, 2015). As expected, this type of waste treatment has its own GHG emissions associated, which vary significantly with the type and amount of feedstock converted. Carbon dioxide is formed during both stages due to bacterial action while CH_4 is intermittently formed in small anaerobic pockets scattered throughout the substrate (Lou et al., 2009; El-Fadel et al., 1997; Amlinger et al., 2004). Nitrous oxide, produced by ammonium oxidation or incomplete denitrification, can also potentially be emitted during composting (Amlinger et al., 2004). Unlike the process of landfill, generally no exploration of the gaseous product of composting is performed. While these emissions are significant, they are not accounted in the carbon balance of the process as the CO_2 is

considered to be of biogenic origin and, therefore, does not contribute to the worldwide GHG emissions. CH_4 and N_2O emissions are negligible, even though the latter has received renewed interest due to the higher global warming potential (GWP) value in comparison to that of CO_2 (Lou et al., 2009). Furthermore, the production and use of compost directly decreases the need for chemical fertilizers eliminating the GHG associated to the production of these compounds. The use of compost also allows for a quicker plant growth, improved soil tillage and consequent utility as well as carbon sequestration in the solid state (Lou et al., 2009).

2.5.2 Anaerobic digestion

Biogas is defined as the gaseous product of the anaerobic degradation of organic material. It is obtained through a relatively easy production process with residual waste generation and is considered a prime manner of converting organic material into a usable form of energy (Awe et al., 2017). Biogas is composed by several chemical compounds, mostly methane (CH_4), CO_2, and minor amounts of CO, hydrogen (H_2) and hydrogen sulphide (H_2S), among others (Awe et al., 2017). The main product of interest in biogas, however, is CH_4, a compound with a relatively high energy content which can be converted readily into electricity (Chandra et al., 2012). The production of biogas consists on the conversion of residues with high moisture content (50-70% wt) originated from varied sources such as agricultural and meat industries, municipal solid waste and wastewaters and industrial organic-rich wastewaters.

Fig 9. Representation of the Clearcove digestion systems and the anaerobic digestion system: A) Biogas enclosure; B) Ground sludge pipe; C) Ground injection pipe; D) Substrate inflow; E)

Effluent substrate; F) Effluent gas; G) Mixer; H) High-pressure valve (adapted from Clearcove systems, 2018).

The production process is comprised by four stages of degradation and conversion: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The first stage consists in the degradation of polymeric material (carbohydrates, proteins and fats) into their respective composing monomers by facultative and anaerobic bacteria. These monomers are then used as carbon and energy source by acidogenic bacteria and converted into H_2, CO_2, alcohols and different organic acids (acetate, butyrate, propionate, etc.). In the acetogenesis phase, acetate is the principal product, being obtained from the combination of exergonic H_2 and CO_2 as well as alcohols and the remaining organic acids. Finally, in the methanogenesis stage, methanol, acetate, H_2 and CO_2 are converted into the product of interest, CH_4. The final biogas contains varying concentrations of CH_4 due to differing bacterial populations and substrate characteristics. Generally, however, CH_4 can be accounted for 45-80% of the final gaseous product.

Anaerobic digestion can usually be performed in either a single or a two-stage process. The single stage production performs the four stages necessary for methane production in one single reactor. It is a rather easier production system to oversee, having far less technical difficulties and requires less initial investment (Kiran et al., 2014). Conversely, two-stage fermentation divides acidogenesis and methanogenesis into separate reactors. The separation of the two stages allows for an optimization of each stage in terms of pH, temperature and nutrients while allowing operation at lower hydraulic retention times (HRTs) and higher loading rates. These factors lead to a faster and more efficient production process (Lee et al., 2010).

The biogas produced during AD cannot be converted directly into energy. The gaseous product is generally riffled with impurities such as particulates, siloxanes or H_2S, and its concentration in CH_4 (50-70% v v^{-1}) is too low in comparison to natural gas (80-95% v v^{-1}) (Cabrita et al., 2015), which invalidates the use of established natural gas systems for the combustion and conversion of CH_4. To counteract this problem, the biogas is submitted to a process denominated upgrading. This process is applied in order to increase CH_4 concentration in the biogas and permit the use of the mentioned natural gas conversion systems. Methods such as water, physical and chemical scrubbing (WATS, PHYS and CHEMS, respectively), pressure swing adsorption (PSA) and membrane separation are preferential for CH_4 upgrading (de Mes et al., 2010).

2.5.3 Biological hydrogen production

The information detailed in the previous chapters underline two factors: the necessity of reducing FW production and the valorisation of produced FW in a sustainable and advantageous manner. The H_2 molecule is seen as an appropriate alternative for more conventional renewable energy sources. It contains a very high energy density (120 MJ Kg^{-1}), is easily attainable through both biological and thermochemical processes and, unlike its counterparts and, even though its biological production is associated to CO_2 production, H_2 conversion into energy is a carbon-free emission process (Ortigueira et al., 2015). Furthermore, H_2 is considered a versatile energy vector, a form of storing renewable energy in an easily transportable chemical form (Edwards et al., 2008) without the consumption of a non-renewable resource and with minimal waste production (Orecchini, 2006). Bar its use as energy source, H_2 can also be used as feedstock for the production of several chemical compounds (ammonia or methanol), upgrading or purification of oil derivates, etc. (Sherif et al., 2014). The term biohydrogen comes into use when hydrogen is either produced biologically (fermentative H_2), through fermentation, or from biological/thermochemical conversion of biomass (Singh et al., 2015). The biological pathways can be undertaken at mesophilic conditions or thermophilic conditions, i.e., temperatures ranging from 20-55 ºC at atmospheric pressures. This factor makes them more easily controllable and its energy requirements lesser than thermochemical alternatives. There are numerous biological H_2 production systems to consider: biophotolysis, photofermentation, dark fermentation and bioelectrohydrogenesis (Miltner et al., 2009). Both biophotolysis and photofermentation are light-dependent energy producing systems. Biophotolysis refers to the direct conversion of solar energy into H_2 and molecular oxygen (O_2) while photofermentation depends on light energy for the degradation of various organic compounds into H_2 and CO_2. Bioelectrohydrogenesis involves the conversion of organic matter into hydrogen through an electrolytic process, taking place in a Microbial Fuel Cell (Cucu et al., 2013).

Dark fermentation (DF) consists on an anaerobic light-independent degradation process, capable of converting polymeric and monomeric sugars into H_2, CO_2 and organic acids (butyrate, acetate, etc.) without the use of O_2 as a final electron accepter. Unlike biophotolysis or photofermentation, DF is a simpler, more direct process with higher

production rates (>1 m^3 h^{-1} m^{-3}), relatively easy to operate and has lower energy requirements (Ren et al., 2011). Additionally, H_2 production through DF can be attained from a vast collection of carbohydrates, many of them easily attainable in low-cost substrates (wastes), while producing several valuable coproducts, which makes it potentially more cost-effective (Venkata et al., 2016). This biological degradation process can be undertaken by various species, facultative or strict anaerobes, the more representative of which being: *Clostridium*, *Prevotella*, *Lactobacillus*, *Selenomonas*, *Enterobacter* and *Megasphaera*. The following picture (Fig 11.) depicts the most common metabolic pathways found in such strains, more specifically, those involved in H_2 production.

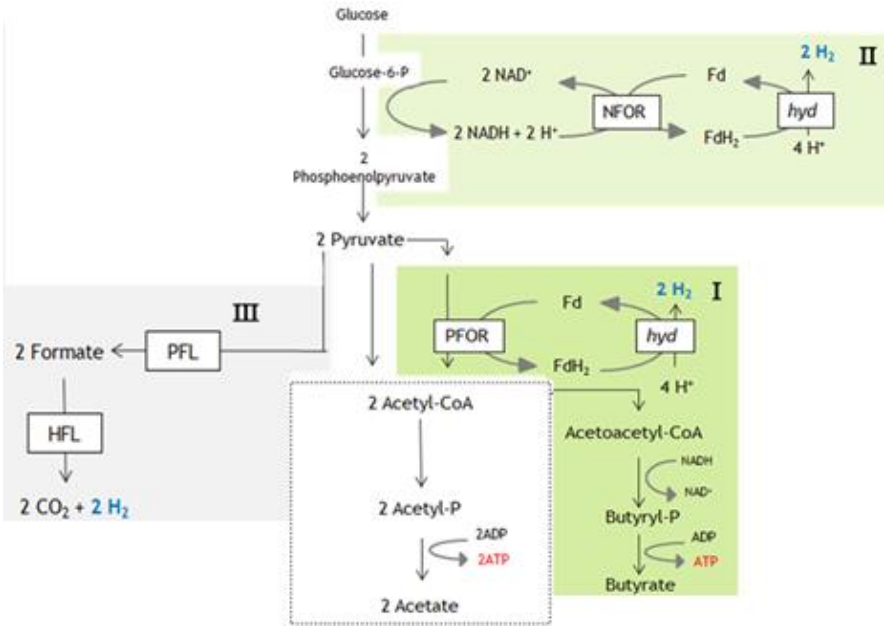

Fig 10. Metabolic pathways for biological H_2 production. **Subpathway I**: Piruvate-ferredoxin oxireductase (Pfor); **subpathway II**: NADH-ferredoxin oxireductase (Nfor); **subpathway III**: pyruvate formate lyase (Pfl) (adapted from Harzevili & Hiligsmann, 2017).

Subpathway I is performed mainly by strict anaerobes such as, for example, *Clostridia*, and is mediated by the pyruvate-ferredoxin oxireductase enzyme (Pfor). Through this conversion process, the glucose molecules are converted through the Embden-Meyerhof-Parnas (EMP) pathway until the formation of pyruvate. During this stage, ATP is generated. Pyruvate is then further converted into acetyl-coenzyme A with the assistance of the enzymatic complex Pfor, during which the reduced form of the ferredoxin is produced (FdH_2). The reoxidization of Fd_{H2} by the cytosolic [FeFe] – hydrogenase, at low H_2 partial pressure, leads to the production of 2 mol of H_2 per mol of

converted pyruvate (Harzevili & Hiligsmann, 2017). The Nfor pathway (subpathway II) intervenes directly in the EMP cycle. During the conversion of glucose into pyruvate, at low H_2 partial pressures, strict anaerobes are capable of catalizing the reduction of NAD^+ through the Nfor enzymatic complex. The regeneration of Nfor by the [FeFe] – hydrogenase leads to an aditional 2 mol H_2 per mol of glucose. Subpathway III depicts a pathway characteristic of facultative anaerobic enteric bacteria. Through the activity of Pyruvate formate lyase (Pfl), pyruvate can be converted in the appropriate conditions, into formate and acetyl-CoA. The acid is then oxidized into CO_2 and the electrons released form this process are directed into the hydrogenase complex (Hydrogen formate lyase, Hfl) which generates H_2 through the reduction of available protons. This pathway generates a maximum yield of 2 mol of H_2 per mol of consumed glucose. Overall, the subpathway I has the potential of achieving higher H_2 yields when compared to its counterparts. However, in practice, there is a very low probability of the bacterial communities of undertaking one single pathway. Summing the above information, the maximum theoretic H_2 yield of DF is 4 mol of H_2 per mol of converted glucose. However, H_2 production is defined by both the type of metabolism undertaken by the bacteria and the oxidation level of the end products. The formation of butyrate and lactate are generally associated with lower levels of H_2 production while acetate will generally implicate higher H_2 levels (Levin et al., 2004). This characteristic leads, in a practical setting, to a shift in metabolism according to environmental conditions, i.e., there is cocurrent production of acetic and butyric acids in a mixed type of metabolism. For example, the bacteria *Clostridium butyricum* is characterized by a mixed metabolism, converting glucose into both acetate and butyrate during growth. As a last addendum to this description, it is important to highlight that glucose can also be converted into lactic acid. Lactate is catalyzed through the action of lactate dehydrogenase with accompanying reoxidation of NADH and yields no H_2 production. Therefore, the production of lactate is generally indicative of a disadvantageous shift in metabolism.

2.6 Summary and Outlook

The present chapter attempted to underline the importance of the food waste thematic and its undeniable impact on the worldwide and national food security. Focus was given to opportunities and advantages of the minimisation of food waste production while referring the current status of its production pattern and the methods for its disposal.

Most importantly, biological hydrogen production was suggested as a possible valorisation method for the conversion of produced food waste. The following chapter will focus on the current state of biological hydrogen production technology and its particular advantages, drawbacks and bottlenecks to be surpassed.

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3. State of the art

3. State of the art

3.1. Food waste valorisation through dark fermentation

The biological conversion of FW is not a novel idea. The treatment of this type of waste throughout the years tended to be fairly straightforward, even under uncontrolled conditions such as landfilling or waste dumping sites, and its remnant materials – such as compost – have long since been used as a nutrient source for agriculture (Chiew et al., 2015). Several studies have been steadily developed over the last two decades focusing on the conversion potential of FW into fermentative H_2. Research focus has been primarily directed to the influence of parameters which may increase the H_2 production rate and yield, such as variation of initial/control pH, substrate pretreatment, substrate composition and mode of operation (Jarunglumlert et al., 2018).

The simplest model for H_2 production from FW focused on the non-sterile mesophilic fermentation without substrate pretreatment. Kim et al. (2009), Pan et al. (2008) and Arslan et al. (2015), successfully implemented this system attaining 4.4, 57.0 and 82.4 ml H_2 g^{-1}_{VS}, respectively. These values are generally lesser than those obtained under thermophilic conditions as it was shown by Kim et al. (2011a). This study compared the fermentation of FW at a temperature range between 35-60 ºC without the addition of bacteria and concluded that the temperature increase shifted the H_2 production by, approximately, 8-fold up to a maximum of 137.2 mL H_2 g^{-1}_{VS}. Additionally, several described thermophilic bacteria can degrade cellulose and hemicellulose such as, for example, *C. thermocellum*. This bacterium has been reported to be able to successfully ferment paper sludge, a cellulose-rich material, producing a maximum of 64 mM H_2 at a temperature of 55 ºC (An et al., 2018). The choice between thermophilic or mesophilic conditions should only be performed after the analysis of the positive impact of temperature on H_2 production as the increase in temperature is associated to a rise in the overall energetic cost of the fermentation.

Table 1. Influence of the temperature and initial pH on the H_2 production potential from FW.

Inoculum	Temperature (ºC)	pH	H_2 yield	Reference
None	25	4.5	4.4 mL g^{-1}_{VS}	Kim et al., 2009

HSSS	35	6.5	122.9 mL carbohydrate-COD^{-1}	Kim et al., 2004
HSSS	37	6.5	310.0 mL g^{-1}_{VS}	Han & Shin, 2004
Seed sludge	50	8.1	57.0 mL g^{-1}_{VS}	Pan et al., 2008
Seed sludge	55	5.5	125.0 mL g^{-1}_{VS}	Shin and Youn, 2005
Seed sludge	55	5.5	205.0 mL g^{-1}_{VS}	Chu et al., 2008
Seed sludge	55	7.1	70.7 mL g^{-1}_{VS}	Algapani et al., 2016

HSSS – heat shocked seed sludge.

Operational and initial pH values are also relevant factors for the proper conversion of FW. According to literature, the optimum pH for acidogenesis varies between 5.5 and 7.0 (Das & Verizoglu, 2001), being heavily inhibited for pH values underneath the referred range. As H_2 production is characterised by the continuous accumulation of organic acids, a higher initial pH supports a higher/more extensive carbohydrate conversion in batch fermentations, without pH control. Kim et al. (2011b) analysed the influence of the initial pH in the fermentation of FW (ranging from 6-9) and determined that the most adequate condition for fermentation was a pH of 8.0, which enabled a maximum H_2 yield of 1.9 mol H_2 mol^{-1}_{sugars}. This value represents approximately 50% of the maximum H_2 theoretical yield (Harzevill and Hiligsmann, 2018). However, in fermentations with higher initial organic loading (OL) the absence of pH control is likely to result in bacterial inhibition well before the complete conversion of the supplied carbohydrates. This factor leads to the use of lower operational pH values while introducing a pH control system which maintains the culture out of inhibitory conditions (Kim et al., 2008).

The inoculum is of utmost importance for a successful H_2 fermentation. Most H_2 production studies opt by taking advantage of the substrate microbiota and bypassing completely the addition of inoculum, or by using mixed cultures. Generally, fermentations without the addition of inoculum display lesser H_2 yields as the substrate might lack the presence of hydrogenogenic bacteria in its bacterial community (Argun and Dao, 2017), i.e., cellular growth is registered but accompanied by the production of non-H_2 related metabolites, such as lactic acid (Harzevill and Hiligsmann, 2018). The mixed inocula used in this context are commonly bacteria communities that are harvested from established large-scale bioreactors such as, for example wastewater treatment

anaerobic reactors. These reactors are generally geared towards CH_4 production which, in practice, requires the inactivation of the hydrogenotrophic bacteria present in the community. This effect can be achieved by making use of the capacity of acidogenic bacteria to produce spores when under stress-inducing fermentation conditions, conversely to methanogenic hydrogenotrophic bacteria (Cohen et al., 1985). Therefore, a simple heat-shock pretreatment (e.g. 90-100 ºC) will be enough to inactivate unwanted microorganisms and potentially increase the H_2 production (Ren et al., 2008). Studies by Kim et al. (2004) and Arslan et al. (2015) used this strategy with varying degrees of success, achieving H_2 production yields from FW of 67.0 and 80.1 mL g^{-1}_{VS}, respectively. Additionally, a similar effect can be caused by inoculum pretreatment with acid or alkaline solutions, aeration or ozonation (Rafieenia et al., 2018). The use of pure microbial cultures as inoculum is also an option though often undervalued due to the inherent need to perform a sterilisation stages prior to fermentation, as contaminants present in both substrate and environment might divert the carbon source towards unwanted metabolic pathways. While sterilisation is energetically costly due to the high-temperature usage, this fact can be counteracted by the higher selective pressure caused by using specific cultures, pushing the fermentation more consistently towards H_2 production (Elsharnouby et al., 2013). Examples of H_2 producing bacteria used for H_2 fermentation are *Clostridium butyricum*, *Enterobacter aerogenes*, *Clostridium beijerinckii*, *Clostridium acetobutylicum* and *Clostridium pasteurianum*, etc. (Hu et al., 2014; Kim et al., 2008; Noike et al., 2002; Yokoi et al., 2002).

Table 2. Influence of the type of culture on the mesophilic H_2 production potential from FW in batch fermentation.

Inoculum	Temperature (ºC)	pH	H_2 yield	Reference
Clostridium butyricum	35	6.8	39.2 mL g^{-1}_{VS}	Hu et al., 2014
Enterobacter aerogenes	35	6.8	8.5 mL g^{-1}_{VS}	Hu et al., 2014
Clostridium beijerinckii	35	6.8	17 mL g^{-1}_{VS}	Hu et al., 2014
Enterobacter cloacae	35	7.0	155.2 mL g^{-1}_{VS}	Xiao et al., 2013
Enterobacter aerogenes	35	7.0	91.4 mL g^{-1}_{VS}	Xiao et al., 2013
Clostridium beijerinckii	35	7.0	128 mL COD^{-1}	Kim et al., 2008

Another important parameter to take into consideration for a successful fermentation is the composition of the substrate and the medium. Food waste is an incredibly eclectic mixture of carbohydrates, proteins, fat and assorted nutrients (metals, ions, etc.), among others (Dinesh et al., 2018). The variety of FW components is generally a positive factor for DF performance. While carbohydrates are the main substrate for DF, proteins, for example, are also incredibly valuable as they act as nitrogen source for bacterial growth (Dinesh et al., 2018). However, while FW can contain readily degradable compounds such as glucose or fructose, it is also highly likely it might be composed by polymeric compounds such as starch and cellulose, for example, for which conversion is less straightforward (Han and Shin, 2004). Many studies focused the introduction of a pretreatment stage for the degradation of polymeric compounds into more readily fermentable substances. Commonly employed pretreatment strategies are as follows: acid, alkaline and enzymatic hydrolysis, steam and hydrothermal pretreatment (Dinesh et al., 2018). As with the application of sterilisation, the use of a pretreatment implies an increase in the energy expenditure as well as the need for additional catalysts when applicable. Therefore, its use must be carefully evaluated after analysis of its influence upon the fermentation.

Table 3. Influence of the pretreatment process on the H_2 production potential from FW.

Pretreatment	Inoculum	Temperature (ºC)	pH	H_2 yield ($mL\ g^{-1}_{VS}$)	Reference
Heat (90 ºC, 20 min)	None	35	7.0	153.5	Kim et al., 2009
Heat (70 ºC, 30 min)	None	37	5.5	70	Elbeshbishy et al., 2011
Ultrasonication (79 kJ g_{TS}^{-1})	None	37	5.5	97	Elbeshbishy et al., 2011
Acid (pH=3.0)	None	37	5.5	55	Elbeshbishy et al., 2011
Ultrasonication/acid (79 kJ g_{TS}^{-1}, pH=3.0)	None	37	5.5	118	Elbeshbishy et al., 2011
Alkaline (pH=11.0)	None	37	5.5	46	Elbeshbishy et al., 2011
Alkaline (pH = 12.5)	HSSS	35	5.3	80.9	Kim et al., 2010
Enzymatic hydrolysis (*A. awamori* and *A. Oryzae*)	HSSS	37	4.6	219.9	Han et al., 2015

HSSS – heat shocked seed sludge.

The nutrients present in the DF substrates, such as nitrogen, phosphorus, assorted metals, etc., must also be characterised and quantified. Like carbohydrates, these nutrients

are fundamental to the bacterial growth and fermentation performance, but they might be present in a chemical form which does not allow its metabolization or in insufficient quantity (Das and Veziroglu, 2008). Low C/N ratios, for example, might remove the need for supplementation of the culture media with additional nitrogen sources, lowering the overall cost of the procedure. In practice, however, the variable nature of FW composition makes this difficult and studies investigating this factor are scarce. Kumar et al. (2015) concluded that the addition of nitrogen sources such as tryptone and yeast extract can have a positive impact on the fermentation of FW. The introduction of yeast extract/tryptone on the fermentation of beverage production wastewater bioaugmented by *E. coli* improved total H_2 production by, approximately, 10 %. However, as to avoid increasing process costs, other studies opted for the use of additional wastes whose content can function as additives. Kim et al. (2004) and Zhu et al. (2008) observed that the use of sewage sludge as co-substrate increased H_2 production up to a maximum of 60.1 and 112 mL H_2 g^{-1}_{VS}, respectively. Kim et al. (2011c) used a similar same co-substrate to circumvent the addition of trace elements, promoting a 13% increase in H_2 production when compared to the non-supplemented assay.

The last factor that must be taken into consideration is the mode of bioreactor operation. The more common types of fermentative operation for H_2 production are: batch, fed-batch, anaerobic sequential batch (ASBR), continuously stirred tank reactor (CSTR), upflow anaerobic sludge blanket reactor (UASB) and plug flow (PFR). Most studies in FW conversion into H_2 focus on the CSTR operating system as it allows for improved mixing and consequent mass transfer, as well as greater control of operational parameters (pH, HRT, etc.) in comparison with the PFR or UASB (Singh et al., 2014). The use of ASBR is also well established, permitting a semi-continuous production process, with periodic removal of inhibiting metabolites and supplementation with required nutrients. Both CSTR and ASBR can theoretically be tailored to keep the culture, either mixed or pure, in an exponential growth stage.

Table 4. H_2 production potential from FW according to mode of bioreactor operation.

Type of operation	Inoculum	Temperature (º C)	pH	H_2 yield (mL g^{-1}_{VS})	Reference
ASBR	HSSS[1]	35	5.3	63.0	Kim and Shin, 2008
ASBR	HSSS[1]	35	5.3	80.9	Kim et al., 2010

CSTR	Seed sludge[2]	35	5.0	261	Reungsang et al., 2013
CSTR	Seed sludge[1]	55	5.5	125.0	Shin and Youn, 2005
CSTR	Seed sludge[3]	55	7.0	16.5	Karlsson et al., 2008
CSTR	Seed sludge[4]	55	5.5	104.5	Algapani et al., 2017
CSTR	Seed sludge[4]	55	5.5	135	Algapani et al., 2018
CSTR	None	35	5.5	96.3 mL H_2 g^{-1} FW_{in}	Alexandropoulou et al., 2018
CSTR	None	35	5.5	101.8 mL H_2 g^{-1} FW_{in}	Alexandropoulou et al., 2018

HSSS – heat shocked seed sludge.

[1] – seed sludge from a wastewater treatment plant anaerobic reactor.
[2] – seed sludge from an upflow anaerobic sludge blanket (UASB) reactor of a brewery company.
[3] – seed sludge from local methanogenic fermenters.
[4] – full scale thermophilic reactor for maize straw treatment.

The HRT influence greatly the performance of the fermentation. Shorter HRTs can be used as selective pressure towards the elimination of methanogenic bacteria which, due to their slower growth rate, are washed out of the system as denoted by Reunsang et al. (2013). The imposition of HRT of 60 hours selected hydrogen producing bacteria (261 mL g^{-1}_{VS}) while removing acetogenic and lactic acid bacteria from the population. Salem et al. (2018) on the other hand, concluded that lower HRT were more conducive to acidogenic fermentation, registering a maximum of 150 mL H_2 g^{-1}_{VS} for an HRT of 16 hours. As with the remaining operating parameters, both the HRT and type of operation must be tailored to the type of substrate and culture in use.

The lack of consensus on the appropriate method for production is undeniable. The variability of both product and microorganisms used for conversion imply that, from study to study, yields, hydrolysis rates and overall H_2 productions are not consistent, resulting in projected results which are not viable or efficient. Furthermore, as the readiness level of this technology is rather low, the technoeconomic analysis of the process is still in its infancy and its impacts are still largely unaccounted for. Han et al. (2015) projected the possibility of using a combination of solid-state fermentation and dark fermentation for conversion of FW into H_2. The study concludes that a process such as the one described would be viable but assumes a H_2 production cost of 22.4 € kg^{-1}, well above the production cost through natural gas steam methane reforming, 1.24 € kg^{-1}

and the projected cost for electrolysis-derived H_2, 2 € kg^{-1} (de Valladares, 2017). To minimize this effect, some studies claim that it is of interest to consider both principal products and co-products in the value analysis of the process (Venkata Mohan et al., 2016), i.e., the organic acids and cellular mass obtained from the DF (hereby designated as digestate/sludge) should also be accounted for. Therefore, instead of considering a hydrogen production process from food waste, one should consider a biorefinery in which the main objective is to obtain as much value as possible from the biomass. Agler et al. (2011) suggests that the application of the concept of biorefinery to DF, i.e., the treatment of every product obtained on the fermentation as possessing intrinsic value. For example, the authors suggest that after the purification and concentration of the produced organic acids (butyrate, acetate, etc.), the recovered water and soluble nutrients can be recycled back into the process for a new fermentation cycle. The acids can be directed into further conversion processes. Sarkar et al. (2018) and Agler et al. (2010) define this venue as the carboxylate platform, a term used for the production of carboxylates as intermediates for the production of bioalcohols (butanol), polymers (such as polyhydroxyalkanoates), biodiesel (through microalgae production) and bioelectricity (through microbial fuel cells). Analysis on the topic will require consideration of the acid production associated bottlenecks such as organic acid separation, methane inhibition and production conditions which are more conclusive to the production of one acid over another.

3.2 Summary and Outlook

The study of H_2 production from FW has been through important breakthroughs but is undoubtedly bogged down by the low yields, low hydrolysis and conversion rates, the need for sterilisation associated the use of pure cultures as inoculum, the low process stability when using mixed microbial cultures as biocatalyst, and the need of temperature and pH control (Jarunglumlert et al., 2018). These issues translate heavily upon the technoeconomical viability of the overall process as well as the analysis of its environmental impacts.

4. Assessment of the adequacy of different agro-industrial wastes and byproducts for fermentative hydrogen production and the particular advantage of carob (*Ceratonia siliqua* L.) pulp

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4. Assessment of the adequacy of different agro-industrial wastes and byproducts for fermentative hydrogen production and the particular advantage of carob (*Ceratonia siliqua* L.) pulp

The present chapter results from the development of the target "Feedstock selection and characterization", consisting on the selection and appreciation of the fermentative potential of several agro-industrial residues produced in the Portuguese territory.

Abstract

The conversion of agro-industrial byproducts and residues wastes to hydrogen (H_2) by *C. butyricum* was compared. Four biomass types were selected: brewery's spent grain (BSG), corn cobs (CC), carob pulp (CP) and wheat straw (WS). The biomasses were delignified and/or saccharified, except for CP which was simply submitted to aqueous extraction, to obtain fermentable solutions with 56.2-168.4 g total sugars L^{-1}. In small-scale comparative assays, the H_2 production from WS, CC, BSG and CP reached 82.6, 126.5, 175.7 and 215.8 mL $g^{-1}_{biomass}$, respectively. The best fermentable substrate (CP) was tested in a pH-controlled batch fermentation. The H_2 production rate was 204 mL $(L\ h)^{-1}$ and a cumulative value of 3.9 L H_2 L^{-1} was achieved, corresponding to a H_2 production yield of 70.0 mL $g^{-1}_{biomass}$ or 1.6 mol $mol^{-1}_{glucose\ equivalents.}$ The experimental data were used to foresight a potential energy generation of 2.4 GWh per year in Portugal, from the use of CP as substrate for H_2 production.

4.1. Introduction

Global warming and issues of national security due to dependence on oil and gas imports have increased the renewable energy research at an unprecedented rate during the last decade (Rogelj, 2016). Regarding biomass use for biofuels, efforts based on the rational use of waste, crop leftovers and agro-industrial byproducts must be undertaken, to avoid any competition between food and energy production (Gírio et al., 2010). Any analysis concerning the production and conversion of biofuels must take into consideration which renewable resources are available at a local and regional level, therefore depending on geographic location, climate specifications and biomass availability (Valentine et al., 2012), while ensuring their possible exploration preserves the natural biodiversity, and soil, fodder and water supply (Gaurav, 2017). For example, the biomass attractiveness of some Mediterranean crops may depend on their drought

resistance, typical of a coast associated dry-land agriculture (Mantineo et al., 2009). In addition, the biomass composition should be a factor of selection, as well as the concentration and ease of depolymerisation of the constituent carbohydrates in the case of further bioconversion, whereas extractable compounds can confer added value to the biomass (Gaurav, 2017; Sanders and Bos, 2012).

Previous studies considering the flora characteristic of/or adaptable to the Mediterranean climate yielded a small selection of several cultures identified as potentially adequate energy crops such as *Ceratonia siliqua* L. or carob tree and brewery's spent grain (BSG). The carob tree is a highly drought resistant species, requires little maintenance and is recommended for afforestation in coastal areas threatened by soil erosion and desertification (Mahtout, 2018). The carob pulp (CP) is the byproduct of galactomannan extraction from the carob seeds and represents about 90% (w w^{-1}) of the total dry weight of the fruit, containing a high percentage of readily soluble sugars (up to 54% w w^{-1}). Brewery's spent grain (BSG) is a byproduct of the beverage industry, being composed by the residue of malt and grain after the production of beer, and shares a comparable content in polysaccharides (40-45% (w w^{-1}) (Mussatto et al., 2006). Unlike carob, barley is not a culture of Mediterranean origin but this cereal crop is well adapted to dry regions (Wahbi and Sinclair, 2005) and the beer industry is representative in countries like Spain, France and Portugal (Eurostat, 2017). Corn cobs (CC) and wheat straw (WS) were included in this study for comparison purposes, as the two cultures accounted for the second highest share (21.0 %) of the cereals produced in the EU-28 (Eurostat, 2017). In their majority, both materials are composed by cellulose and hemicellulose at, approximately, 45% and 35% (w w^{-1}) in CC, and 30% and 50% (w w^{-1}) in WS, respectively (Sun and Cheng, 2002).

The suggested feedstocks – CP, BSG, CC and WS - are considerably rich in carbohydrates, a characteristic that makes them suitable as substrate for dark fermentation (DF) by microbial consortia or microorganisms like *Enterobacter*, *Clostridium* or *Bacillus* (Harzevili and Hiligsmann, 2017) for hydrogen (H_2) production. Hydrogen possesses a high energy content (120 MJ kg^{-1}), is easily convertible into energy by combustion or into electricity through the use of fuel cells, and generates no greenhouse gases in its conversion (Dutta, 2014). The fermentative H_2 production is accompanied by the production of a vast array of organic acids which are considered high-value products and can be further valorised *e.g.* for photoproduction of H_2 (Luongo et al., 2017) or for the production of polyhydroxyalkanoates for bioplastics (Moita et al., 2014).

Furthermore, this H_2 production process requires neither light or O_2, can convert a vast array of carbon sources, including waste streams, and achieves higher H_2 production rates when compared to other biological H_2 production systems (Veras et al., 2017). Naturally, the fermentative H_2 production also has limitations, which are mostly associated with the low production yields and the cost of the fermentable raw materials (Boodhun et al., 2017). The strategies used to overcome these problems include, for example, the integration of dark and photofermentation into a single production system (Ghosh et al., 2018), the application of different reactor configurations, flow dynamics (Show et al., 2012) or cell immobilisation (Sivagurunathan et al., 2016) and the removal of dissolved H_2 (Mandal et al., 2006). Additionally, metabolic engineering and gene regulation strategies can be employed to increase conversion efficiency and mesophilic pure cultures can be replaced by thermophilic strains or microbial consortia as biocatalysts as the latter can achieve higher production yields and rates (Harzevili and Hiligsmann, 2017). The use of carbohydrate-rich byproducts or waste biomass as fermentation substrate is generally accepted as the best option to reduce the overall process costs (Rai et al., 2018).

Current sustainability concerns about resources scarcity and the preservation of natural biodiversity make it important to benchmark the locally best-positioned waste feedstocks for the production of bioenergy and bioproducts. As such, this study focused on comparing the H_2 fermentative performance of waste lignocellulosic biomasses adapted to the characteristics of the Mediterranean climate, and to select the best feedstock for which the saccharification and conversion yields may boost a future process scale-up. *C. butyricum* was chosen as a model microorganism by its robustness, its capability to attain high H_2 production yields and rates, the possibility of using a vast range of substrates, and the fact that it can be cultured efficiently at mild mesophilic conditions (Chen et al. (2005)). By the first time, the potential of CP for fermentative hydrogen production was clearly evidenced by a thorough comparison with other Mediterranean waste biomass counterparts.

4.2. Material and Methods

4.2.1. Raw biomass

CC, BSG and WS were obtained from Companhia das Lezírias (Samora Correia, Portugal), Central Society of Beers and Beverages (SCC, Vialonga, Portugal) and National Plant Breeding Station (ENMP, Elvas, Portugal), respectively. The materials

were dried to reduce moisture below 10% (w w^{-1}) and ground to a particle size of less than 3 mm (CC) or approximately 0.5 mm (BSG and WS). The CP kibbles were obtained from Chorondo & Filhos Lda. (Loulé, Portugal).

4.2.2. Biomass pretreatment and saccharification

The methods for the pretreatment and hydrolysis of each feedstock were selected from the literature (Table 1) (Dominguez et al., 1997; Eken-Sarago et al., 1998). Wheat straw and CC required a previous delignification stage. Carob pulp was submitted to an aqueous extraction to obtain a sugar-rich extract. The feedstock was mixed with water in a 1:2 solid to liquid (g to mL) ratio and incubated at 25 ºC for 6 hours (150 rpm). The remaining mixture was pressed for separation of the aqueous phase and posterior filtration of the liquid for removal of particulates (Lima et al., 2012). All hydrolysates were neutralised with NaOH.

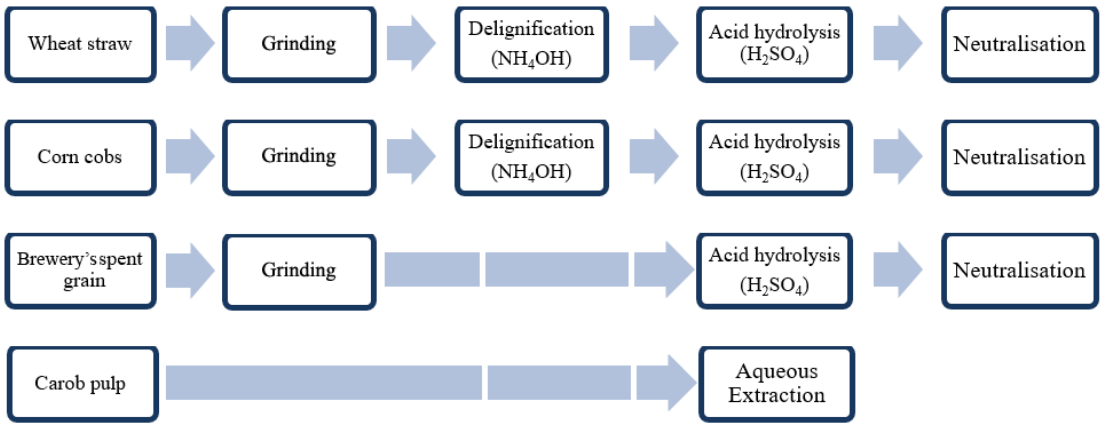

Fig. 1 – Graphical representation of the pretreatment and saccharification stages applied to the studied feedstock: Brewery's spent grain, Corn cobs, Carob pulp and Wheat straw.

The hydrolysates and the CP aqueous extract were filter sterilised (0.2 µm, Pall Life Sciences, USA) into serum flasks, stoppered with butyl rubber stoppers and aluminium caps. The gas phase was aseptically replaced by N_2 and the hydrolysates were used as carbon and energy source in the fermentations.

Table 1. Pretreatment and hydrolysis or aqueous extraction applied to the respective feedstock biomass

Substrate	Pretreatment and hydrolysis or extraction	Solid/liquid ratio (g to mL)	Temperature (ºC)	Time	Reference
Brewery's spent grain	Hydrolysis with H_2SO_4 10% (w v^{-1})	1:8	120	17 min	(modified from Mussato et al., 2006)
Carob pulp	Aqueous extraction	1:2	25	16 hours	(Lima et al., 2012)
Corn Cob	Delignification with NH_4OH 10% (w v^{-1})	1:5	26	24 hours	(Dominguez et al., 1997)
	Hydrolysis with H_2SO_4 2.94% (w v^{-1})	1:4	120	30 min	(Eken-Sarago et al., 1998)
Wheat Straw	Delignification with NH_4OH 10% (w v^{-1})	1:5	26	24 hours	(Dominguez et al., 1997)
	Hydrolysis with H_2SO_4 1.2% (w v^{-1})	1:7	130	150 min	(Dominguez et al., 1997)

4.2.3. Bacterial strain and culture media

The bacterial strain used in this study was *Clostridium butyricum* DSM 10702, from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *C. butyricum* was pre-cultured in Reinforced Clostridial Medium (RCM) (Difco laboratories, Le Pont de Claix, France). The batch fermentations in serum bottles were performed in basal modified medium (BM1) with the following composition (per litre of 50 mM phosphate buffer, pH 6.8): 5 g trypticase without dextrose, 5 g yeast nitrogen base, 0.56 g cysteine-HCl, 10 mL solution A (100.0 g L^{-1} NH_4Cl, 10.0 g L^{-1} $MgCl_2.6H_2O$, 10.0 g L^{-1} $CaCl_2.2H_2O$), 2 mL solution B (200.0 g L^{-1} $K_2HPO_4.3H_2O$), 2 mL solution C (0.5 g L^{-1} resazurin) and 10 mL solution D (500 mg L^{-1} $Na_2.EDTA.2H_2O$, 150 mg L^{-1} $CoCl_2.6H_2O$, 100 mg L^{-1} $MnCl_2.4H_2O$, 100 mg L^{-1} $FeSO_4.7H_2O$, 100 mg L^{-1} $ZuCl_2$, 40 mg L^{-1} $AlCl_3.6H_2O$, 30 mg L^{-1} $Na_2WO_4.2H_2O$, 20 mg L^{-1} $CuCl_2.2H_2O$, 20 mg L^{-1} $NiSO_4.6H_2O$, 10 mg L^{-1} H_2SeO_3, 10 mg L^{-1} H_3BO_3 and 10 mg L^{-1} $Na_2MoO_4.2H_2O$). In the fermentations with pH control, the nutrients were minimised to a C:N ratio of 3:1 (Minimum Mineral Medium, MMM), containing 12.0 g NH_4Cl, 3.3 mg $FeSO_4.7H_2O$, 0.56 g cysteine-$HCl.H_2O$ and 1 mg resazurin per litre of 50 mM phosphate buffer (pH 6.8), prepared under anoxic conditions, with replacement of the gas phase by N_2.

4.2.4. Comparative batch serum bottle fermentations

The comparative small-scale fermentations were performed in 120 mL serum flasks filled with 20 mL of BM1 supplemented with the respective carbon and energy source at approximately 4 ± 0.5 g total sugars L^{-1} due to physical limitations of the serum bottles. *C. butyricum* pre-cultured in RCM at 37 ºC for 16 h was used for inoculation at 2.5% (v v^{-1}). The bottles were incubated at 37 ºC, 150 rpm, for a total of 48 hours. Three replicates were prepared for each gas and liquid sampling time and the results were expressed as mean ± standard deviation (SD).

4.2.5. Batch fermentation with pH control

The batch fermentation was performed in a 1.65 L lab scale double jacketed bioreactor with a working volume of 0.5 L, equipped with a pH electrode (Metter Toledo, Columbus, Ohio, USA), agitation control (Labinco, Breda, The Netherlands), and neoprene tubbing for one inlet for nitrogen gas, a second inlet for the hydrolysate or solution used as carbon and energy source, one exit for the effluent biogas connected to the biogas collecting system, and one additional exit for the collection of liquid samples. A pH controller (SGI, California, USA) for the automatic addition of sterile 2 M NaOH was used to control the pH to a minimum of 5.5 ± 0.1 throughout the fermentation runtime. The biogas produced in the bioreactor was continuously collected in inverted stoppered serum flasks filled with NaOH (250 mM) for CO_2 stripping, and the production volume was ascertained through the liquid displacement method (Ortigueira et al., 2015a).

The fermentation medium was supplemented with 48 mL of filter sterilised and anoxic carob extract corresponding to approximately 20 g L^{-1} total sugars. The medium was inoculated with *C. butyricum* at 5 % (v v^{-1}), and the temperature and agitation were kept at 37 ºC and 150 rpm, respectively. Approximately 2.8 L of produced biogas were collected in the inverted serum flasks for 23 h until the cessation of biogas production, whereas the liquid samples were collected from the bioreactor every two hours. Three independent batch fermentations were repeated using the same conditions. An ANOVA (single factor) test was used for statistical significance analysis, and a significant difference was considered at a level of $p \leq 0.05$.

4.2.6. Analytical methods

4.2.6.1. Chemical characterisation

The moisture of the samples was determined by oven drying at 100 ºC until constant weight and ash content was determined by thermal treatment in a muffle furnace at 550 ºC for 6 hours (Browning, 1987). The protein content was estimated by the Kjeldahl method using 6.25 as the conversion factor of total nitrogen into crude protein (Horwitz and Latimer, 2005). Fat content was determined after ether extraction in a Soxhlet system (Sukhija and Palmquist, 1988). Total sugars were quantified after treatment with H_2SO_4 (720 g kg^{-1}) according to standard methods (Browning, 1987). The total sugars were determined by the phenol–sulphuric acid method (Dubois et al., 1956).

4.2.6.2. Cell biomass and pH quantification

Liquid fermentation samples were filtered through 0.2 µm membrane filters (Pall Life Sciences, USA) which were then used to determine cell biomass after filter drying at 100 ºC for 16 hours. The pH of each triplicate in the small-scale fermentation assay was measured with a pH meter (Micro pH 2002, Barcelona, Spain).

4.2.6.3. Sugars and soluble metabolites quantification

Acetate, butyrate, formate, lactate, 5-hydroxymethyl furfural (5-HMF), furfural, sucrose and monosaccharide concentrations were determined in the cell-free supernatants in a HPLC system (LaChrom, Merck, Germany) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, USA) and a refraction index (RI) detector (LaChrom L-7490). The temperature of the column and the RI detector were kept constant at 50 °C and 45 ºC, respectively, and samples were eluted using 5 mM H_2SO_4 (flow rate = 0.4 mL min^{-1}). For sucrose, glucose, fructose and pinitol quantification in the carob pulp extract an Aminex HPX-87P, at 85 °C, 0.6 mL min^{-1}, and water as eluent was used. Solutions of each soluble metabolite were used as external standards.

4.2.6.4. Analysis of the produced biogas

Biogas samples were collected from the stoppered serum flasks through the butyl rubber stoppers by means of a air-tight syringe rated for gas chromatography (GC) analysis. A GC (Varian 430-GC) equipped with a thermal conductivity detector (TCD)

was used. H_2 and CO_2 analysis were performed using a fused silica column (Select Permanent Gases/CO_2-Molsieve 5A/Borabound Q Tandem #CP 7430). The injector and column were operated at 80 °C and the detector at 120 °C. Argon was the carrier gas at a rate of 32.4 mL min^{-1}. The GC column was kept at 30 – 60 ºC, the injector at 60 ºC and the TCD at 150 ºC. The molar concentration of H_2 and CO_2 in the produced biogas was calculated as described elsewhere (Ortigueira et al., 2015b). The volume of the produced biogas in the serum flasks was measured after injection of a 100 mL glass syringe, being equivalent to the displacement of the barrel.

4.3. Results and Discussion

4.3.1. Biomass characterisation

The selection of the feedstocks for H_2 production considered the characteristics of the Mediterranean climate and the native or well adapted vegetation types to this climate. The dry summers and the scarcity of soil nutrients which are characteristic of Mediterranean regions induce the accumulation of metabolites and water-soluble sugars in the plant tissues (Sardans and Peñuelas, 2013). Consequently, the native biomasses tend to be rich in carbohydrates, which are prime substrate for H_2 fermentative production. In order to evaluate the fermentative potential, each biomass type was chemically characterised. The results are disclosed in table 2.

Table 2. Chemical composition of the lignocellulosic fraction, protein, fat and ash content of the selected feedstocks.

Compound (%$_{d.w.}$)	**Brewery's spent grain**	**Carob pulp**	**Corn cobs**	**Wheat straw**
Cellulose	25.6	ND	39.9	38.9
Hemicelluloses	23.5	ND	41.3	29.9
Total sugars[1]	ND	46.6	ND	ND
Crude protein	26.5	5.4	3.4	0.9
Total fat	0.4	0.6	nd	1.4
Lignin	9.4	32.9 [3]	14.1	18.8
Ash	3.5	3.5	0.4	6.9
Total phenols[2]	ND	0.8 [3]	ND	ND
Others (by difference)	11.1	10.2	0.9	3.2

[1] Total sugars expressed as percentage (w w^{-1}) of glucose equivalents ($Glc_{equiv.}$)

[2] Total phenols expressed as percentage (w w^{-1}) of gallic acid equivalents ($GA_{equiv.}$).

[3] Reis, 2013.

nd, not detected; ND, not determined

The CP used in this study was composed by approximately 47% (w w^{-1}) of sugars, mostly sucrose, glucose and fructose. CC and WS possess a similar cellulose concentration, averaging 39% (w w^{-1}), while CC displayed a higher hemicellulose concentration (Table 2). BSG showed lower cellulose and hemicellulose concentration, 25.6 and 23.5% (w w^{-1}), respectively, than the former feedstocks. Carob pulp, BSG, CC and WS also possess a considerable amount of lignin. Lignin cannot easily be biodegraded anaerobically and its presence in the feedstock diminishes the effectiveness of milder pretreatment processes, affecting the overall carbon conversion (Sun and Cheng, 2002). BSG contained the highest protein content, 26.5 (w w^{-1}). This is representative of a high nitrogen content, an essential nutrient for bacterial growth, and its presence in the feedstock may reduce the supplementation needs of the fermentation media.

4.3.2. Biomass pretreatment and saccharification

Biomass saccharification was performed by acid hydrolysis for all the materials except CP that was submitted to a simple aqueous extraction. Fig 1 summarises the pretreatment and the acid hydrolysis or extraction stages to which each biomass type was submitted. Prior to hydrolysis, and as suggested by data from the literature, CC and WS were submitted to delignification to indirectly improve the breakdown of the cellulosic structure, as lignin stabilises and hardens the feedstock structure (Dominguez et al., 1997). Previous studies showed that BSG was successfully saccharified after acid hydrolysis without prior delignification (Mussatto et al., 2006). BSG was composed by similar concentrations of cellulose and hemicellulose (Table 2). Published data underlined that dilute acid hydrolysis with mild temperature and acid conditions are efficient in the degradation of BSG hemicellulosic fraction (Mussatto and Roberto, 2005). Nonetheless, in this study, the conditions of acid hydrolysis applied to BSG for bioethanol production were more severe than those already reported. In turn, WS and CC were submitted to an initial delignification with NH_4OH (10% (w v^{-1})) (Dominguez et al.,

1997). The acid hydrolysis conditions chosen for each biomass type were extracted or modified from the literature (Table 1), and the option for low acid concentrations and short hydrolysis time to avoid the production of inhibitory sugar degradation products was chosen. The CP extract was prepared by aqueous extraction of the soluble sugars as described elsewhere (Lima et al., 2012). The acid hydrolysates required pH neutralisation with NaOH before its addition to the fermentation media. The hydrolysates and aqueous extract produced were characterised in terms of sugars and sugar degradation compounds (Table 3).

Table 3. Concentration of sugars and sugar degradation compounds in the liquid fractions obtained after biomass delignification, and/or acid hydrolysis, or aqueous extraction.

Compound (g L^{-1})	**Brewery's spent grain**	**Carob pulp**	**Corn cobs**	**Wheat straw**
Sucrose	nd	91.8	nd	nd
Glucose	19.9	25.2	2.6	6.0
Xylose	13.5	nd	43.0	29.0
Arabinose	7.3	nd	6.2	6.0
Fructose	nd	21.4	nd	nd
Total sugars[1]	56.2	168.4	73.3	66.0
Pinitol	ND	14.1	NQ	NQ
Acetic acid	1.9	nd	nd	nd
Formic acid	nd	nd	nd	nd
5-HMF	nd	NQ	nd	nd
Furfural	0.5	NQ	0.7	0.2
Total phenols[2]	NQ	0.3	NQ	NQ

[1] Total sugars expressed in g L^{-1} of glucose equivalents ($Glc_{equiv.}$)

[2], Expressed as percentage (w w^{-1}) of gallic acid equivalents ($Ga_{equiv.}$)

nd, not detected

NQ, not quantified

A detailed analysis of the monosaccharides composition in the CC and WS hydrolysates identified xylose as the main sugar component of the liquid fraction as well as smaller fractions of glucose. This is consistent with the fact that less severe saccharification conditions succeed in the solubilisation of hemicelluloses but are

insufficient for an extensive hydrolysis of cellulose (Dominguez et al., 1997; Mussatto and Roberto, 2005). The aqueous extraction of CP sugars enabled a maximum of 87.5% of sugar solubilisation. Conversely, lower sugar recoveries of 35.1% and 38.6%, and 42.4% were achieved for WS, CC and BSG, respectively, mostly because of the increased complexity of the carbohydrate matrix (cellulose, hemicellulose and lignin) in these feedstocks when compared with CP (sucrose). Minor amounts of furfural were detected in BSG, CC and WS hydrolysates, but not exceeding the 1 g L^{-1} threshold indicated by Akobi et al. (2017) as the necessary concentration for the inhibition of mesophilic acidogenic cultures. The hydrolysis of BSG produced a sugar solution with 13.5 g L^{-1} of xylose and 19.9 g L^{-1} of glucose. A concentration of 1.9 g L^{-1} acetate was also detected. The CP extract contained sucrose, glucose and fructose at 91.8, 25.2 and 21.4 g L^{-1}, respectively, and corresponded to the most concentrated sugar solution obtained from the tested feedstocks. Pinitol was also present in the CP extract at a significant concentration, 14.1 g L^{-1}, as well as phenolic compounds (0.3 g L^{-1} GAE) solubilised by the extraction process. These compounds were quantified as gallic acid equivalents, the predominant extractable phenols in CP (Papagiannopoulos et al., 2004). Phenolic compounds exert an inhibitory action over *C. butyricum* metabolic activity and, consequently, the time and temperature conditions used for CP aqueous extraction must result from a compromise for the maximisation of the soluble sugar extraction while minimising the extraction of soluble phenols. *Clostridium* sp. are metabolically versatile and have the capacity to degrade a wide range of organic materials including to a certain extent some aromatic compounds. According to Tai et al. (2010), *C. butyricum* suffers no significant inhibition up to 1 g L^{-1} phenol, a concentration clearly higher than the one determined in this study.

4.3.3. Comparative batch fermentations by *C. butyricum*

The hydrolysates and the aqueous extract produced from the different feedstocks were tested for H_2 production in comparative batch fermentations by *C. butyricum*. Fig 2 depicts cell growth, sugar consumption and H_2 and organic acids cumulative production.

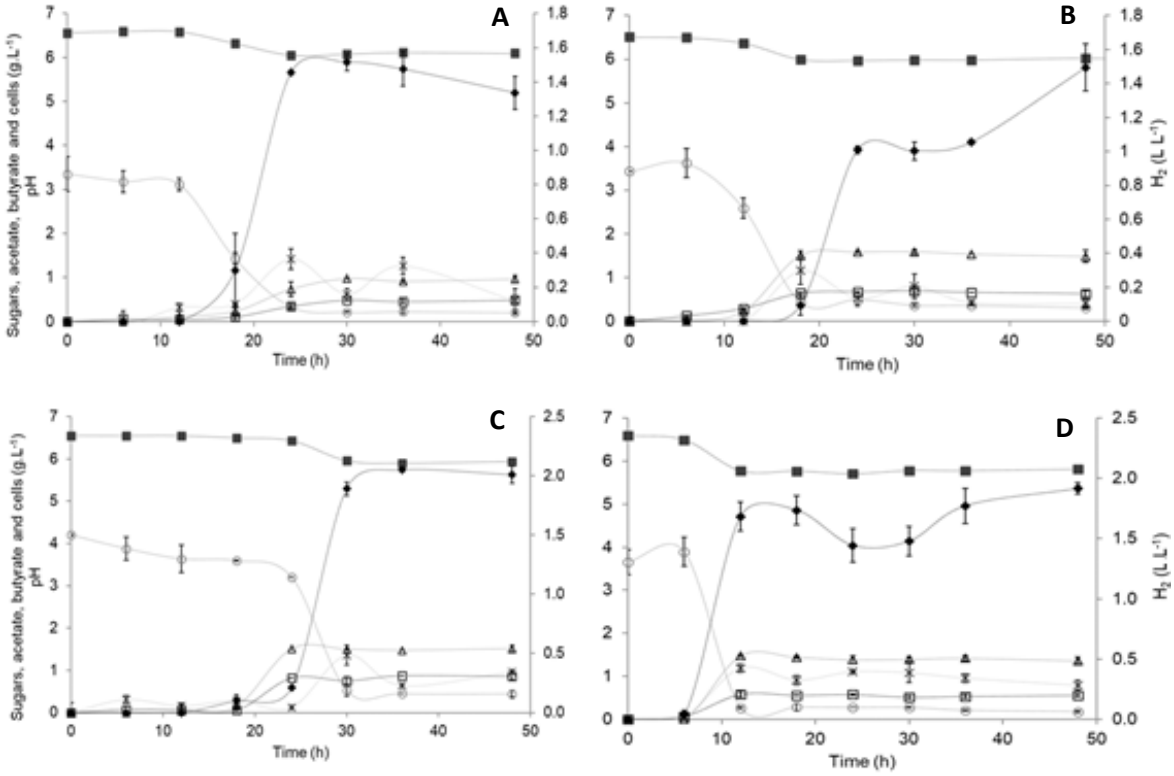

Fig. 2 – Time-course of H_2 production, sugar consumption, butyrate and acetate production, and cell dry weight using different feedstock as carbon and energy source: (A) Wheat straw; B) Brewery's spent grain; C) Corn cobs; D) Carob pulp) o – total sugars; ■ – pH; × – cells; Δ - Butyrate; □ - Acetate; ♦ – H_2).

The fermentations of WS, BSG and CC hydrolysates exhibited longer lag phases of 18, 19 and 24 hours, respectively, while the fermentation of CP extract started approximately 5 hours after inoculation (Fig. 2). In the case of WS and CC, this can be due to the delignification with NH_4OH, as it might have remained partially adsorbed on the feedstock and persisted in the hydrolysate in the form of ammonium and hydroxide ions. Conversely, the longer lag phase observed in the BSG fermentation was likely a consequence of the use of a concentrated acid solution in the saccharification stage and the subsequent neutralisation with NaOH before fermentation. The addition of sodium may generate an extracellular concentration that requires energy from the cells to balance the Na^+ across the membrane, thus affecting *C. butyricum* growth and decreasing the rate of H_2 production (Lee et al., 2012). Hydrogen production started immediately after the respective lag phases, increased rapidly in all assays and was accompanied by organic acids production and substantial pH decrease (Fig. 2).

Table 4 registers the results of the different fermentation assays by *C. butyricum*. The Anova analysis of the H_2 production rates and cumulative H_2 production showed that

the five fermentation assays were significantly different ($p<\alpha$) from each other. The total H_2 production expressed per mass unit of feedstock incorporates key differences associated with the adequacy of each biomass type for bioconversion by DF. Accordingly, the fermentation of CP was the most successful, reaching a maximum of 215.8 mL H_2 $g^{-1}_{d.w.}$, followed by the fermentation of BSG that reached 175.7 mL H_2 $g^{-1}_{d.w.}$ (Table 4). The fermentations of CC and WS achieved H_2 yields of 126.5 and 82.6 mL H_2 $g^{-1}_{d.w}$. These results are directly linked with the biomass composition in carbohydrates and the efficiency of the saccharification process. The lower values were associated with higher polysaccharide complexity and the probable need for more severe saccharification conditions. This was the case of WS and CC, where the saccharification degree was 35.1 and 38.6%, respectively, contrasting with the value of 87.5% in the case of CP. Nonetheless, the H_2 production yields achieved in the various fermentation assays were comparable to those already registered in literature. Wheat straw and CC have been extensively tested in dark fermentation experiments, with maximum H_2 production yields in the range of 79.5 mL g^{-1} of WS xylan, 44.7 mL g^{-1} of WS dry biomass, and 119 mL g^{-1} of CC (Chen et al., 2005; Ivanova et al., 2009; Valdez-Vazquez et al., 2015).

Table 4. Results of the fermentation by *C. butyricum* of the different sugar-rich liquid fractions obtained after biomass delignification, and/or acid hydrolysis, or aqueous extraction.

Biomass feedstock	Sugar consumption (%)[1]	Cumulative H_2 production (L L^{-1})[2]	$Y_{p/s}$ H_2 (mL $g^{-1}_{d.w.}$)[3]	Q_p H_2 (mL $(L.h)^{-1}$)[4]	H_2/CO_2 (mol mol^{-1})[5]	Butyrate production (mol L^{-1})[6]
Brewery's spent grain	90.2 ± 0.5	1.3±0.0	175.7±1.1	56.1±0.7	1.7±0.0	0.018±0.004
Carob pulp	94.3±1.0	1.9±0.1	215.8±8.0	140.3±0.7	1.6±0.0	0.016±0.001
Corn cob	91.1±0.2	2.1±0.0	126.5±1.7	56.9±0.6	1.8±0.0	0.017±0.004
Wheat straw	94.7±0.4	1.4±0.0	82.6±1.9	49.7±0.8	1.7±0.0	0.012±0.000

[1] Total sugars consumption (%) – percentage of initial sugars consumed.

[2] Cumulative H_2 production (L L^{-1}) – maximum volume of H_2 produced (L) per litre of medium (L).

[3] $Y_{p/s}$ H_2 (mL $g_{d.w.}^{-1}$) – H_2 yield: ratio between the maximum volume of H_2 produced (mL) and feedstock dry weight supplied ($g_{d.w.}$).

[4] Q_p H_2 (mol $(L.h)^{-1}$) – H_2 production rate: ratio of H_2 concentration (mL L^{-1}_{medium}) and fermentation time (h).

[5] H_2/CO_2 (mol mol^{-1}) – ratio between H_2 (mol H_2) and CO_2 (mol CO_2) in the produced biogas.

[6] Butyrate production (mol L^{-1}) – maximum moles of butyrate produced (mol) per litre of medium (L)

In a closed system without pH control as the gas tight serum flasks, the initial sugar concentration must be low to prevent excessive gas production and guarantee as much as possible the sugars depletion. For this reason, in the comparative experiments, the initial sugar concentration did not exceed 5 g L^{-1} and all the fermentations achieved more than 90% of total sugar consumption. The fermentations supplemented with CC hydrolysate and CP extract achieved the highest cumulative H_2 production, 2.1 and 1.9 L L^{-1}, respectively. Lesser productions were achieved in the fermentations of BSG and WS hydrolysates, 1.3 and 1.4 L H_2 L^{-1}, respectively.

The scale-up of fermentative hydrogen must necessarily address the co-production of organic acids which can find industrial application and confer economic benefits to the overall carbon-conversion process. Acetate and butyrate were quantified in the fermentation supernatants of all the assays, averaging between 9 - 13 and 6 - 18 mmol L^{-1}, respectively. According to Mizuno et al. (2000), the byproducts formation is heavily dependent on the H_2 partial pressure in the reaction vessel. Low acetate to butyrate ratios indicate that the metabolism of *C. butyricum* has shifted towards the production of butyrate. This behaviour is associated with a high H_2 partial pressure in the serum flask headspace (Mizuno et al., 2000). Therefore, the ratio of acetate to butyrate helps to assess the state of H_2 production by pure microbial cultures in such gas tight systems. Lowest acetate to butyrate ratios of 0.71, 0.74 and 0.78 were attained in the fermentation of CP, BSG and CC, respectively, which is coherent with the relative high H_2 accumulation displayed in the three assays.

The most adequate substrate for H_2 fermentation was evaluated according to the parameters discussed above. Whereas the highest value of cumulative H_2 production was attained in the fermentation of CC hydrolysate, it was not significantly different from that obtained with CP extract. Comparatively, the fermentation of CP produced both to the shortest lag period and the smallest time required to achieve the maximum H_2 production, of 4 and 12 hours, respectively. The suitability of CP for fermentative H_2 production was also demonstrated by the highest production yield per mass of feedstock that was

achieved, and it is additionally reinforced by the fact that a simple aqueous extraction is efficient to obtain a sugar-rich fermentable solution. Considering these results the follow-up bioreactor fermentation was performed with the use of CP extract as carbon and energy source.

4.3.4. Bioreactor batch fermentation of the carob pulp extract by *C. butyricum*

Three independent batch fermentations were performed in a 1.65 L bioreactor. The larger scale applied of the fermentation assay so as the application of a pH control to a minimum of 5.5 ± 0.1 (Chen et al., 2005) enabled the concentration of total sugars to be increased up to approximately 20 g L^{-1}. Biogas samples were collected continuously while liquid samples were collected with intervals of 2 hours. Anova analysis of the H_2 production rates and cumulative H_2 production obtained showed no significant differences in the triplicates ($p>\alpha$). Therefore, Fig 3 represents the typical profiles of H_2 and organic acids production, sugar consumption and pH.

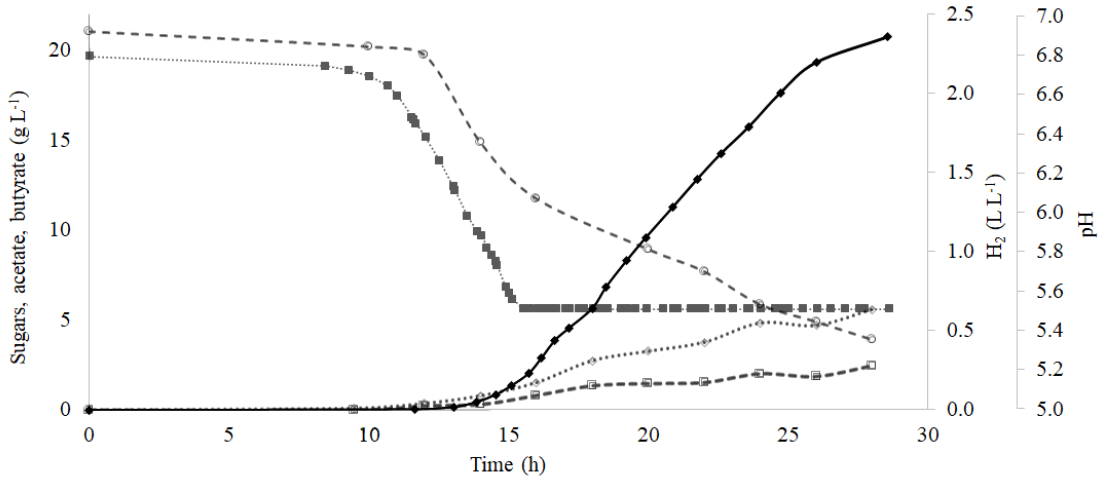

Fig. 3 – Time-course of H_2 production, sugar consumption, and butyrate and acetate production by *C. butyricum* through conversion of carob pulp extract: o – Total sugars; ■ – pH; Δ - Butyrate; □ - Acetate; ♦ – H_2.

Hydrogen production was detected 11.6 hours after the inoculation of the bioreactor medium with *C. butyricum* and increased exponentially for approximately 24 hours (Fig. 3). The H_2 production rate achieved 204 mL $(L\ h)^{-1}$ (Table 5), a value which corresponds to an increase of more than two times the rate registered in the serum flasks. After 28 hours, 4.2 L biogas L^{-1} were produced with a final H_2 concentration of 70% and 92% (v v^{-1}) prior and after CO_2 stripping, respectively. The percentage of H_2 in the produced

biogas almost doubled that of the small-scale fermentation. This factor can be detrimental, as the excessive H_2 partial pressure might cause product inhibition (Beckers et al., 2015), which however tends to be compensated by the absence of overpressure in the bioreactor when compared to the serum flasks. The total H_2 collected in the inverted serum flasks plus the H_2 volume accumulated in the headspace of the bioreactor totalised 3.9 L L^{-1}.

Table 5 – Results of the serum flask and bioreactor fermentations of the CP extract by *C. butyricum.*

Parameters	**Serum flasks**	**Biorreactor**
Fermentation time (hours)	12.0	28.0
H_2 yield per unit feedstock (mL H_2 $g^{-1}_{d.w.)}$	215.8	70.0
H_2 production rate (mL $(L.h)^{-1)}$	140.3	204.0
Acetate / butyrate ratio (mol mol^{-1})	0.71	0.64

Approximately 89.8% of the total sugars in the media were metabolised by *C. butyricum*. This incomplete sugar conversion might have been partially caused by the increase of organic acid concentration in the medium, inhibiting *C. butyricum* activity (Yokoi et al., 1997). By the end of the batch fermentation assay, a H_2 production yield of 70.0 mL $g_{d.w.}^{-1}$ of CP was attained, corresponding to a H_2 molar yield of 1.6 mol (mol of glucose equivalents)$^{-1}$. Fountoulakis et al. (2014) developed a continuous production process based on the conversion of carob pods by a thermally treated microbial consortium which achieved a steady H_2 production yield of 0.43 mol (mol of sugar)$^{-1}$. The major difference from the present study is that carob pods were used instead of CP and the aqueous extraction was performed at 70 ºC, which may have facilitated the solubilisation of inhibiting phenolic compounds (Lima et al., 2012).

The organic acids produced during the fermentation of CP extract by *C. butyricum* were majorly acetate and butyrate up to a concentration of 40.9 and 63.6 mmol L^{-1}, respectively. Traces of lactate were only detected after the beginning of the exponential growth phase up to a maximum of 8.6 mmol L^{-1}. Compared to the small-scale fermentation, a small decrease of the acetate to butyrate ratio to 0.64 occurred. This result is characteristic of a high H_2 partial pressure in the system, which is associated with the production of butyric acid by *C. butyricum*, as already denoted on similar assays with other carbon and energy sources (Ortigueira et al., 2015a). The referred acids, particularly acetate and butyrate, can be further converted to H_2 through photofermentation or through

microbial electrolysis (Luongo et al., 2017; Marone et al., 2017). These options increase substantially the overall H_2 yield of the conversion process.

4.3.5. Potential for H_2 production and energy generation – a case study

The purpose of this study was to provide a first look to the possibility of producing H_2 through DF using byproducts originated from agricultural cultures or agro-industrial activities well established in the Mediterranean region. The choice for a feedstock for bioenergy production should not be uniquely based on the production values (both production rate and total production yield). An ideal carbon and energy source should comply with a high H_2 production yield, low process energy input, low process costs and nutrient requirements, low production of polluting byproducts and lack of contaminants in its composition (McKendry, 2002). The majority of the feedstocks tested in this study fulfilled these prerequisites, but CP extract emerged clearly as the best fermentable substrate. In view of these considerations, the potential of CP as agro-industrial byproduct for bioenergy production was evaluated at a regional level, based in a case-study for the Portuguese reality that considered the importance and dimension of the carob culture. Portugal is the fourth largest worldwide carob producer after Spain, Italy and Morocco, presenting increasing production values from 2005 to 2011 which culminated in a total production of 21,736 tonnes of carob fruit in 2014 (FAO, 2017). Until recently, the culture of carob was performed almost exclusively because of the fruit seeds, while the remaining material, CP, is processed to animal feed. It is possible, instead, to redirect the CP for fermentative production, and in particular for H_2 production. Considering that the carob fruit comprises 90% (w w^{-1}) of pulp (Battle and Tous, 1997), a total of 19,562 tonnes of CP should be available as fermentation substrate based on the FAO data for Portugal. Similarly, the equivalent theoretical energy production from CP in the country was estimated. The yields obtained in the bioreactor batch fermentations were used to calculate the potential H_2 production, so as the H_2 low heating value of 120 MJ kg^{-1} and the H_2 density of 0.0899 kg m^{-3} (Dutta, 2014). The value of 70% for the average energy conversion efficiency was retrieved from the literature (Kirubakaran et al., 2009). Considering the Portuguese reality, the estimated value of potential H_2 production from CP was 101.7 tonnes H_2 per year, which corresponds to an energy output of 2.4 GWh. This value is equivalent to 76% of the total amount of the electric energy obtained from biomass in the year of 2018 in Portugal (Pordata, 2020). The potential of such an energy

production process from an agro-industrial byproduct as CP would thus decisively boost the share of biomass in the Portuguese renewable energy map.

4.4. Conclusions

Four agro-industrial feedstocks were elected for fermentative H_2 production by *C. butyricum*. Carob pulp produced the best results in comparative small-scale fermentation experiments. The system scale-up increased the H_2 production rate and the cumulative H_2 production. The higher fermentation yields that were obtained by the use of CP as fermentation substrate, associated with the simpler process of sugars solubilisation, pointed out that this agro-industrial byproduct would best support the development of a bioenergy production system in Mediterranean regions. Future studies should evaluate further the scale-up feasibility and economic viability of the use of CP as fermentable substrate for bioenergy and bioproducts.

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5. Improving the non-sterile food waste bioconversion to hydrogen by microwave pretreatment and bioaugmentation with *C. butyricum*

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5. Improving the non-sterile food waste bioconversion to hydrogen by microwave pretreatment and bioaugmentation with *C. butyricum*

The present chapter results from the development of the target "Biorefinery concept and optimisation of operational parameters", focusing on the design and optimisation of a simple and direct method for FW conversion which takes into account the possible intervention of the waste producer in the waste treatment process.

Abstract

This work targeted the energy recovery from food waste (FW), most specifically catering industry wastes (CIW), aiming at the implementation of a potentially participative process of CIW conditioning before the non-sterile CIW biological conversion to hydrogen (H_2). CIW conversion was initially performed under sterile conditions, achieving a maximum H_2 productivity of 249.5 ± 24.6 mL H_2 (L h)$^{-1}$ and a total H_2 production to 4.1 ± 0.2 L L^{-1}. The non-sterile operation was implemented as a way of process simplification, but the total H_2 production decreased by 59% due to the CIW native microorganisms. To counteract this effect, CIW was submitted to acid, microwave (MW), and combined acid and MW pretreatment. The application of 4 minutes MW, 550 W, efficiently controlled the CIW microbial counts. The *Clostridium butyricum* bioaugmented conversion of MW-pretreated CIW accelerated the H_2 production to 406.2 ± 8.1 mL (L h)$^{-1}$ and peaked the total H_2 production and conversion yield to 4.6 ± 0.5 L L^{-1} and 234.6 ± 55.6 mL g^{-1} sugar, respectively. These results exceeded in 63, 14 and 4%, respectively, the H_2 productivity, total production and conversion yield obtained under sterile conditions, and are encouraging for the future implementation of increasingly responsible waste valorisation practices.

5.1. Introduction

According to the intergovernmental Food and Agriculture Organisation of the United Nations (FAO), part of the worldwide food production does not actually reach the consumer, being wasted or lost well before the consumption stage (Searchinger and Heimlich, 2015). FAO estimates that approximately 1.3 billion tonnes of food produced worldwide were wasted in 2012, a value that is equivalent to a total waste of 24% of all produced food (Lipinski et al., 2013). This wastage represents not only a lost opportunity for hunger mitigation and improvement of social equality, as it also has a very distinct

and unsurmountable impact on the environment, increasing both carbon footprint and water/nutrients wastage, as well as instigating land use change and the associated biodiversity loss (Scherhaufer et al., 2018). While strategies for FW reduction are already in place, even a 50% reduction of the global FW seems a faraway goal due to financial, social and technological hindrances. Until such reduction is feasible, it would be of upmost importance to devise clean and sustainable FW valorisation (Lipinski et al., 2013). Dark fermentation (DF) is one of such processes. Dark fermentation is a biological process undertaken by strict anaerobic bacteria based on the conversion of carbohydrates to hydrogen (H_2) and a vast array of valuable byproducts, such as organic acids (Mohan et al., 2016). Its main product, H_2, is a versatile energy carrier and its conversion into useful energy is a carbon-free emission process (Sherif et al., 2014). Dark fermentation enables the highest productivities (>1 m^3 h^{-1} m^{-3}) amongst the biological H_2 production processes, has low energy requirements, and is easy to operate (Ren et al., 2011). The conversion of FW to H_2 through DF is already well-established (Kanchanasuta and Sillaparassamee, 2017), reaching productivity values as high as 334 and 353 mL H_2 (L $h)^{-1}$ (Moreno-Andrade et al., 2015; Lee et al., 2014) by mixed and pure microbial cultures, respectively. Additionally, the FW conversion process into H_2 can also be performed and improved through bioaugmentation, which involves the introduction of specialised microorganisms into the native microbial community of the substrate. Ideally, these microorganisms should primarily employ the metabolic pathway for converting carbohydrates to H_2 and be able to metabolise a wide range of substrates (Kumar et al., 2016). For example, Goud et al. (2014) concluded that the addition of *Pseudomonas stutzeri* to an anaerobic seed sludge during the fermentation of FW improved the overall productivity from 1.3 mL (L $h)^{-1}$ to a maximum value of 52 mL (L $h)^{-1}$. Similarly, microorganisms such as *Clostridium sp*., *Clostridium butyricum*, *Escherichia coli* and *Ethanoigenens harbinense* have been used as bioaugmenters with relative success (Kumar et al., 2016). There are, however, still circumstantial issues which delay the process scale-up. The major issues with this biochemical conversion reside on the need for additional stages of feedstock pretreatment, coupled with the requirement of complex media supplementation and operation under strict pH and temperature settings. As FW is highly putrescible and will suffer rapid degradation without proper contamination control, the initial FW contamination should be diminished or eliminated prior to fermentation. Common pretreatment examples are sonication, thermal, acid or alkaline treatment, microwave application or ozonation (Rafieenia et al. 2018; Salem et al., 2018).

Elbeshbishy et al., (2011) concluded that the synergy between pretreatments should also be taken into consideration when aiming for a successful fermentation. The use of pretreatments is a risk *versus* reward scenario. The impact in production has to surpass the negative effect of the pretreatment energy, the possible inhibitors formed by the pretreatment process and chemical requirements to guarantee the viability of the global conversion process. Therefore, the pretreatment should be rapid, simple, flexible, *i.e.* applicable to different types of organic waste, avoid the generation of toxic compounds or inhibiting products and display low energy requirements. Ideally, at the FW consumption level, the necessary separation and processing prior to FW fermentation should be easily adaptable to be performed at household level or communal collection points in order to involve each FW producer more thoroughly in the subsequent waste valorisation stage (Vittuari et al., 2016).

The present study focused on the development of a simplified procedure for CIW pretreatment and fermentation for H_2 production that enables direct household or community intervention at its initial stage, while simultaneously achieving a high conversion yield. The minimisation of the nutrient supplementation, sterilisation waive and bioaugmentation with *C. butyricum* were evaluated. A stage of CIW pretreatment was introduced for native microbial control and conversion yield improvement. It is expected that the proposed pretreatment will be simple enough to begin with the participation of the waste producers themselves.

5.2. Material and Methods

5.2.1. Food waste collection

The food waste used in this research, more specifically, the catering industry waste (CIW) was collected during a period of 8 hours (during 1 workday) in a local restaurant that serves mainly fish dishes, cephalopods and shellfish, located on the southern area of metropolitan Lisbon (Trafaria, Almada, November 2016). Bones and other unidentified solid materials, impossible to mash in a laboratory setting, were removed from the collected CIW and disposed of. The remaining material was thoroughly mashed into a pulp and mixed until a standard consistency was attained, for homogenisation. The processed sample was aliquoted and used directly for characterisation, pretreatment, or was stored in closed plastic containers at -20 °C for subsequent fermentation.

5.2.2. Bacterial strain and culture media

The bacterial strain used in this study was *Clostridium butyricum* DSM 10702, from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *C. butyricum* was pre-cultured in Reinforced Clostridial Medium (RCM) (Difco laboratories, Le Pont de Claix, France). The batch fermentations in small-scale serum bottles were performed in basal modified medium (BM1) with the following composition (per litre of 50 mM phosphate buffer, pH 6.8): 5 g trypticase without dextrose, 5 g yeast nitrogen base, 0.56 g cysteine-HCl, 10 mL solution A (100.0 g L^{-1} NH_4Cl, 10.0 g L^{-1} $MgCl_2.6H_2O$, 10.0 g L^{-1} $CaCl_2$. $2H_2O$), 2 mL solution B (200.0 g L^{-1} $K_2HPO_4.3H_2O$), 2 mL solution C (0.5 g L^{-1} resazurin) and 10 mL solution D (500 mg L^{-1} $Na_2.EDTA.2H_2O$, 150 mg L^{-1} $CoCl_2.6H_2O$, 100 mg L^{-1} $MnCl_2.4H_2O$, 100 mg L^{-1} $FeSO_4.7H_2O$, 100 mg L^{-1} $ZuCl_2$, 40 mg L^{-1} $AlCl_3.6H_2O$, 30 mg L^{-1} $Na_2WO_4.2H_2O$, 20 mg L^{-1} $CuCl_2.2H_2O$, 20 mg L^{-1} $NiSO_4.6H_2O$, 10 mg L^{-1} H_2SeO_3, 10 mg L^{-1} H_3BO_3 and 10 mg L^{-1} $Na_2MoO_4.2H_2O$) (Ortigueira et al., 2018). Bench scale fermentations with pH control were performed with tap water or Minimum Mineral Medium (MMM), a simplified medium in which the nutrients were minimised to a C:N ratio of 3:1. The medium contained per litre of distilled water: 12.0 g NH_4Cl, 3.3 mg $FeSO_4.7H_2O$, 0.56 g cysteine-$HCl.H_2O$ and 1 mg resazurin, prepared under anoxic conditions, replacing the gas phase by N_2.

5.2.3. Fermentative systems

5.2.3.1. Batch fermentation with pH control

The batch fermentations were performed in a 1.65 L lab-scale double jacketed bioreactor with a working volume of 0.5 L, equipped with a pH electrode (Mettler Toledo, Ohio, USA) and controller (SGI, California, USA), stirring (Labinco, Breda, The Netherlands) and assorted inlets and outlets for addition and removal of liquid and gaseous samples as described elsewhere (Ortigueira et al., 2018). The pH control was set to 5.5 ± 0.1 through the automatic addition of NaOH 2 M. The batch assays were performed as follows: 100 g of wet CIW (resulting in, approximately, a sugar concentration of 20 g L^{-1} total sugars) were suspended in tap water or MMM medium up to a total volume of 500 mL. This mixture was introduced into the bioreactor and degassed with N_2 for 45 minutes. In the S and 2S fermentations, the bioreactor was sterilised in

autoclave at 121 °C for 1 hour. The medium was inoculated with *C. butyricum* at 5% (v v^{-1}), and the temperature and agitation were kept at 37 ºC and 150 rpm, respectively. Liquid samples were collected from the bioreactor every two hours. The collection of the produced biogas was continuous, performed into inverted NaOH-filled serum bottles (250 mM) stoppered with air-tight butyl rubber stoppers. The bottles were manually substituted when the NaOH solution was replaced by the produced biogas (about 90% vol. of liquid removal from the flask), according to the water-displacement method (Ortigueira et al., 2015). The collection of biogas was performed until the pH value stabilised and the biogas production was negligible. Therefore, the duration of each experimental assay varied from 18-28 hours, depending on the time required for each conversion.

Five operational conditions were tested:

- Sterile non-supplemented (CIW + tap water) fermentation with addition of *C. butyricum* as H_2 producing microorganism (**S**).
- Sterile and supplemented (CIW + MMM) fermentation with addition of *C. butyricum* (**2S**).
- Non-sterile and supplemented (CIW + MMM) fermentation with addition of *C. butyricum* (**NSS**).
- Non-sterile and supplemented (CIW + MMM) fermentation without addition of *C. butyricum* (**NSS-Neg**).
- Non-sterile and supplemented fermentation of microwave treated CIW with addition of *C. butyricum* (**NSS-MW**).

Three independent batch fermentations were performed for each experimental condition. An ANOVA (single factor) test was used for statistical significance analysis, and a significant difference was considered at a level of $p \leq 0.05$. The represented kinetic parameters per condition correspond to the average obtained from the results in each of the 3 repetitions.

5.2.3.2. Comparative small-scale batch fermentations

The comparative small-scale fermentations were performed in 120 mL serum bottles filled with 20 mL of BM1 and supplemented with the respective untreated or pretreated CIW as carbon and energy source. The initial sugar concentration was approximately 20 g total sugars L^{-1} and the acidified CIW was neutralised to pH 7 with

NaOH prior to supplementation. *C. butyricum* pre-cultured in RCM at 37 ºC for 16 h was used for BM1 inoculation at 2.5% (v v^{-1}). The serum bottles were incubated at 37 ºC, 150 rpm. Three replicates were prepared for each gas and liquid sampling time and the results were expressed as mean ± standard deviation (SD).

5.2.4. Food waste pretreatment

The pretreatments applied to CIW were the acid pretreatment (AC), the microwave pretreatment (MW) and the combination of both MW and AC (MW+AC). The AC pretreatment consisted in the addition of sulphuric acid (H_2SO_4, 98% (w w^{-1})) to the CIW batch until a final pH value of 1.0 was attained. This batch was incubated for 24 hours at room temperature. The acidified CIW was neutralised to pH 7 with sodium hydroxide (NaOH) prior to the supplementation with the fermentation media. In the MW pretreatment, 400 g of CIW in containers partially covered to minimise water and volatiles evaporation were submitted to an electromagnetic radiation of 550 W for 2, 3, 4 or 5 minutes. The samples were weighted before and after each treatment, and the moisture content was determined. To select the duration of the MW pretreatment capable of producing the highest decrease in CIW native microbial numbers, PCA medium (Scharlau Chemie SA, Spain) was used as a non-selective medium for aerobic plate counts (Geeraerts et al., 2018). One gram of untreated or pretreated CIW was diluted in 10 mL of sterile deionised water, further serially diluted (10 fold) to 10^8, aliquots of 0.2 mL of each suspension were spread on PCA plates in duplicate and incubated at 25 and 37 °C for 19, 43, 67, 135 and 163 h. The number of colonies growing on all the plates displaying 30 to 300 colonies/plate were counted and converted to the number of colony forming units (CFU) per mL of suspension by CFU/mL= CFU * dilution factor * 1/aliquot.

The combined MW and AC (MW+AC) pretreatment consisted in the sequential application of MW-4 min and acidification, i.e., the CIW was subjected to electromagnetic radiation of 550 W for 4 minutes before acidification with H_2SO_4 for 24 hours and neutralisation before fermentation (adapted from Eswari et al., 2016). To evaluate the impact of the pretreatments on CIW native contamination, the untreated CIW and the MW-4 min, AC, and MW+AC pretreated samples were placed in capped plastic containers at room temperature for 24, 48, 72, 96 and 144 hours. After that, serial dilutions

of these samples were plated in PCA, incubated at room temperature for 72 hours, and the CFU mL^{-1} were assessed, as described earlier.

5.2.5. Analytical methods

5.2.5.1. CIW chemical characterisation

The CIW samples were characterised for moisture, ash, crude protein, total fat and sugar concentration and the results are displayed on Table 1.

Table 1. Proximal composition of the processed CIW used in the fermentation experiments.

CIW components	% dry weight
Total carbohydrates	42 ± 4.1
Crude protein	24 ± 0.1
Fat	22 ± 0.1
Ash	2 ± 0.2
Others	10

Moisture and ash were determined by oven drying (100 ºC, 12 hours) followed by organic component volatilisation (550 ºC, 6 hours) according to standard methods (Horwitz and Latimer, 2005). The protein content was estimated by the Kjeldahl method using 6.25 as the conversion factor of total nitrogen into crude protein (Horwitz and Latimer, 2005). Total fat was determined after ether extraction in a Soxhlet system (Sukhija and Palmquist, 1988). Total sugars were determined after treatment with H_2SO_4 (720 g kg^{-1}) according to standard methods (Browning, 1987) and quantified by the anthrone method (adapted from Southgate, 1969) as follows: 500 µl of sugar solution sample were mixed with 1 mL of anthrone reagent (125 mg of anthrone per 100 mL of H_2SO_4) and digested for 14 minutes at 100 ºC. 300 µl of the digested solution were added per well (96-well microplate) for absorbance analysis (625 nm). Glucose solutions (0-125 mg L^{-1}) were used as standards and digested as described. All the measurements indicated were performed in triplicate.

5.2.5.2. Characterisation of the fermentation products

The liquid fermentation samples were centrifuged for the removal of solid residues prior to the performance of a liquid-liquid extraction for oil separation. The samples were mixed with hexane in a 1:2 ratio and vortexed during 5 minutes for appropriate dissolution into the organic phase. After phase separation, the aqueous phase was removed, filtered (0.2 µm) and analysed by HPLC (LaChrom, Merck, Germany) for the quantification of acetic, butyric and lactic acids. The HPLC system was equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, USA) and a refraction index (RI) detector (LaChrom L-7490). The temperature of the column and the RI detector were kept constant at 50 °C and 45 ºC, respectively, and samples were eluted using H_2SO_4 5 mM (flow rate = 0.4 mL min^{-1}). Solutions of carboxylic acids were used as external standards.

The biogas samples were collected through the butyl rubber stoppers of the serum bottles by means of a stoppered syringe rated for gas chromatography (GC). A GC (Varian 430-GC) equipped with a thermal conductivity detector (TCD) was used. H_2 and carbon dioxide (CO_2) analysis were performed using a fused silica column (Select Permanent Gases/CO_2-Molsieve 5A/Borabound Q Tandem #CP 7430). The injector and column were operated at 80 °C and the detector at 120 °C. Argon was the carrier gas at a rate of 32.4 mL min^{-1}. The GC column was kept at 30 – 60 ºC, the injector at 60 ºC and the TCD at 150 ºC.

5.2.5.3. Fermentation data

The lag phase of the fermentation assay corresponded to the time necessary for the H_2 production to be detected. The total H_2 production was calculated as the sum of the volume of H_2 collected in the inverted serum bottles, herein expressed as cumulative H_2 production, and the volume of H_2 which remained inside the bioreactor headspace at the end of the fermentation assay. The molar concentration of H_2 and CO_2 (mmol) was calculated through the Peng-Robinson equation (Ortigueira et al., 2015). Hydrogen productivity was estimated from the graphical representation of the cumulative H_2 production (L L^{-1}) *versus* time (hours), as the slope of the exponential production period. Molar H_2 yield was defined as the ratio between the total amount of H_2 (mol) produced throughout the experiment and the total sugars (mol), expressed as glucose equivalents, consumed in the same period of time. Volumetric H_2 yield was defined as the ratio between the total H_2 volume (mL) produced and the mass of CIW (g of volatile solids)

supplied to the culture medium in the same period of time. Residual sugar was considered to be the percentage of the initial sugar concentration which remained in the culture medium after fermentation.

5.3. Results and discussion

5.3.1. Effect of minimum medium supplementation on CIW conversion to H_2 by *C. butyricum*

The untreated restaurant CIW was used as substrate for the batch fermentative H_2 production by *C. butyricum*, under sterile conditions. The typical H_2 production profiles obtained in S and 2S fermentations are displayed in Fig. 1, as well as the acetate and butyrate concentration, as byproducts.

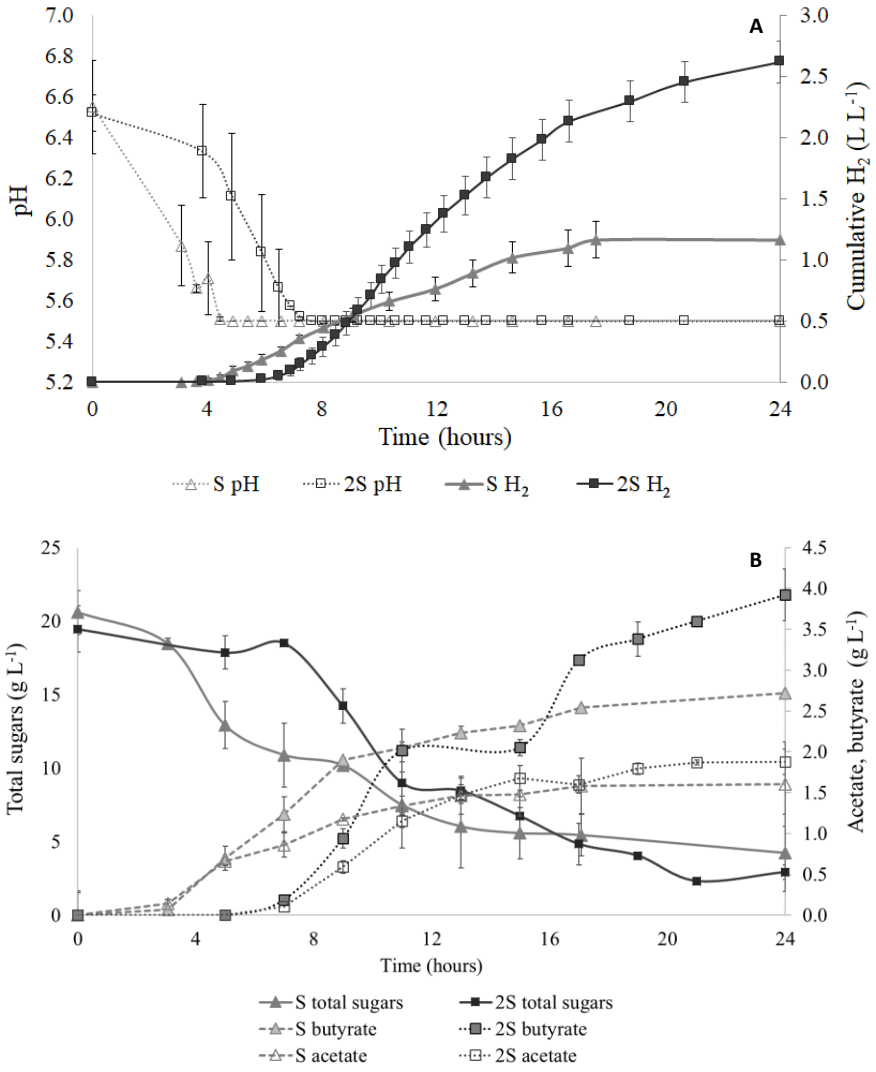

Fig 1. A) Time-course of the cumulative H_2 production and pH variation during the FW fermentation by *C. butyricum* in non-supplemented (S) (▲ – Cumulative H_2; △ – pH) or

supplemented (2S) sterile conditions (■ – Cumulative H_2; □ – pH). B) Time-course of the total sugar consumption and acid production in non-supplemented (S) (▲ – total sugars; △ – butyrate; △ – acetate) or supplemented (2S) sterile conditions (■ – total sugars; ■ – butyrate; □ – acetate).

On the S assay, the conversion of CIW started at 3.8 ± 0.6 hours and was signalled by triggering of the exponential H_2 production (Fig. 1A) accompanied by the pH decrease from the initial value of 6.8 down to 5.5. The biogas production became negligible (below 10 mL biogas h^{-1}) after 17.3 hours of fermentation. The maximum productivity was 134.0 ± 12.3 mL H_2 (L h) $^{-1}$, registered between 6-10 hours, and the total H_2 production reached 2.2 ± 0.2 L L^{-1}. The sugar concentration steadily decreased up to 7.6 g L^{-1} in approximately 15 hours and remained unchanged until the end of the assay (Fig. 1B). The overall sugar consumption amounted to 81.8 ± 10.5% (dry weight). Compared with the S assay, the supplementation of the fermentation medium (2S fermentation) increased both H_2 productivity and the total H_2 production, achieving 249.5 ± 24.6 mL (L h)$^{-1}$ and 4.1 ± 0.2 L L^{-1}, respectively (Fig. 1A). This improvement supports the assumption that the CIW alone does not contain all the nutrients required for an efficient bacterial growth. However, the lag phase increased by approximately 3 hours (6.9 ± 1.2 hours), which extended the overall fermentation time to 23 hours. The sugar consumption of the 2S fermentation was 86.5 ± 5.1% (dry weight), representing a non-significant increase when compared to S (Fig. 1B). The possibility of the remnant sugars being composed by polymeric carbohydrates such as cellulose or hemicellulose, components of the vegetable material present in the CIW sample, which were not depolymerised during the sterilisation stage was suggested. As *C. butyricum* cannot degrade these components directly, it is likely that these components persist in the unfermented fraction (Ortigueira et al., 2015).

Organic acids, mainly acetic and butyric acid, were produced in both S and 2S fermentations. In the former, both acids were produced throughout the fermentation runtime at a similar productivity (0.19 ± 0.03 and 0.31 ± 0.01 g (L h) $^{-1}$ for acetate and butyrate, respectively), culminating in a final butyrate-to-acetate molar ratio of 1.0 ± 0.2. Conversely, in the 2S fermentation while both acids were produced simultaneously (0.22 ± 0.04 and 0.35 ± 0.02 g (L h)$^{-1}$ of acetate and butyrate, respectively), there was a clear increase of the final butyrate-to-acetate molar ratio to 1.5 ± 0.2. This increase is characteristic of a fermentative system under high H_2 partial pressure (Ortigueira et al., 2018).

5.3.2. Effect of sterilisation waive and bioaugmentation of *C. butyricum* on CIW conversion to H_2

The processes described in section 5.3.1 implied the use of an initial sterilisation stage and the maintenance of asepsis during the CIW fermentation. To simplify the conversion process, the removal of this stage was evaluated. The performance of the non-sterile system was tested with (NSS) or without (NSS-Neg) the addition of *C. butyricum* as biocatalyst. The results of the two fermentations are displayed in Fig. 2.

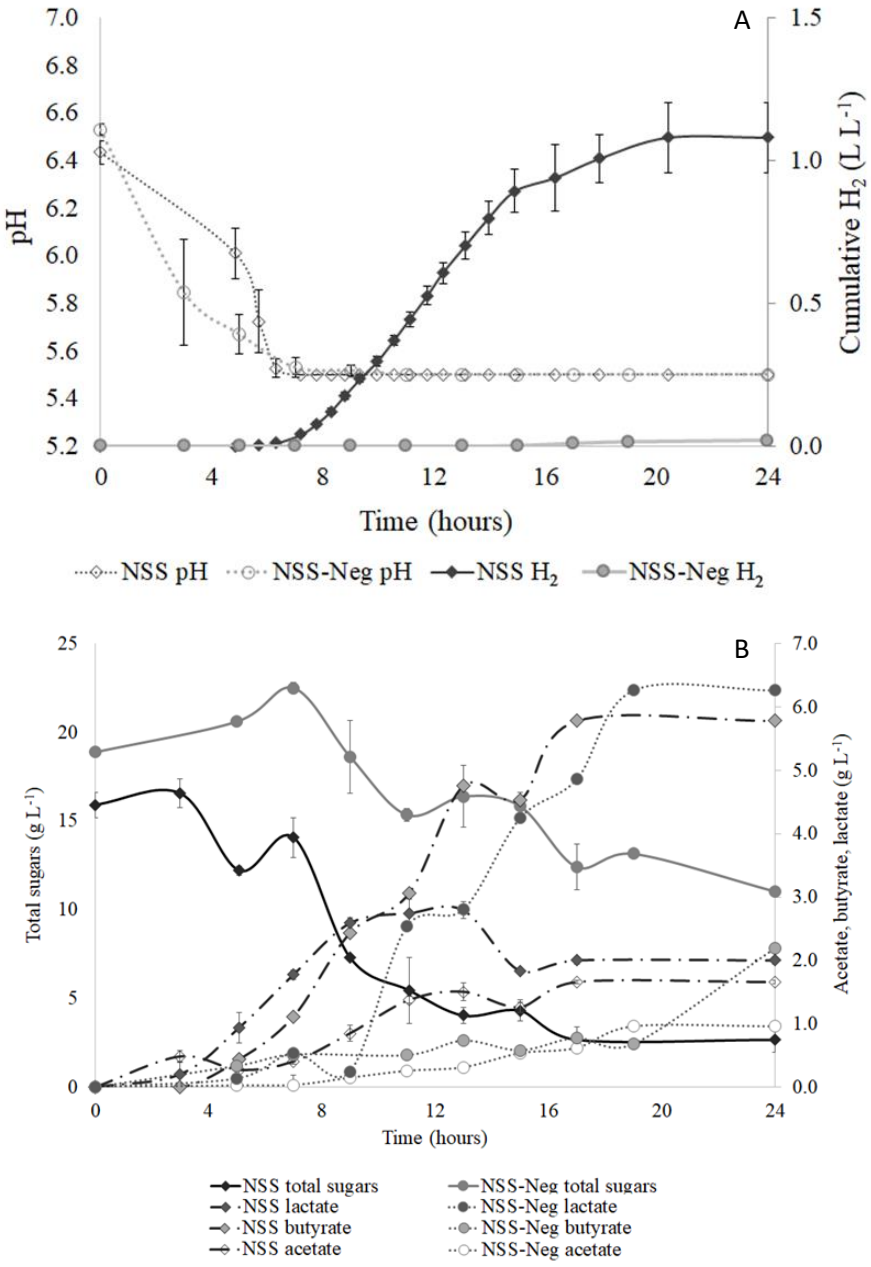

Fig 2. A) Time-course of the cumulative H_2 production and pH variation during CIW fermentation in supplemented non-sterile conditions with the addition of *C. butyricum* (NSS) (◆ – Cumulative H_2; ◇ – pH) or without (NSS-Neg) (● – Cumulative H_2; ○ – pH). B) Time-course of the total

sugar consumption and acid production in supplemented non-sterile conditions with the addition of *C. butyricum* (NSS) (◆– total sugars; ◆ – lactate; ◇– butyrate; ◇– acetate) and without (NSS-Neg) (● – total sugars; ● – lactate; ● – butyrate; ○ – acetate).

In comparison with the sterile fermentations, the lag phase for H_2 production in the NSS assay was 6.4 ± 0.1 hours and remained similar to the 2S fermentation (Fig. 2A). Conversely, the decrease of the pH value began much earlier, which was indicative of the growth of the CIW native microbial community. The maximum H_2 productivity was registered between 8-14 hours and peaked at 128.1 ± 15.1 mL $(L\ h)^{-1}$. The productivity of the NSS fermentation was not significantly different from that of S, but it corresponded to approximately half of the productivity values obtained by Kanchanasuta et al. (2017), under non-sterile conditions. This is likely due to the use of synthetic CIW by these authors, which is expected to have a lower native contamination degree than that of a restaurant-collected feedstock. Under non-sterile conditions, the total H_2 production by *C. butyricum* decreased 23 and 59% in comparison with S and 2S, respectively, although approximately 81.9 ± 5.5 % of the carbohydrates supplied were metabolised (Fig. 2B). The organic acid production pattern was also distinctively different. Unlike the sterile assays, butyric and acetic acid production started well before H_2 production (at, approximately, 4 hours of fermentation), indicating the production of both acids by the CIW microorganisms (Fig. 2B). Additionally, the NSS also displayed lactic acid production. Lactate was quantified between 3-11 hours of fermentation, peaking at 11 hours with a maximum concentration of 2.8 ± 0.04 g L^{-1} before decreasing to 2.0 ± 0.03 g L^{-1}.

To assess the basal activity of the CIW native microbial community, the non-sterile conditions were tested without the *C. butyricum* addition, as NSS-Neg fermentation. An experimental time course of 24 hours was set, to compare with the previous experiments. Sugar consumption started around 5 hours, accompanied by biogas production, the latter of which being composed almost exclusively by CO_2. Hydrogen production was only registered after 15 hours and reached a maximum total production and productivity of 0.09 ± 0.07 L L^{-1} (at 24 h) and 1.7 ± 1.0 mL $(L\ h)^{-1}$ (between 15-24 h), respectively (Fig. 2A; Table 2). No methane (CH_4) was detected in the timespan of the experiment. A persistent increase in lactate concentration was noted throughout the fermentation runtime reaching a maximum of 6.3 ± 0.0 g L^{-1} before the assay was concluded. Lactic acid production justifies the decrease in the concentration of total sugars but it does not favour H_2 production (Harzevili and Hiligsmann, 2018). It is also important to highlight that the

carbon source supplied was not exhausted, reaching a sugar consumption of 46.3 ± 0.9 %.

The kinetic and stoichiometric parameters of the four fermentations – S, 2S, NSS, NSS-Neg - are summarised in Table 2. The highest H_2 productivity was achieved in the 2S fermentation, as well as the maximum value of total H_2 production and conversion yield. Comparing the two sterile fermentations 2S and S, the supplementation of CIW with nitrogen, iron and phosphorus produced a positive effect, increasing both yield and total production, while slightly increasing sugar consumption. I.e. it allowed for a higher feedstock conversion to H_2 and efficiency of the system. Conversely, the elimination of the sterilisation caused a decrease in all H_2 production parameters, mainly in the yield and productivity. While the efficiency of the H_2 production system decreased as, undoubtedly, the contaminants degraded the substrate into other products, the portfolio and concentration of the organic acids produced increased.

Table 2. Kinetic and stoichiometric parameters of the CIW fermentations for H_2 production under sterile (S), sterile and supplemented (2S), non-sterile and supplemented (NSS-Neg), and non-sterile, supplemented and *C. butyricum* bioaugmented (NSS) conditions.

Fermentation	Lag phase (hours)	H_2 productivity (ml (L h)$^{-1}$)	Total H_2 production (L L^{-1})	H_2 conversion yield (mL (g_{sugar})$^{-1}$)	Butyrate-to-acetate ratio	Sugar consumption (%)
S	3.8 ± 0.6	134.0 ± 12.3	2.2 ± 0.2	123.1 ± 6.8	1.0 ± 0.2	82.0 ± 10.5
2S	6.9 ± 1.2	249.5 ± 24.6	4.1 ± 0.2	226.1 ± 13.6	1.5 ± 0.2	86.5 ± 5.1
NSS-Neg	14.8 ± 0.2	1.7 ± 1.0	0.09 ± 0.07	0.9 ± 0.0	1.4 ± 0.0	46.3 ± 0.9
NSS	6.4 ± 0.1	128.1 ± 15.1	1.7 ± 0.1	127.0 ± 25.4	2.2 ± 0.1	81.9 ± 5.5

As observed, the non-sterile conversion of the feedstock is possible, given time and appropriate settings, but it is not as efficient as the sterile condition, nor leads to the best outcome of the conversion process (higher production of H_2, butyric and acetic acid). It is fair to assume that the majority of the CIW native microorganisms did not convert carbohydrates through the DF metabolic pathways associated to H_2 production and that the addition of *C. butyricum* was effective as bioaugmentation strategy. However, *C. butyricum* was unable to ingrain itself to the native CIW microbial community so that the

H_2 production yield increased significantly to values comparable to S or 2S. From a feedstock conversion standpoint, the microbiome of the non-sterile system was efficient, but not for H_2 production.

5.3.3. Food waste pretreatment and impact on H_2 fermentation

Food waste pretreatments, such as the application of temperature, acid or alkaline conditions, aeration, ozonation, ultrasonication, microwaves, etc., have been commonly studied as a way of increasing the substrate biodegradability (Rafieenia et al., 2017). However, one of the main problems with the non-sterile conversion of CIW resides in the contamination of the sample. In this study, the pretreatment options aimed to reduce the CIW microbial numbers while simultaneously using simple procedures that may be initiated by the waste producer at the point of waste generation, with incubations at room temperature, using day-to-day and easy-to-handle equipment easily available within the household, condominium or even restaurants. Ozonation and ultrasonication were discarded from the study due to the inability to apply these methods easily outside of a laboratory or industrial setting (Chauhan, 2018). The application of aeration for CIW contamination control was studied previously, concluding that this pretreatment lowered the overall H_2 yield by 19% when compared to the untreated samples (Rafieenia et al., 2017). Conversely, it was ascertained by Elbeshbishy et al. (2011) that alkaline pretreatment impacted negatively on H_2 production from CIW when compared to the use of, for example, concentrated acid. Therefore, to reduce the degree of CIW contamination, potentially degrade polymeric materials and improve H_2 production under non-sterile conditions, three CIW pretreatments were tested in small-scale bioreactors: the application of microwaves (**MW)**, acidification by addition of H_2SO_4 (**AC)** and the combination of microwave pretreatment and acidification (**MW + AC)**.

To select the most effective MW pretreatment, CIW was submitted to a constant power of 550 W for 2, 3, 4 or 5 min. The CIW contamination was evaluated by the microbial counts in PCA after incubation at 25 and 37 ºC (Table 3).

Table 3. Microbial counts of the CIW after MW pretreatment for 0, 2, 3, 4 or 5 minutes and incubation at 25 ºC and 37 ºC.

Incubation time at 25 °C (h)	Pretreatment time (min) 0	2	3	4	5
19	$1.3 \pm 0.1 \times 10^8$	$4.0 \pm 0.6 \times 10^7$	$1.7 \pm 0.7 \times 10^6$	n.d.	n.d.
43	$2.6 \pm 0.4 \times 10^8$	$5.2 \pm 0.3 \times 10^7$	$2.6 \pm 0.2 \times 10^6$	n.d.	n.d.
67	$3.2 \pm 0.4 \times 10^8$	$5.4 \pm 0.4 \times 10^7$	$2.8 \pm 0.2 \times 10^6$	n.d.	n.d.
135	$3.4 \pm 0.4 \times 10^8$	$6.2 \pm 0.2 \times 10^7$	$3.0 \pm 0.2 \times 10^6$	n.d.	n.d.
163	$3.4 \pm 0.4 \times 10^8$	$6.3 \pm 0.7 \times 10^7$	$3.0 \pm 0.2 \times 10^6$	n.d.	n.d.

Incubation time at 37 °C (h)	Pretreatment time (min) 0	2	3	4	5
19	$2.1 \pm 0.4 \times 10^8$	$7.8 \pm 4.0 \times 10^7$	$3.1 \pm 0.6 \times 10^6$	n.d.	n.d.
43	$2.7 \pm 0.3 \times 10^8$	$9.3 \pm 3.0 \times 10^7$	$3.7 \pm 0.2 \times 10^6$	n.d.	n.d.
67	$2.9 \pm 0.3 \times 10^8$	$1.0 \pm 0.2 \times 10^8$	$3.9 \pm 0.1 \times 10^6$	n.d.	n.d.
135	$2.9 \pm 0.3 \times 10^8$	$1.0 \pm 0.2 \times 10^8$	$4.1 \pm 0.2 \times 10^6$	n.d.	n.d.
163	$3.0 \pm 0.2 \times 10^8$	$1.1 \pm 0.2 \times 10^8$	$4.2 \pm 0.1 \times 10^6$	n.d.	n.d.

(n.d., not detected)

The untreated CIW samples showed a high microbial proliferation (10^8 CFU mL^{-1}) after 19 hours of incubation at 25 and 37 ºC. In general, the MW pretreatment caused a clear loss of microbial viability, but the time of MW exposure affected differently the CIW microbial counts. The MW-3min pretreatment decreased the CFU mL^{-1} in 2-log at both incubation temperatures when compared to untreated CIW. No colonies were detected in the case of 4 or 5 minutes of MW application. Therefore, the MW pretreatment of 4 minutes at 550 W was elected as the most effective and was used in the subsequent assays.

The effects of MW (MW-4 min) application, CIW acidification (AC), and MW application combined with acidification (MW-4 min+AC) on the number of CIW microorganisms were compared. Considering the most probable temperature for the domestic CIW conditioning before fermentation, the microwaved and/or acidified CIW was maintained at room temperature (approximately 20-25 ºC) in covered plastic

containers over 24, 48, 72, 96 and 144 hours. The colony counts in PCA after each incubation period were compared (Fig. 3 (A)).

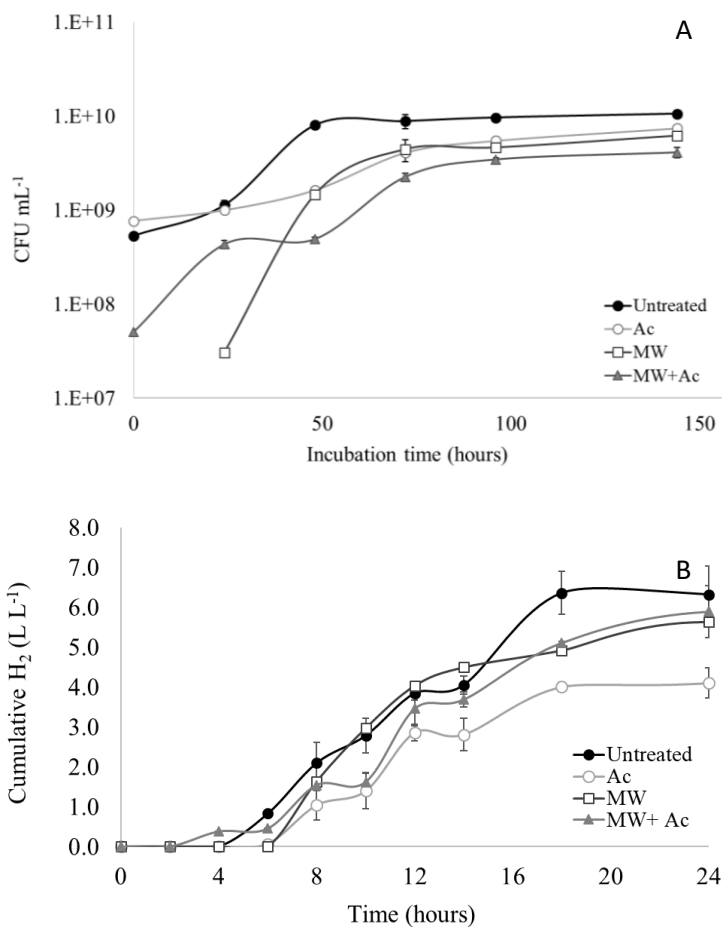

Fig 3. Evaluation of the influence of the CIW pretreatments in contamination control and H_2 production. A) Microbial counts of untreated or pretreated CIW after incubation at room temperature (● – untreated; ○ – AC; □ – MW-4 min; ▲ – MW-4 min+AC). B) Time-course of H_2 cumulative production in small-scale reactors by *C. butyricum* from untreated and pretreated CIW (● – untreated; ○ – AC; □ – MW-4 min; ▲ – MW-4 min+AC).

The application of MW-4 min ceased or caused a clear delay on microbial proliferation immediately after pretreatment (0 hours) or after 24 h at room temperature, respectively. The addition of acid to MW-4 min pretreated CIW helped to control the microbial counts below 10^9 CFU mL^{-1} after 48 h but was less effective than just MW in the first 24 hours. The effect of the acid pretreatment was mostly marked after 48 hours, when the microbial counts were closer to the ones obtained with the application of MW than those of untreated CIW. After 72 hours at room temperature, all the counts were ≥

10^9 CFU mL^{-1}, and remained unchanged at 96 and 144 hours. Foreseeing a procedure that includes the initial handling and conditioning of CIW in the household, longer storage periods of putrescible waste are undesirable and limited by physical setting constraints (Xiao and Siu, 2018). Accordingly, the first 24 hours can be considered the most important for the efficient control of the CIW microbial community and it is possible to rank the effectiveness of each pretreatment in terms of CFU mL^{-1} decrease in this period, as follows: MW-4 min>MW-4 min+AC>AC or untreated CIW. However, the decisive factor in the evaluation of the CIW pretreatment performance is ultimately the impact on H_2 production. Accordingly, *C. butyricum* was used as biocatalyst in a series of comparative small-scale fermentations in serum bottles using untreated and pretreated CIW as carbon and energy source (Fig 3 (B)). The analysis of the sugar solubilisation showed that the CIW pretreatment induced higher polymeric degradation and increased the concentration of initial sugars. The untreated CIW attained a maximum solubilisation of 52% (w/w). This value increased up to 65.2, 66.8 and 67.2% after AC, MW+AC-4-min and MW-4-min pretreatment, respectively. In the comparative fermentations, the soluble sugar concentration decreased during the first 12 hours of fermentation (data not shown) accompanying the bulk of H_2 production and stabilising at, approximately, 24 hours of fermentation.

As seen on Fig. 3 (B), the cumulative H_2 production by *C. butyricum* mirrored the results from the bench-scale assay despite the pretreatment applied. The highest productivity was registered with the MW-4 min pretreatment, 569 mL H_2 $(L\ h)^{-1}$. Untreated CIW, AC and MW+AC-4 min achieved productivities of 442, 325 and 364 mL H_2 $(L\ h)^{-1}$, respectively. The use of acid without MW and subsequent neutralisation had an inhibitory effect in the bacterial growth, decreasing the H_2 productivity and cumulative production (Fig. 3 (B)). This effect, associated with the cost and the environmental consequences of acid residues disposal relegates AC to a second place. Conversely, the use of MW-4 min not only diminished CIW contamination, as it had a more positive effect on the H_2 conversion rate of CIW by *C. butyricum*. With these conclusions in mind, MW-4 min was elected as a potential CIW pretreatment for enhancement of the non-sterile fermentation system.

5.3.4. Performance of the non-sterile *C. butyricum* bioaugmented H_2 fermentation of MW pretreated CIW

The non-sterile and *C. butyricum* bioaugmented fermentation of CIW submitted to pretreatment with MW-4 min (NSS-MW) was selected as the most appropriate to support the objective of this work: the development of a potentially participative process of CIW handling and conversion to H_2. The performance of this system was evaluated (Fig. 4).

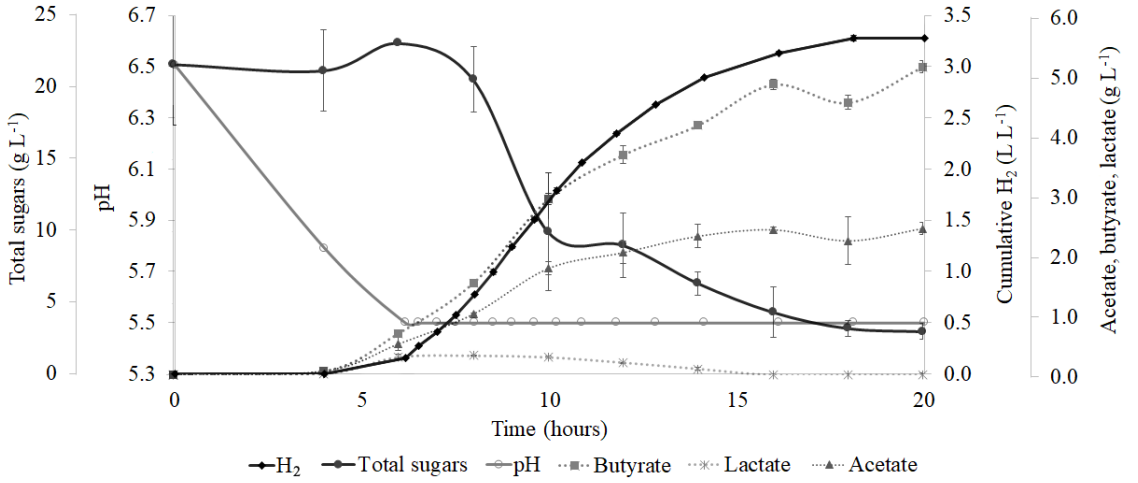

Fig 4. Time-course of the cumulative H_2 production, total sugar consumption, and acetic, butyric and lactic acid production from non-sterile supplemented and pretreated CIW (NSS-MW) (◆ - cumulative H_2; ● – total sugar concentration; ○ – pH**;** ■ - butyrate; ※- lactate; ▲ - acetate).

The application of the MW-4 min pretreatment produced positive effects immediately. Compared to the 2S fermentation, the lag phase was reduced in approximately 1 hour to an average value of 5.6 ± 0.1 hours. The exponential H_2 production lasted between 5.6-12 hours of fermentation and reached a maximum productivity value of 406.2 ± 8.1 mL H_2 (L h)$^{-1}$. This value was double the value of the one obtained in the 2S assay and the one registered by Kanchanasuta et al. (2017) in non-sterile fermentations. It is also not significantly different from the productivity registered in starch fermentations under sterile conditions (Ortigueira et al., 2015). I.e., the MW pretreatment appears to have favoured the substrate biodegradability, possibly by increased solubilisation of nutrients (Eswari et al, 2016), and improved the fermentative rate and yield. The total H_2 production and yield peaked at 4.6 ± 0.5 L H_2 L^{-1} and 234.6 ± 55.6 mL (g sugar) $^{-1}$ or 98.8 ± 10.2 mL (g volatile solids)$^{-1}$, respectively. Overall, the conversion of CIW to H_2 was improved by 47 and 13% compared to S and 2S, respectively. This result illustrates the positive impact of the MW pretreatment on the

fermentative yield *vs*. sterile fermentations by *C. butyricum* such as, for example, the H_2 yield of 39.2 mL (g volatile solids)$^{-1}$ registered by Hu et al. (2014). The efficacy of the MW pretreatment is also positive, when compared to other systems such as the use of aerobic aeration (Rafieenia et al., 2017) and hydrothermic pretreatment (Ding et al., 2017). In these two studies an average production of 44 and 43 mL H_2 (g volatile solids)$^{-1}$, respectively, was reached after CIW pretreatment. These values are similar to those obtained in the S fermentation (46.4 ± 4.7 mL (g volatile solids)$^{-1}$). Although the values obtained in this work did not reach 118 mL H_2 (g volatile solids)$^{-1}$ obtained by Elbeshbishy et al. (2011) with the combined CIW pretreatment by ultrasonication and acid, the importance of the operational simplicity of a MW pretreatment and the ease of implementation in small scale installations by non-specialists should not be overlooked. The MW pretreatment was performed with the main objective of decreasing the CIW native microbial counts which diverted the carbon-source to non-H_2 producing pathways. One of the characteristics registered in the non-sterile assays (both NSS and NSS-Neg) was the tendency of the CIW native microbial community to produce lactic acid. The application of MW decreased lactate to a minimum of 0.09 ± 0.03 g L^{-1}, representing a reduction of 98.5 and 96.2 % in comparison with the NSS-Neg and NSS assays, respectively. Conversely, acetate and butyrate productions were improved (acetate concentration of 2.5 ± 0.1 g L^{-1} and butyrate concentration of 5.2 ± 0.1 g L^{-1}), reaching a final butyrate-to-acetate molar ratio of 1.34 ± 0.03. These values are equivalent to a production of 58 g kg_{VS}^{-1} and 113 g kg_{VS}^{-1} of acetate and butyrate, respectively.

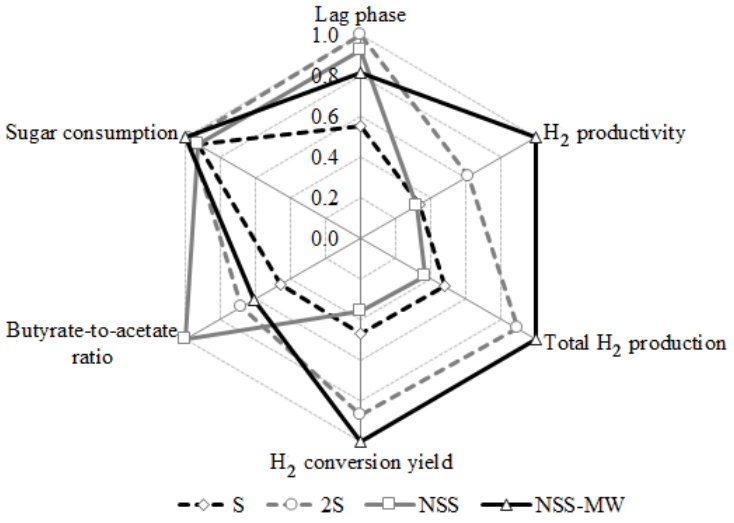

Fig 5. – Radar chart for the comparative analysis of the S (◇), 2S (○), NSS (□) and NSS-MW (Δ) fermentations for H_2 production in respect to 6 fermentative parameters: lag phase, H_2

productivity, total H_2 production, H_2 conversion yield, butyrate-to-acetate molar ratio and sugar consumption. Values were calculated as the ratio between the parameter value for a given fermentation and the respective maximum parameter value obtained in all fermentations (example: ratio between the H_2 production for S fermentation and the H_2 production for NSS-MW fermentation).

Table 4. Prediction of the potential energy production from CIW according to kinetic parameters of the non-sterile, supplemented and *C. butyricum* bioaugmented (NSS) fermentation, and non-sterile, supplemented, MW pretreated and *C. butyricum* bioaugmented (NSS-MW) fermentation, and based on the estimated CIW generated in Portugal in 2012.

Fermentation	H_2 conversion yield ($L\ H_2\ (kg_{VS})^{-1}$)	Potential energy production ($kJ\ kg_{VS}^{-1}$)	Potential energy production per year ($GWh\ year^{-1}$)[a]
NSS	35.6 ± 1.4	268.5 ± 10.6	17.8
NSS-MW	98.8 ± 10.2	746.3 ± 77.0	49.6

[a] Based on an analysis performed for the Portuguese territory, with the estimated amount of CIW generated in the year 2012 (Baptista et al., 2012).

The radar chart (Fig. 5) compares the four fermentation conditions - S, 2S, NSS and NSS-MW – according to 6 critical fermentation parameters: the duration of the lag phase, H_2 productivity, total H_2 production, H_2 conversion yield, butyrate-to-acetate ratio and sugar consumption. The carbohydrate fraction of the CIW sample was successfully metabolised and fermented in all the conditions, and it reached a maximum consumption of approximately 88% in the NSS-MW fermentation. The introduction of supplementation (2S *vs* S fermentation) had a positive impact on H_2 production, increasing the total production and conversion yield, while shifting the metabolism towards the production of butyric acid, as verified by the increase of the butyrate-to-acetate ratio. The simplification of the system by removing the sterilisation stage in NSS fermentation caused a visible reduction in the H_2 production, productivity and conversion yield when compared to the 2S fermentation. This effect resulted from the activity of the CIW native community, which shifted the consumption of carbohydrate to other diverse products, such as lactic acid. The application of MW in the NSS-MW fermentation successfully counteracted this: it led to the highest H_2 production values (total production, productivity and conversion yield), while achieving the second lowest lag phase and

butyrate-to-acetate ratio. Considering the comparative analysis of the critical kinetic and stoichiometric fermentation parameters, the application of the MW pretreatment lead undoubtedly to the most efficient and simple CIW conversion system.

Based on the production yields of the NSS and NSS-MW fermentations, the H_2 low heating value of 120 MJ kg^{-1} and the H_2 density of 0.0899 kg m^{-3}, the potential energy balance of the CIW to H_2 conversion was estimated (Heywood, 2018). The value of 70% for the average energy conversion efficiency was retrieved from the literature (Kirubakaran et al., 2009). The results are depicted in Table 4. The introduction of the MW pretreatment caused an increase in the energy consumption of the overall process of approx. 132 kJ kg_{VS}^{-1} (4 min application of 550W, 37 Wh kg_{VS}^{-1}) when compared to the NSS fermentation. However, this value was offset by an increase in the total H_2 production in the NSS-MW fermentation, which amounted to a maximum energy production increase of 477.8 kJ kg_{VS}^{-1}. These results were used to estimate the H_2 production at a larger scale, taking the Portuguese territory as an example. Accordingly, Baptista et al. (2012) estimated that 1,031 thousand tons of FW were generated in Portugal in 2012. Considering an average moisture and ash sum of 77% (w/w), this amount of FW is equivalent to 239 thousand tons volatile solids per year. Using the highest H_2 production yield of the present study, the conversion of this FW could potentially lead to an annual energy production of 49.6 GWh. This value is equivalent to 15.7 times the value of electricity obtained from biomass for the year of 2018 (Pordata, 2020). Comparatively, Dung et al. (2014) estimated a vastly superior energy production of 1,872 GWh $year^{-1}$ from a total of 1,000 thousand tons of FW generated in the Republic of Ireland (data estimated from 2009-2013) by the combination of DF and photo-fermentation. In spite of this difference, it should be emphasized that the focus of this study was also to design a FW handling and conversion process which, being relatively simple, could be started by the FW producer on a decentralised level and help to reinforce the self-responsiveness concerning waste generation.

5.4. Conclusions

The FW fermentation to H_2 by *C. butyricum* was successfully performed under sterile and non-sterile conditions. The best fermentation performance, according to the

higher H_2 production parameters (productivity, yield and total production) required nutrient supplementation and sterilisation. Under non-sterile conditions, the fermentation efficiency visibly decreased. This effect was counteracted by the application of a MW pretreatment to the FW, a method that is possible to undertake at household or community level. This pretreatment, along with the addition of *C. butyricum* as an H_2 producing microorganism, effectively limited the FW native microbial counts and improved the H_2 production and substrate conversion. A maximum H_2 yield and productivity of 98.8 ± 10.2 mL (g volatile solids)$^{-1}$ and 406.2 ± 8.1 mL (L h)$^{-1}$, respectively, were achieved. These values represent an increase of 47 and 13% in the substrate conversion yield compared to the S and 2S fermentations that were performed under sterile conditions.

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6. Optimisation of nitrogen content on the acidogenic fermentation of food waste

Ortigueira J, Silva C, Moura P.

Dark fermentation sludge as nitrogen source for hydrogen production from food waste *in Wastes: Solutions, Treatments and Opportunities III: Selected Papers from the 5th International Conference Wastes 2019, September 4-6, 2019, Lisbon, Portugal 2019;301. CRC Press, Taylor & Francis Group, Boca Raton, Florida, USA*

6. Optimisation of nitrogen content on the acidogenic fermentation of food waste

The present chapter results from the development of the target "Biorefinery concept and optimisation of operational parameters", focusing on the optimisation of the operational parameters of the acidogenic fermentation of FW, particularly the nitrogen source and content.

Abstract

The biological conversion of food waste (FW) into hydrogen (H_2) by anaerobic fermentation is associated with high production costs and complex supplementation requirements. The present study had two main objects, focused on the simplification of the H_2 production through dark fermentation (DF): the reuse of its residual solid fraction, herein referred as DF-sludge, as nitrogen source for a subsequent conversion of a catering industry waste (CIW) and the minimisation of the initial nitrogen content. The non-sterile FW fermentation with addition of *C. butyricum* as H_2-producing microorganism were performed under supplementation with two nitrogen sources (ammonium chloride (NH_4Cl) or DF-sludge) and two nitrogen concentrations (default and 66% nitrogen reduction). The maximum biogas productivity, H_2 production yield and H_2 cumulative production were obtained with the DF sludge supplementation, reaching values of 433.3 ± 34.3 mL biogas $(L\ h)^{-1}$, 194.2 ± 24.4 mL $H_2\ g^{-1}_{\text{total sugars}}$ and 3.2 ± 0.0 L $H_2\ L^{-1}$, respectively. The use of DF sludge improved the fermentation efficiency on H_2 production by 40 %, underlining the impact of nutrient recycling in *C. butyricum* fermentative performance.

6.1. Introduction

Over the past decade, the scientific community has emphasized the environmental and economic impact of wasted food products (Scherhaufer et al. 2018). The act of throwing out food due to inefficient production and consumption practices, reaches farther than the mere gesture. It represents the loss of all resources required for its production as well as those necessary for its proper treatment and disposal. According to the Food and Agriculture Organization of the United Nations, approximately 88 billion

tonnes of food waste (FW) were discarded in the 28 countries of the European Union in 2013, a wastage that represents up to 186 Mt CO_{2eq} carbon emissions (Scherhaufer et al. 2018). While FW prevention is a required target in the majority of legislation packages dealing with this problem (Corrado & Sala 2018), it is not feasible to assume that it will be possible to reduce or eliminate this type of waste in a nearby future. Therefore, several studies focus the possibility of FW valorisation.

Food waste is a highly heterogeneous mixture of chemical components, containing water, carbohydrates, proteins, fat, among others. This composition makes it an interesting substrate for dark fermentation (DF), a biological process which consists on the anaerobic conversion of carbohydrates into hydrogen (H_2), organic acids, such as butyrate and acetate, and compost (Ortigueira et al. 2019). Hydrogen is considered to be an extremely interesting bioenergy carrier as it has a considerably high energy density (120 MJ kg^{-1}), is storable at -253 ºC in the liquid form or in the gaseous form at high pressures of 300-700 bar, and its combustion is not associated to carbon or sulphur emissions (Dutta et al. 2014). However, the low production yields which are normally associated with biological conversion systems tend to inflate the production costs and difficult the implementation at industrial scale. One strategy commonly used for costs reduction involves the minimisation of the nutrient requirements in the culture media or its replacement by cheaper alternatives. The first condition can be achieved by optimising the minimum nutrient requirements that have no impact on the fermentation yield. The second requires an easily attainable source, readily available in large quantities, renewable and environmentally friendly (Han et al. 2016). Dark fermentation sludge can be defined as the solid residue obtained after completion of the biological conversion process, being composed by substrate leftovers that remain in the fermentation sludge, produced metabolites possibly adsorbed to the solid residues, and cellular biomass (Moser-Engeler et al. 1998). Chemically, DF-sludge is composed by a considerable carbon fraction and also nitrogen. The latter is an essential nutrient for bacterial growth and necessary for the correct conversion performance through DF. In fact, the compost that is traditionally produced from DF sludge after maturation and stabilisation has a high concentration in nitrogen (Wilson & Novak 2009).

This study analysed the effect of replacing NH_4Cl as nitrogen source by DF nitrogen-rich sludge on the fermentative conversion of FW, specifically food waste acquired in the catering industry service CIW, to H_2. The main objective was not only to

reduce process costs, but also to promote the reincorporation of nutrients and achieve disposal savings.

6.2. Material and Methods

The food waste utilised in this study, more specifically, the catering industry waste (CIW) was collected from a local restaurant during a period of 8 hours, located in the southern area of metropolitan Lisbon (Trafaria, Almada, November 2016). The chemical characterisation was performed after bones removal, mashing and homogenisation of the samples. The processed samples were then stored at -20 ºC prior to characterisation and fermentation. The CIW samples were characterised in terms of water, total sugars, crude protein, total fat and ash. Moisture and ash were determined by oven drying (100 ºC, 12 hours) followed by organic component volatilisation (550 ºC, 6 hours) according to standard methods (Horwitz & Latimer 2005). The protein content was estimated by the Kjeldahl method using 6.25 as the conversion factor of total nitrogen into crude protein (Horwitz & Latimer 2005). Total fat was determined after ether extraction in a Soxhlet system (Sukhija & Palmquist 1988). Total sugars were determined through an adapted anthrone method as published elsewhere (Ortigueira et al. 2018). The CIW fermentation assays were divided in two series, referred to as NH_4Cl assay and DF-sludge assay, according to the nitrogen source used. Three independent batch fermentations were performed for each experimental condition, and where prepared as follows:

NH_4Cl assay – 60.8 g CIW were submitted to microwave pretreatment (550 W for 4 mins) (Ortigueira et al., 2019a). The pretreated sample was suspended up to a total volume of 500 mL in Minimum Mineral Medium (MMM), containing per litre of 100 mM phosphate buffer (pH 6.8): NH_4Cl, 12 g, 3.3 mg $FeSO_4.7H_2O$, 0.56 g cysteine-$HCl.H_2O$ and 1 mg resazurin (Ortigueira et al. 2018).

DF sludge assay – 60.8 g CIW were mixed with 14 g of DF-sludge obtained from a previous non-sterile, *C. butyricum* bioaugmented, CIW fermentation. The mixture was submitted to microwave pretreatment as described above, and then suspended in phosphate buffer, pH 6.8, up to total volume of 500 mL.

66% reduction assay – 60.8 g CIW were submitted to microwave pretreatment (550 W for 4 mins) (Ortigueira et al., 2019a). The pretreated sample was suspended up to a total

volume of 500 mL in Minimum Mineral Medium - Low Nitrogen (MMM-LN), containing per litre of 100 mM phosphate buffer (pH 6.8): NH_4Cl, 4 g, 3.3 mg $FeSO_4.7H_2O$, 0.56 g cysteine-$HCl.H_2O$ and 1 mg resazurin (Ortigueira et al. 2018).

The initial concentration of total sugars was 20 g L^{-1} all performed assays were undertaken under non-sterile conditions. The bacterial culture *C. butyricum* DSM 10702 from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) was used as additional biocatalyst. *C. butyricum* was pre-cultured in Reinforced Clostridial Medium (RCM) (Difco laboratories, Le Pont de Claix, France) and the cells in exponential growth phase were inoculated at 5% (v v^{-1}) in each fermentation medium that was contained in a 1.65 L bioreactor to be operated at pH 5.5, 37 ºC and 150 rpm. The produced biogas at the outlet of the bioreactor was routed through a gas washing bottle containing NaOH 250 mM, for CO_2 stripping, after which was continuously quantified by means of a gas flowmeter (μflow, Bioprocess Control, Lund, Sweden) and collected in gas sampling bags (SKC sample bags, 263-03, SKC, PA, USA). The produced biogas in the sampling bags, herein expressed as cumulative biogas, and the biogas in the bioreactor headspace at the end of the fermentation were characterised by gas chromatography (GC). A chromatographer (Varian 430-GC) equipped with a thermal conductivity detector (TCD) and a fused silica column (Select Permanent Gases/CO2-Molsieve 5A/Borabound Q Tandem #CP 7430) was used in the following operational conditions: the injector and column were operated at 80 °C and the detector at 120 °C with argon as the carrier gas, at a flow rate of 32.4 mL min^{-1}. The GC column was kept at 30 – 60 ºC, the injector at 60 ºC and the TCD at 150 ºC. The liquid fermentation samples were processed for oil removal by hexane extraction, using a 1:2 sample/hexane ratio, and the organic acids were subsequently quantified by high performance liquid chromatography (HPLC). The HPLC system was equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, USA) and a refraction index (RI) detector (LaChrom L-7490). The temperature of the column and the RI detector were kept constant at 50 °C and 45 ºC, respectively, and the samples were eluted using H_2SO_4 5 mM at a flow rate of 0.5 mL min^{-1}. Ammonia concentration (mM) was quantified with a Crison ion selective electrode as published elsewhere (Queirós et al., 2018). The total H_2 production was calculated as the sum of the H_2 volume in the biogas sampling bags and the volume of H_2 in the biogas that remained inside the bioreactor headspace at the end of each fermentation assay. The H_2 productivity was estimated from the graphical

representation of the cumulative biogas production (L L^{-1}) versus time (hours), as the slope of the exponential production period, and using the percentage of H_2 (% vol.) in the total volume of biogas produced. The volumetric H_2 yield was defined as the ratio between the total H_2 volume (mL) produced and the mass of CIW volatile solids (g $_{VS}$) supplied to the culture medium in each assay. Total sugars consumption was defined as the percentage of initial sugars consumed.

6.3. Results and discussion

6.3.1. Food waste and dark fermentation-sludge characterisation

The use of CIW as substrate for DF is highly dependent on an appropriate chemical composition, particularly the carbohydrate composition and chemical form. Highly polymerised components such as cellulose, hemicelluloses and starch might be more recalcitrant for direct bioconversion and usually require an additional pretreatment and/or saccharification stage prior to fermentation. Additionally, a high nitrogen content in the CIW may circumvent the need for media supplementation. The chemical characterisation of the CIW used in the present work is presented in Table 1.

Table 1. Proximate composition of the CIW and DF-sludge samples.

Component	CIW % (w w^{-1})	DF-Sludge % (w w^{-1})
Moisture	74.4	80.9 ± 3.9
Total sugars*	62.1 ± 0.1	58.2 ± 0.9
Crude protein*	10.4 ± 0.2	14.0 ± 4.7
Total fat*	26.3 ± 2.2	nd
Ash*	1.2 ± 0.1	nd

*, dry weight
nd, not determined

While the chemical variability is one of the main characteristics of this kind of samples, the CIW collected for this work was considered to be a good representative of the typical summer Portuguese diet. It was composed by residues of cooked vegetables, fish skins/bones/scraps and a visibly high fraction of starch-rich leftovers, such as bread,

rice and potato scraps. The high concentration of total sugars suggests the sample to be quite adequate for DF conversion (Table 1). Furthermore, the carbon:nitrogen molar ratio was 2.9:1 which was comparable to the ratio of 3:1 of CIW samples used in previous fermentations (Ortigueira et al. 2019a). However, in that study it was found that not all the crude protein supplied by the substrate was converted by *C. butyricum*, which led to the need of supplementing the fermentation medium with NH_4Cl.

The DF-sludge used in the present study corresponded to the solid fraction obtained at the end of a CIW non-sterile fermentation with addition of *C. butyricum* as H_2-producing microorganism (Ortigueira et al. 2019a). It contained mainly residual carbohydrates that were not metabolised by the microbial population, remaining bacterial population and organic acids, particularly butyric and acetic acid, that were present in the liquid fermentate and were possibly adsorbed to the remnant solids. The DF-sludge was separated from the liquid fermentate through centrifugation, kept wet to minimise additional drying costs and then rerouted into the DF sludge assay. The microwave pretreatment applied to the sludge could, theoretically, breakdown the cellular material, reduce contamination and degrade residual carbohydrate components.

6.3.2. Comparative CIW fermentation with NH_4Cl or DF-sludge supplementation

The fermentability of the CIW samples for H_2 production was evaluated in a series of batch experiments conducted in a bench-scale bioreactor with pH control. The results of the fermentations supplemented with NH_4Cl or DF-sludge are shown in Fig 1.

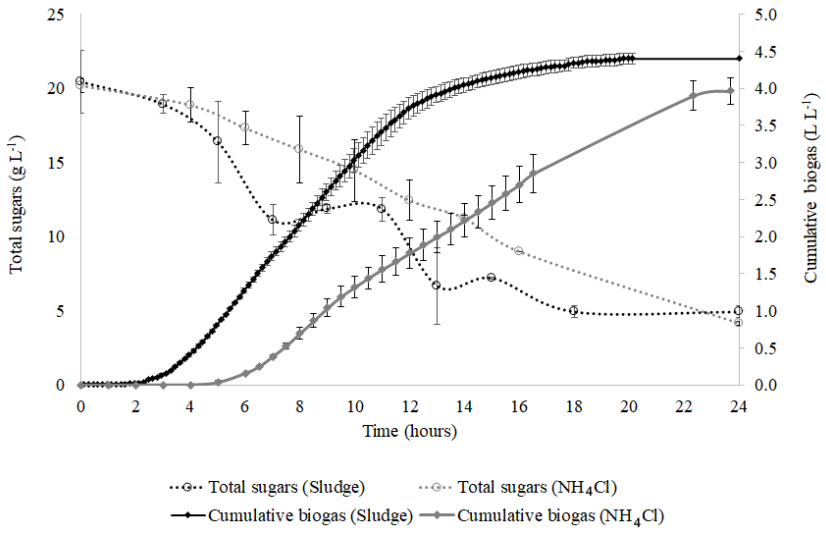

Fig 1. Non-sterile CIW mesophilic fermentation, at pH 5.5, 37 ºC and 150 rpm, with addition of *C. butyricum* as biocatalyst and NH_4Cl or DF-sludge supplementation: time-course of the cumulative biogas production and sugar consumption (NH_4Cl suppl.: ♦ –biogas; ○ – total sugars) (DF-sludge suppl.: ♦ – biogas; ○ – total sugars).

The change in the nitrogen source altered visibly the fermentation start-up. The lag phase for biogas production in the DF-sludge assay decreased to 3 hours, while in the NH_4Cl assay it started only 5 hours after the inoculation of *C. butyricum*. The maximum biogas productivity was achieved between 3 and 12 hours of operation in the DF-sludge assay, reaching a maximum value of 433.3 ± 34.3 mL biogas $(L\ h)^{-1}$ (Table 2). This result represents an increase of almost 64% when compared to the NH_4Cl assay that achieved a productivity of 264.4 ± 13.8 mL biogas $(L\ h)^{-1}$ from 7 to 16 hours of fermentation. It was suggested that both the decrease in the lag phase and the higher productivity obtained in the DF-sludge assay were likely due to the presence of enzymatic material in the sludge, which enabled the bacterial population to use the polymeric components more readily than in the NH_4Cl assay. The improvement in both these fermentation parameters reduced the process time in the DF-sludge assay, which is confirmed by the negligible biogas production, *i.e.* below 10 mL $(L\ h)^{-1}$, after 18 hours of fermentation.

Table 2. Results of the non-sterile CIW fermentation with addition of *C. butyricum* and NH_4Cl or DF-sludge supplementation.

Supplementation	Total sugars consumption	Total biogas production	Max. biogas productivity	H_2 concentration	H_2 production yield	
	(%)	($L\ L^{-1}$)	$(mL\ (L\ h)^{-1})$	(% vol)	$(mL\ g^{-1}_{VS})$	$(mL\ g^{-1}_{total\ sugars})$
NH_4Cl	79.3 ± 0.0	4.1 ± 0.1	264.4 ± 13.8	55.6 ± 0.1	77.9 ± 4.1	139.1 ± 2.6
DF-sludge	75.9 ± 0.0	4.4 ± 0.1	433.3 ± 34.3	73.0 ± 0.0	111.9 ± 1.6	194.2 ± 24.4

The replacement of NH_4Cl by DF-sludge impacted significantly in the H_2 concentration of the produced biogas (Table 2) and increased the total H_2 production by 41%. This can be explained by a faster adaptation of *C. butyricum* to the DF-sludge composition possibly due to the inhibitory effect that sludge-adsorbed organic acids may exert over the CIW native microbial community. As there were no significant differences in both the initial sugar concentration (Fig 1) and the total sugars consumption (Table 2) between the assays, it was assumed that the metabolic behaviour of the microbial

population in the DF-sludge assay was more efficient. This pattern was reflected in the H_2 yield that reached a maximum of 194.2 ± 24.4 mL g $_{\text{total sugars}}^{-1}$ (Table 2) with DF-sludge supplementation. It was not possible in any fermentation condition to completely convert the carbohydrates supplied, and the final concentration of total sugars was approximately 5 g L^{-1} (Fig 1). This may indicate the presence of polymeric components that *C. butyricum*, in association with the CIW native microbiota, cannot metabolise without additional substrate pretreatment and/or saccharification apart from the exposure to MW as performed in the present study.

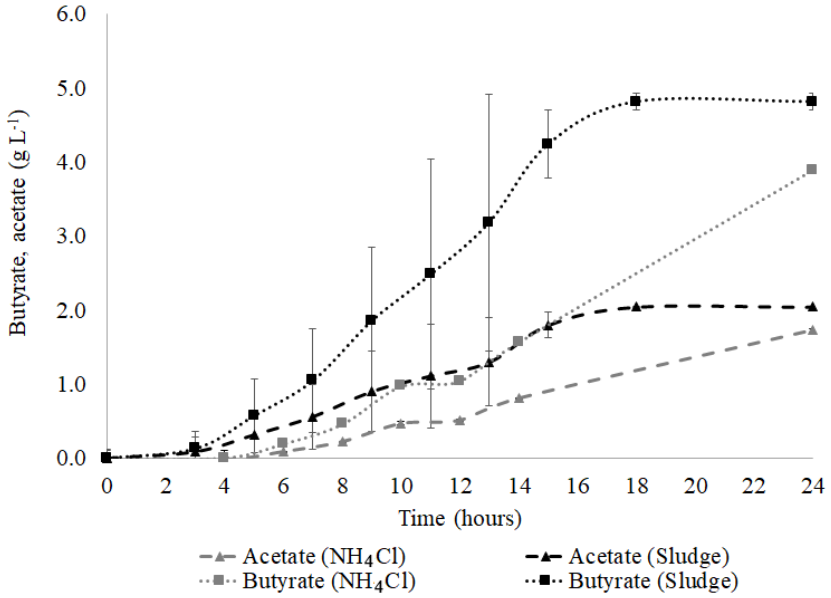

Fig 2. Non-sterile CIW fermentation, at pH 5.5, 37 ºC and 150 rpm, with addition of *C. butyricum* as biocatalyst and NH_4Cl or DF-sludge supplementation: time-course of acetic and butyric acid production (NH_4Cl suppl.: ▲ - acetate; ■ - butyrate) (DF-sludge suppl.: ▲- acetate; ■ - butyrate).

Hydrogen production was accompanied by the production of organic acids, particularly acetate and butyrate, which started in the first 3 h (DF-sludge assay) or 5 h (NH_4Cl assay) after the inoculation of *C. butyricum* (Fig 2). Apparently, there was no microbial inhibition by the presence of organic acids in the sludge biomass. Once again, the production of both acids was higher in the DF-sludge assay, where the production of butyric acid reached a maximum of 4.8 ± 0.1 g L^{-1}. The butyrate-to-acetate molar ratio in the DF-sludge assay was accordingly higher (1.49 ± 0.0 mol mol^{-1}), which illustrates a situation of high H_2 partial pressure in the bioreactor headspace (Ortigueira et al., 2018). In that case, the concentration of H_2 in the produced biogas reached 62 % vol. before CO_2

stripping and increased to 73 % vol. after stripping (Table 2). Lactate was detected at a maximum concentration of 0.5 g L^{-1} throughout the DF-sludge assay runtime up to 12 hours (data not shown), point after which it steadily decreased to 0.06 g L^{-1}. This behaviour was not mirrored in NH_4Cl assay, in which the lactate concentration increased steadily up to the end of the fermentation, reaching a maximum concentration of 0.4 g L^{-1}.

According to the obtained results, the DF sludge has the correct characteristics to serve as potential nitrogen-rich supplement for the CIW conversion through DF. Furthermore, the rerouting of this solid effluent stream to a new fermentation process has two main positive impacts: reducing the need of using NH_4Cl as external resource in the H_2 and/or organic acids production stage, and reducing the cost and environmental burden associated with the conversion of the fermentation sludge into compost, including the required stabilisation, drying and transportation prior to its use as fertiliser. Therefore, it is likely that the replacement of the nitrogen source by this environmental friendly option can lead to a significant decrease in the overall operation costs in scalable CIW bioconversion processes.

6.3.3. Optimisation of the nitrogen content of the feed

The culture medium used in the present study was a minimum media (MMM), containing only essential nutrients for microbial growth which are not available in the substrate, either because they are present in a form that bacteria cannot convert or because their concentration is too low. The analysis of the fermentate obtained after the acidogenic fermentation of CIW registered a residual nitrogen concentration of 182.6 mM of NH_4^+ (equivalent to 9.7 g NH_4Cl L^{-1}), i.e., the initial amount of NH_4Cl supplied to the culture was not completely exhausted during bacterial growth. This amount can be reduced to avoid unnecessary nutrient waste. Taking this value into consideration, the initial NH_4Cl concentration in the MMM was reduced by 66%, as to reduce the residual concentration and still allow for nitrogen surplus. The fermentation results of the default and the reduced nitrogen content assays were compared. The results are depicted on Fig 3.

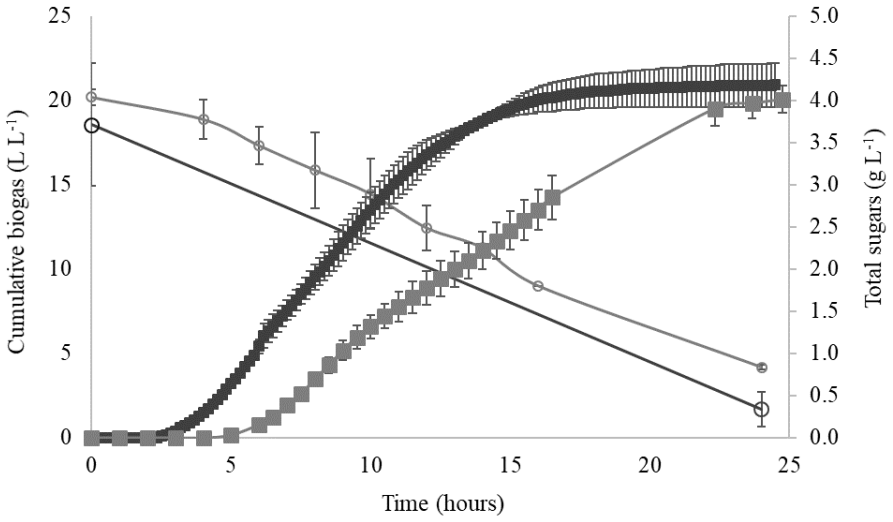

Fig 3. Non-sterile CIW mesophilic fermentation, at pH 5.5, 37 ºC and 150 rpm, with addition of *C. butyricum* as biocatalyst with or without a reduction in the NH_4Cl supplementation: time-course of the cumulative biogas production and sugar consumption (NH_4Cl suppl.: ■ – biogas; ○ – total sugars) (66% reduction in NH_4Cl suppl.: ■ – biogas; ○ – total sugars).

The reduction of the initial NH_4Cl concentration produced a biogas production profile similar to the one registered on the DF-sludge assay (Fig 1). The lag phase was shortened to approximately 3 hours after which biogas production was immediately detected. The total biogas production steadied at the volume of 4.2 L biogas L^{-1} and leading to a final H_2 production of 4.1 L L^{-1}. The biogas productivity increased significantly, up to a maximum value of 420.1 ± 11.9 mL $(L\ h)^{-1}$. This value represents an increase of 59 % compared to the normal NH_4Cl supplementation. The productivity increase cannot be attributed to the presence of additional enzymatic material recirculated into the fermentation bioreactor. In this case, the reduction of the NH_4Cl concentration implicated solely a decrease in $NH4^+$ and Cl^- initially present in the fermentation medium. Therefore, the increase in biogas productivity might indicate that either one or both ionic compounds were likely present at a concentration sufficiently high to exert an inhibitory effect on the bacterial community. Studies on the effect of ionic compounds on bacterial growth indicate a possible association between the presence of the chloride ion and the inhibition of *C. butyricum* growth (Shockey and Borger, 1991). This reason which might explain the positive impact of its removal from the fermentation media.

Table 3. Results of the non-sterile CIW fermentation with addition of *C. butyricum* and with or without a reduction in the NH_4Cl supplementation

Supplementation	Total sugars consumption	Total biogas production	Max. biogas productivity	H_2 concentration	H_2 production yield	
	(%)	(L L^{-1})	(mL (L $h)^{-1}$)	(% vol)	(mL g^{-1}_{VS})	(mL $g^{-1}_{total\ sugars}$)
Non-reduced NH_4Cl	79.3 ± 0.0	4.1 ± 0.1	264.4 ± 13.8	55.6 ± 0.1	77.9 ± 4.1	139.1 ± 2.6
66% reduction NH_4Cl	90.1 ± 3.3	4.2 ± 0.1	420.1 ± 11.9	62.7 ± 5.5	141.9 ± 18.1	221. 4 ± 50.4

Although the N supplementation was reduced, the H_2 production yield reached 141.9 ± 18.1 mL g^{-1}_{VS} and was comparable to the values of 115.8, 118.0, 134.0, and 150.5 mL g^{-1}_{VS} obtained in similar fermentation conditions (Wang et al., 2010, Elbeshbishy et al., 2011, Tawfik et al., 2015, Sattor et al., 2015). Finally, butyric and acetic acids were produced in concentrations of 4.2 and 1.9 g L^{-1}, respectively, producing a final butyrate-to-acetate molar ratio of 1.6. Trace amounts of lactic acid were detected only at the beginning of the fermentation, and were depleted after 24 hours. Considering the obtained results, it can be concluded that the 66% reduction of NH_4Cl supplementation improved the fermentation productivity. Additionally, the use of a medium with lower nitrogen concentration will result in acid-rich fermentates more adequate for the subsequent conversion to polyhydroxyalkanoates as trace amounts of nitrogen in the fermentate will induce bacterial growth fermentation instead of polymer accumulation. Lower nitrogen content will likely lead to higher polymer production (Aragão et al., 1996).

6.4. Conclusions

The two optimisation settings imposed upon the non-sterile CIW fermentations bioaugmented with *C. butyricum*, namely the replacement of NH_4Cl by DF-sludge and a reduction of 66% in the initial NH_4Cl concentration, produced a positive impact on the process productivity Maximum biogas productivity was registered in the DF-sludge assay which increased by 64% when compared to the default condition. In the same assay, the H_2 concentration achieved 73% vol. in the produced biogas and yielded 194.2 ± 24.4 mL H_2 g_{sugar}^{-1}, which represented a 40% increase when compared to the NH_4Cl assay. Butyric acid was the major cometabolite in all performed assays and exhibited a production

profile similar to that of biogas. The present study showed that, in compliance with circular economy practices, the recycling of nutrients in non-sterile CIW fermentations for H_2 production increases the overall process efficiency.

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7. Food waste biorefineries: Stability of a non-sterile food waste acidogenic fermentation system with CO_2 sequestration integrated with a PEM fuel cell

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7. Food waste biorefineries: Stability of a non-sterile food waste acidogenic fermentation system with CO_2 sequestration integrated with a PEM fuel cell

This chapter fits in with the objectives "Biorefinery concept and optimisation of operational parameters", "Biohydrogen conversion" and "Global warming potential assessment", focusing on the change of batch operation mode to CSTR for H_2 production, the conversion of H_2 to electricity, and the technical evaluation of the process by scale-up simulation and quantification of the global warming potential.

Abstract

The present study focused on the integration of the non-sterile acidogenic conversion of food waste (FW) to hydrogen (H_2) with the subsequent electricity generation in a proton-exchange membrane fuel cell (PEMFC), and the assessment of the Global Warming Potential (GWP) of the overall process. The acidogenic conversion of FW performed in a CSTR bioreactor produced 91.3 ± 0.1 L L^{-1} of H_2 at an average H_2 productivity of 257.4 ± 54.6 mL $(L\ h)^{-1}$ during the 16 days of operation. Butyric and acetic acid were simultaneously produced at average concentrations of 3.6 ± 0.5 and 1.6 ± 0.3 g L^{-1}, respectively The CO_2 from biogas product was solubilised in a NaOH solution and the resulting H_2-rich stream was fed to a PEMFC, attaining the yield of 1.7 Wh L^{-1}. The scale-up simulation was based on conversion yields obtained with bench-scale processes and used to assess the global warming potential. Two of the developed scenarios, which considered the reuse of dark-fermentation sludge as nitrogen source in the acidogenic fermentation, diminished GWP emissions by 63.8% and 64.3% when compared to the default condition, attaining an average of 5907 tons of CO_2 per biomass selectively collected per year in the best-case scenario.

7.1. Introduction

Food waste (FW) is defined as food removed from the food production supply chain by voluntary or involuntary decision without a defined purpose (European Union, 2019). It is a direct result of human activity, being generated independently of social, cultural and economic variables (Lipinski et al., 2013). The analysis of FW generation patterns is made difficult by the lack of data about national waste quantification which does not permit the identification of the critical loss or waste points on the food production supply

line. According to available data, the results from quantitative studies vary greatly depending on selection factors such as: accounting for inedible food and liquid waste, destination of the waste and distinction between waste/loss and avoidable/unavoidable (Corrado and Sala, 2018). The FW production range in EU-28 was estimated to be between 158-298 kg capita^{-1} year^{-1} (Alexander et al. 2017; Kenma et al., 2017; Stenmark et al., 2016; Tisserant et al., 2017). The conventional approach for FW treatment and disposal permitted deposition of FW in landfill (Kim et al., 2010). According to EU legislation, waste disposed of in landfill must be reduced by 10% up to 2030, as leachates and gaseous emissions produced by the disposal system have severe harmful consequences for the environment (European Parliament, 2018). In its place a more circular approach to the FW problem has been taken, in which there is a primary focus on a decrease of FW production through an increase of social awareness (i.e. reduction of avoidable waste) and use of the unavoidable waste a potential substrate for the production of energy, platform-chemicals for industry, fertilizers, etc (De Menna, 2019). Anaerobic conversion, particularly dark fermentation (DF), has been suggested as an appropriate bioconversion path for FW (Ortigueira et al., 2019a). This system has as prime objective the conversion of carbohydrate-containing biomass into hydrogen (H_2), while achieving productivity values of more than 1 m^3 h^{-1} m^{-3} (Ren et al., 2011). Simultaneously, DF generates organic acids and sludge for which valorisation solutions such as the fermentation to produce bioplastic precursors and the maturation to a nutrient-rich compost, respectively, are well-stablished (Lakeh et al, 2019). Hydrogen is an energy carrier with a high energy content (120 MJ kg^{-1}), which can be efficiently converted into electricity through the use of proton-exchange membrane fuel cells (PEMFC). These cells consist in electrochemical devices typically composed by a membrane electrode assembly of one anodic and cathodic layer separated by an electrolyte membrane, which produce electricity through the conversion of H_2 and O_2. The PEMFC can operate at relatively low temperatures (25-80 ºC), tolerate CO_2 in the fuel gas and do not generate carbon or sulphur emissions (Abdi et al., 2017). The conversion efficiency in regular PEMFC varies from 40-50% (Kirubakaran et al., 2009) according to the operational temperature and the H_2 pressure (Lin et al., 2006). The ideal operational settings for these cells require impurity free fuel, i.e., the absence of CO, H_2S, NH_3, NO_x, among others, as compounds can react with the various components of the membrane electrode assembly (MEA). This will lead to poisoning of the electrode catalyst, increasing the resistance of the solid electrolyte and weakening the mass transfer due to alterations on the catalyst structure

and hydrophobility conditions, and leading to an overall efficiency loss (Cheng et al., 2007). The listed impurities are generally consequence of the conventional H_2 production processes, particularly by hydrocarbons or methane reforming, but they are uncommon in H_2-rich biogas produced through DF. Koruglu et al. (2019) investigated the conversion of biogas with an H_2 fraction between 33-60% in electricity by a PEMFC and correlated the presence of increasing amounts of CO_2 with a decrease in power density, suggesting that fermentation parameters should be optimised towards H_2 enrichment in the final biogas (Lin et al., 2006). The removal of CO_2 in the biogas prior to conversion through pressure swing adsorption or liquid adsorption produced positive results in the efficiency of the cell performance (Rhaman et al., 2016).

The present study addressing electricity generation from fermentative H_2, process scale-up and GWP assessment was divided in two parts. The first dealt with the integration of a continuous non-sterile acidogenic CIW fermentation system and a PEMFC for electricity production, with the full characterisation of the process input and output streams (FW, N, NaOH, acids, sludge, H and C). The CSTR operation was performed under non-sterile conditions with incorporation of a substrate pretreatment step for contamination control and a CO_2 stripping stage for H_2 enrichment of the produced biogas. The H_2-enriched biogas was fed to a PEMFC and compared with the cell performance with commercial H_2 at 25 and 50 ºC. The second part of the study sought to set-up the basis for an industrial process structured under a biorefinery model, where the food waste conversion to multiple valuable products was modelled. The lab-scale data were used in association with data from the literature concerning energy consumption values for the stages of industrial mashing, microwave application, bioreactor operation and centrifugation. The amount of 35 000 ton of FW collected annually by a Portuguese waste treatment management operator that receives separately collected waste from restaurants, food markets, hotels and school canteens in the Lisbon metropolitan was used as reference. The direct energy consumption and CO_{2eq} emissions generated by the conventional FW treatment to produce electricity and compost performed by the waste management operator were compared with six scenarios developed for the scaled-up biorefinery. The CO_{2eq} emissions of FW landfilling were also estimated.

7.2. Material and Methods

7.2.1. Food waste collection and conditioning

The FW used in this study was gathered from a local seafood restaurant located in the metropolitan Lisbon area, more precisely Trafaria, Almada, on July 2018, and corresponded to the customary service food scraps and leftovers from raw food preparation, i.e., designated as catering industry waste (CIW). The collection lasted for 8 hours of a regular day of activity of the establishment. Materials that could not be mashed within laboratorial setting such as bones and hard seeds were removed from the mixture. The remaining material was mashed thoroughly, homogenised until a typical consistency was attained and then stored at -20 ºC until further use. Samples of this material were characterized for moisture, carbohydrates, crude protein, fat and ash. The results of this analysis are displayed in table 1.

Table 1. Proximal composition of the processed CIW used in the fermentation experiments, by dry weight (d.w.).

Component	% d.w.
Total sugars	62.1 ± 0.1
Crude protein	10.4 ± 0.2
Total fat	26.3 ± 2.2
Ash	1.2 ± 0.1

7.2.2. Bioreactor operation under non-sterile conditions

The bioreactor apparatus consisted in a 1.65 L bench-scale double jacketed glass reactor with a working volume of 0.5 L. The air-tight reactor was equipped with a pH sensor (Mettler Toledo, Ohio, USA) and controller (SGI, California, USA), and inlets/outlets appropriate for the following events: removal of gaseous product, removal of fermentate, addition of fresh medium and carbon source, pH control and addition of inoculum. The bioreactor is illustrated below (Fig 1).

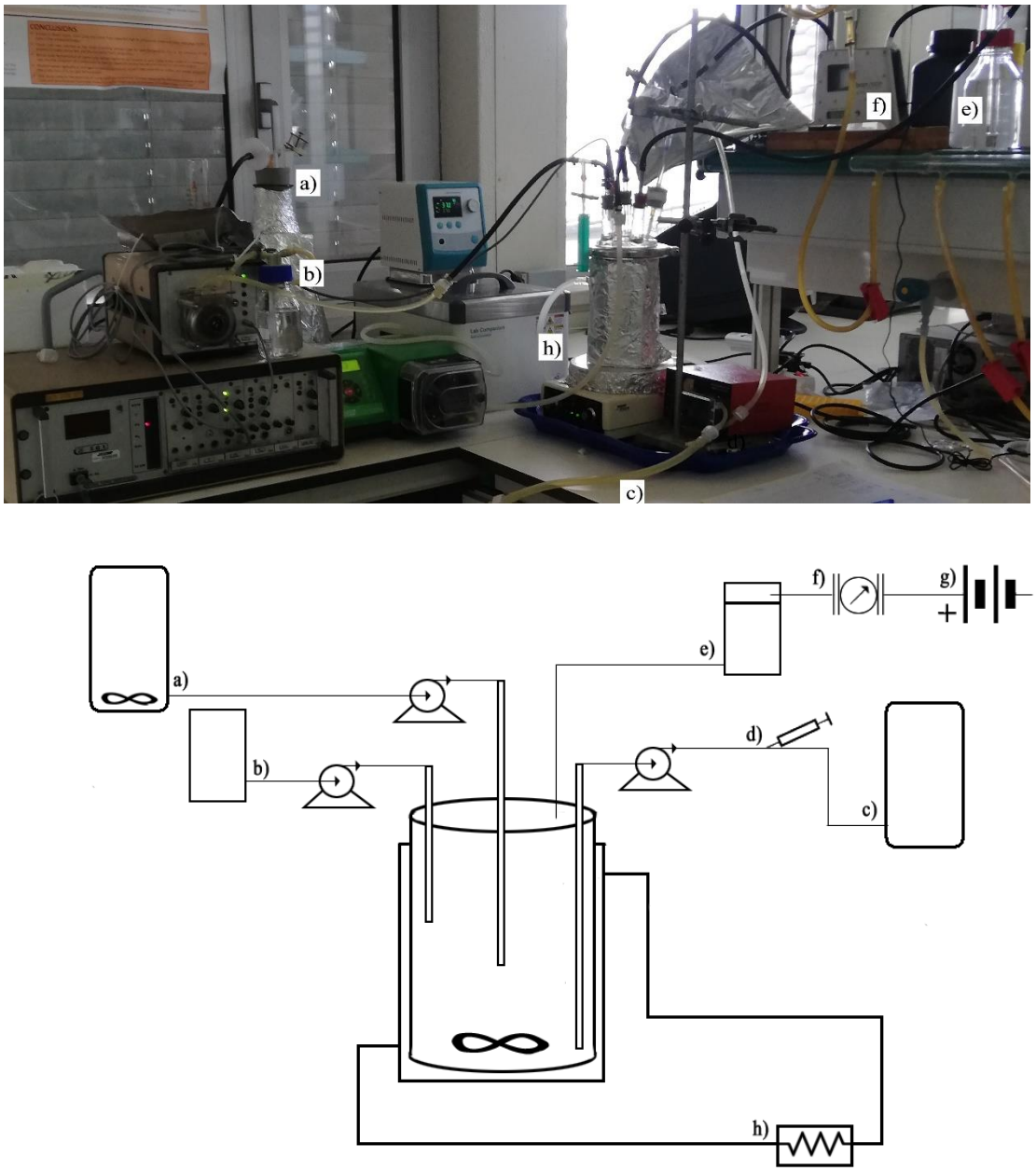

Fig 1. Schematic representation of the CSTR bioreactor: a) CIW + MMM feed; b) NaOH solution for pH control; c) effluent; d) sampling port; e) NaOH scrubber for CO_2 sequestration; f) Flowmeter; g) Proton-exchange membrane fuel cell (PEMFC); h) Water bath for temperature control.

The operational settings were kept constant throughout the experiment. pH control setpoint was defined as 5.5 ± 0.1 and controlled through the periodic addition of NaOH solution (2M). The temperature was maintained at 37 ºC and stirring was adjusted to 300 rpm to avoid biomass settling inside the vessel.

The start-up of the process was performed as follows: 60.75 g of humid CIW (correspondent to approximately 10 g of total sugars, final total sugars concentration of

20 g L^{-1}) were subjected to microwave pretreatment (MW) as described elsewhere (Ortigueira et al., 2019a). This mixture was suspended in minimum mineral medium as already published (MMM (per L of phosphate buffer 100 mM: 12 g NH_4Cl, 3.28 mg $FeSO_4.7H_2O$, 0.56 g Cysteine-HCl), up to a total volume of 500 mL (resulting in a concentration of total sugars of 20 g L^{-1}) and degassed with N_2 for 1 hour (Ortigueira et al., 2019a). *Clostridium butyricum* DSM 10702, from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) was used to inoculate the non-sterile mixture at a volumetric concentration of 5% (v/v). This inoculum was pre-cultured in Reinforced Clostridial Medium (RCM) (Difco laboratories, Le Pont de Claix, France) for 16 hours prior to the fermentation start-up. After 24 hours of operation, the operation mode was shifted from batch to CSTR.

The CSTR operation was performed as follows: 516.4 g of humid biomass was subjected to MW pretreatment as described previously (Ortigueira et al., 2019a). The resulting material was suspended in MMM medium up to a total volume of 5 L, resulting in a final concentration of total sugars of 17 g L^{-1}. The referred solution, hereby defined as feed solution, was used to fill the bioreactor communicating vessel, degassed with N_2 and then added to the reactor through the appropriate inlet at the flow of 67.8 mL h^{-1}. The hydraulic flow was equivalent to a hydraulic retention time (HRT) of 7.4 hours and the addition of 1.15 $g_{\text{total sugars}}$ h^{-1}. The fermentate was removed from the bioreactor at the same flow, to avoid culture volume variation. The liquid samples were collected through the appropriate outlet every 24 hours for characterisation and quantification of total sugars and soluble metabolites by HPLC. The gaseous product, mainly composed by H_2 and CO_2, generated inside the bioreactor was continuously conducted through a scrubber with NaOH solution (2 M) for CO_2 sequestration prior to the passage through a flowmeter (μflow, Bioprocess Control) for data collection. The outlet of the apparatus was connected to appropriate air-tight gas collecting bags (Flexfoil sample bags, SKC, Dorset, United Kingdom) for the final collection of the H_2-enriched biogas.

7.2.3. Fuel cell on-set and characterisation

A lab-scale proton-exchange membrane fuel cell (PEMFC) (Parker TekStak Fuel Cell, Parker Hannifin, Cleveland, USA) was integrated with the CSTR bioreactor. The fuel cell was composed by graphite composite plates, a Nafion membrane (10.89 cm^2 of

membrane area) and platinum-ruthenium catalysts. Prior to use, the PEMFC was assembled according to the instructions provided with the device and inserted into the experimental setup depicted on Fig 2.

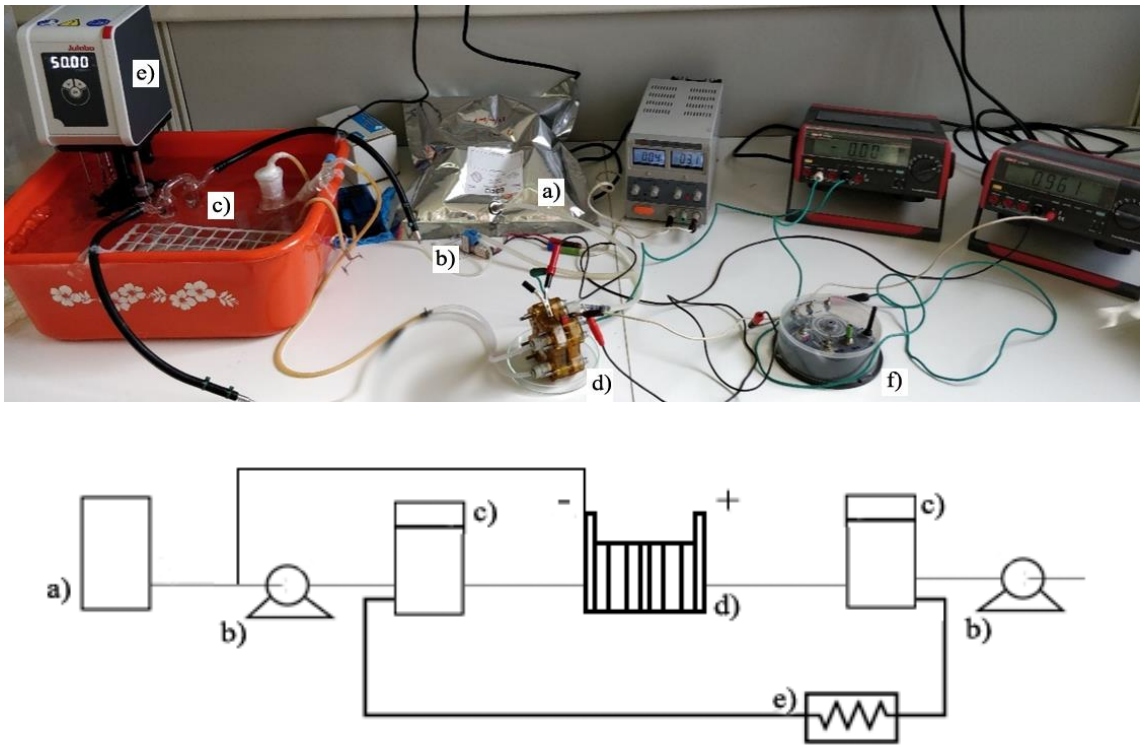

Fig 2. Schematic representation of the fuel-cell apparatus: a) Fermentative H_2 sample; b) air pump; c) gas washing bottle; d) Fuel cell; e) Water bath for temperature control, f) potentiometer.

The PEMFC was connected in series with a potentiometer and in parallel with an ammeter and voltmeter (Fig 2). The biogas sampling bag (a) was attached to an air pump for flow regulation (b) and forced through a water bath where the gas was humidified and heated to operational temperature (c). Atmospheric air was subjected to the same conditions. The PEMFC was tested with commercial H_2 (approximately 100% H_2 purity) and fermentative H_2 under two operational temperatures (25 and 50 ºC). The content of the feed gas varied according to the fermentation performance, being composed mainly by H_2 and vestigial traces of N_2. The biogas was kept humid and added sequentially for improvement and characterisation of fuel cell performance, respectively. Polarization and power density curves were drawn with the obtained data in order to attain the relation between bioH_2 flow ($\dot{m_{H2}}$) and power (P). Power is measured by voltage (V) and current (I),

$$P[W] = VI \quad (1)$$

Hydrogen flow is obtained from Larminie et al. (2013).

$$\dot{m_{H2}}\left[\frac{mL}{h}\right] = \dot{n_{h2}} * \frac{RT}{P}\left[\frac{m^3}{mol}\right] * 10^6\left[\frac{mL}{m^3}\right] * 3600\left[\frac{s}{h}\right] \quad (2)$$

were, $\dot{n_{h2}}$ is molar mass flow (mol s^{-1})

$$\dot{n_{h2}} = \frac{I}{2F}\left[\frac{mol}{s}\right] \quad (3)$$

F is the Faraday's constant (96485.33 A mol^{-1} s^{-1}), I is the current (A), R is the universal gas constant (8.314 kJ $kmol^{-1}$ K^{-1}), T is the trial temperature and P the ambient pressure (100 kPa). The efficiency η of the PEMFC can be obtained through equation 4:

$$\eta = \frac{P}{HHV * \dot{n_{h2}}} \quad (4)$$

Where HHV represents the higher heating value of hydrogen, assuming the water is removed from the system in liquid form (- 287230 J mol^{-1}).

The target ration of P/$\dot{m_{H2}}$can be derived by using the equations above,

$$\frac{P}{\dot{m_{H2}}}\left(W/_{L/h}\right) = \frac{\eta * HHV}{\frac{RT}{P} * 10^6 * 3.6}$$

For STP and $\eta = 0.5$, this procedure retrieves 1.96. Rahman et al. (2016) also considers a fuel utilization coefficient, μf, of 0.95, reducing the $\frac{P}{\dot{m_{H2}}}\left(W/_{L/h}\right)$ to 1.86. This value was experimentally validated. A total of ten assays were performed with bioH_2 and commercial H_2 at the temperatures of 25 and 50 ºC were made. These experiments amount to 142 voltage and current data points.

7.2.4. Analytical methods

7.2.4.1. Food waste proximal characterisation

The CIW samples were characterised for total sugars and fat, crude protein, moisture and ash according to standard methods (Horwitz and Latimer, 2005). Total sugars were determined after acidic pretreatment with H_2SO_4 (720 g kg^{-1}) according to standard methods (Horwitz and Latimer, 2005) followed by quantification through an adapted anthrone method as already published (Ortigueira et al., 2019a).

7.2.4.2. Characterisation of the fermentation products

Samples of the collection bags were analysed in a gas chromatography (GC) (Varian 430-GC) equipped with a thermal conductivity detector (TCD). The H_2 and CO_2 quantification was performed using a fused silica column (Select Permanent Gases/CO2-Molsieve 5A/Borabound Q Tandem #CP 7430). The injector and oven were operated at 80 and 70 °C, respectively and the detector at 120 °C. Argon was the carrier gas at a rate of 32.4 mL min^{-1}. The TCD was kept at 220 ºC.

The liquid samples were purged of solid residues by centrifugation (15000 rpm, 5 mins) and vestigial oil/fat was subsequently removed by hexane extraction (Ortigueira et al., 2019a). The resulting aqueous phase was filtered (0.2 μm) and analysed in a high-performance liquid chromatography (HPLC) system (LaChrom, Merck, Germany) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, USA) and a refraction index (RI) detector (LaChrom L-7490). The temperature of the column and the RI detector were kept constant at 50 °C and 45 ºC, respectively, and samples were eluted using H_2SO_4 5 mM (flow rate = 0.5 mL min^{-1}). Solutions of carboxylic acids were used as external standards. The solid fraction (sludge) was characterised according to nitrogen and total sugar content according to standard methods (Horwitz and Latimer, 2005) and a modified anthrone method as published elsewhere (Ortigueira et al., 2019a). Elemental characterisation of the feed and sludge were performed according to standard methods (ISO 18122:2015, ISO 16948:2015, EN 15289:2011, ionic chromatography) as already published (Ortigueira et al., 2019b). The analysis is summarised in table 2.

Table 2. Elemental characterisation of the feed supplied to the CSTR and the produced sludge.

% d.w.	Feed (CIW+MMM)	Sludge
Carbon	22.7	8.4
Hydrogen	5.5	3.6

Nitrogen	7.1	6.6
Chloride	3.2	0.6
Sulphur	0.065	0.05
Ash	1.2	54.3

7.2.4.3. Growth and fermentation parameters

The lag phase of the fermentation assay corresponded to the time necessary for the biogas production to be detected. The total H_2 production was calculated as the sum of the volume of H_2 collected in the collection bags during the course of the experiment, herein expressed as cumulative H_2 production, and the volume of H_2 which remained inside the bioreactor headspace at the end of the fermentation assay. The molar concentration of H_2 and CO_2 (mmol) was calculated through the Peng-Robinson equation (Ortigueira et al., 2015). Hydrogen productivity was estimated from the graphical representation of the cumulative H_2 production (L L^{-1}) *versus* time (hours), as the slope of the production tendency obtained for each 24-hour period. The molar H_2 yield was defined as the ratio between the total amount of H_2 (mol) produced throughout the experiment and the consumed total sugars (mol), expressed as glucose equivalents, and the volumetric H_2 yield was defined as the ratio between the total H_2 volume (mL) produced and the mass of CIW (g of volatile solids) supplied to the culture medium. Both parameters were calculated in 24-hour intervals.

7.2.5. Scale-up

The design of the industrial system considers the following stages: mashing, microwave pretreatment, acidogenic fermentation, gas stripping, centrifugation, electricity production from obtained biohydrogen and recirculation of sludge. The scaled-up system is depicted in Fig 3.

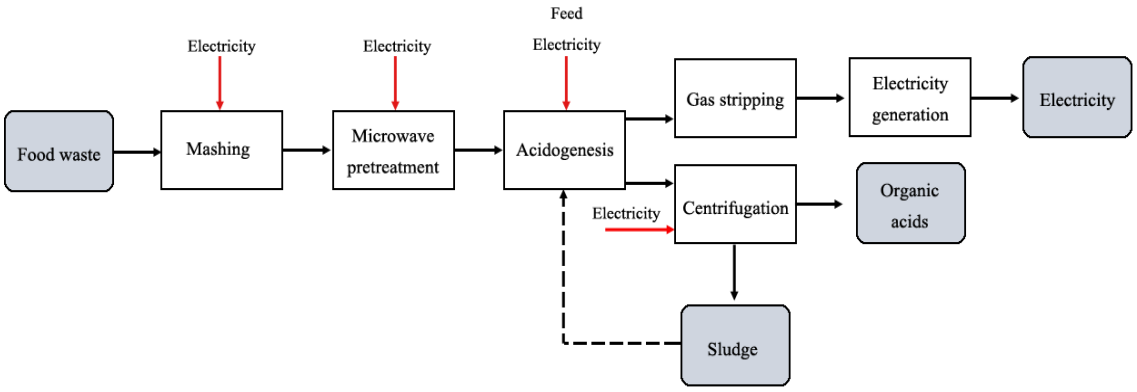

Fig 3. Schematic representation of the proposed industrial FW biorefinery

The scale-up was performed with basis on the selective collection of organic waste in the municipality of Lisbon for the year of 2018 (Valorsul, 2018). Considering the collection was performed from markets and canteens, green residues were considered negligible and the overall collection was equivaled to food waste and assumed to be the available substrate for acidogenic conversion. Therefore, the functional unit (FU) was defined as the amount of selectively collected organic waste in 365 days of operation, i.e., 35000 tons $year^{-1}$. Yields from lab-scale system were kept constant during scale-up, i.e., FW ton h^{-1} will produce L H_2 h^{-1}. This hydrogen flow will operate the PEMFC system. An inverter was introduced into the system after the PEMFC in order to convert direct current (DC current) obtained through the fuel cell operation into alternate current (AC current). The efficiency of the inverter was defined as 94% (Nishikawa et al., 2008).

The energy consumption data used for scale-up calculations was based on literature review registered on Table 3.

Table 3. Energy consumption of the industrial equipment considered for the scaled-up model of the biorefinery.

Industrial stage	Energy consumption	Reference
Industrial masher	1.1 kWh ton^{-1} biomass	de Marco et al., 2018
Microwave	168 kWh ton^{-1} biomass	Olatunde et al., 2017
Reactor	5.4 kWh m^{-3} feed	Collet et al., 2011
Centrifugation	5.5 kWh ton^{-1} biomass	Soda et al., 2010

7.2.6. Reference system for FW valorisation

The *Valorsul* company is responsible for the urban solid wastes (USW) collection and treatment from the Lisbon and West regions of Portugal, dealing with circa 20% of the USW generated in Portugal (1.3 kg of urban waste per capita per day). The waste treatment system is composed by, among others, an organic anaerobic digestion unit (OADU) with an installed capacity of 28 tonnes h^{-1}, i.e., capable of converting 1200 ton of waste into biogas per year. This particular stage consumes between 4 to 6 GWh $year^{-1}$ with a total yearly production of 8-12 GWh of electricity, through biogas burning, and 9800-14700 ton of compost. The biomass used in the process is selectively collected from restaurants, food markets, hotels and school canteens. The OADU unit is, by definition, a biorefinery as shown in a simplified schematic on Fig 3, involving various stages of pretreatment, drying, weighting, dehydration, etc. The referred installation will be compared with the system proposed in this study, based on the lab-scale results. The compost nitrogen content was obtained from the commercial information available online (2.3 %).

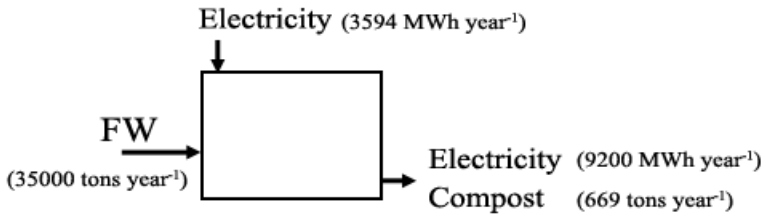

Fig 4. Schematic of the organic anaerobic digestion unit (OADU) from Lisbon valorisation unit (Lisbon, Portugal, 2018), reference biological treatment of FW.

The case in which FW is not valorised and allowed to decompose completely without human intervention, is reported only to support the discussion. In order to calculate the biogenic CO_2 of this process, the elemental characterisation of the feed (22.7 % $_{d.w.}$, see table 2) was applied to the FU. According to this calculus, a total of 2018 tonnes of C are introduced into the system for conversion. Without valorisation, the entirety of the carbon present in the biomass is converted into CO_2 leading to a maximum yield of 0.21 tons of CO_2 per ton of biomass or 7350 tons of CO_2 $year^{-1}$. It is considered the unfavourable condition, in which no product is derived from the process and the entirety of carbon content is lost as biogenic CO_2 emission.

7.2.7. System comparison

The production data obtained in the experimental stage were utilized as basis for the projection of a possible scaled-up production and compared with the data obtained from the reference biological conversion system (Fig 4). In order to compare the alternative proposed systems with the reference system in terms of direct energy consumption and global warming potential ($GWP_{100\ years}$), a consequential approach is followed, i.e, marginal supply and demand on affected markets is taken into consideration and allocation is avoided by system expansion. In practice, the same input of FW was considered and its associated outputs such as electricity (MWh $year^{-1}$) and compost (tons $year^{-1}$). Excess electricity, compost and additional byproducts are considered as credits (-) or burdens (+) in the alternative systems. The CO_{2eq} values for the consumables of the processes were taken from Ecoinvent 3 and other sources of literature to obtain a range of emissions instead of a unique quantity. Several cases of study were considered for analysis: Food waste without valorisation, valorisation through anaerobic digestion (reference) and the following scenarios with basis on the FW acidogenic biorefinery:

- **Scenario 1**. Default. FW is processed into hydrogen, organic acids (butyrate, acetate, lactate and formate) and sludge. Microwave pretreatment is performed in the waste treatment facility. Biogenic CO_2 is adsorbed during the gas stripping stage with associated sodium bicarbonate production.
- **Scenario 2**. FW is processed into hydrogen, organic acids (butyrate, acetate, lactate and formate) and sludge. Microwave pretreatment is performed at the household level. Biogenic CO_2 is adsorbed during the gas stripping stage with associated sodium bicarbonate production.
- **Scenario 3**. FW is processed into hydrogen and organic acids (butyrate, acetate, lactate and formate). Sludge is recirculated into the system as nitrogen source. Microwave pretreatment is performed at the household level. Biogenic CO_2 is adsorbed during the gas stripping stage with associated sodium bicarbonate production.
- **Scenario 4**. FW is processed into electricity and organic acids (butyrate, acetate, lactate and formate). Sludge is recirculated into the system as nitrogen source. Microwave pretreatment is performed at the household level. Biogenic CO_2 is

adsorbed during the gas stripping stage with associated sodium bicarbonate production.

- **Scenario 5**. FW is processed into hydrogen and organic acids (butyrate, acetate, lactate and formate). Sludge is recirculated into the system as nitrogen source. Microwave pretreatment is performed at the household level. Biogenic CO_2 is not sequestered.
- **Scenario 6**. FW is processed into electricity and organic acids (butyrate, acetate, lactate and formate). Sludge is recirculated into the system as nitrogen source. Microwave pretreatment is performed at the household level. Biogenic CO_2 is not sequestered.

The reference and alternative scenarios are depicted in Fig 5, including input and output information.

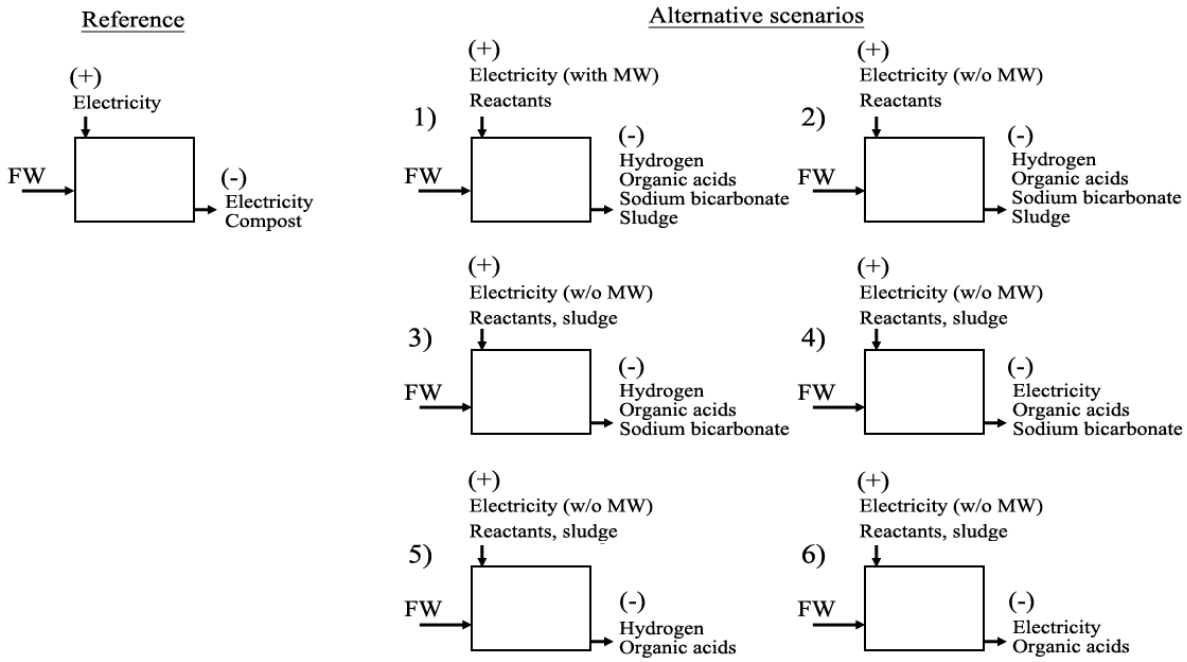

Fig 5. Schematic representation of the reference and scaled-up alternative systems and considered market influences for equivalency. credits (-) or burdens (+) for CO_{2eq}.

Table 4. Global warming potential of the main consumables and products identified in the different processes.

Item	Value kg CO_{2eq} kg^{-1}	Average kg CO_{2eq} kg^{-1}	Reference
Acetic acid	1.11-1.57	1.34 ± 0.23	Ecoinvent v3 database, 2016
Ammonium chloride	1.37-3.29	2.33 ± 0.96	Ecoinvent v3 database, 2016
Butyric acid	3.31-3.46	3.38 ± 0.07	Own calculations
Disodium phosphate	1.58	1.58 ± 0.00	Henvry et al., 2015
Electricity	296-524	377.5 ± 81.5	European Environmental agency database, 2019
Fertilizer (N)	4.62-5.88	5.25 ± 0.63	Ecoinvent v3 database, 2016 UZOS South-West Wastewater Treatment Plant annual data
Formic acid	1.43-2.91	2.17 ± 0.74	
Commercial hydrogen	0.97-12.9	6.94 ± 5.97	WTT JRC 2014 database, Ecoinvent v3 database, 2016 UZOS South-West Wastewater Treatment Plant annual data
Iron sulphate	0.17-0.23	0.20 ± 0.03	Ecoinvent v3 database, 2016
Lactic acid	3.97-4.95	4.46 ± 0.49	Ecoinvent v3 database, 2016
Monosodium phosphate	2.32-2.96	2.64 ± 0.32	Ecoinvent v3 database, 2016
Sodium bicarbonate	0.24-1.17	0.71 ± 0.47	Ecoinvent v3 database, 2016 UZOS South-West Wastewater Treatment Plant annual data
Sodium hydroxide	0.414-1.21	0.81 ± 0.40	Ecoinvent v3 database, Cetinkaya et al., 2011

The calculations were performed with basis on average GWP values prior to a range evaluation to account for uncertainty. Electricity carbon intensity is highly dependent on the country and year. To capture this effect on the calculations, results are depicted as a function of the electricity carbon intensity (European Environmental Agency, 2019). The products from the fermentation process were considered in the scenarios if produced in a significant quantity (> 1 g L^{-1} in the fermentate) and have widespread commercial importance, particularly for the chemical and pharmaceutical industry. The value of GWP for butyric acid value was not available in literature or available databases, therefore being estimated from the biological process described upon this document through

economic allocation based on scenarios 1 and 2. Table 5 depicts the current commercial prices for product and byproducts of the fermentative process and calculated GWP according to the economic allocation procedure.

Table 5. Input data for economic allocation and corresponding GWP per considered product for scenario 1 and 2.

Fermentation products	Price (€ kg^{-1})[1]	Proceeds (M€ $year^{-1}$)	GWP based on scenario 1	GWP based on scenario 2
Hydrogen	12.5	0.7	0.7	0.7
Acetic acid	28.7	16.7	1.6	1.7
Butyric acid	58.9	79.0	3.3	3.5
Lactic acid	62.5	45.6	3.5	3.7
Formic acid	24.5	9.6	1.4	1.4
Sludge	5.3	52.1	0.3	0.3
Sodium bicarbonate	53.9	290.7	3.0	3.2

[1] prices referenced chemicalbook.com.

The value for GWP of the L-cysteine hydrochloride was not available in literature and could not be estimated due to lack of data on its production. Hence, it was not considered. The compost and sludge obtained from the reference and acidogenic systems, respectively, were compared to fertilizer according to their nitrogen content (see section 2.6 and table 2). The range of GWP for electricity was imposed after analysis of the CO_2 intensity data for the year of 2016 (European Environmental Agency, 2019). The current electricity mix (CEM) corresponds to the best possible scenario, in which a higher fraction of renewable energy sources contribute to the electricity mix (2016 data). A low-renewable electricity mix (LREM) was also considered, corresponding to the worst-case scenario in which a larger fraction of non-renewable energy sources are used (1990 data).

7.3. Results and Discussion

7.3.1. Stability of continuous fermentative H_2 production from pretreated and bioaugmented CIW in a lab-scale setting

The continuous non-sterile H_2 production from highly contaminated substrates, such as FW, is highly influenced by the diversity and activity of the substrate native microorganisms that enter into the bioconversion system. The coexistence of different metabolic pathways may divert the conversion of sugars to undesired final products and

cause the decline of H_2 production. Promising results were obtained with the application of microwaves (MW) to CIW as a mean of reducing the initial contamination of the substrate before the batch fermentation for H_2 production (Ortigueira et al., 2019a). However, it is important to accurately assess the mid-term impact of such a MW pretreatment on the stability and performance of non-sterile bioaugmented fermentations operated under continuous mode. With this purpose, a CSTR bioreactor was operated for a total of 16 days for the non-sterile acidogenic conversion of MW-pretreated CIW. The profiles of the daily and cumulative biogas and H_2 production in the bioreactor are illustrated in Fig 6.

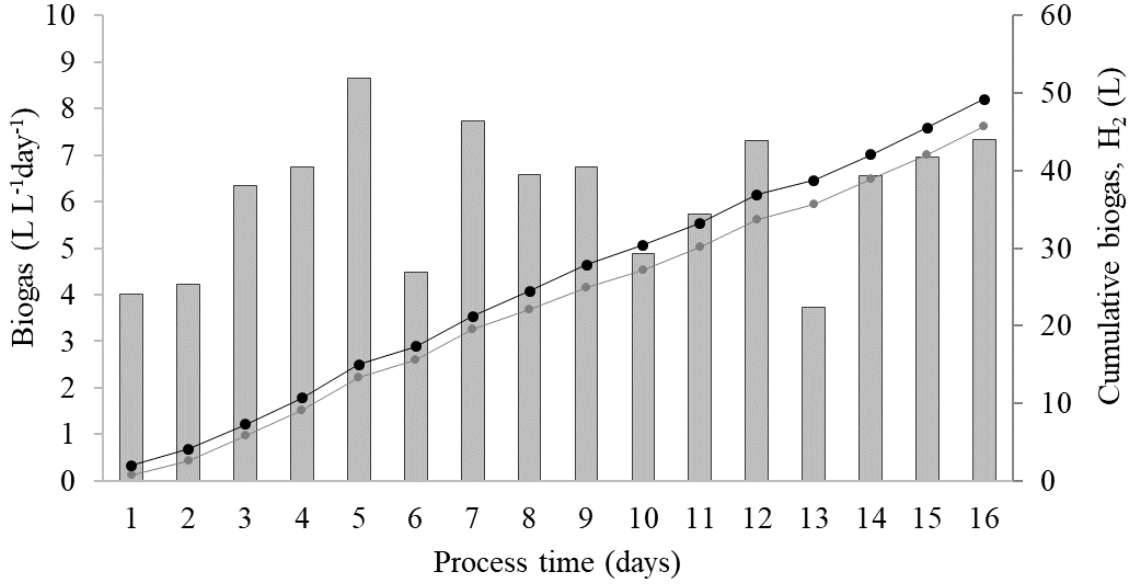

Fig 6. Daily biogas production (columns) and time-course of accumulated biogas and H_2 (biogas – ●; H_2 – ●) in a CSTR fed with MW-pretreated CIW, under non-sterile conditions and with addition of *C. butyricum* as biocatalyst.

The feed and HRT imposed upon the system were based on the consumption rate of total sugars by *C. butyricum* that was determined in a previous study (Ortigueira et al., 2019a). Accordingly, the feed suspension was introduced into the reactor at a flow rate of 1.15 g_{sugars} h^{-1} under a constant HRT of 7.4 hours. This value is consistent with the information reported by Sivagurunathan et al. (2016), that low values of HRT are conducive to more efficient acidogenic fermentations. Theoretically, a low HRT maintains the acidogenic microorganisms in exponential growth and minimises the proliferation of H_2-consuming microorganisms in the mixed culture. The results obtained from day 2-5 (Fig 6) show that the bacterial community was still under adaptation to the new hydraulic regimen, marked by a large increment in the volumetric biogas production

from 4.0 up to 8.7 L (L day)$^{-1}$. At that point, the concentration of H_2 in the produced biogas achieved 98% vol. after CO_2 stripping. Subsequently, the H_2 production was consistently maintained throughout the course of the experiment, steadying at an average volumetric production of 6.1 ± 1.3 L H_2 (L day)$^{-1}$ and an H_2 concentration in the output biogas of 95 ± 7 % vol. The exception to the tendency displayed occurred in day 6 and 13, wherein problems with the stirring of the feed stream were experienced, caused by an infrequent accumulation of solid particulates. These results are dissimilar to those registered by Kanchanasuta et al. (2017). The authors found that when *C. butyricum* strain TISTR 1032 was used as biocatalyst in non-sterile fed-batch fermentations of food waste, the registered total H_2 production was higher during the first substrate feed (24 hours of operation). During the second feed, a reduction of approximately 70% in total H_2 production was denoted. In the present study, the H_2 productivity and production yield, while variable, were consistent throughout the experiment. With exception of day 6 and 13 of operation (Fig. 6), the system was able to deliver incessantly a highly H_2-enriched biogas in the range of 4.9 to 8.7 L (L day)$^{-1}$ from day 5 to day 16 of process time. This result supports the assumption that the MW pretreatment, while not able to eliminate all the CIW native microorganisms, was able to partially control the activity of native microorganisms and maintain the H_2 production by *C. butyricum* in the microbial consortium. No CH_4 was detected in the biogas produced, i.e., the methanogenic activity was supressed or inexpressive either by the application of the MW pretreatment or by the low HRT imposed upon the system (Alexandropoulou et al., 2018).

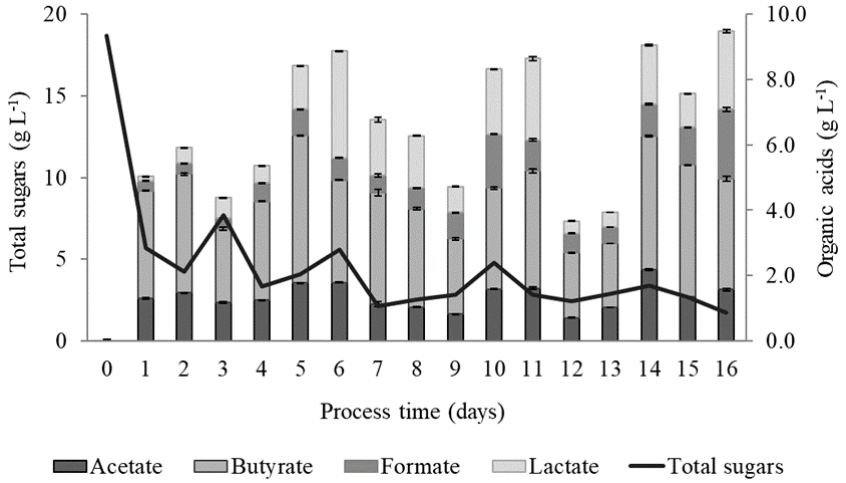

Fig 7. Time-course of total sugars consumption (line) and organic acids production (acetate – ■; butyrate – ■; formate – ■; lactate – ■) in a CSTR fed with MW-pretreated CIW, under non-sterile conditions and with the addition of *C. butyricum* as biocatalyst.

Sugar consumption under CSTR operation increased visibly through the fermentation cycles, up to a maximum of 94% (w/w) by day 16 (Table 5). This was likely associated with the adaptation of the microbial consortium to the substrate and accumulation of enzymatic compounds in the fermentation medium, which enabled the degradation of polymeric carbohydrates to a greater extend when compared to the first 48 h of fermentation. This may also be attributed to the persistence of native microorganisms of the CIW-feed, which may play a role in cross-feeding or exhibiting enzymatic complementarity to *C. butyricum*. An average H_2 yield of 74.9 ± 15.8 mL H_2 g_{vs}^{-1} or 110.7 ± 22.5 mL H_2 $g_{total\ sugars}^{-1}$ was obtained during the steady-state phase of the CSTR operation, between day 3 and day 16 (Table 6). These values represent a clear decrease when compared to the yields obtained previously under batch operation (Ortigueira et al., 2019a). They point to the presence of microbial populations that deviate carbohydrates to non-H_2 producing metabolic pathways. To counteract this effect, several authors suggested an increase of the fermentation temperature, and Shin and Youn (2005) obtained H_2 yields in the range of 125 L H_2 kg^{-1} $_{VS}$ under thermophilic conditions. In the present study this option was disregarded as it would be associated with higher energetic costs. A second option was suggested in the same study which would reside on the use of mixed culture adaptation to the substrate, imposing a low HRT or a high operational temperature as selective pressure to supress the activity of undesirable microorganisms. Qin et al. (2019), De Gioannis et al., (2017) and Algapani et al. (2018) obtained H_2 yields of 50, 56.5 and 135 L kg^{-1} $_{VS}$, respectively, with the latter strategy, the last value being considerably higher than that obtained in the present study. However, the yield per unit of substrate biomass is highly dependent on the chemical composition and it is likely that an increase in carbohydrate concentration may impact significantly the overall production yield. Conversely, the H_2 productivity registered in the present study reached 6.1 ± 1.3 L $(L\ day)^{-1}$, which is considerably higher than 3 L $(L\ day)^{-1}$ obtained by Algapani et al. (2018).

Previous studies on the fermentation of carbohydrate-containing biomass by *C. butyricum* identified butyric and acetic acid as the main byproducts of sugar conversion (Ortigueira et al., 2019a). The concentration of organic acids throughout the operation of the CSTR averaged 5.2 ± 0.8 g L^{-1}, being composed mainly by acetate and butyrate, which corresponded to approximately 19 and 44% of the total acids produced, respectively. The average butyrate-to-acetate ratio was 1.9 ± 0.4 (Table 6), indicating the slight shift

towards butyrate production which is generally associated with high H_2 partial pressure (Ortigueira et al., 2019a). Lactate was also produced, particularly after day 5 (Fig. 7). Unlike acetate and butyrate, lactate is not linked with H_2 production and was associated to the presence of microbial contaminants in the substrate (Harzevilli & Hiligsmann, 2018). The MW pretreatment was originally introduced to control the substrate contamination, particularly by lactic acid bacteria which deviate the carbon source to undesirable metabolic pathways. However, this effect became less apparent after the first 5 days of fermentation, resulting in a highly variable lactic acid concentration (0.4 - 3.2 g L^{-1}) up to the end of the process time. These results lead to the conclusion that lactic acid bacteria persisted in the substrate after pretreatment and in the reactor during fermentation.

Table 6. Average production parameters obtained during the 16 days of operation of the CSTR fermentative system for CIW conversion.

Total sugars consumption (%)	Total H_2 production (L L^{-1})	Average H_2 productivity (mL L h^{-1})	H_2 in the biogas (% vol)	H_2 yield (L kg^{-1}_{VS})	Butyrate-to-acetate ratio
94.1 ± 2.6	91.3 ± 0.1	257.4 ± 54.6	95.8 ± 1.0	74.9 ± 15.8	1.9 ± 0.4

The results of H_2 production showed a stable performance of the CSTR throughout the process runtime, although some metabolic activity directed to the production of lactic acid was denoted.

7.3.2. Electricity generation from biohydrogen, lab-scale setting

The biogas produced during the operation of the CSTR was composed by CO_2 and H_2, likely in a humidified state due evaporation of the culture media. The CO_2 sequestering stage dissolved all produced CO_2 in a supplied solution of NaOH with the associated production of dissolved sodium bicarbonate and originating an H_2-enriched biogas sample (average of 95% purity). This sample was fed to a PEMFC at two operational temperatures, 25 and 50 ºC. For comparison purposes, the same experimental setup was undertaken with commercial H_2 (>99% purity) in order to ascertain if biogas

characteristics would impact PEMFC performance. The experimental polarization curve and efficiency are depicted in Fig. 8 and 9 for $BioH_2$ and commercial H_2.

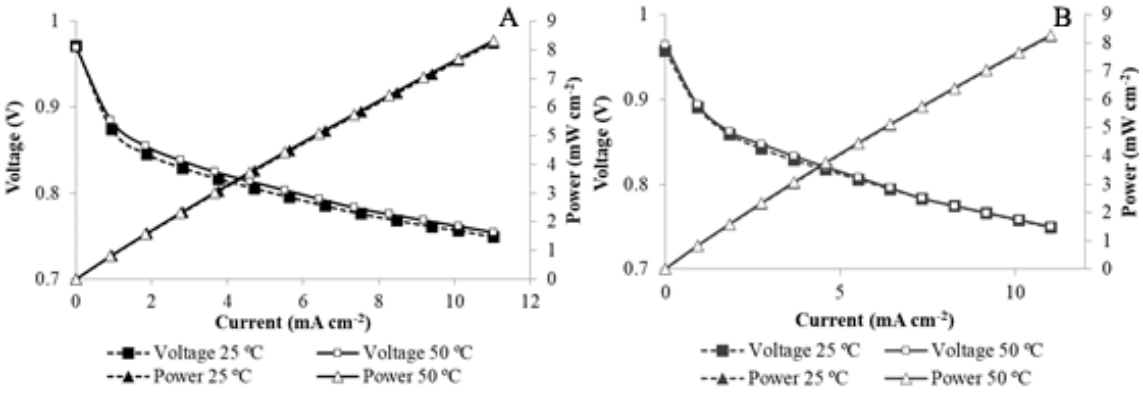

Fig 8. Polarization and power density curves for the lab-scale fuel cell operated with: A) $BioH_2$ produced during the operation of the CSTR; B) Commercial H_2.

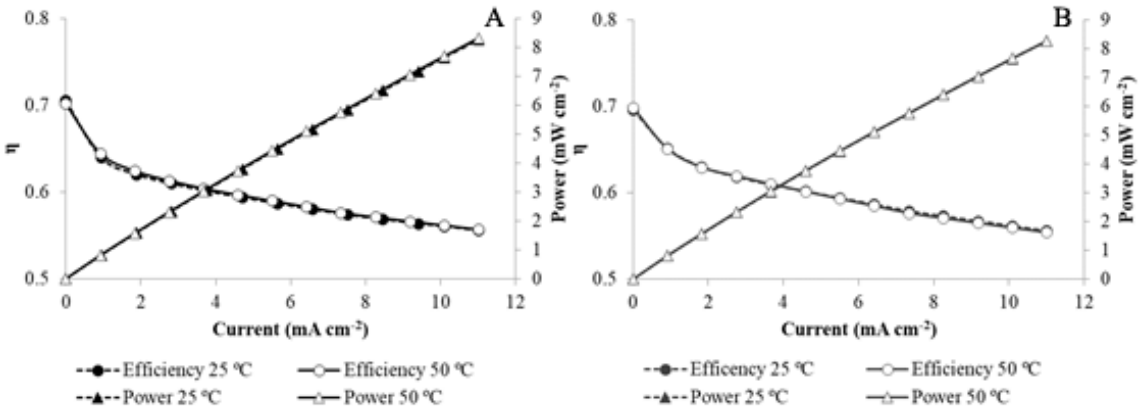

Fig 9. Representation of the fuel cell efficiency data versus current and power for the lab-scale fuel cell operated with: A) $BioH_2$ produced during the operation of the CSTR; B) Commercial H_2.

Fig. 8A compared the information attained through the operation of the PEMFC with the $BioH_2$ at the two operational temperatures. Values of voltage measured for both systems registered a slight increase with the increasing temperature but the variation was not considered to be statistically significant. In fact, the same effect is denoted upon analysis of the power measurements for both temperatures which, once more, proved to be nearly indistinguishable. This result seems to contradict known literature which underlines that temperature increase should impact the PEMFC performance (Lin et al., 2006). This deviation in behaviour might be indicative of a required further increase in temperature (80-100 ºC) or a result of the operational setting. The stage for biogas/air

temperature increase should have, theoretically, increased the overall temperature of the gas and of the PEMFC. However, the PEMFC itself as well as connecting pathways were not subjected to any type of temperature increase, possibly counteracting the effect of the higher gas temperature. As it is, the analysis of the results does not indicate any advantage related to the use of 50 ºC as operational temperature. Comparison between 8A and 8B revealed no significant statistical difference between the type of fuel, i.e., the performance of the PEMFC did not vary with the origin of the gas. This result is indicative of the absence of serious contaminants in the biogas, particularly CO and H_2S which are often produced in other biological systems (Rahman et al., 2016). The removal of the CO_2 sequestration stage should, theoretically, have no influence in the performance of the described PEMFC as CO_2 is not described as contaminant of the membrane. 9A and 9B depict the variation in efficiency according to the type of gas and operational temperature and, as previously observed, there was no significant alteration in efficiency for the considered variables. To avoid unnecessary energy expenditures associated with the temperature increase, 25 ºC was elected as the appropriate operational temperature. In this condition, the efficiency registered varied between 50-70%, attaining its minimum value at maximum power. The experimental data was used to quantify the ratio between power and hydrogen flow, 1.7 W L^{-1} h^{-1}. This value is consistent with literature values such as 1.86 W L^{-1} h^{-1} obtained by Rahman et al., (2016).

7.3.3. Scale-up and system comparison

The alternative systems were dimensioned according to the specifications of biomass feed, i.e., the facility will need to process 35000 tonnes of biomass per year (defined FU). This value is equivalent to, approximately, 4 tonnes of biomass per hour, which indicates a total volumetric flow of feed (i.e., biomass and fermentative medium) of 930.6 m^3 per hour. The ratio between volumetric feed flow and the reactor volume was kept from the lab-scale to the scale-up (0.14). All unitary operations will be based upon the value of feed indicated, assuming lab-scale yields are kept constant. The power and hydrogen flow tendency were assumed to be the same as the one obtained in the lab-scale, as depicted in the previous section (Fig 8). According to lab data, each litre of H_2 consumed, will produce 1.7 Wh of electricity. The industrial PEMFC dimensions are obtained by knowing the maximum industrial hydrogen flow with η = 50%, for maximum

power. This is, for the 72.7 m^3 H_2 h^{-1} production predicted from the scale-up, the stack maximum power should be 123.5 kW. For this production to be viable, two bioreactors would be required with an overall volume of 200 m^3 (useful volume of 143 m^3, assuming a reactor occupation of approximately 70%). Moreover, as the laboratorial equipment does not have the capacity for the volumes considered in this study and lack the increasing efficiency effect usually denoted in large-scale equipment, the energy requirements for the system were obtained from literature (refer to table 2). Mass and energy balances were made available for the reference system by the ValorSul company (see Fig 4). The energy consumption pattern was considered the same for all analysed scenarios for four of the five unitary operations: grinding/mashing, fermentation, gas stripping and centrifugation. Scenario 1 analyses the impact of the stage of contamination control (microwave) if performed at the industry level. Results of the energy consumption per stage analysis are depicted in table 7.

Table 7. Direct energy consumption of the alternative valorisation system (Scenario #1-Default).

Stage	Energy (MWh per year)
1. Grinding/mashing	28.2
2. Microwave	4269.0
3. Fermentation	1328.7
4. Gas stripping	0.0
5. Centrifugation	2644.6
Total	8270.5

The intensive energy stages are temperature related, such as the microwave stage for contamination control and the fermentative stage which requires strict temperature control. In fact, the comparison between scenario 1 and 2 indicated that the microwave pretreatment stage is the main critical point of the overall process. The energy consumption in Scenario 1 reaches a maximum of 8271 MWh $year^{-1}$, approximately 2.3 times superior to that which is reported for the reference (3594 MWh $year^{-1}$). This situation can be potentially avoided if the microwave pretreatment is undertaken at the household level as suggested in a previous iteration of the system (Ortigueira et al., 2019a). If this change is considered, there is only a 11.3% increase in energy consumption due to the application of acidogenesis when compared with the reference system.

Table 8. Inventory for the reference and alternative systems, per FU: 1. Default; 2. Default without microwave pretreatment; 3. Sludge recirculation to H_2; 4. Sludge recirculation to electricity; 5. Sludge recirculation without CO_2 sequestration to H_2; 6. Sludge recirculation without CO_2 sequestration to electricity.

		Reference biological treatment	Scenario					
			1	2	3	4	5	6
Inputs (tons)	Biomass input per year (FU)	35000	35000	35000	35000	35000	35000	35000
	Iron sulphate	-	1114	1114	1114	1114	1114	1114
	Sodium hydroxide	-	4159	4159	4159	4159	1359	1359
	Ammonium chloride[1]	-	4076	4076	15	15	15	15
	Cysteine-HCl [1]	-	190	190	190	190	190	190
	Disodium phosphate[1]	-	2962	2962	2962	2962	2962	2962
	Monosodium phosphate[1]	-	2391	2391	2391	2391	2391	2391
Inputs (MWh)	Electricity	3594	8271	4002	4002	4002	4002	4002
Outputs (tons)	Hydrogen	-	57	57	57	-	57	-
	Acetic acid	-	583	583	583	583	583	583
	Butyric acid	-	1340	1340	1340	1340	1340	1340
	Lactic acid	-	730	730	730	730	730	730
	Formic acid	-	393	393	393	393	393	393
	Sodium bicarbonate	-	3710	3710	3710	3710	-	-
	Compost/sludge	669	9835	9835	-	-	-	-
	Carbon dioxide	6829	-	1540	-	-	-	-
Outputs (MWh)	Electricity	9200	-	-	-	1078	-	1078

* components of minimum mineral medium (MMM)

The reference biological treatment (FW to electricity and compost) process is based on the anaerobic digestion of the biomass into biogas and compost. Biogas is burned in a combined heat and power unit (CHP) to produce electricity and heat for the process. The main product considered by the referred process is electricity and compost

and the only input accounted for is electricity (Valorsul, 2018). Conversely, the alternative process relies heavily upon different byproducts for possible viability, particularly the organic acids obtained from the fermentative process (butyric, acetic, formic and lactic acid) which have considerable importance in both the chemical and pharmaceutical industries. The contribution of each input and output was associated with its respective global warming potential through the quantification of the CO_{2eq} parameter. The GWP data was calculated according to emission factors obtained from the average of minimum and maximum emission factors attained from literature and specialized databases (see table 4). Fig 10 depicts the GWP potential of the various scenarios for the alternative valorisation process, taking into consideration the various contributions per input and output (credits (-) or burdens (+)). It serves the purpose of identifying the highest contribution stages to CO_{2eq}.

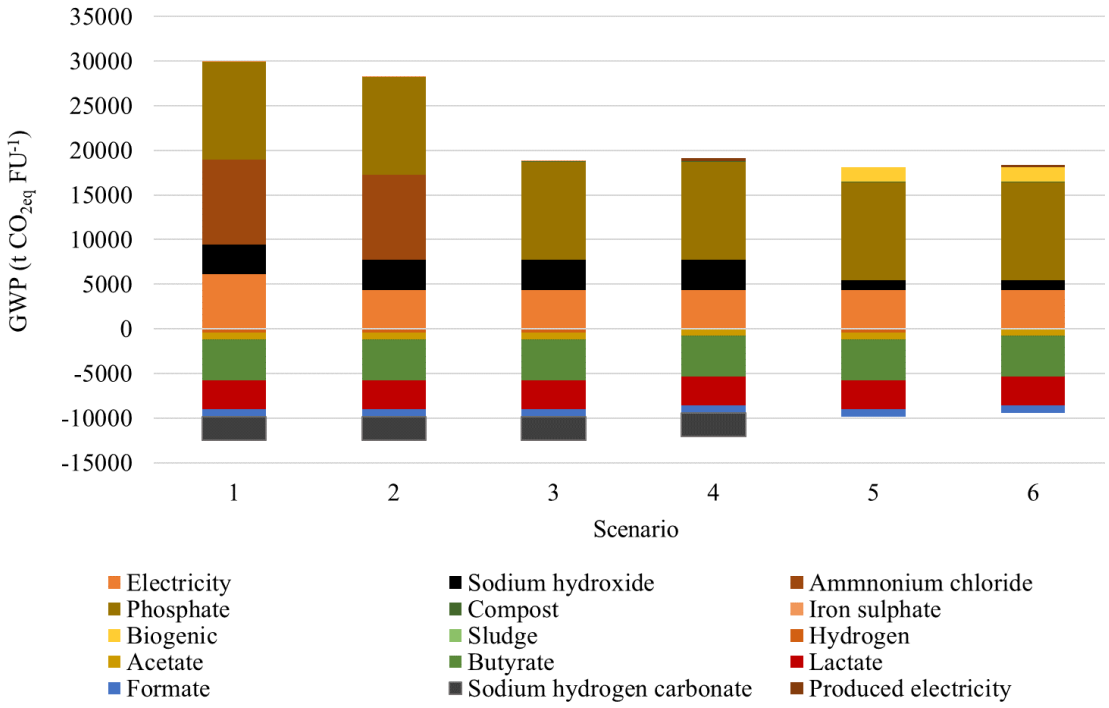

Fig 10. Global warming potential of the alternative valorisation system according to the described scenarios, per FU: 1. Default; 2. Default without microwave pretreatment; 3. Sludge recirculation to H_2; 4. Sludge recirculation to electricity; 5. Sludge recirculation without CO_2 sequestration to H_2; 6. Sludge recirculation without CO_2 sequestration to electricity.

The default condition (Sc#1) implies the use of the defined media described in section 2.2, particularly the use of phosphates as buffer and ammonium chloride as nitrogen source and both compounds have relatively high associated emissions. Nitrogen cannot be removed completely as it is a vital compound for cellular growth

and this particular substrate does not have enough nitrogen content for the optimal development of *C. butyricum* (Ortigueira et al., 2019a; Ortigueira et al., 2019b). It was postulated that the byproduct acidogenic sludge can be recirculated into the bioreactor and used as source of nitrogen (Ortigueira et al., 2019b). The application of this change to fermentation parameters decreased the GWP emissions by 64.3 and 63.8% for Sc#3 and Sc#4, respectively, and was considered the most advantageous condition. Conversely, the removal of the buffer solution is not ideal as the fermentation is highly dependent on pH for optimum results. The absence of buffer leads to high variations in the pH value of the culture, caused by the fermentative acid production and the addition of NaOH solution for pH control. This variation can influence negatively the cellular viability. A previous study explored the effects of the replacement of buffer solution with tap water and the removal of the nitrogen source on the fermentation of FW concluded that none of both studied factors invalided the conversion though the removal of both had a negative impact in performance (Ortigueira et al., 2019a). Nevertheless, further experiments must be undertaken to fully evaluate the need for buffer solution and its effects. Sodium hydroxide is generally associated with high carbon emissions (Thannimalay et al., 2013). In this process, it is used for both CO_2 capture and solubilization as well as pH control. The removal of CO_2 capture eliminates a secondary product – sodium bicarbonate – while adding the quantification for the biogenic CO_2 produced during fermentation. Overall, the impact of this measure is negative, increasing GWP values by approximately 30%. The last two scenarios were undertaken to evaluate the effect of account for the final conversion of hydrogen into electricity through the use of a fuel cell. The comparison between the scenarios 3 and 4 indicated an increase of 2.3% in GWP when electricity was considered the final product of the valorisation system. When the emission of the electricity mix was not considered to be of renewable origin, i.e., 524 kg CO_{2eq} MWh^{-1}, the system reacted more favourably, reaching a maximum decrease of 1.9% in GWP.

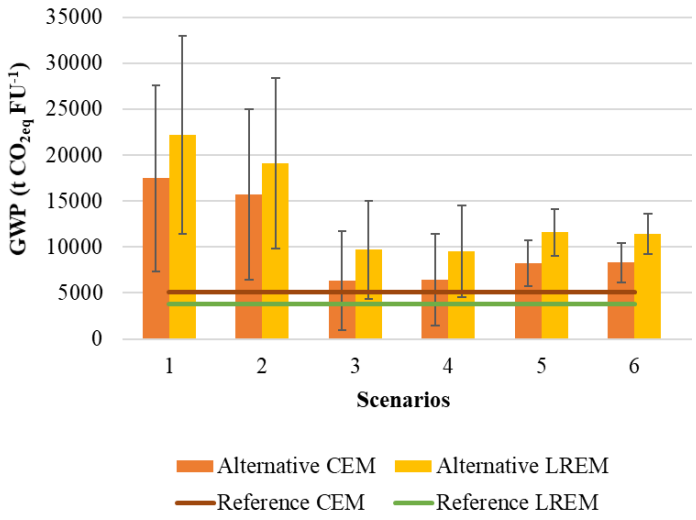

Fig 11. Total GWP calculated for the reference and alternative scenarios considering the electricity mix: current electricity mix (CEM) and low-renewable electricity mix (LREM).

The energy consumption of the anaerobic digestion and the acidogenic fermentation are not dissimilar, as discussed previously. Therefore, the impact of the energy sources considered for the electricity mix will influence the input emissions of the reference and alternative systems in a similar manner. The same cannot be stated for the evaluation of the product credits. As the reference considers electricity as its main product, the increase in non-renewable fraction in the electricity mix will result in 25% reduction of the overall GWP value. Conversely, the increase in emissions of the electricity mix impacts negatively all the scenarios considered for the alternative system due to emission input increase. The analysis lead to the conclusion that the removal of NH_4Cl from the fermentation media (scenario 3) reduced greatly the GWP value of the system. Taking into consideration the uncertainty of the GWP inputs, we obtained a range of values for the GWP of scenario #3, 3499 - 14191 tonnes of CO_{2eq}. The uncertainty should be addressed in order to evaluate if the alternative system would have a positive impact when compared to the reference.

7.4. Conclusions

The fermentation of FW by *C. butyricum* was undertaken successfully under non-sterile conditions in a CSTR. The predominance of the biocatalyst was favoured by the application of a microwave pretreatment to FW for substrate contamination control, and

by operational settings that favour *C. butyricum* activity, such as low HRT and adjustment of the optimum pH and temperature. During the 15 days of process time the average H_2 production remained stable at 6.1 ± 1.3 L H_2 (L day)$^{-1}$, with an average composition of 95% (v/v) of H_2 in the produced biogas and an average yield of 74.9 ± 15.8 mL H_2 g^{-1}_{VS}. The principal cometabolites produced were butyric and acetic acid, averaging 5.2 ± 0.8 g L^{-1} in the fermentate. The biogas produced in the CSTR was enriched in H_2 by CO_2 stripping and fed to a PEMFC at two operational temperatures, for electricity generation. No significant differences in the cell performance were recorded under all conditions tested, nor when compared to synthetic H_2. The data obtained during the experimental procedure were used to model a process scale-up and for the estimation of GWP values according to six scenarios. The best scenario (#3), i.e., with a lower GWP of 3499 - 14191 tonnes of CO_{2eq}, was the one in which the fermentation nitrogen source was replaced by recycled DF-sludge.

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8. Conclusions

8. Conclusions

Food waste has long been considered an important issue of larger and modern societies, representing one third of the food produced in the world for human consumption (Lipinski et al., 2013). The increasing tendency for its production coupled with the associated waste of resources and carbon emissions, do not allow for its continuous neglect. Quantification of food waste is still elusive due to lack of formal quantification systems which are common to the different stages, from production to retailing and consumption. For example, in the Portuguese territory and according to (2012) data, approximately one million of tonnes are produced and disposed of, potentially without recovery of value.

This thesis explored the potential of FW valorisation under a biorefinery scope for the production of energy, sludge (for use as fertiliser or as nutrient source) and chemical precursors, particularly through the application of dark fermentation (acidogenic fermentation), an anaerobic fermentation process whose main products are hydrogen, organic acids (butyric, acetic, lactic, formic acids) and sludge, and its improvement, optimization and potential pairing with other FW processing options. The research process was based on the following stages:

- Evaluation of the substrate array and quantity available in the Portuguese territory according to two predefined typologies (agro-industrial residues and/or byproducts, and catering industry waste), selection of appropriate substrates for acidogenic conversion and quantification of those selected;
- Evaluation and comparison of the fermentability of the model substrates, particularly under sterile conditions in small scale-assays. Selection of the best substrate for larger-scale fermentation;
- Bench-scale, pH-controlled, sterile fermentations using both typology substrates. Assessment of the effect of sterilisation waive on the acidogenic fermentations. Application of different methods for substrate contamination control and effects on the fermentation performance. Optimisation of the operation parameters, particularly nitrogen supplementation;
- Acidogenic fermentation of a model substrate in continuously stirred tank reactor.
- Integration of a PEMFC in the fermentation system for the conversion of produced H_2-rich biogas into electricity;

- Quantification of the direct energy consumption and direct and indirect greenhouse gas emissions through estimation of emitted carbon dioxide equivalents (CO_{2eq}).

One of the major contributions of this thesis was the development of a simple valorisation process focused on the use of waste/residue/byproduct streams whose current valorisation leads to low-value products. The various stages of the process are reliant on the waste producer intervention, particularly for organics separation and preliminary waste handling preferably with simple and household adaptable procedures. The acidogenic biorefinery model described in this work could, theoretically, be applied to other carbohydrate-rich residues with only slight adaptations as, for example, the type of pretreatment applied according to the biomass chemical structure.

One of the objectives of this work was to underline the lack of waste quantification data at the different stages of the food production line and the lack of alternatives to conventional valorisation processes for biowaste, making room for the improvement of the current FW treatment and disposal practices.

Research question 1: *Is there a significant food waste potential in the Portuguese territory?*

There is no actual quantification of the food waste generated in all its various sources in the Portuguese territory. The project PERDA estimated that approximately one million tonnes of food waste per year were produced from the various phases of the food production supply line (Baptista et al., 2012). The analysis of the available data identified strong inadequacies in the food production stage, underlining efficiency problems during harvest, storage and post-harvest processing in the agricultural production and severe issues associated with transport of animal fed. Food processing, on the other hand, was identified as highly efficient, with small losses usually connected to accidental mechanical damage, start-up stages, end of production, etc. The food losses during distribution and consumption shared much of its causes, lack of or bad storage, lack of proper stock management, inappropriate handling or lack of information concerning the 'use-by' or 'best before' nomenclatures.

The actual data on the organic waste production in the Portuguese territory was obtained in reports made available by municipal urban waste management (MSW)

systems. In the year of 2017, 5 Mtonnes of municipal solid waste (MSW) were quantified in the continental Portuguese territory (Marçal & Teixeira, 2017). The chemical characterisation of this material yielded an approximate organic content of 36% (w/w), resulting in a total organic waste production of 1.8 Mtonnes per year. Approximately 0.98 Mtonnes of this highly biodegradable, carbon-rich material were not subjected to valorisation, being instead disposed or landfilled.

Research question 2: *How does Clostridium butyricum perform in terms of hydrogen and organic acid production when using food waste as carbon and energy source*?

To address the suitability of food waste as substrate for acidogenic fermentation, the following typologies of waste were selected: agro-industrial residues and/or byproducts (AG) and catering industry waste (CIW). Four AG materials were selected according to the production relevance in the Portuguese territory (corn cob, carob pulp, brewery's spent grain and wheat straw) and used as substrate in small-scale comparative fermentation assays performed with *C. butyricum*. The fermentability of the materials was highly dependent on the chemical characterisation, particularly the composition in carbohydrates and their associated easiness of solubilisation or saccharification. Higher concentrations of cellulose, hemicellulose and lignin were generally indicative of more hardly fermentable substrates that require increasingly severe pretreatments to obtain sugar-rich solutions. The four AG substrates were successfully converted into hydrogen, organic acids and sludge. Carob pulp was elected as the most suitable model substrate for the conversion to H_2 as its sugars are easily solubilised through a simple water-extraction process, conversely to the other substrates which required harsh pretreatments at high temperatures.

The evaluation of the *C. butyricum* performance while using carob pulp (CP) and catering industry waste (CIW) as carbon and energy source was performed after scale-up, namely a 25-fold volume increase from the small-scale to bench-scale. Bench-scale assays were undertaken in sterile conditions with nutrient supplementation, i.e., addition of nitrogen, iron and phosphorus to guarantee minimum bacterial growth requirements. The cumulative H_2 production, productivity and yield attained with both substrates were comparable; however, the maximum values of 4.1 ± 0. 2 L H_2 L^{-1}, 249.5 ± 24.6 mL H_2 $(L\ h)^{-1}$ and 86.5 ± 0.1 ml H_2 g^{-1}_{VS}, respectively, were registered in the fermentation of CIW. A simplification of the fermentation process was required to proceed with the scale-

up and to shift from batch to continuous mode of operation. The sterilisation stage was identified as the most problematic of the overall process, as it is heavily associated with high-energy requirements. The removal of this stage reduced the overall H_2 production by 59% from CIW due to the presence of CIW native microorganisms that compete for carbohydrates. The application of microwaves (MW-pretreatment) to CIW was tested as a simple process for contamination control. The combination of the microwave pretreatment with the addition of *C. butyricum* as biocatalyst improved significantly the performance of the fermentation, up to a maximum of 98.8 ± 10.2 mL H_2 (g volatile solids)$^{-1}$. This value represented an increase of 14% and 64% in the substrate conversion yield when compared to sterile and non-sterile conditions performed with *C. butyricum* but without MW-pretreatment, respectively.

Research question 3: *Is it technologically possible to set up a lab-scale acidogenic fermentation in continuous mode?*

The major problem identified in the operation of the continuous stirred reactor (CSTR) supplied with FW as substrate and *C. butyricum* as biocatalyst, for H_2 production, was to the capability of this microorganism to succeed in a bioreactor whose operational settings did not include a prior sterilisation stage. According to experimental data obtained in previous research, the CIW native microorganisms counts diminished substantially with the application of the microwave pretreatment. However, the complete elimination of the native community was not expected, and additional contamination is expected to be introduced into the bioreactor from daily handling and operation of the non-sterile system.

The CSTR was performed under the optimum fermentation parameters for *C. butyricum* in order to favour its growth, i.e., to serve as positive selective pressure. The optimum temperature and pH were defined as 37 ºC and 5.5, respectively, while the feed flow and hydraulic retention time adopted for the operation were modelled based on the consumption rate of total sugars by *C. butyricum* (1.15 g_{sugars} h^{-1}, HRT = 7.4 hours). The acidogenic CSTR bioreactor was operated for a total of 16 days for the non-sterile conversion of MW-pretreated CIW and the experimental results showed consistent H_2 production throughout the process runtime. In fact, the initial H_2 production was lower during the start-up stage of the bioreactor. After a 5-day period, the H_2 production was

consistently maintained throughout the course of the experiment, steadying at an average volumetric production of 6.1 ± 1.3 L H_2 (L day)$^{-1}$ and an H_2 concentration in the output biogas of 95 ± 7 % vol. The slow increase of the H_2 production and its stabilisation seemed to indicate the persistence of the *C. butyricum* in the bacterial community inside the bioreactor. The production of H_2 was accompanied by the production of organic acids, particularly butyric, acetic, formic and lactic acid. In a previous section, lactic acid was associated to the presence of microorganisms undesirable for H_2 production, and its production in the CSTR was indicative of the persistence of such microorganisms throughout bioreactor operation. With these results in mind, it was concluded that the microwave pretreatment was undoubtedly efficient, as its application enabled some level of substrate contamination control inside the bioreactor, to limit the activity of CIW native microbiota and indirectly favour the growth of *C. butyricum*. It was also possible to conclude that, according to the fermentation set-up designed in this study, the production of a H_2-rich, highly pure biogas from CIW in a continuous manner is feasible.

Research question 4: *How does the biohydrogen produced at ambient temperature and pressure, with moisture, affect a proton exchange fuel cell performance*?

The biogas produced during the CSTR was directly converted into electricity in a proton-exchange membrane fuel cell. The main limitation associated with this process integration stage concerned the chemical composition of biogas, i.e. the possibility that other minor biogas compounds could contaminate the PEMFC membrane and compromise the cell operation. To evaluate this possibility, the produced biogas was submitted to CO_2 sequestration so that the PEMFC feed gas was mainly composed by H_2 (95 ± 7 % (v/v)) and traces of nitrogen (bioH_2). The use of the bioH_2 in the cell was tested at two operational temperatures, 25 and 50 ºC, and compared with the use of commercial H_2 (>99% (v/v)). The operational temperature had no significant impact in the PEMFC performance, registering solely a slight voltage increase of approximately 2% when operating at the temperature of 50 ºC. There was no significant difference between the use of bioH_2 and its commercial counterpart. The electricity produced from bioH_2 operation was 1.7 kWh L^{-1} H_2.

Research question 5: *Will the fermentation byproducts be suitable for bioplastic production*?

The production of bioplastic, polyhydroxyalkanoates (PHA), is highly dependent on the chemical composition of the substrate. In the particular case of PHA accumulation, the fermentation process requires a substrate solution rich in organic acids, for example butyric acid, and low nitrogen or phosphorus concentration. The later requirement is necessary because the presence of these nutrients in the fermentative medium will lead PHA-producing microorganisms to direct the provided carbon source to cell growth rather than to the accumulation of PHA in their cytoplasm. Taking this fact into consideration, two modifications were tested to enable the use of the fermentate from the acidogenic fermentation as substrate for PHA production: the replacement of the original nitrogen source by the solid residue, or sludge, obtained during the dark fermentation (DF-sludge) and the decrease of the initial nitrogen supplementation. The reuse of the DF-sludge would enable the removal of the NH_4Cl from the fermentation, as an emission-heavy component in the biorefinery model. The DF-sludge reuse lead to a quicker process start-up that was attributed to enzymes recycling from previous acidogenic fermentations, and the productivity of 433 ± 34.3 mL biogas $(L\ h)^{-1}$. The lower concentration of the initial nitrogen concentration also impacted positively the *C. butyricum* performance, shown by the increase in biogas productivity of 59 %, 420 ± 11.9 mL biogas $(L\ h)^{-1}$, in comparison with the default condition. In conclusion, the initial nitrogen supplementation can be reduced without negative impact on H_2 and organic acids production. This simple modification should, theoretically, enable the production of an organic acid-rich fermentate with better quality to be used as substrate for PHA production.

Research question 6: *How does the virtual food waste biorefinery energy demand and global warming potential compares with conventional food waste valorisation treatment*?

The previous results explored successfully the feasibility of a simplified CIW bench-scale conversion system by acidogenic fermentation. It was required, however, to compare the possible benefits of the application of the acidogenic-based biorefinery with established technologies of FW treatment and disposal. The metric chosen was the quantification of the global warming potential (GWP) through the quantification of the CO_{2eq} emissions (direct and indirect).

The case study considered was based on the data from a waste management system operating in Lisbon for the selective collection and valorisation of FW by anaerobic digestion (reference). The amount of selectively collected FW (35000 tonnes) was used as basis for the scale-up modelling. The mathematical process estimated the potential production of H_2, organic acids, sludge and sodium bicarbonate which could be obtained from the referred biomass by acidogenic fermentation, as well as the associated energy and nutrients consumption, and CO_{2eq} emissions of the global process according to six scenarios: #1. Default (acidogenic fermentation to H_2 with microwave pretreatment); #2. Default without microwave pretreatment; #3. Sludge recirculation to H_2; #4. Sludge recirculation to electricity; #5. Sludge recirculation without CO_2 sequestration to H_2; #6. Sludge recirculation without CO_2 sequestration to electricity.

The analysis of the default system (#1) lead to a GWP quantification of 16550 tonnes of CO_{2eq} FU^{-1}. The analysis of the scenario #1 permitted the identification of two major emission sources. One source associated to the fermentative medium itself, which was composed by phosphate compounds and NH_4Cl. As registered in both chapter 6 and in the previous research question (5), the decrease or replacement of the nitrogen source by DF-sludge were successfully applied. The application of the later change to the analysis, i.e., the reuse of DF-sludge as nitrogen source in replacement of the NH_4Cl decreased the GWP emissions by 64.3%. The phosphate component of the media was used as pH buffer to control pH variations due to the production of acids and the subsequent addition of NaOH for pH control. Therefore, in the scope of this thesis, the exemption of the phosphate component could not be viably performed. The energy consumption of the acidogenic fermentation was also identified as the source of increased CO_{2eq} emissions. The microwave pretreatment of the substrate implied an increase in the energy consumption of approximately 50% when compared to the reference. This effect would be counteracted if the application of MW was performed at the household, at the point of waste production, instead of in the installation of FW processing. In fact, this change would decrease the overall energy consumption of the biorefinery and promote the participation of the waste producer in the process of FW valorisation, potentially influencing future behaviour. The exclusion of the microwave pretreatment from the industrial plant decreased GWP emissions by 7.6%. The comparison between the considered scenarios also took into consideration the final product of the biorefinery, hydrogen or electricity. The fuel cell integration for electricity production in the biorefinery (electricity as final product) lead to an increase of 3% in GWP when compared

to the default (H_2 as final product). Finally, the scenario #3 that considered the replacement of the nitrogen source by DF-sludge, was defined as the best-case scenario out of the 6 scenarios devised in this study. The DF-sludge reuse pointed to a GWP range between 3499 and 14191 tonnes of CO_{2eq} (61-231 tonnes of CO_{2eq} per tonne of produced H_2). Further analysis should be performed in order to reduce uncertainty.

8.1 Ongoing studies and guidelines for further research

The research work developed in this thesis uncovered a series of possibilities but also limitations for future research. The analysis of the fermentative process, particularly after optimisation and GWP quantification, underlined future needs for nutrient minimisation, particularly the phosphate and nitrogen sources of the medium. The operation of the CSTR should be performed for longer operational periods (over a minimum of 1-2 months) to evaluate the microbial evolution, the persistence of *C. butyricum* in the bioreactor consortium and the overall efficiency of the MW-pretreatment as method of contamination control. The bioreactor should also be tested with additional and diversified FW sources or combinations thereof and, if possible, submitted to a pilot scale-up for H_2 yield and production readjustments.

The conversion of the bioH_2 into electricity undertaken in this study did not benefit significantly of the operational temperature variations, possibly due to inefficiency of the heating system. Therefore, a more efficient temperature control should be implemented in future work, perchance increasing PEMFC cell stack, in order to evaluate correctly the influence of the temperature on the performance of the cell. Furthermore, the valorisation of cometabolites, such as the butyric and acetic acid, was not undertaken extensively. The biological conversion of the organic acids into PHA is presently under development, and the preliminary results were not included in the present text due to their early-stage of analysis. Further work should be performed in order to increase the efficiency of the fermentate upgrading to PHA and optimise methods for separating the produced polymeric material from the cells.

As the last venue of research, additional impact categories of the life cycle assessment should be undertaken (ex: acidification potential, toxicity, abiotic depletion), conjugated with a techno-economic and scale-up analysis.

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APPENDIX I: Research indicators

1. Articles in internationally reviewed scientific journals

1.1. Published

Ortigueira J, Silva C, Moura P. Assessment of the adequacy of different Mediterranean waste biomass types for fermentative hydrogen production and the particular advantage of carob (*Ceratonia siliqua* L.) pulp. Int. J. Hydrogen Energy. 2018;43(16), 7773-7783.

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1.2. Under submission or in final preparation for submission

Ortigueira J, Pacheco M, Trancoso A, Farrancha P, Ferreira J, Silva C, Moura P. Food waste biorefineries: Stability of a non-sterile food waste acidogenic fermentation system with CO_2 sequestration integrated with a PEM fuel cell. Under final preparation for submission, Journal of Cleaner Production, December 2019.

2. Book chapters

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3. Refereed conferences and proceedings

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4. Awards

Toste de Azevedo 2018, awarding academic and research studies for the development of systems or technologies focused on the use of hydrogen as energy vector for energetic

sustainability. Awarded by the “Associação Portuguesa para a promoção do Hidrogénio” (AP2H2, https://www.ap2h2.pt/)

2019 Fermentation travel awards, awarded by Fermentation (ISSN 2311-5637), https://www.mdpi.com/journal/fermentation/awards