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INSTITUTO SUPERIOR TÉCNICO

Production of first- and second-generation bioethanol by yeasts: global warming potential and fermentation productivity issues

Rui Cruz Pacheco

Supervisor: Doctor Carla Alexandra Monteiro da Silva

Co-Supervisor: Doctor Isabel Maria de Sá-Correia Leite de Almeida

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RESUMO

Na transição para economias de baixo carbono, o bioetanol apresenta-se como uma alternativa aos combustíveis fósseis. A produção de bioetanol pode usar biomassa de primeira-geração (1G), mas isso implica competição com cultivos alimentares, além de requererem grandes áreas, quantidades de água, e fertilizantes; biomassa de segunda-geração (2G) aproveitando desperdícios agrícolas e outros resíduos. Contudo, há uma necessidade de pré-tratamentos para a libertação de açúcares fermentáveis, mas estes tratamentos podem também libertar compostos inibidores. Sendo a fermentação um processo exotérmico, a temperatura também deve ser considerada no desenho e instalação de processos industriais de produção de bioetanol, considerando a gama de temperaturas óptimas para actividade da levedura. Adicionalmente, a avaliação destes processos, do laboratório para escala industrial, considerando consumo de energia e emissão de gases com efeito de estufa é essencial, numa perspectiva de avaliação de sustentabilidade.

É apresentada uma revisão sobre o panorama de produção de etanol de cana de açúcar, no Brasil, realçando-se a importância da conjugação de biorrefinarias 1G e 2G. Além disso, mostra-se uma avaliação da sustentabilidade do tupinambo como produto agrícola para energia, em termos do Potencial de Aquecimento Global (expresso em CO_2eq), considerando variações em hipóteses assumidas na metodologia de Análise de Ciclo de Vida (LCA), e os seus impactos na variação dos resultados. Verificou-se que a fermentação de sumo de tupinambo pode ser uma forma de produção de etanol competitiva, mas cujo impacto ambiental ao nível de emissões de CO_2eq é dependente dos factores considerados na análise. Esta influência nos resultados de LCA apresenta uma necessidade de mais estudos integrativos, no estabelecimento de cadeias de produção industriais, com vista a processos de decisão mais informados.

De modo a analisar factores limitantes da produtividade de fermentações 1G e 2G, foram comparados os perfis de fermentação alcoólica por estirpes de *Saccharomyces cerevisiae* (ou derivadas desta espécie), a diferentes temperaturas (30 ºC, 35 ºC e 40 ºC) e/ou concentrações de açúcares (glucose e/ou xilose). A influência do gene mutado *HAA1** na tolerância a temperaturas elevadas foi também testada numa estirpe industrial de levedura, analisando-se variações no crescimento e produtividade de etanol, em comparação com a estirpe sem o referido gene.

Palavras-chave: emissões biogénicas; alteração do uso de solo; Análise de Ciclo de Vida dinâmica; temperatura; glucose/xilose

ABSTRACT

In the transition to low-carbon economies, bioethanol appears as an interesting alternative to fossil fuels. Bioethanol production can use first-generation biomass, but this implies a competition with food crops, besides being water- and land-intensive, and using fertilizers; second-generation biomass takes advantage of agriculture wastes and other residues. Nevertheless, there is a need for pre-treatments to release fermentable sugars, but these can also release inhibitory compounds. As fermentation is an exothermic process, temperature must be considered in the design and installation of industrial bioethanol processes, considering the optimal temperature ranges for yeast activity. Also, the evaluation of these processes, from laboratory- to industrial-scale, considering energy consumption and greenhouse gases emissions is essential, from a perspective of sustainability evaluation.

A review of the ethanol production panorama is presented, highlighting the importance of the conjugation of first- and second-generation biorefineries. Additionally, it is shown an evaluation of the sustainability of Jerusalem artichoke as an energy crop. The Global Warming Potential (expressed in CO_2eq) of this case-study is studied, considering variations in Life Cycle Assessment (LCA) methodology hypothesis, and their impacts on the results. It was found that fermentation of Jerusalem artichoke juice can be a competitive type of ethanol production, but which the environmental impacts of the processes, related to greenhouse gases emissions varies depending on the considered factors in the analysis. This influence on LCA in the results raises the need for more integrative studies, in the establishment of industrial production chains, envisaging more informed decision processes.

Limiting factors of 1G and 2G fermentation productivity were examined by comparing the alcoholic fermentation profiles of different strains of *Saccharomyces cerevisiae* (or derived from this species), at different temperatures (30 ºC, 35 ºC, and 40 ºC) and/or sugar concentrations (glucose and/or xylose). The influence of the mutated *HAA1** gene in the tolerance to high temperatures was also tested in an industrial yeast strain, assessing variations in the yeast growth and ethanol productivity, when compared with the strain without the referred gene.

Keywords: biogenic emissions; direct land-use change; dynamic Life Cycle Assessment; temperature; glucose/xylose

RESUMO ALARGADO

Na transição para economias de baixo carbono, o bioetanol surge como um uma interessante alternativa a combustíveis fósseis. Assim, a necessidade de etanol produzido a partir de fermentação de fontes renováveis está a aumentar. A produção de bioetanol pode usar biomassa de primeira-geração, mas isto implica competição com agricultura alimentar, além de requerer um uso intensivo de água e área de cultivo, podendo também necessitar de fertilizantes; biomassa de segunda-geração aproveita desperdícios agrícolas e outros resíduos. Contudo, há uma necessidade de pré-tratamentos para a libertação de açúcares fermentáveis, mas estes pré-tratamentos podem também libertar compostos que actuam como inibidores da fermentação. Como a fermentação é um processo exotérmico, a temperatura deve também ser tida em consideração no desenho e instalação de processos industriais de produção de bioetanol, considerando as gamas de temperaturas óptimas para a actividade da levedura. Adicionalmente, a avaliação destes processos, desde a escala laboratorial até a escala industrial, considerando consumos de energia e emissões de gases de efeitos de estufa, é essencial.

Neste trabalho, é apresentada uma revisão sobre a sustentabilidade da cana-de-açúcar para fins energéticos. Em operações ao longo do ano, um moinho de cana-de-açúcar, que produz bioetanol, açúcar e bio-resíduos para usar como fonte de energia, pode usar outro tipo de matéria-prima além da cana-de-açúcar. Resíduos de cana-de-açúcar são, na sua maioria, queimados para a produção de calor e electricidade para alimentar o moinho ou vender excedentes de electricidade para a rede. Contudo, esses resíduos podem também ser usados como matéria-prima para outros tipos de produtos, no contexto de uma biorrefinaria de lenhocelulósicos. A sustentabilidade de uma refinaria de cana-de-açúcar foi avaliada, concluindo-se que a integração de biorrefinarias de primeira- e segunda-geração pode ser favorável, quer a um nível tecno-económico, quer ao nível de métricas de aquecimento global, reduzindo as emissões de gases de efeitos de estufa até 60%. Ao mesmo tempo, sugere-se a manipulação de leveduras para um aumento da produção de etanol como factor de interesse para melhoramento de sistemas de produção.

A sustentabilidade do tupinambo como matéria-prima alternativa de primeira-geração para a produção de etanol foi também avaliada. O uso de materiais lenhocelulósicos ainda não é economicamente viável, especialmente devido aos baixos rendimentos de fermentações de segunda-geração. Assim, há um aumento de interesse em matérias primas que não requerem terras aráveis nem elevados consumos de água, duas das maiores desvantagens das principais matérias-primas de primeira-geração, como a cana-de-açúcar, milho ou beterraba. O tupinambo consegue crescer em terrenos marginais e é resistente à seca, além de normalmente não requerer fertilizantes ou pesticidas, mostrando-se como uma

matéria-prima de interesse para fermentação. O tupinambo foi usado para testar duas estratégias de fermentação para produção de bioetanol, e o bioprocessamento consolidado com a levedura *Zygosaccharomyces bailii* Talf1 foi a que apresentou maior produtividade de etanol e menor consumo de electricidade por volume de produto. Assim, este processo foi considerado para aumento de escala, com base em análise de dados energéticos e produtividade de etanol. Foram definidos diferentes cenários industriais para emissões de gases de efeito de estufa e consumo de energia por litro de etanol, usando critérios adequados numa abordagem *cradle-to-gate*: sem alteração do uso de solo, sem CO_2 biogénico e sem crédito atribuído a co-produtos, a comparação do consumo total de energia e emissões de CO_2eq de etanol a partir de tupinambo (9 $MJ.L^{-1}_{etanol}$; 679 gCO_2 /L_{etanol}), com cana-de-açúcar/beterraba (42/29 $MJ.L^{-1}_{etanol}$; 731/735 gCO_2 /L_{etanol}), e com refinaria de gasolina (15 $MJ.L^{-1}_{etanol}$eq; 1154 gCO_2 /L_{etanol}eq).

A sustentabilidade ambiental de processos de produção de bioetanol é normalmente medida pela métrica de Potencial de Aquecimento Global, para um horizonte temporal de 100 anos. Foi efectuada uma revisão sobre as emissões de gases de efeitos de estufa de diferentes processos de produção de bioetanol, resultando numa gama de valores de 0.31 a 5.55 gCO_2eq/L_{etanol}. Dada a disparidade de resultados para o mesmo produto, o cenário à escala industrial descrito acima foi usado para avaliar o impacto de escolhas metodológicas nas emissões de CO_2eq da produção de bioetanol: LCA convencional *versus* dinâmico; diferentes métodos de análise de impacto (TRACI, IPCC, ILCD, IMPACT, EDIP, e CML); mix eléctrico da zona geográfica/país para diferentes localizações de fábrica; diferenças nos factores de CO_2eq para CH_4 e N_2O devidas a actualizações nos relatórios do Painel Internacional sobre as Alterações Climáticas (IPCC) (5 relatórios até à data de publicação deste trabalho), diferentes tempos de operação da fábrica, e futuros melhoramentos de produtividade. Os resultados demonstraram que o mix eléctrico, relacionado com a localização da fábrica e perfil de produção de electricidade de cada país, e *land-use* são os factores com maior efeito (até 800% de desvio). O uso de factores de equivalência de CO_2 dos relatórios do IPCC teve a menor influência (menos de 3% de desvio). A consideração de emissões biogénicas (sequestro de CO_2 durante a fase de agricultura e emissões de CO_2 durante a fase de fermentação) e diferentes métodos de alocação também mostraram ser influentes na análise, com uma variação de valores até 250%.

Tendo em conta a necessidade de responder a múltiplos factores que podem limitar o desempenho fermentativo da levedura na produção de bioetanol de primeira- ou segunda-geração, ou mesmo levar à paragem da fermentação, limitando a produtividade de estirpes de *Saccharomyces cerevisiae*, comparou-se o perfil de fermentação de diferentes estirpes de *S. cerevisiae*, naturais ou sujeitas a engenharia genética, em diferentes condições ambientais relevantes. As estirpes foram cultivadas a diferentes temperaturas (sub-óptima, óptima e

supra-óptima), em misturas de açúcar com diferentes concentrações de glucose e xilose (simulando o conteúdo de açúcares de biomassa lenhocelulósica). Foram usadas três temperaturas de crescimento diferentes (30 ºC, 35 ºC e 40 ºC), a temperatura mais elevada, conjugada com o efeito do etanol produzido acumulado, levou a uma diminuição da produção de biomassa celular e de etanol a partir de glucose, para as estirpes *S. cerevisiae* BY4741 e Ethanol Red, apesar da última, uma estirpe industrial, ter demonstrado uma maior tolerância a estes stresses conjugados. O crescimento em mistura de açúcares (glucose e xilose) foi testado usando uma estirpe *S. cerevisiae* capaz de catabolizar xilose, GSE16-T18_HAA1*. Esta estirpe expressa a via do metabolismo da xilose, baseada na enzima xilose isomerase (com origem em *Clostridium phytofermentans*), e possui um gene mutado de *HAA1*, que codifica um activador transcricional cuja expresso aumenta a tolerância a ácido acético, de modo a ser mais eficaz no aumento da tolerância a esse ácido fraco. Neste caso, a fermentação a 40 ºC resultou também num decréscimo da produção de etanol, quando comparado com as outras duas temperaturas, devido a uma metabolização mais reduzida da xilose após o consumo de glucose e acumulação do etanol produzido. Esta estirpe foi também usada em experiências considerando diferentes concentrações de glucose e xilose no meio (100+80/100+37 $g.L^{-1}_{glucose+xilose}$) simulando o conteúdo de açúcares em matéria-prima de segunda-geração. A concentração de xilose no meio apenas começa a diminuir após o esgotamento de glucose, que exerce repressão catabólica.

O gene mutado *HAA1** foi inserido num plasmídeo com marca de selecção, que foi usado para transformação da estirpe Ethanol Red. Testou-se a influência da expressão deste gene para fenótipos de tolerância a temperaturas elevadas, comparado com a estirpe sem o gene *HAA1**. Paralelamente, a performance fermentativa das leveduras transformadas foi avaliada, permitindo inferir sobre a vantagem da expressão do gene *HAA1** em fermentações a elevadas temperaturas (41 ºC).

Esta tese realça a necessidade de melhoramentos informados de processos de produção de etanol, especialmente relacionados com cenários de escala industrial, com foco no impacto das emissões de gases de efeito de estuda dos referidos processos, considerando matéria-prima de primeira- e segunda-geração. Apresentam-se também estudos dos impactos de factores limitadores de fermentação na produção de etanol por *S. cerevisiae*, relativamente à importância do uso de estirpes tolerantes a temperaturas elevadas (que são frequentemente desejadas em processos industriais), que consumam açúcares normalmente presentes em biomassa lenhocelulósica, e que apresentem resistência ao efeito conjugado da temperatura elevada e etanol produzido, e outros stresses químicos, por exemplo, causados por ácido acético.

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LIST OF ACRONYMS, ABBREVIATIONS AND MOLECULES

In alphabetical/numerical order.

®	registered trademark
µ	growth rate constant
1G	first generation
2G	second generation
3G	third generation
AGWP	absolute global warming potential
AMP	adenoside mono-phosphate
AR	assessment reports (from Intergovernmental Panel on Climate Change)
ATP	adenosine tri-phosphate
C	carbon (molecular)
CAP	capacity (reactor)
CBP	consolidated bioprocessing
cLCA	conventional life cycle assessment
CO	carbon monoxide
CO_2	carbon dioxide
CO_2eq	CO_2 equivalent
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DBD	DNA binding domain
dLCA	dynamic life cycle assessment
DLUC	direct land-use change
DNA	deoxyribonucleic acid
Dt	diameter of tank (reactor)
EC	energy consumption
EDTA	ethylenediamine tetra-acetic acid
EF	emission factor
EtOH	ethanol/bioethanol
FU	functional unit
GHG	greenhouse gas(es)
GREET	Greenhouse Gases, Regulated Emissions, and Energy Use in transportation
Gt	gigatons
GWP	Global Warming Potential
H_2SO_4	sulfuric acid
HPLC	high performance liquid chromatography
HS	head space

HT	height of tank (reactor)
ISO	International Standardization Organization
JA	Jerusalem artichoke
JAJ	Jerusalem artichoke juice
JAT	Jerusalem artichoke tubers
JRC	Joint Research Center
JSEB	joint sugarcane ethanol biodiesel
LCA	Life Cycle Assessment
LHV	lower heating value
LNEG	Laboratório Nacional de Energia e Geologia (Portugal)
LUC	land use change
MAGAP	Ecuadorian Ministry for Agriculture, Livestock, Aquaculture and Fisheries
N	nitrogen (molecular)
nBR	number of bioreactors
NOx	nitrous oxides
$OD_{600\ nm}$	optical density (at 600 nanometers)
P	power
PCR	polymerase chain reaction
PEP	prospective economic performance
Po	power number
PPP	pentose-phosphate pathway
RED	Renewable Energy Directive
RF	radiative forcing
RFS	Renewable Fuel Standard
ROS	reactive oxygen species
SEC	specific energy consumption
SOC	soil organic carbon
TEA	technoeconomic assessment
TH	time horizon
™	trademark
UK	United Kingdom
US	United States of America
VHG	very high gravity (fermentation)
WS	working space
WTT	well-to-tank
WTW	well-to-wheel
WV	working volume

XDH	xylitol dehydrogenase
XI	xylose isomerase
XK	xylulokinase
XR	xylose reductase
YMA	yeast malt agar
YMB	yeast malt broth
YPD	yeast extract-peptone-dextrose (medium)
η	efficiency
ρ	density

LIST OF UNITS

In alphabetical order.

%	percentage
cm	centimeter
ºC	degree Centigrade
$g.kWh^{-1}$	gram per kilowatt hour
$g.L^{-1}$	gram per liter
$g.L^{-1}.h^{-1}$	gram per liter per hour
$g.MJ^{-1}$	gram per megajoule
$g.ton^{-1}$	gram per ton
h	hour
ha	hectare
kg	kilogram
$kg.L^{-1}$	kilogram per liter
$kg.ha^{-1}.year^{-1}$	kilogram per hectare per year
$L.kg^{-1}$	liter per kilogram
$L.L^{-1}$	liter per liter
$L.ha^{-1}.year^{-1}$	liter per hectare per year
$L.ton^{-1}$	liter per ton
M	molar
m^2	square meter
m^3	cubic meter
$m^3.ha^{-1}.year^{-1}$	cubic meter per hectare per year
$MJ.L^{-1}$	megajoule per liter
mL	milliliters
mM	millimolar
nm	nanometers
rpm	revolutions per minute
$ton.ha^{-1}$	ton per hectare
$ton.ton^{-1}$	ton per ton (mass/mass)
TWh	terawatt hour
U/mL	units of enzyme per milliliter $U.mL^{-1}$
V	volts
$W.m^{-2}.kg^{-1}$	watt per square meter per kilogram (of gas)

NOTES

Regarding the scope of the work, the words “ethanol” and “bioethanol” will be used interchangeably, unless explicitly clarified as different in the text.

For reading clarity and simplicity, except for the first reference in the text, D-glucose, D-xylose, and L-arabinose will be written simply as glucose, xylose, and arabinose, respectively.

Depending on the context, for clarity purposes, some values will be presented in units other than the ones from the International System of Units.

OUTLINE OF THE DOCUMENT

The thesis document is organized into six major sections:

- Section 1 – Introduction of the thematic of ethanol production, its importance, current drawbacks and need of studies. Highlighting of the general objectives of the research.
- Section 2 – Highlights and review of the published work on the sustainability of sugarcane as an energy crop.
- Section 3 – Description of the published work evaluating Jerusalem artichoke as a feedstock for industrial fermentation processes, and its carbon dioxide emissions.
- Section 4 – Description of the published work for the assessment of the Global Warming Potential of a case-study (described in the previous section), and the influence of the variation of several parameters in Life Cycle Assessment works.
- Section 5 – Description of work on yeast strains, evaluating ethanol productivity under different stress conditions and with a carbon source common in lignocellulosic hydrolysates. Assessment of the influence of a gene of interest in the phenotype of thermotolerance and fermentative processes at high temperatures.
- Section 6 – General discussion of the thesis work, main conclusions, and future perspectives.

SECTION 1

GENERAL INTRODUCTION AND MOTIVATION

1. GENERAL INTRODUCTION AND MOTIVATIONS

1.1 THE NEED FOR ETHANOL AS A FUEL

Ethanol can be used as a solvent, raw material for the production of chemicals and for human use (e.g. disinfectant), but is mainly used as a fuel.[1] It can be synthetically produced from petroleum, but this process is losing terrain to biological fermentation processes, from renewable sources.[1]

Biofuels have been considered as a relevant alternative for the replacement of fossil fuels, in the transition process to low carbon economies.[2] Globally, transportation and industry are two sectors with the biggest consumption of energy, of which 97% has its origin on oil.[3] In the last two decades, around the world, governments have been dynamically promoting the identification, development, and commercialization of technologies for the production of alternative fuels,[4] including ethanol.[5] Being used as an automotive fuel for over a century,[6] ethanol has an estimated global production of 100 million tons,[7] being the largest-volume product in the industrial biotechnology,[8] and considered to have environmental (it has a high oxygen content, which might lead to a reduction of emission of particles by combustion motors)[9,10] and energy security benefits, when compared to gasoline.[11–18] Actually, bioethanol is the main renewable biofuel used in the transportation sector.[19] Moreover, it also reduces countries' reliance on oil imports and eases uncertainties caused by fluctuations in oil price.[9]

Ethanol is mostly produced by fermentation of cane sugar, hydrolyzed corn starch, maize, wheat, and sugarbeet, with the yeast *Saccharomyces cerevisiae*. This type of fermentation originates the so-called "first generation" (1G) bioethanol,[20,21] and the processes have a high ethanol yields (above 90% of the theoretical maximum yield of 510 kg per kilogram of hexose sugar), ethanol titers up to 21% (weight-weight), and volumetric productivities of 2 to 3 $kg \cdot m^{-3} \cdot h^{-1}$.[8] The global output of ethanol has been growing and by 2030 it is expected to provide up to 7% of the energy used in the transport sector.[22]

1.2. ETHANOL FROM ALTERNATIVE SOURCES

Bioethanol is considered to take part in reducing resource use and global warming, even though it has been suggested that it can have unfavorable impacts on acidification, human toxicity and ecological toxicity, occurring mainly during the growth and processing of biomass.[23] Feedstock production can be land and water-intensive, and fertilizers and pesticides can enter water sources, situations that are often discarded from impact studies.[23]

Brazil and the United States of America already use and produce bioethanol in large scale, and European countries are increasing its use,[24] but feedstock for 1G ethanol comes

mainly from dedicated energy crops (such as sugarcane in Brazil) or cultivated crops that could otherwise be used for food and animal feed.[24,25] Per year, about 13 billion liters of ethanol are produced from sugarcane in Brazil, but prices are still too high for sugar to be considered a viable feedstock in other countries,[23] such as the United Kingdom, which has committed to producing 10% biofuel for transportation by 2020.[26]

The drawbacks of 1G biofuels, such as competition with food, low yields per hectare, and damage to ecosystems, can partly be overcome by the utilization of second-generation (2G) biofuels.[23] Lately, significant efforts have been made to produce bioethanol from non-food lignocellulosic biomass, found in several wastes and residues. Replacement of fossil fuels can be greatly supported by ethanol originated from crop residues, sugarcane bagasse and agricultural waste from bioenergy crops.[27,28]

Representing a vast and renewable reservoir for transport energy, lignocellulosic materials are the most abundant organic materials in the biosphere.[29–32] Annually, there is an estimated availability of 1.3 billion dry tons of lignocellulosic biomass, coming from forestry and agricultural residues, municipal solid waste and woody and grassy crops, that can support ethanol production, a value that could be translated in a reduction of petroleum consumption of around 30%, by the US.[23,33]

Lignocellulosics are not only available in large quantities (as, for example, co-products of food production), but can also be used to produce other chemical compounds of high value and heat.[34] The material does not compete with food crops and its use takes care of plant residues in an environmentally sustainable process, reducing costs of waste disposal.[35–37]

It has been estimated that a production of around 49.1 gigaliters per year can be achieved, which is around 16 times higher than the current production.[38,39] Lignocellulose-based biorefineries have been seen as future candidates to supply the global demand for sustainable energy sources, in order to replace non-renewable sources used nowadays, and solve the transport energy deficit and greenhouse gas (GHG) emission problems, with good potential to decrease environmental impacts when compared to conventional processes.[40–44]

In the last decades, significant technological advances have been made to handle the potential of lignocellulosic feedstock for 2G ethanol production, but the process is not yet economically viable.[5,45]

Third generation ethanol, using algae as biomass, has also proven to be interesting, due to the high concentration of sugar and ease of accumulation of rich biomass, while generally not being used as food. However, the technology to fully harness the potential of this type of feedstock is, almost as much as 2G, still far for being economically viable.[46,47]

1.3. PROBLEMS WITH FERMENTATION PROCESSES

Bioethanol production, either from first- or second-generation biomass, involves a series of factors that can lead to sub-par productivities or economic viability, especially considering that, for a long-case scenario, it is considered to be done in large-scale industrial settings. Some of the most recurrent problems to be addressed are the fermentation temperatures (both for 1G and 2G biomass), the consumption of all available sugars in the medium, and the deleterious effects of chemical compounds that inhibit fermentation.

1.3.1. EFFECTS OF TEMPERATURE ON ETHANOL FERMENTATION

Temperature affects all cellular metabolic functions. An increase from low temperatures (≈27 ºC) to 40 ºC can lead to a great reduction of protein synthesis. Also, pre-treatment of 2G biomass utilizes enzymes with optimal temperatures higher than 45 ºC. These temperatures are desirable to reduce contamination and cooling processes. However, most yeast strains do tolerate temperatures up to 42 ºC, but only in the absence of the typical stress factors of alcoholic fermentation.[48]

Increasing industrial ethanol fermentation rates is often done through intensification of the process. One commonly used improvement relies on the very-high-gravity (VHG) technology. A higher concentration of ethanol, in conjunction with supra-optimal temperatures for that level of stress leads to a change in the kinetic of growth and fermentation profiles.[49] Therefore, the thermal adaptation of the fermenting yeasts must be done, as well as the improvement of intrinsic yeast thermotolerance. Indeed, it has been shown that for a specific *S. cerevisiae* strain and conditions, a concentration of 6% (w/v) of ethanol depressed the optimum temperature for growth from 37 ºC to 25 ºC, the final maximum temperature for growth from 40 ºC to 33 ºC and the maximum temperature for growth from 44 ºC to 36 ºC.[50] Although the fermentation is less sensitive to the same ethanol concentrations than growth, the conjugated effect of ethanol and temperature in growth and fermentation is, qualitatively, the same. Furthermore, since fermentation is an exothermic process, the growth/fermentation profiles of each strain and/or process conditions are among the most important determinants of ethanol (and other stresses) toxicity and tolerance in yeasts.[51,52]

While higher temperatures can maximize the initial stages of conversion of sugars, the later stages of the fermentation require lower temperatures to diminish the inhibitory level and cellular damage caused by the accumulation of ethanol in the medium.[49] Cooling down the systems, nevertheless, comes with its costs. Yeast strains capable of effectively producing ethanol at high temperatures are, therefore, of interest. Not only a reduction of cooling expenses can be obtained, but there is also a decrease in the risk of contamination by other

microorganisms.[53,54] However, optimal yeast growth in the absence of stresses is observed around 35 ºC, while the referred fermentation processes preferably take place at around 40 ºC.[54] The thermotolerance of *S. cerevisiae* strains is a highly desirable trait for sustainable fermentation both in 1G and 2G ethanol production processes, as higher temperatures in the fermentation range are also desirable for hydrolyzing enzymes to act.[55]

Accumulation of ethanol can also inhibit fermentation processes. High concentrations of ethanol in the medium can increase plasma membrane permeability, leading to a disruption of sorting and signaling functions, as well as delays in the cell cycle.[56] Conjugated with high temperatures, ethanol aggravates the inactivation of some enzymes, such as alcohol dehydrogenase. [52] High concentrations of ethanol in the medium can also disrupt proton efflux, decreasing acid resistance by affecting the activity of plasma membrane H^+ATPase for the regulation of internal pH.[52]

A conjugation of the accumulation of toxic chemicals released by pre-treatment processes and fermentation can aggravate the energy requirements of the cell, rendering difficult the active excretion of toxic chemicals.

1.3.2. XYLOSE CONSUMPTION IN THE PRESENCE OR ABSENCE OF GLUCOSE

Second-generation bioethanol is foreseen as a major impact on transportation fuel markets, without the negative impacts commonly associated with the production and use of 1G fuels. However, lignocellulose is a more complex substrate than 1G feedstock.[23]

Lignocellulosic substrates are mainly composed of cellulose, hemicellulose, pectin, lignin, and carbohydrates. D-glucose is the major sugar component, but pentose carbohydrates such as L- arabinose, and D-xylose, are present at significant levels.[57] Lignocellulosics can have great potential but are not devoid of major challenges to become a mainstream feedstock for bioethanol production:

- The release of fermentable sugars requires a pre-treatment step involving extreme physicochemical conditions and hydrolytic enzymes;[58,59]
- Pre-treatment processes can result in the release of fermentation inhibitors;[60,61]
- Non-manipulated *S. cerevisiae* strains cannot ferment pentose sugars.[62,63]

The fibrous nature of the plant cell wall is highly difficult to deconstruct into fermentable sugars.[64] Conventional processes for 2G bioethanol production include extreme chemical/physical pre-treatment and enzyme-catalyzed hydrolysis of the biomass in order to release arabinose and xylose.[25,65,66] The final composition of the hydrolysates may vary, but plant biomass can usually consist of ≈30% to 40% of xylose.[64,66]

S. cerevisiae is the most commonly used microorganism for industrial production of bioethanol due not only to its high capacity of fermenting hexose sugars, but also its tolerance to ethanol and other inhibitors, ease of storage and transport, no expensive nutritional needs, ability to produce high ethanol concentrations (above 15%), not expensive, production of low levels of by-products and osmotolerance. Additionally, yeast cells can be recycled during sequential fermentations and there is a comprehensive physiological and molecular knowledge and its genetic manipulability is relatively easy.[67–69] However, this yeast species is naturally not able to ferment pentose sugars, particularly xylose.[64]

To take full advantage of the carbon content of lignocellulosic biomass, biorefineries require microorganisms that can not only ferment hexose sugars but also xylose, preferably simultaneously.[70] Co-consumption is desired to reduce fermentation time and achieve industrial economically-viable productivities.[59] This is challenging because, even though some native hexose transporters can uptake xylose from mixed sugar media, glucose is yeast's preferable sugar,[66,70–72] also because the microorganism lacks specific transporters for the xylose.[73] Xylose is usually only consumed after significant depletion of the glucose concentration in the medium, exhibiting a diauxic growth and fermentation.[74] Eventually, by the time glucose concentration decreases enough to allow xylose assimilation, ethanol concentration is already high enough to reduce the xylose fermentation rate.[75] The depletion of important metabolites and cofactors used by the cells to resist the stressful conditions in the hydrolysate medium is an additional limitation.[76]

Even if the yeast has a disrupted glucose metabolism, this sugar can still have an inhibitory effect over xylose, as the hexose transporters still favor glucose uptake.[77] The presence of glucose prompts a genome-wide transcriptional response, called "glucose repression", that leads to the down-regulation of the expression of genes involved in respiration and metabolism of alternative carbon sources.[78–80]

This comes as a problem if there is an intent to make bioethanol production processes economically viable, with high-efficiency conversion of hemicellulose.[81] Some non-*Saccharomyces* yeasts, such as *Kluveromyces marxianus*, *Scheffersomyces* (*Pichia*) *stipites*, *Pachysolen tannophilus*, and *Candida shehatae* are able to ferment pentose sugars, but they have not been considered for large-scale processes.[82,83]

Effective conversion of cellulosic biomass into bioethanol will require yeasts that can efficiently use xylose as well as glucose.[84,85] Considering all the limitations imposed by the use of lignocellulosic material, most of the work on pentose fermentation by yeasts has been focused on genetically-modified *S. cerevisiae* strains.[66]

Additionally, other compounds can be released or formed during biomass pre-treatment, besides sugars, and several of them can inhibit yeast metabolism.[66] Organic acids (mainly acetic acid, but also formic acid)[86–88], furaldehydes (furfural and 5-hydroxymethyl-

furfural) and phenolic derivatives can prolong the lag phase,[89] as well as negatively affecting growth and fermentation rates, decreasing the longevity of the microorganisms and ethanol productivity, which can lead to significant economic losses due to the reduction of efficiency of production plants.[90–92] The inhibitors also have a greater effect on xylose fermentation than on hexose fermentation.[93]

Hydrolysates also carry with them chemical inhibitors of growth and fermentation, that can act synergistically. Furfural can cause cell damage since it leads to the accumulation of reactive oxygen species. Phenolic compounds can change the protein-to-lipid ratio of the yeast cell membrane, affecting its integrity.[94–96]

Acetic acid, the most important inhibitor, a weak carboxylic acid that originated from the deacetylation of hemicellulose during the pre-treatment of 2G substrates.[27,97–100] In industrial processes for bioethanol production from this type of substrates, acetic acid concentrations can, theoretically, reach 11.2 $g.L^{-1}$, not considering accumulation caused by the recycling of process streams. In pre-treated corn stover hydrolysate, concentrations of 13 $g.L^{-1}$ have been reported.[101] The undissociated acid form can easily diffuse across the cell membrane, and in the intracellular medium it rapidly dissociates due to its pH, with the consequent decrease in fermentation rate.[102–105]

1.4. Overcoming the limitations of fermentation

1.4.1. Improving xylose fermentation

Effective xylose fermentation is a polygenic trait, challenging engineering strategies for the development of improved yeast strains.[24] Most progress has been made through approaches where the genetic changes responsible for such improvement generally remain unknown.[24]

Consumption and degradation of xylose in microorganisms can happen in three different ways, but only two have been successfully integrated into *S. cerevisiae* (Figure 1). In the oxidative pathway, a NAD(P)H-dependent XR, encoded by XYL1, reduces xylose to xylitol.[106] Xylitol is then oxidized into 5-xylulose by a NADP+-dependent XDH, encoded by XYL2.[107] On the other hand, in the xylose isomerase (XI) pathway, characteristic of bacteria, xylose is directly converted to 5-xylulose.[108] Then, in both pathways, 5P-xylulose enters the PPP and glycolysis.[66] In the third known pathway, described in *Archae*, xylose is oxidized by xylose dehydrogenase, xylonate dehydratase, 2-keto-3-deoxyxylonate, and alfa-ketoglutarate

semialdehyde dehydrogenase.[109] Involving so many genes, this pathway is yet to be engineered into *S. cerevisiae*.[66,110]

Orthologous encoding functional XR and XDH have been found in *S. cerevisiae*, but still, the yeast cannot grow with xylose as sole carbon source,[111,112] and overexpression of native aldose reductase and xylitol dehydrogenase genes allowed just for limited growth in xylose.[113] Hence, expression of XR/XDH heterologous pathways (for example, from the naturally xylose-assimilating yeast *S. stipitis*) in *S. cerevisiae* has been attempted but, while XR preferentially uses NAD(P)H as the cofactor, the exclusive use of NAD+ by XDH is an important limitation[108] since it leads to a cofactor imbalance that leads to xylitol excretion and thus, reducing carbon assimilation and ethanol production.[66] The addition of an external electron acceptor to the fermentation media,[114,115] the connection of furaldehyde reduction with xylose metabolism,[116] modification of the ammonium assimilation pathway,[117] change in carbon fluxes through a recombinant phosphoketolase pathway in a xylose-consuming strain,[118] and the change in cofactor preferences of XR and XDH[118] are some of the strategies that have been used to solve the cofactor imbalance problem, usually resulting in low yields of xylitol in the engineered strains.

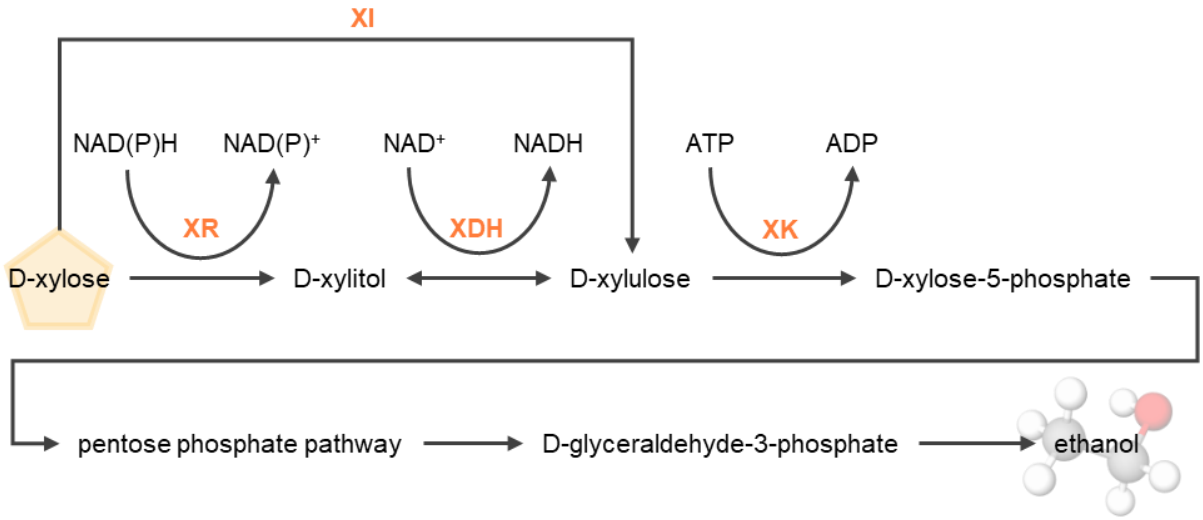

Figure 1. Xylose metabolic pathways integrated into *S. cerevisae*. Arrows indicate the direction of the chemical reactions; orange color highlights the reaction mediators. (Adapted from Moysés et al., 2016)

Heterologous pathways can also be engineered. Some non-*Saccharomyces* yeasts can ferment pentoses. *Scheffersomyces (Pichia) stipites* is one example, even though its characteristics do not propose them for large-scale processes. Still, its XR/XDH pathway is commonly used to engineer yeast for xylose fermentation.[66] As referred above, the preferential use of NAD(P)H by XR, and the exclusive use of NAD^+ by XDH can, however, raise a limitation on this possible solution for xylose consumption by *S. cerevisiae*. These factors prompt a

cofactor imbalance that results in the excretion of xylitol, reducing carbon assimilation and therefore, ethanol production.[66,108] Addition of external electron acceptors to the media, connection of furaldehyde reduction with xylose metabolism, modification of the ammonium assimilation pathway, redirection of carbon fluxes from a phosphoketolase pathway in xylose-consuming strains, and change of cofactor preference of reductase and dehydrogenase are all alternative strategies of engineering to solve this problem, but all of them result in low-yield of xylitol production by the new strains.[114–119]

To overcome this problem, the introduction of an XI pathway comes as an interesting alternative, as it does not require pyridine nucleotide co-factors. Several scenarios for this engineering were previously tested with low to moderate success. Expression of prokaryotic XI, encoded by *xylA*, in *S. cerevisiae* showed no activity, due to protein misfolding, post-translational modification, sub-optimal intracellular pH, among others.[62,66] A successful recombination of a XI pathway into *S. cerevisiae* was only obtained with the expression of *xylA* from *Piromyces* sp E2, although the new strain showed no growth in anaerobiosis and low growth rates under aerobic conditions in xylose.[120,121] These low rates have been attributed to xylitol formation, by the action of Gre3p and limited activity of XK and other PPP enzymes.[122]

An attempt to overcome these limitations was made by deletion of *GRE3* and overexpression of genes encoding XK, ribulose 5-phosphate isomerase, ribulose 5-phosphate epimerase, transketolase, and transaldolase, in a strain previously engineered to grow on xylose. In this case, xylitol production was not observed.[123,124]

The activity of Gre3, a yeast endogenous non-specific aldose reductase, and the limited activity of xylulokinase (XK) and non-oxidative pentose-phosphate pathway (PPP) enzymes have been attributed to the low rates of xylose fermentation, due to xylitol formation.[122] To solve this case, works have been made with strains with deleted *GRE3* and overexpressing genes encoding XK, ribulose 5-phosphate isomerase, ribulose 5-phosphate epimerase, transketolase, and transaldolase, resulting in an absence of production of xylitol and increased ethanol yield.[122,124] In other instances, the oxidoreductase pathway, which includes a xylose reductase (XR) and a xylitol dehydrogenase (XDH), was introduced in *S. cerevisiae* to convert xylose to xylulose via xylitol. Xylulose can then be used in the non-oxidative PPP via the endogenous XK and take part in the central carbon metabolism of the cell.[70,125] Alternatively, the expression in yeast of *xylA* from *Clostridium phytofermentans*, which has low susceptibility to xylitol inhibition, resulted in an ethanol yield of over 80%.[126]

Other genetic information agents can also be involved in glucose repression. Transcriptional repressors Mig1, Cat8, and the Ssn6/Tup1 complex prevent transcription of genes involved in gluconeogenesis and metabolism of alternative carbon sources, which are subjected to glucose repression.[127–129] The kinase Snf1 and phosphatases Glc7/Reg1 mediate

the activities of these repressors as intracellular sensing mechanisms,[128–130] but membrane sensors such as Snf3 and Rgt2 also take part in the complex network of repressing signalization and regulation, sensing extracellular sugar concentrations and internalizing signals.[129,131]

Expression of *xyl*A from *Burkholderia cenocepacia* has also shown interesting results, with high ethanol yield and no accumulation of xylitol and co-consumption of glucose under anaerobic conditions.[132,133]

Different recombinants capable of fermenting xylose were obtained in several experiences, with different approaches, but the most successful show that adaptation and evolution of strains in xylose are important to screen XI activity in *S. cerevisiae*.[66] These strains seem to have an advantage over oxireductase-based strains, as they do not subject the cell to extra burdens in terms of cofactor requirements in the biosynthetic pathways for biofuels.[134,135]

Even when expressing a heterologous xylose assimilation pathway, glucose can still outcompete xylose for uptake by hexose transporters.[71,77,136,137] The reduced accumulation of xylose in the cell is a bottleneck for downstream metabolism. Reducing or eliminating glucose inhibition of xylose transport through hexose transporters and increasing xylose transport has been the focus of metabolic engineering studies to overpass that limitation.[136,138–143] However, it has been shown that simultaneous consumption of glucose and xylose does not result from mutations in sugar transporters but rather in hexokinases and glucokinases that reduce the glucose metabolic flux.[129] Also, high concentrations of glucose (>36 $g.L^{-1}$) have been shown to greatly repress transcription of *HXT5* and *HXT7*, which encode the main transporters responsible for xylose uptake when xylose is the only carbon source in the medium, or when glucose concentration is low. At the same time, this low hexose amount is associated with a decreased transcription of *HXT1* and *HXT4*, leading to the expression of *HXT5* and *HXT7*. Small amounts of glucose (0.5 $g.L^{-1}$) seem to improve xylose utilization, when compared with a medium with xylose as only carbon source, probably due to the induced increase of expression of *HXT7* by this low hexose condition. Glucose also represses the transcription of *GAL2*, but with constitutive expression, Gal2 and Hxt7 show the highest rates of xylose uptake, of all endogenous hexose transporters.[66,71]

Simultaneous saccharification (the breaking of carbohydrates into monosaccharides) and fermentation, and consolidated bioprocessing (the process of combination of saccharolytic enzyme production and secretion, polysaccharide hydrolysis, and fermentation on a single operation)[144] have been tested as alternative bioethanol production configurations. However, they are still not appropriate for large-scale production scenarios, due to the high optimal

temperature for fungal enzyme activity and low productivity of heterologously expressed hydrolases in *S. cerevisiae*.[145,146]

Still, the performance of the best available xylose-fermenting/inhibitor tolerant strains is yet to reach efficient fermentation of lignocellulosic hydrolysates.[147]

1.4.2. COUNTERACTING THE INHIBITORY EFFECTS OF CHEMICAL STRESSES

To surpass the negative effects of inhibitory chemical compounds, present in the hydrolysate biomass, several approaches have been used. Treatment with the use of reducing agents, activated charcoal, overliming, anion exchanger, evaporation, enzymatic treatment with peroxidase and laccase, *in situ* detoxification by fermenting microbes, extraction by solvents, extraction through membrane, and yeast adaptation through repeated sequential fermentations are examples of attempted strategies for reduction of their inhibitory effects in fermentation.[99,100,148–154] Precipitation and evaporation are two of the most used processes to decrease inhibition by acetic acid. The precipitation technique results in the neutralization of the acid, while the evaporation technique takes advantage of its volatility.[99,150] Acetic acid can also be removed via membrane extraction, as it is not easily eliminated from the hydrolysate by solvent extraction.[154]

1.4.2.1. *HAA1* AS AN ACETIC ACID RESISTANCE DETERMINANT

In recent years, the *HAA1* gene (Homolog of Ace1 transcriptional Activator) has been described as an important determinant of resistance to acetic acid, controlling yeast genomic expression processes in response to this acid and to formic acid, also present in lignocellulosic hydrolysates. [86,155]

The *S. cerevisiae* transcription factor Haa1 was first identified based on the DNA binding domain (DBD) homology with the Cup2 (alias Ace1) DBD, a copper regulated transcription factor.[156] The Haa1 DBD is formed by 123 amino acid residues at the N-terminal and has a conserved zinc module and a set of four cysteine clusters, which are organized in a consensus sequence forming the copper regulatory domain, in a way closely similar to the Cup2 DBD. The similarities led to the hypothesizing that Haa1 could have a role in copper homeostasis, yet the transcription factor could not be demonstrated as having a part in tolerance to copper in *S. cerevisiae*. In fact, unlike its homologs, the function of *HAA1* is not dependent on the copper status of the cell.[156] Haa1 has, nevertheless, proven influence in other processes. It is the main subject in genomic expression reprogramming in response to stress caused by the presence of acetic acid, transcriptionally activating (direct or indirectly) about 80% of the acetic acid-responsive genes in yeast, a majority of those necessary for

maximum tolerance to the acid.[155,157] Expression of the gene decreases the duration of the adaptation period of yeast when suddenly exposed to toxic concentrations of acetic acid.[158] Targets of Haa1 are involved in an array of essential biological functions: gene transcription, metabolism of lipids, carbohydrates and amino acids, processing of nucleic acids, cell wall remodeling and resistance to multiple drugs.[155,158]

In the cell, Haa1 binds to the promoter of its target genes at an acetic acid responsive element. *TPO2* and *TPO3* are relevant targets; they code for two plasma membrane transporters of the Major Facilitator Superfamily and are proposed to mediate the efflux of acetate from the interior of the cell, in yeast under acetic acid stress.[155,158,159] A 6.8-fold expression of *TPO2* and was observed in a yeast strain overexpressing *HAA1*.[110]

The cell wall-related secretory glycoprotein and the plasma membrane coding genes *YPG1* and *YRO2*, respectively, are also regulated by Haa1 and are required for tolerance to acetic acid.[155,156,160,161] Likewise, Haa1 is indirectly related in the regulation of Pma1, as *HRK1*, a kinase associated with phosphorylation of the membrane proteins and activation of the H^+-ATPase Pma1, and *HSP30*, a plasma membrane heat shock protein suggested to be a negative regulator of Pma1, are part of its regulon.[162,163]

When the laboratory yeast strain BY4741, harbors *S. stipits* XR and XDH genes, the overexpression of *HAA1* led to a 2.3-fold increase of expression of *YPC1* than the control strain.[164] Ypc1 is involved in the sphingolipid metabolism, directly influencing the good assembly of the vacuolar membrane V-ATPase pump.[164] Increased expression of *YPC1* under stress by acetic acid may lead to increased activity of the proton pump, increasing tolerance to the acid.[165–167]

Yeast strains overexpressing the *HAA1* gene (see section 5) also gave insights on the sugar consumption problematic. In the referred strains, the transcription level of *TOS3* (regulated by Haa1) was increased 4.9-fold than in a control strain. Tos3 is an activator of *SNF1*, which codes for an AMP-activated protein kinase involved in the carbohydrate metabolism, and is required for the switch from glucose to other carbon sources.[168–170]

1.5. IMPORTANCE OF IMPACT ANALYSIS OF PRODUCTION PROCESSES

Bioenergy from lignocellulosic biomass has many benefits and is usually seen as carbon neutral, but processes such as transportation and growth of feedstock have associated environmental impacts. There is a need for sustainability assessments as important elements to develop the processes to produce this type of energy.[171]

Life Cycle Assessment (LCA) is considered amongst the scientific community as one of the most appropriate methodologies to evaluate the environmental impacts of biofuel production processes, allowing for the identification of opportunities for improvement.[39,172] A

LCA is able to provide an overview as complete and detailed as possible over the interactions of anthropogenic activities with the environment and eventually be a source of knowledge to allow effective strategic planning of production scenarios.[39,173,174]

Ideally, in LCA studies, environmental and economic impacts of all stages of a production chain are taken into consideration, and so it has been increasingly used in works with conventional and renewable energy generation systems.[175] Taken this background in consideration, fossil energy and GHG (and carbon) releases into the atmosphere have been a major focus of analysis.[176]

In a scenario of bioethanol production from lignocellulosic materials, a LCA study should be done to evaluate the environmental impacts of the system and to calculate the ecological benefits from the replacement of a conventional system. The results can be used by policymakers and consumers, to choose the eco-friendliest fuel, considering the different involved variables.[39,177]

With LCA, the life cycle of a product, service or production system is analyzed, following the framework provided by the International Standardization Organization (ISO) norms 14040 and 14044. Preferably, it analyses the environmental burden of a product or process (or products or processes) from their production, through use and finally disposal or recycling. Four steps constitute the basis of the methodology:[171]

1- Definition of goal and scope;
2- Inventory analysis;
3- Impact assessment
4- Interpretation.

1) The first step is where the system boundaries are defined, to enunciate which processes and materials are to be included in the system. The functional unit (FU) is selected to express the function of the system in a quantitative manner, enabling comparison of different systems (e.g., kilogram of sugarcane, liter of produced ethanol, traveled kilometer by car, etc.).[171] 2) For the inventory stage, data about system inputs and outputs is gathered, specifically, values for energy consumption, materials used, amount of products and co-products, and emission/release of pollutants and wastes as consequence of the production. If the process eventually gives origin to other products than the one(s) to be analyzed, allocation is done to allocate environmental burdens between main products and co-products,[171] although the ISO 14040 normative suggests this process is not used, as it can lead to further uncertainty of results.[178] 3) The inventory results are analyzed for their potential impact on a given number of environmental issues (depending on the selected LCA method). These issues are classified into categories depending on their potential for long-term damage to the environment. 4) The resulting scores show the potential relative severity of the studies subject

on an environmental impact category (e.g., ozone depletion, climate change, human toxicity, etc.).[171]

LCA, despite its advantages as a scientific tool, can lead to conflicting results between studies, usually due to the use of different FU, system boundaries, data or methods.[171] Also, final results for the same given product or process can differ, depending on the selection and importance given to different parameters, such as factory location, land use issues, use (or not) of dynamic evaluation, and factory/system lifetime.[177] However, even though system boundaries of biomass ethanol can diverge between studies, it is suggested that a comprehensive LCA contemplates the production and harvesting of feedstock, transportation of biomass to the biorefineries, conversion of biomass to ethanol, and burn of ethanol as fuel.[179]

On the analysis of ethanol production from lignocellulosic materials, a gap in LCA studies has been noticed. Most studies do not account for the impacts originated by the production and use of pre-treatment chemicals, enzymes and nutrients. In fact, it was estimated that up to one-third of GHG emissions over the 2G bioethanol life cycle corresponds to enzymes and chemicals.[180] Improvements on fermentation processes, namely the engineering of yeast strains, should be taken under LCA studies to assess its practical effectiveness on an industrial scale.

1.6. OBJECTIVES

This thesis intends to deliver an encompassing work on both biological improvement of bioethanol production processes and assessment of large-scale feasibility of ethanol production in terms of environmental impacts (Global Warming Potential), considering alternatives for the enhancement of methods, such as use of alternative 1G feedstock or development of viable 2G production processes.

Hence, the proposed works aim to fulfill the following objectives:

- Assess the viability of using an alternative feedstock (Jerusalem artichoke) in fermentation processes, by selecting the best laboratory-scale case study and gauge impact of fertilizers, land-use change and biogenic emissions from fermentation in the GWP of a modeled industrial scenario.
- Track the influences of several factors (methods, inputs, energy characteristics) in LCA studies related to Global Warming Potential of fermentation processes.
    - New/alternative processes of ethanol production must be submitted to impact analysis to assess their environmental viability and/or improvements over conventional processes.
    - LCA studies considering different methodologies, functional units, countries' electricity mixes and factories' operational time can lead to different results for the analysis of the same processes. A sensitivity analysis must be performed to assess each factor's influence on the results.
- Verify the influence of temperature, sugar concentrations (glucose and xylose), chemical stresses and genetic background of *S. cerevisiae* strains in the production of ethanol.
    - To exploit the potential of 1G and 2G feedstock, the effects of several existing constrains on alcoholic fermentation should be considered and analyzed. Performance assessment of strains in sub-optimal, optimal and above optimal temperatures must be done, as well as ethanol productivity assays using a mixture of xylose and glucose as carbon sources and acetic acid as a relevant chemical stress, simulating the challenging conditions observed during lignocellulosic substrate fermentations.
- Improvement of yeast strains' performance facing temperature stress as one of the limitations in lignocellulosic hydrolysates fermentation, through the transformation of yeast strains with a gene of interest.

SECTION 2

SUSTAINABILITY OF SUGARCANE FOR ENERGY PURPOSES

2. SUSTAINABILITY OF SUGARCANE FOR ENERGY PURPOSES

The work in this section is included in the published book chapter:
Carla Silva, **Rui Pacheco**, Danilo Arcentales and Fernando Santos, 2019, *Sustainability of sugarcane for energy purposes*. In Sugarcane Biorefinery, Technology and Perspectives. Academic Press. ISBN: 97801281142363

The publication comes as a joint work for an overview over fermentation landscapes using different types of feedstock as the basis for energy products. It results in an analysis showing the interest of flexibility of bioethanol production processes, in terms of what types of feedstock should be used for improved sustainability, as well as suggesting the need for biological enhancement of yeast strains for fermentation purposes. The author, Rui Pacheco, contributed to this work by making a review on 1G and 2G ethanol, emissions and energy flows of processes, GHG emissions related to ethanol production and input/output inventory.

2.1. INTRODUCTION

The pursuit of fossil-free systems with minimum waste disposal covering food, energy, materials and chemicals production is a major driver for the development of biorefineries where all these products could be produced simultaneously. The idea to have a dedicated unit to produce exclusively biofuels should be avoided, or its benefits over biorefinery should be proven. To select the most feasible biorefinery configuration, economic as well as environmental aspects must be studied. The former relates to a technoeconomic assessment (TEA), the latter is usually tackled in an LCA framework.

Both TEA and LCA studies are designed to address a specific question and contain different assumptions, data sources and uncertainties. Therefore, it is not surprising that the results vary widely across the studies and care must be taken in making direct comparisons between them. For example, a different geographical location/different country will have different labor, insurance of equipment and materials costs, different electricity and product prices, different currencies, different electricity generation mixes, different transportation/distribution distances, and even different agriculture productivities induced by different climate/weather conditions. Additionally, different studies may refer to different chronologies (different reference dollars or euros) and, different IPCC assessment report CO_2eq values. The following table includes the 100-year time horizon global warming potentials (GWP) relative to CO_2. The AR5 values are the most recent, but AR2, AR3 and AR4 report values are also listed because they are sometimes used for inventory and reporting purposes.

	Designation	Chemical formula	AR2-1996	AR3-2001	AR4-2007	AR5-2013
Common among carbon footprint	carbon dioxide	CO_2	1	1	1	1
	methane	CH_4	21	23	25	28
	nitrous oxide	N_2O	310	296	298	265

Table 1. Differences in global warming potentials for 100-year time horizon. (source: IPCC Assessment Reports)

Historically, the culture of sugarcane has been considered as the main economic activity since the Brazilian colonial period, when slave labor was still used. Since then, with the rise in sugar consumption, the economic importance of the crop has been further increased. At this time, sugarcane mills in Brazil produced only sugar. The Pró-Álcool program (1975) induced the increase in distilleries and sugarcane mills producing both sugar and ethanol in an integrated fashion. More recently, electricity production is also a by-product of the sugarcane mills and, therefore the actual sugarcane mill is a biorefinery, producing food, electricity and biofuel. Figure 2 shows the sugarcane production historical evolution, the ethanol production and the electricity sold to the grid. The number of registered flex fuel cars is also represented to show its link with ethanol availability and sugarcane industry growth.

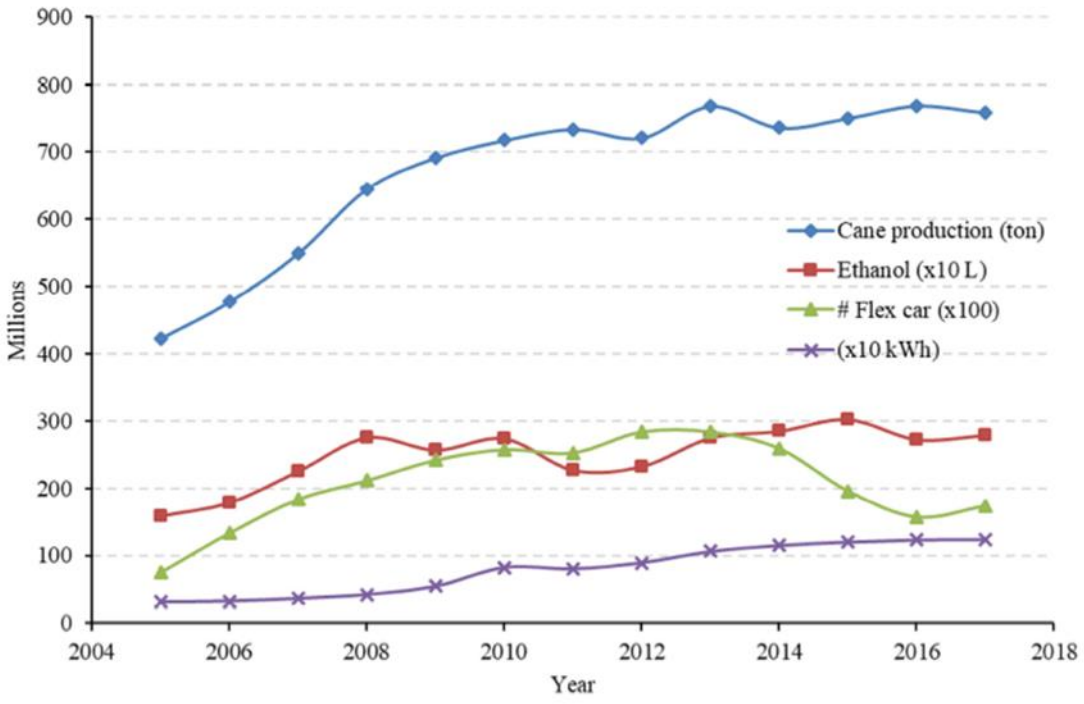

Figure 2. Historical trends showing the increase in cane production, ethanol production, number of flex fuel vehicles registered and surplus electricity from bagasse.[181–184]

From 2005 to 2014, co-generated electric energy dispatch increased from 1.1 TWh to 19.4 TWh, and biomass became the third electric energy generation source in Brazil, after hydro and fossil sources.[185]

In terms of sustainable assessment of the sugarcane mills, for example, TEA and LCA could both be used to find out the merit of a sugarcane biorefinery in comparison with the alternatives to produce ethanol and other co-products.

Several studies were reviewed from literature to have the inventory values of Table 2, mainly from Brazil Center-South region.[11,18,185,186] The quantification of wastes is stressed out because they can be further processed in the sugarcane biorefinery. Regarding straw and tips, studies recommend the use of about 50% of the total collected to maintain the field, control erosion, and to keep moisture, temperature, and soil fertility. The remaining 50% used for burning in boilers, producing electric energy. In addition, used to produce cellulosic ethanol, also known as 2G ethanol. The sugarcane wastes from harvesting (straw) and juice extraction (bagasse) are produced in large quantities, about 280 million tons of bagasse and straw per year, and they are likely to increase in the near future as new industrial plants are implemented. Currently, sugarcane residues are mostly burned for the production of heat and electricity at the sugar mill. However, they could also be used as feedstocks for the production of other high-value products in the context of the lignocellulosic biorefinery.[187] This would be a 2G biorefinery integrated with the first generation (1G), whose schematic is represented in Figure 3.

For year-round operation, the sugar mill could adopt other feedstocks, besides sugarcane. Energy cane and sweet sorghum have different harvest times but can be processed for bioethanol using the same equipment.[188]

The CO_2 intensity of electricity in Brazil is low due to the high percentage of renewables, c.a. 73 g CO_2eq/kWh carbon intensity;[189] electricity used in the processes, 14 kWh/ton cane[11] is nowadays mainly suppressed by bagasse co-generation and surplus electricity is sold to the grid, being a product of the biorefinery and no longer an input.

The use of LUC, when the natural landscape is transformed by human activity, and biogenic emissions of CO_2 as a result of sugar fermentation processes, can also be taken into account when analyzing production processes.[190]The values of fossil energy consumption and CO_2eq emissions with no LUC, no biogenic emissions from fermentation and credits to surplus electricity are, from the literature: for the reference case,[18] 2008 data for sugar production, fossil energy use 721 kJ/kg_{sugar} and 234 g CO_2eq/kg_{sugar}, respectively. For the ethanol life cycle, these values are 80 kJ/MJ and 21.3 g CO_2eq/MJ. Other study for the Center-South Region of Brazil claims 202-238 MJ/MJ_{EtOH} and 24 g CO_2eq/MJ.[2] Considering both agricultural and industrial phases, the total emissions of hydrous and anhydrous ethanol production for

2005/2006 were evaluated as 417 and 436 g CO_2eq/L, respectively,[11] or, 19.8-20.7 g CO_2eq/MJ (density 0.785 kg.L^{-1}, lower heating value 26.9 MJ/kg,[191] respectively.

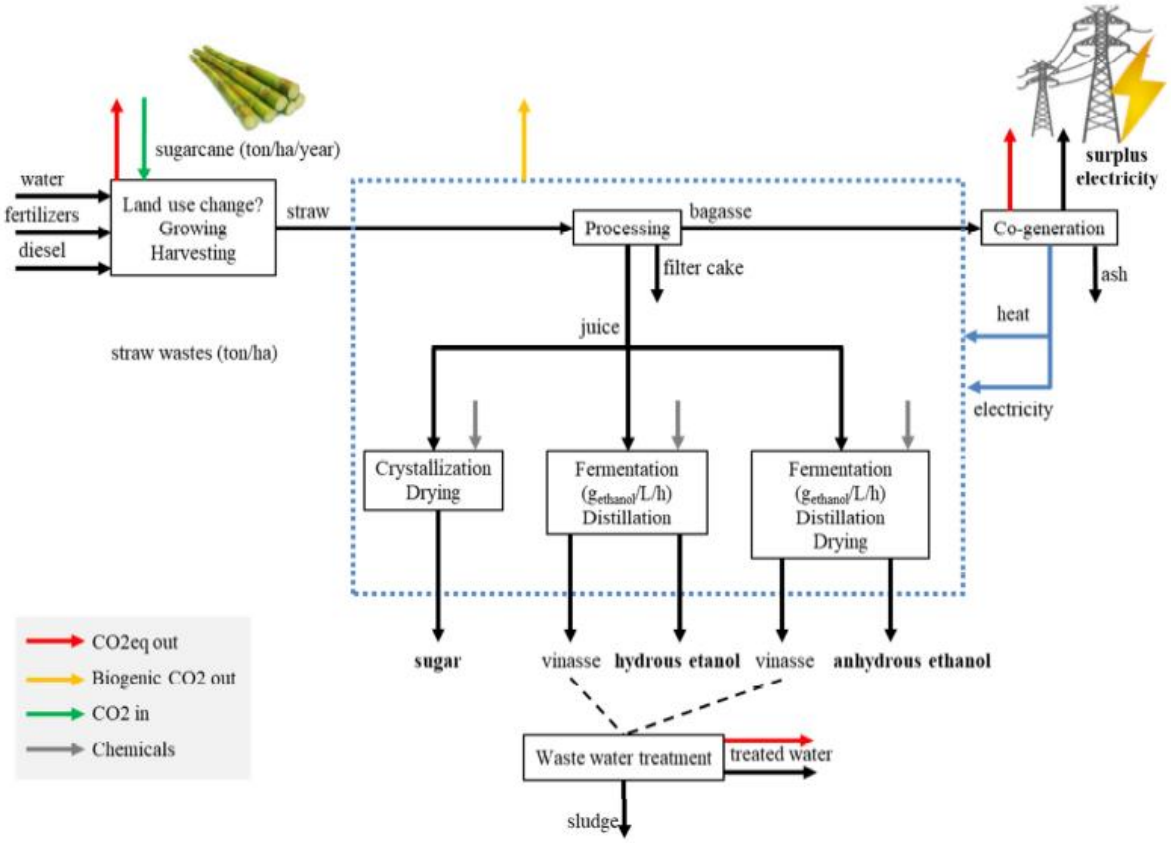

Figure 3. Process material, emissions and energy flows for a sugarcane traditional 1G biorefinery mill (can be used for sugarcane, energy cane and sweet sorghum).[192] Partition of 40% sugar, 39% hydrous ethanol and 21% anhydrous ethanol.[18]

Other possible biorefineries could be projected to further convert the process residues to 2G ethanol or even 3G ethanol. Those several virtual biorefineries are usually simulated by using the AspenPlus® software to get all virtual materials and energy flows.[193] These virtual inventories are then used in the LCA and TEA analysis. For example,[194] comparison of three biorefineries in terms of the internal return rate (IRR), a TEA metric. Fifty percent of the straw waste (crop residues) was used in the soil and the other 50% used in the cogeneration unit to produce extra electricity to the grid (biorefinery #1, Table 3), and to produce ethanol from cellulose (biorefinery #2, Table 3) and from cellulose and hemicellulose (biorefinery #3, Table 3). More specifically, 50% of sugarcane juice was used for sugar production and the other part of the juice, together with molasses from sugar processing, was used to produce ethanol. Furthermore, 50% of the sugarcane crop residues were hydrolyzed together with surplus of bagasse (amount that was not used to attend the process heat and electricity demand) to produce 2G ethanol, and crop residues were used to attend energy supply of the process. No

LCA global warming impact assessment was made, and only the value of CO_2 uptake 178 kg of carbon per 1000 kg of sugarcane stalks during the plant growing phase is mentioned.

Previous studies[189] used TEA and LCA metrics to select the best integrated sugarcane-based biorefineries. They were integrated biorefineries producing 1G and 2G ethanol, sugar, molasses (for animal feed) and electricity in the context of Brazil. The metrics prospective economic performance (PEP) (for TEA) and climate change impact (CO_2eq) are used in a reference system that produces gasoline (allowing a vehicle to move the same km), sugar, animal feed and electricity and in the integrated biorefineries.

Stage	Inputs		Outputs	
Agriculture[186]	nitrogen (N) phosphate (P_2O_5) potassium (K_2O) limestone ($CaCO_3$) herbicide insecticide cropping practices harvesting water	57.7 $kg.ha^{-1}.year^{-1}$ 20.63 $kg.ha^{-1}.year^{-1}$ 30.39 $kg.ha^{-1}.year^{-1}$ 400 $kg.ha^{-1}.year^{-1}$ 5.00 $kg.ha^{-1}.year^{-1}$ 2.00 $kg.ha^{-1}.year^{-1}$ 33.49 $L_{diese}L.ha^{-1}.year^{-1}$ 49.09 $L_{diese}L.ha^{-1}.year^{-1}$ 140 $m^3.ha^{-1}.year^{-1}$	sugargane	89 ton/ha/year
Agriculture[18]	274 $L_{diese}L.ha^{-1}.year^{-1}$ N P_2O_5 K_2O $CaCO_3$ herbicide insecticide	 777 $g.ton^{-1}_{cane}$ 249 $g.ton^{-1}_{cane}$ 980 $g.ton^{-1}_{cane}$ 5183 $g.ton^{-1}_{cane}$ 44 $g.ton^{-1}_{cane}$ 3 $g.ton^{-1}_{cane}$	sugarcane cane trash yield	86.7 ton/ha/year 140 kg_{dry}/ton_{cane}
Industrial[186]	lime phosphoric acid H_2SO_4 lubricants	1066.52 $g.ton^{-1}_{cane}$ 23.28 $g.ton^{-1}_{cane}$ 6.31 $g.L^{-1}_{EtOH}$ 13.93 $g.ton^{-1}_{cane}$	bagasse filtercake stillage ethanol	0.27 $ton.ton^{-1}_{cane}$ 24 $kg.ton^{-1}_{cane}$ 14 $L.L^{-1}_{EtOH}$ 85 $L.ton^{-1}_{cane}$
Industrial[18]	lubricants sulfur lime sulfuric acid fermentation	10.3 $g.ton^{-1}_{cane}$ 156 $g.ton^{-1}_{cane}$ 880 $g.ton^{-1}_{cane}$ 7.4 $g.L^{-1}$	stillage filtercake boiler co-generation ash soot bagasse ethanol electricity surplus	11 $L.L^{-1}$ 31 $kg.ton^{-1}_{cane}$ 2 $kg.ton^{-1}_{cane}$ 12 $kg.ton^{-1}_{cane}$ 0.264 $ton.ton^{-1}_{cane}$ 86.7 $L.ton^{-1}_{cane}$ 10.7 kWh/ton_{cane}

Table 2. Input-output inventory for 1G biorefinery from different sources in the literature. The uptake of CO_2 from sugarcane culture adopted is 653 kg of CO_2 per ton of sugarcane. [6]

	Biorefinery #1 optimized 1G	Biorefinery #2 integrated optimized 1G and 2G	Biorefinery #3 future integrated optimized 1G and 2G
Ethanol yield ($L.ton^{-1}_{cane}$)	89.3	110.7	131.5
Electricity surplus to the grid (kWh/ton_{cane})	185.8	92.8	72.7

US$2009/L	0.288	0.317	0.253
IRR per year	16.9%	12.2%	18.4%

Table 3. TEA analysis, based on IRR metric, of virtual biorefinery from sugarcane. [8]

They concluded that integrated 1G2G biorefinery reduced 56-59% the CO_2eq emissions in comparison with the reference system and even got lower emissions than a dedicated 1G mill. The TEA metric showed that the 1G mill had the highest economic performance, followed by the 1G2G with four main products: ethanol, sugar, electricity and animal feed. This example compares a system basket of products with other with the same products and is not only a well-to-wheel (WTW) of the liquid fuel.

Other example found in literature considers sugarcane ethanol and palm oil biodiesel joint production in a biorefinery (joint sugarcane ethanol biodiesel - JSEB) whose products are: ethanol, glycerin, kernel oil and cake, and electricity to the Brazilian grid. In this case, no sugar was produced.[186] The biodiesel internally produced was used to suppress the diesel fuel needs in managing soils, harvesting and transportation. Life cycle CO_2eq emissions of ethanol, based on the traditional system were 20.2 g.MJ^{-1}, without LUC emissions. In comparison, CO_2eq emissions of ethanol produced by the factory were 15.6 g.MJ^{-1}, a 23% reduction in life cycle emissions when compared to the traditional system. These examples show a cradle-to-gate comparison of biorefinery systems. 1G2G3G ethanol biorefinery integration systems, e.g. exploring microalgae to consume the CO_2 released in sub-processes and used as raw material for ethanol production is not yet fully explored[195] and could be a topic of future research. The only study found regarding algae use to collect the biogenic CO_2 from fermentation and produce biodiesel to use in all agriculture operations was in an ethanol distillery.[192] The authors showed a potential reduction of 10-50% of CO_2eq emissions in comparison compared to a traditional Brazilian sugarcane ethanol distillery. Despite this intense research in Brazil, other American countries are likely to follow the Brazilian example. For instance, the major sources of biomass in Ecuador are agricultural residues, animal manure, woody biomass, industrial effluents and municipal solid wastes.[196] The Ecuadorian's agricultural production benefits from its geographic location and favorable weather conditions having on average a 12-hours day luminosity.[197]

Biofuels production in Ecuador is poor comparing to Brazil's. Ecuadorian sugarcane production is mostly concentrated in the coastal region, closely to Guayaquil and it has been like that since the 90's decade. For the year 2001, Ecuador counted with a sugarcane cultivated area of 69,085 hectares. After 4 years, the total cultivated area increased to 135,000 ha., (75 000 ha. for sugar) whereof, solely 10 000 ha were dedicated for ethanol production. For the same year, the Ecuadorian Ministry for Agriculture, Livestock, Aquaculture and Fisheries (MAGAP, acronym in Spanish), estimated that Ecuador had 675 932 ha. of suitable land for sugarcane production. A year later, by 2006, the total sugarcane cultivated area

increased to 147 270 ha. This year it was determined that almost 60% of the national sugarcane crops belonged to individual farmers and the remaining 40% belonged Valdez and San Carlos sugar companies.[198] In the year 2009, Ecuador had a daily capacity to produce 136 000 liters of ethanol, resulting in an estimated production of 50 million liters per year.[199] The use of ethanol on fuels started in Guayaquil and Duran by 2010, where the Ecuadorian government launched a pilot program denominated Ecopaís fuel (E-5), a blended gasoline type Extra of 85 octanes with 5% ethanol processed through sugarcane production. Initially, the government aimed to increase the blend of gasoline with ethanol from 5% to 15% by 2016. For this reason, the government started promoting sugarcane production in small and large-scale. After the introduction of Ecopaís, it was possible to observe a decrease in emissions of CO, NOx and HC.[200] In comparison with the previous year (2011), Ecuador increased by 10% the sugarcane cultivated area, having an installed capacity to distill around 185 000 liters of alcohol per day. From that number of liters, uniquely 20 000 liters per day were used for the production of ethanol.[197] Therefore, alcohol production was around 20.5 million liters in 2012, where 39% of the total amount was used for biofuels production.[201] The installed capacity by 2012 was 36 million liters of ethanol. However, in order to reach the target of having a blended gasoline with 15% (400 million liters of ethanol per year), it is a requirement to sow larger extensions of sugarcane and build new distilleries.

2.2. FLEX-FUEL VEHICLE TECHNOLOGIES AND WTW

When the comparison is the liquid fuel ethanol production system with gasoline refining to use in a flex-fuel vehicle, a well-to-wheel framework is used. For example, in Europe, the carbon footprint (as CO_2eq) of ethanol from sugarcane produced in Brazil does not account for LUC, nor biogenic CO_2 emissions. Table 3 shows the well-to-tank part of energy and CO_2 balance. From here, the "Primary energy input ($1+MJ_{expended}$)/MJ_{final}) is 3.09 MJ/MJ_{EtOH}" and "total CO_2 emission 24.8 gCO_2eq/ MJ_{EtOH}". This means in E5 to E85 blend in a gasoline car WTW values of 26-37 MJ_{fossil}/100 km and 36-53 gCO_2eq/km a reduction of more than 60% of the CO_2 equivalent emissions.[202]

With the expanded Greenhouse Gases, Regulated Emissions, and Energy Use in Transportation model (GREET), a study[203] examined the WTW energy use and CO_2eq emissions of sugarcane-derived ethanol produced in Brazil and used to fuel light-duty vehicles in the U.S.A. The sugarcane-to-ethanol pathway evaluated in the GREET model comprises fertilizer production, sugarcane farming, sugarcane transportation, and sugarcane ethanol production in Brazil; ethanol transportation to U.S.A. ports and then to U.S.A. refueling stations; and ethanol use in E85 vehicles. Results for sugarcane ethanol were compared with those for petroleum gasoline. Their analysis showed that sugarcane ethanol can reduce CO_2eq by 78%.

This value was recently updated to 40– 62%.[204] Both Europe and U.S.A. advantages in using ethanol from sugarcane produced in Brazilian biorefineries explored in conventional internal combustion engine vehicles, using 85% anhydrous ethanol blend with gasoline, or hydrous ethanol 5-10% blend with gasoline. As seen in Figure 2, flex-fuel technology could be other way to boost ethanol consumption and stimulate more efficient sugar cane from 1G2G3G biorefineries. With the future electrification of the road vehicles the plug-in hybrid flex-fuel technology could be the best choice in terms of fuel flexibility (ethanol-gasoline-electricity), decarbonization, and use of endogenous resources.[205]

Brazil product to the European context		**Energy expended (MJ/MJ_{EtOH})**	**GHG emissions (g CO_2eq/MJ_{EtOH})**			
Standard steps	**Actual steps**		**Total**	**as CO_2**	**as CH_4**	**as N_2O**
production & conditioning at source	sugarcane cultivation + local transport	0.09	17.9	6.54	3.33	7.99
transformation at source	EtOH production	1.88	-1.4	-1.73	-0.06	0.36
	of which credit for electricity from surplus bagasse	-0.06	-2.5	-2.5	-0.14	-0.03
transportation to market	EtOH long distance transport	0.09	6.7	6.62	0.03	0.02
transformation near market	n.a.	-				
conditioning & distribution	distribution	0.02	1.1	1.11	0.01	0.02
	dispensing at retail site	0.01	0.5	0.48	0.03	0.00
Total WTT		**2.09**	**24.8**			

Table 4.Brazilian sugarcane to ethanol for use in Europe. Excess bagasse used for electricity production. SCET1 pathway from Edwards *et al*. (2013)[202] with inventory data mostly from Macedo *et al.* (2008)[11]. IPCC 100 values from AR4.

Looking at the well-to-tank (WTT) part, for the same industrial emission burden, the higher the ethanol productivity, the better. Depending on the feedstock and fermentation conditions (e.g. temperature, agitation speed, and initial sugar concentration), ethanol productivity values can range from 0.13 to 6.55 $g_{EtOH}/L_{bioreactor\ medium}/h$. To increase ethanol production in industrial scenarios, engineered yeast strains can be used. Those may have enhanced tolerance to ethanol concentration in the medium, pH fluctuations, temperature, osmotic stress, toxic inhibitors, and/or have a capacity to perform alcoholic fermentation of xylose (a pentose) additionally to glucose (a hexose). An industrial value of this productivity, which actually occurs in sugarcane mills, is not publicly known. If we try to estimate to 1G mills: sugarcane juice density varies between 1044.5 and 1189.5 kg/m^3,[206] and from the Table 2 inventory for 1G mills, bagasse and filter cake amount ~ 290 kg/1000 kg_{cane} This means an availability of ~ 215 L_{juice}/ton_{cane}. If we take the bioreactor medium as 215 L and assume 60 h fermentation, this would mean a 1G productivity of ~5 $g_{EtOH}/L_{bioreactor\ medium}/h$. These are very rough claims and of course should be taken with caution. So, if this ethanol productivity was

doubled by means of engineered yeast and because results of the CO_2eq are expressed as per production of ethanol, the doubled production would mean half the emission burden. The yeast capability to process pentose will boost 2G ethanol production.

2.3. CONCLUSIONS

Integrated 1G2G biorefineries, processing food (sugar), ethanol (biofuel), heat and electricity from bagasse (bioenergy for self-consumption) and surplus electricity to the Brazilian grid seems to be favorable from the point of view of tecnhoeconomic metrics and the global warming metric. Typically, integrated biorefineries show reductions in CO_2eq of 23-60%. The engineering yeast to further increase ethanol productivity for the same conditions will improve the well-to-tank values used to compare with conventional gasoline vehicles because the metric is expressed as MJ/MJethanol and gCO_2eq/MJ_{EtOH}. For 1G mills, the latter is typically on the order of 20 $g.MJ^{-1}$, without land use issues and disregarding biogenic emissions.

SECTION 3

EVALUATION OF JERUSALEM ARTICHOKE AS A SUSTAINABLE ENERGY CROP TO BIOETHANOL: ENERGY AND CO_2eq EMISSIONS MODELING FOR AN INDUSTRIAL SCENARIO

3. EVALUATION OF JERUSALEM ARTICHOKE AS A SUSTAINABLE ENERGY CROP TO BIOETHANOL: ENERGY AND CO_2EQ EMISSIONS MODELING FOR AN INDUSTRIAL SCENARIO

The work in this section is included in the published manuscript:
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The publication is the result of a collaboration with the Laboratório Nacional de Energia e Geologia (Lisboa, Portugal), where the main biological processes for ethanol production were made. The processes were to be evaluated on an environmental level, for energy and emission analysis, and considering the used feedstock as an alternative of interest for industrial-scale fermentation processes. The author, Rui Pacheco, contributed to this work by assessing the system inventory, processing the scale-up model, accounting for biogenic CO_2 emissions, and final energy and emissions analysis.

3.1. INTRODUCTION

Environmental issues, such as greenhouse gas (GHG) emissions and the depletion of fossil fuel reserves are still driving research on renewable sources for energy and chemicals. Biomass can be converted into ethanol through either biochemical (hydrolysis and fermentation) or thermochemical conversion processes (gasification and catalytic synthesis).[207] There is a multitude of feedstock for biomass conversion to bioethanol. According to the Renewable Fuels Association, worldwide ethanol production is dominated by the US (corn feedstock) and Brazil (sugarcane feedstock) that produce 85% of the world's ethanol (c.a. 92 billion liters). Europe is the third main producer (sugarbeet feedstock). According to 2014 statistics of the European Renewable Ethanol Association (ePURE) there are 8799 million liters of installed production capacity in Europe.[208] Minimum installed capacity stands for Denmark with 5 million liters and maximum goes to France with 2318 million liters. The main feedstocks used for the production of renewable ethanol in the European Union are wheat (34%) and maize (42%), followed by sugarbeet (17%).[209]

Despite its applications to hygiene and wine industry, bioethanol is also playing an important role as a substitute for the gasoline fuel in pure or blended form (e.g. E27 to E100 in Brazil, and E15 and E85 in US).[210] The importance of bioethanol as an industrial product has generated a great deal of research in increasing the ethanol fermentation yield,[211–213] and, at

the same time, in increasing its sustainability when compared to its fossil fuel competitor. The improvement of the bioconversion efficiency is an important issue and it is already been tackled.[214–216] Without this improvement, biofuels could not be market competitive.

The benefits in gasoline substitution are measured in avoided GHG emissions, i.e. GHG emission reduction in percentage. In Europe and in the US there are already directives that biofuels must meet in order to be qualified as a biofuel usable in the transportation sector: the Renewable Energy Directive (RED) and the Renewable Fuel Standard (RFS).[26,217] In both directives, the life cycle assessment methodology must be followed to compute the GHG savings from the reference fossil system. In Europe, from January 2017, at least a 50% reduction must be achieved. In the US, feedstocks different than corn starch (kernels) must reduce life cycle GHG by 50%, cellulosic and agricultural waste feedstocks must reduce at least 60% (for biomass-based biodiesel 50%).

An important methodological issue is the way co-products/by-products share energy consumption and emissions with the main product (ethanol or gasoline). The RED directive uses energetic allocation both for ethanol and gasoline. It will be quite interesting to compare both without any allocation, and considering biogenic emissions and land used change (LUC).

At present, industrial bioethanol is essentially produced from crops such as corn, sugarcane, and sugarbeet. However, concerns have been raised about its production-related to food shortage because such crops must be planted on farmland.[218,219] Moreover, these agricultural crops consume a significantly high amount of water and nutrients. An important question is whether we apply scarce water resources for food or for fuel. This requires detailed information about how much water is needed to produce food and fuel.[220]

Although biomass is a renewable energy source, the development of biofuels raises important issues such as: food *versus* fuel, land availability and environmental impact (e.g. soil erosion, water shortage, pollution from pesticides and problems with overuse of fertilizers), and a need for measures to be put in place to ensure sustainability (e.g. certification systems for verifying origin of biofuels, cultivation criteria).[209]

Therefore, the production of biofuels from both food and energy crops is limited by the availability of land, water, energy and co-product yields, and sustainability considerations, such as the life-time accountancy of CO_2 emissions. In this context, feedstocks that do not depend on fertile farmland, pesticides, water irrigation and presenting high yields, are ideal energy crops towards bioethanol production. One of such important feedstocks is the Jerusalem artichoke (*Helianthus tuberosus* L), JA, also known as topinambour, a perennial tuberous plant demanding low cultivation costs. Owing to its unique agronomic traits such as tolerance to drought and salt stresses and high resistance to frost and plant diseases, JA can grow in marginal lands and does not require soil fertilization, so it does not compete for arable lands with food crops.[212,221–224] The major carbohydrate accumulated in JA tubers (JAT) is inulin, a

poly-disperse fructan polymer that can be hydrolyzed by microbial inulinases to fermentable sugars, essentially fructose. JA, dahlia, and chicory are the major sources of inulin for industrial scale,[225] but JA is superior to the other inulin-accumulating crops, in terms of its outputs of biomass production, inulin content, and tolerance to a relatively wide range of environmental conditions.[226] The polysaccharides in JA carry a substantial amount of energy that can be partly accessed through bioconversion into storable fuels, such as bioethanol. Inulin or its hydrolysates with high fructose content can be converted into ethanol by microorganisms.

Consolidated bioprocessing (CBP) of JAT for ethanol production is one of the most promising conceptual designs towards the development of sustainable cost-effective biofuel technology. In this strategy, the inulin hydrolysis and further fermentation occur in a single step using one microorganism as sole biocatalyst. Indeed, CBP strains have the advantage of producing the necessary enzymatic machinery to hydrolyze the JAT inulin and simultaneously converting the fermentable sugars into ethanol. However, there are some drawbacks to the development of this promising technology towards bioethanol, such as the scarcity of efficient CBP strains able to directly convert inulin to high levels of ethanol.

A novel CBP strain, the yeast *Zygosaccharomyces bailii* strain Talf1 was isolated at Laboratório Nacional de Energia e Geologia, Portugal.[227] The strain, first yeast belonging to the *Zygosaccharomyces* genus described as an inulinase-producing microorganism, presents high inulinase activity and produces high ethanol levels directly from commercial inulin by CBP approach,[227] attaining an ethanol yield better or within the same range as that obtained by the best CBP strains described in recent studies of CBP for ethanol production from JAT juice (JAJ).[212,228,229] Moreover, characteristics such as fructophilic behavior and high resistance to ethanol attributed to *Z. bailii* species[230] are also relevant features that grant advantages to *Z. bailii* Talf1 over the other described strains able of CBP from JAT inulin towards ethanol. Contrary to the non-frutophilic CBP strains, *Z. bailii* Talf1 yeast can preferentially assimilate the high amount of fructose produced from inulin hydrolysis, which can contribute to more efficient ethanol production and to the novelty of the process.

In this work, aiming to evaluate if JA may be considered a sustainable energy crop to bioethanol production, several issues were considered. In this context, in a first stage, the cultivation of JA in non-arable land using no fertilizers, no pesticides and no water irrigation (only rain) was performed, followed by manual harvesting of the tubers, to get a sense on its productivity under such conditions. Further ultimate analysis to know the carbon sequestered, and experimental laboratory-scale trials to convert JAT directly to ethanol were then carried out. Indeed, two different strategies, a CBP using the novel inulinase-producing yeast *Z. bailii* strain Talf1 *versus* simultaneous saccharification and fermentation (SSF) with Talf1 inulinases and *S. cerevisiae* CCMI 885 as the ethanologenic strain were used and further compared for the best bioethanol yields. On a second stage, the best process strategy will serve as the basis

for an industrial-scale extrapolation. Usually, an intermediate pilot-plant is built prior to industrial applications, but in this study, a modeling approach to avoid this interim step is sketched by assuming the same laboratory-scale productivity and dimensioning the impeller for reactor agitation. Then, the extrapolated industrial ethanol is compared against the fossil equivalent system and another industrial ethanol system in a cradle-to-gate approach. For a fair comparison of energy consumption, GHG emissions, and freshwater use, for the first time, the three industrial systems: fossil gasoline, sugarcane, and JAT are reported without allocation (energy, economy, mass) or system expansion. The FU is 1 L of ethanol equivalent (L_{EtOH} eq). Additionally, since LUC can be a factor in CO_2 atmospheric concentration, and, thus, it is a contributor to global climate change, LUC impacts from non-arable to JA cropland are considered to have further insights. The results are expected to hopefully guide appropriate actions on research, industry and/or policymakers in the context of ethanol production.

3.2. MATERIALS AND METHODS

3.2.1. CULTIVATION OF JERUSALEM ARTICHOKE AND LAND-USE CHANGE

About 120 JA plants were cultivated about 30 cm apart within a 12 m^2 area of a forest soil at Oleiros, Castelo Branco, Portugal, from April to early November, without any water irrigation, besides local precipitation, or fertilizer/pesticide supply. The harvested JAT were washed and further mashed in a juice extractor machine (Ceado™ ES-500) for inulin-rich JAJ extraction. The JAJ was kept at -20 ºC until further use. Moreover, a portion of JAT was sliced, dried at 60 ºC for 48 h and further ground to a powder. The carbon and nitrogen content of tubers powder was determined: 38.1% and 1.02%, respectively, using an elemental analyzer EuroVector (model EuroEA 3000, Italy). Additional acid characterization revealed also about 77.4% (w/w) inulin (glucose+fructose) in this dried JAT powder, with a moisture content of about 5% (w/W), 3.24% lignin and 6% ash. In general, on a dry weight basis, JAT contains 68 to 83% fructans (inulin), 15 to 16% proteins, 13% insoluble fibers and 5% ash.[226]

Regarding direct LUC (DLUC), before JA plantation the land would have a certain carbon pool, litter and woody debris, soil organic matter and aboveground (e.g. branches, leaves) and below-ground (e.g. roots). Carbon stock varies in depth and according to the original land type. So, JA plantation can potentially lead to an alteration in the soil's carbon stock (SOC: soil organic carbon). According to IPCC guidelines,[231] the emission factor (EF) for year *t* for deforestation for stratum *x* and driver *y*, is a function $EF_{def(x, y, t)}$ expressed in tons of CO_2 equivalent per hectare (t CO_2eq/ha):

Equation 1.

$$EF_{\mathrm{def}\,(x,y,t)} = (C_{bio.pre(x)} - C_{bio.post(t,y)} - C_{wp} + \Delta SOC_{(t)}) \times 44 \div 12$$

Or, in condensed form,

Equation 2.

$$EF_{LUC} = \frac{C_{stored\ prior\ to\ cultivation} - C_{stored\ after\ cultivation} \times \frac{44}{12} + CO_2eq_{(non\ CO_2 gas\ from\ burning, land\ clearance)}}{Duration\ of\ cultivation\ in\ years\ (the\ IPCC's\ default\ value\ is\ 20\ years)}$$

$C_{bio.pre(x)}$ is the carbon stock in biomass in stratum *x*, prior to deforestation, *t* C/ha. According to Guo and Gifford,[232] soil carbon stocks decline after land-use changes from native forest to crop by 50%. In this experiment, no biomass was removed prior to the plantation. Therefore, this previous carbon was unaltered. The default value for temperate continental forest is 87 t C/ha.

$C_{bio.post(t,y)}$ is the carbon stock in biomass in year *t* post-deforestation, for driver *y*, *t* C/ha. In this case, new biomass enters the land, the JA. The carbon dioxide uptake during the photosynthesis of the feedstock (biomass) is for a simple sugar: $6CO_2 + 12H_2O$ + light + biomass = $C_6H_{12}O_6 + 6\ O_2 + 6H_2O$. The overall CO_2 uptake for JA (leaves, tubers, stalk) can be known by the ultimate analysis of the tubers and assuming they represent over 90% of the plant weight (1.28 to 3.27 kg/plant).[233] Therefore, CO_2 uptake during feedstock cultivation may be roughly estimated by using its carbon content (38.1%) and assuming CO_2 as the only carbon source (12 g C per each 44 g CO_2) by using the following formula: CO_2 uptake ($g/g_{dry\ biomass}$) = C(5)/100 x 44/12. Regarding JAT, there is 38.1 g C in 100 g of feedstock, so 38.1 x 44/12 = 139 g CO_2 per 100 g of feedstock or 1.39 g $CO_2/g_{dry\ tubers}$. Considering that JAT consists of about 80% water and 20% dry matter (proteins, carbohydrate, insoluble fibers, ash),[234] this means that wet biomass uptake will be 1.39 x 0.2 = 0.28 g CO_2/g_{tubers}. Assuming 1 g of JAT means 1/0.9 g of plant, carbon uptake would be 0.25 g CO_2/g_{JAT}. Combining this value with the productivity in $g_{JA}ton.ha^{-1}$ it is obtained the final value for the formula.

C_{wp} is the carbon stock in long-term harvested wood products following deforestation, t C/ha. Again, this value is zero because no biomass was removed. ΔSOC(t) is the change in soil carbon stocks in year *t* following deforestation, *t* C/ha. 44/12 is the conversion factor from carbon to CO_2. L_{fire} is the value of emissions from burning harvesting wastes, including non-CO_2 gases, N_2O and CH_4, expressed in CO_2 equivalents, t CO_2eq/ha. In this study, no fire took place for clearing the land and, therefore, this value is zero.

Hence, in this specific case study, the LUC impact on CO_2 was considered only due to ΔSOC(t) and $C_{bio.post(t,y)}$. The change in soil carbon stocks is assumed to occur over a 20-year time period, at which time a new steady state for a given land use is reached.[231]

$\Delta SOC(t) = SOC_{REF} - SOC_{REF} \times F_{LU} \times F_{MG} \times F_I$, where SOC_{REF} is the reference soil organic carbon stock under natural vegetation in the 0-30 cm topsoil layer (t C/ha); the value for SOC_{REF} can be obtained from the IPCC default value which will vary depending on the climate region and soil type of the area concerned. The specific value of SOC from measurement or literature can also be used in the calculation. The three dimensionless factors that appear are related to the stock change factor for the land-use system for a particular land-use (dimensionless F_{LU}), stock change factor for the land management regime (dimensionless F_{MG}) and stock change factor for input of organic matter (dimensionless F_I). Assuming IPCC Tier 1 methodology,[231] the ranges for the default values considered are: 31 to 117 tons C/ha for 0 to 30 cm depth for SOC_{REF}, 0.48 to 1.00 for F_{LU}, 1.02 to 1.22 for F_{MG}, and 0.91 to 1.38 for F_I. The inclusion of the EF_{LUC} would, therefore, give a range of uncertainty in the results. Despite this uncertainty, this case study will be a favorable scenario because cultivation did not use fertilizers. Therefore, no N nutrient enters the boundary, which is the clear advantage of JAT, avoiding high N_2O emissions at the culture stage (c.a. 2.6 kg CO_2eq/kg N, formula 1 + 0.0125 x N),[235] which could represent as much as 38% of overall culture CO_2 emissions.[236]

In addition, to evaluate the effect of N-fertilizer on increased CO_2 equivalent emissions from the culture stage, as well as the supposedly increased productivity of JAT, several studies that cover N-fertilizer effect on JAT productivity were accounted for[237,238] and such information was used to sketch “what -if” scenarios for N-fertilizer introduction, yield variations, and LUC and N_2O effect on overall CO_2 emissions.

3.2.2. COMMERCIAL CULTURE MEDIA AND CHEMICALS

Complete culture media were used: Yeast Malt Agar (YMA) and Yeast Malt Broth (YMB) from Sigma™. All media were sterilized by autoclaving at 121 ºC for 15 minutes. All other reagents were of the highest grade commercially available.

3.2.3. MICROORGANISMS

In this work, two ethanologenic strains were used: *Z. bailii* strain Talf1, a novel inulinase-producer yeast recently isolated from carob pulp kibbles[227] and *S. cerevisiae* CCMI 885 (Laboratório Nacional de Energia e Geologia (LNEG) culture collection, Portugal). Both yeast strains are maintained on YMA slants at 4 ºC and sub-cultured monthly for laboratory routine, and also they are maintained at -20 ºC by addition of 30% (v/v) glycerol to previously grown cultures in YMB.

3.2.4. BIOETHANOL PRODUCTION THROUGH CBP

Prior to the CBP assays (Figure 4 – CBP pathway), strain Talf1 was transferred from a fresh YMA slant to 100 mL of YMB in 500 mL Erlenmeyer flask and cultivated aerobically for 24 h at 25 ºC and 150 rpm for the seed production. After, for ethanol production directly from JA juice (JAJ), 10% (v/v) inoculum (cells obtained by centrifuging the grown culture at 7500 rpm for 10 min) was used to inoculate 500 mL Erlenmeyer flasks containing 150 mL of JAJ (≈ 130 $g.L^{-1}$ hydrolysable fermentable sugars, pH 6 ± 0.5) as carbon source the only nutrient source, and then the cultures were incubated at 25 ºC, without shaking, for 8 days. The assays were carried out in triplicates. The time course profiles of microbial growths, ethanol production, and sugars consumption were evaluated.

3.2.5. BIOETHANOL PRODUCTION THROUGH SSF

Prior to the SSF assays (Figure 4 – SSF pathway), *S. cerevisiae* CCMI 885 was transferred from a fresh YMA slant to 100 m of YMB in 500 mL Erlenmeyer flask and cultivated aerobically for 24 h at 25 ºC and 150 rpm for the seed production. For ethanol production from JAJ, it was used the same procedure described above, but supplementing the fermentative medium with 5% (v/) of Talf1 crude enzymatic extract with inulinase activity (8.67 U/mL)[227]. So, 150 mL of the fermentative medium in 500 mL Erlenmeyer flasks was inoculated with 10% v/v of inoculum, previously grown in YMB, and then the cultures were incubated at 25 ºC, without shaking for 8 days. The assays were carried out in triplicates. The time course profiles of microbial growths, ethanol production, and sugars consumption were evaluated.

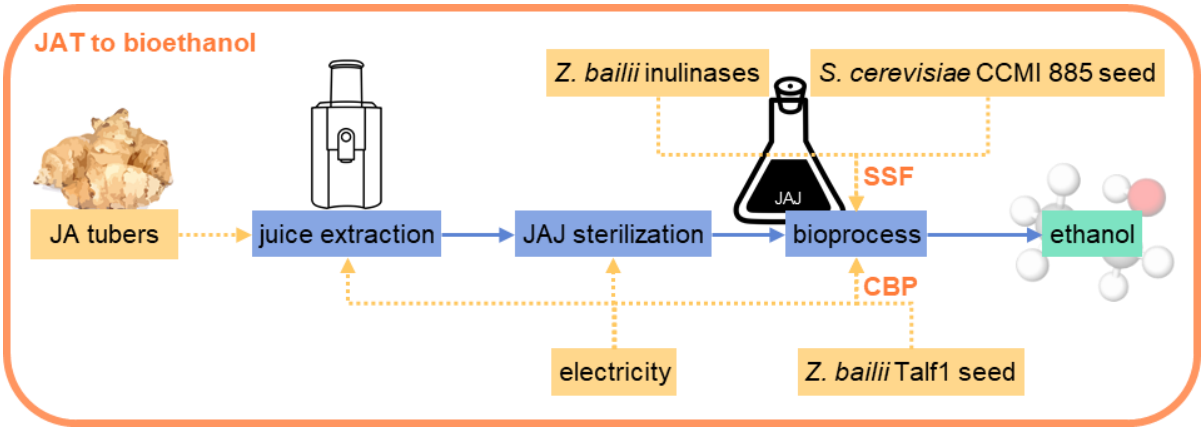

Figure 4. Laboratory-scale plant processes for bioethanol production from Jerusalem artichoke tubers.

3.2.6. ANALYTICAL METHODS

The cell growth was monitored by measuring the optical density of culture broth samples at 600 nm ($OD_{600\ nm}$), using a spectrophotometer (Genesys™ 20, Thermo Scientific™). The maximum growth rates (μ_{max}) were calculated through linear regression of the first three points of the exponential phase, using Excel software (Microsoft® Office Excel®, 2007 for Windows™).

Sugars and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) using a Waters Sugar-Pak™ I column (Bio-Rad Laboratories™ operating at 75 ºC with Ca-EDTA at 50 mL/min.

In the batch fermentations for bioethanol production from JAJ, the remaining amount of inulin in the fermentative broths (no hydrolyzed inulin by Talf1 inulinases) was quantified by performing acid hydrolysis of the JAJ inulin present in each broth sample. These hydrolyzes were carried out incubating the samples at 55 ºC and pH 2 for 96 h. Then the hydrolysates were analyzed for sugar content (glucose and fructose) by HPLC using an Aminex HPX-87H™ column (Bio-Rad Laboratories™) operating at 50 ºC with 5 mM H_2SO_4 as mobile phase, at a flow rate of 0.4 mL/min.

For both bioprocesses, direct electricity consumption measurements of the preparation and fermentation steps were performed by using the Analog Discovery™ oscilloscope and WaveForms™ software (Diligent®). To get the final energy consumption data, the following equation was used:

Equation 3.

$$E_{equipment} = \sum_{i=1}^{i=n} \left(\int_{0}^{\Delta t_i} V_i(t) \times I_i(t) dt \right)$$

$E_{equipment}$ is the direct energy consumed by the *n* equipment from the laboratory-scale apparatus in joule; *V* is the instantaneous voltage in volts; and *I* is the instantaneous current in ampere. The direct energy requirements for each bioprocess (CBP of SSF) are settled by the sum of all the equipment data. Inventories of energy consumption are presented for both bioprocesses for comparison of energy efficiency.

3.2.7. LABORATORY-SCALE TO INDUSTRIAL-SCALE MODELING: DIRECT ENERGY AND CO_2EQ EMISSIONS CONSIDERATIONS

Several SSF/CBP processes for ethanol fermentation from JAT have been reported in the last two decades.[226–229,239] The most widely used ethanologenic microorganism in industrial processes is the *S. cerevisiae* yeast, which has proved to be very robust (high ethanol

tolerance and high rate of fermentation activity) and well suited to the fermentation of several substrates/hydrolysates.[58] In this context, *S. cerevisiae* strains have been extensively used for ethanol fermentation studies from JAT either in SSF processes coupled with inulinases or inulinase-producing microorganisms, such as *Aspergillus niger*[240] and *Kluveromyces fragilis*;[236] or in CBP processes after gene-engineered to produce effective inulinases (e.g. *Saccharomyces* sp W0,[224] *S. cerevisiae* DQ1[228]). So far, the best results obtained for bioethanol production from JAT were those reported by Guo et al.[228] and Ge and Zhang,[240] considering the CBP and SSF approaches, respectively (30 ºC). Guo et al.[228] using *S. cerevisiae* DQ1 as CBP strain were also able to get 128.7 $g.L^{-1}$ of ethanol within 80 h, corresponding to a productivity of ≈1.61 $g.L^{-1}.h^{-1}$ and a sugar consumption over 96%. Ge and Zhang,[240] coupling *S. cerevisiae* Z-06 and *A. niger* SL-09 obtained the highest ethanol production reported, 155 $g.L^{-1}$ of ethanol within 48 h using 98% of total sugars,[226] which corresponds to a productivity of 3.23 $g.L^{-1}.h^{-1}$. In both studies, a fed-batch approach was used to load JAT flour up to ≈350 $g.L^{-1}$ JAT inulin, permitting in this way the fermentation of a high level of total sugars and consequently the achievement of high levels of bioethanol.

Based on the highest ethanol productivity, from JAT, achieved so far (3.23 $g.L^{-1}.h^{-1}$ up to 155 $g.L^{-1}$)[226] or the ethanol productivity obtained in this work, if higher, an ultimate scale-up scenario for the best conceptual strategy will be further modeling, assuming that ethanol has a density of 0.789 $kg.L^{-1}$ and a lower heating value (LHV) of ≈27 MJ/kg.[191] So, an industrial scenario towards the production of 10 million liters (ML) is sketched in Figure 5. The total bioreactor volume can be divided into the working space (WS) and the headspace (HS) typically with a ratio of 0.25 (HS/WS) or WS with 70 to 80% of the total fermenter volume.[241] For such a large volume reactor an agitation system is required. The agitator impeller usually is a Rushton turbine connected to a shaft and electrical engine. Diameter of impeller to diameter of tank, Da/Dt, is typically 1/3 (Figure 5).

A single-phase (i.e. 240 V) drive motor can be used with small reactors. However, for large reactors, a 3 phase motor (i.e. 430 V) should be used. The latter will tend to require less current and therefore generate less heat. In this work, the main assumptions for the industrial scale-up are homogeneity over the whole reactor and the same productivity in $g.L^{-1}.h^{-1}$ as in laboratory-scale, fixed capacity (CAP), number of bioreactors (nBR) equal to 4 and the vessel is a standard cylinder with diameter of tank (Dt) equal to its height (Ht). The following equations would apply (for 8760 working hours per year):

Equation 4.

$$CAP = \frac{productivity}{\rho_{EtOH}} \times 8760 \times nBR \times WV \equiv WV\ (working\ volume) = \frac{CAP}{\frac{productivity}{\rho_{EtOH}} \times 8760 \times nBR}$$

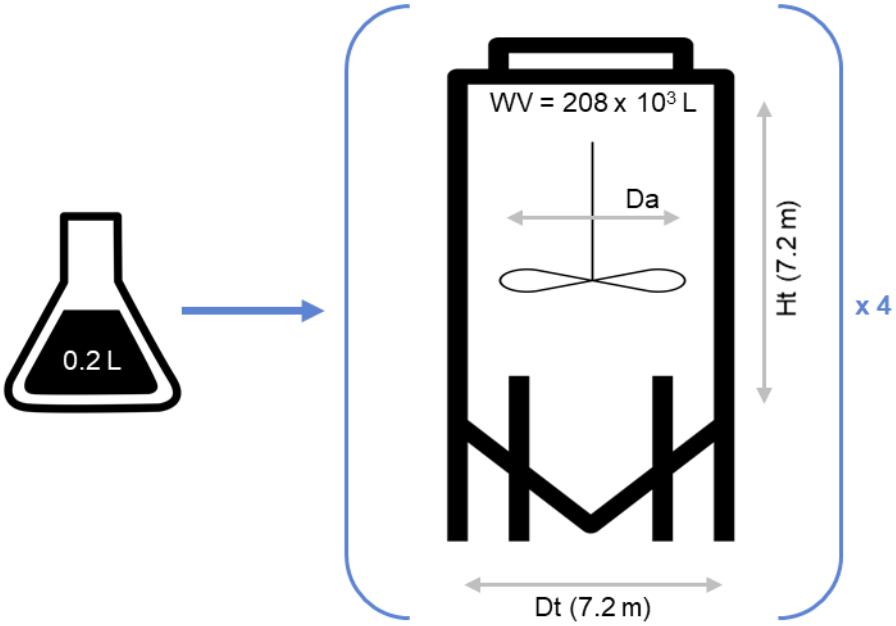

Figure 5. Scale-up considerations (4 reactors to 10 ML bioethanol/year) without intermediate step, with main dimensions referred on scheme (DT = Ht = 7.2 m; WV = 208,000 L; see Table 5). Power and speed requirements depend on the scale-up approach.[242] Constant power/volume, constant Reynolds (Re), provide similar flow patterns and constant rotational speed (N) giving constant mixing times or constant top speed and constant shear.

Ethanol production rate (L_{EtOH}/LWV/h)	0.0014
Bioreactor working volume (WV in L)	208,000
Bioreactor head volume (HV in L)	83,000
Bioreactor diameter (Dt in m) = Bioreactor height (Ht in m)	7.2
Turbine diameter (Da in m)	2.4
Rotational speed (rpm)	200, 350, 500
Electrical motor power requirements (MW)	11.3
Energy requirements (kWh/L_{EtOH})	2.7 – 41.6

Table 5. Production plant tank, with capacity (CAP) to produce 10 ML of ethanol.

Equation 5.

$$CO_{2\,(preparation+processing)}[g/L_{EtOH}]$$

$$= \frac{[14\ kWh/t[JAT]] \times \frac{0.21 \times 10^{-3}}{0.15}[t_{JAT}/L_{juice}]}{Yield\left[\frac{kg_{EtOH}}{L_{juice}}\right] \times \frac{1}{\rho}\left[\frac{L_{EtOH}}{kg_{EtOH}}\right]} \times CO_{2\,grid}\left[\frac{g}{kWh}\right] + 750\left[\frac{g}{L_{EtOH}}\right]$$

$$+ 4.73 \times EF_{natural\ gas}$$

Equation 6.

$$HV = 0.25 \times WV$$

Equation 7.

$$\pi(\frac{Dt}{2})^2 \times Ht = WV + HV$$

Equation 8.

$$Da(impeller\ diameter) = \frac{1}{3} \times Dt$$

Equation 9.

$$P(power) = Po \times \rho \times N^3 \times Da^5 \cong N^3 \times Da^5$$

Where Po = Power Number, typically higher than 0.75;[243] ρ = density of the liquid, kg/m^3 (>1) and N is the impeller rotational speed (200 to 500 rpm).

Table 5 shows the parameters used to model a 10 ML production capacity plant with 4 bioreactors totalizing 291,000 L of total working and head volume per vessel. Finally, direct energy consumption (EC) is calculated by multiplying power required, P, and the annual working hours over the electrical motor efficiency plus transmission, η_{motor}, which was assumed to be high (0.95):

Equation 10.

$$EC_{fermentation} = \frac{P \times 8760 \times 10^{-3}}{\eta_{motor}} (kWh)$$

Fermentation specific energy consumption (SEC) will be a function of rotational speed and diameter of the impellers, as well as the ethanol productivity.

Equation 11.

$$SEC_{fermentation}[MJ/L_{EtOH}] = \frac{EC_{fermentation}[kWh] \times 3.6[MJ/kWh]}{productivity[\frac{g}{L_{EtOH}h}] \times WV \times 8760}$$

Regarding the direct biogenic CO_2 emissions, it can be assumed that the fermentation is translated in the following chemical equation, for a simple sugar ($C_6H_{12}O_6$): $C_6H_{12}O_6$ + yeast → $2C_5H_5OH + 2CO_2$ + heat, and consider one mol of CO_2 release to one mol of ethanol (C_2H_5OH), or in g.L^{-1}, CO_2 molar mass/ethanol molar mass x ethanol density (44 / 46 x 789 = 750 g.L^{-1}). Otherwise measured, this ratio will be assumed. Another direct contribution to CO_2 is the burning of natural gas for the distillation process. Indirect CO_2 emissions have to do with electricity power plants that are based on combustion to heat water (Rankine cycle).

For a generic sugar, the chemical formula would be $C_nH_{2n}O_n$, 3<n<7.[244] The electricity needs for juice extraction are 14 kWh/ton of JAT and natural gas energy for distillation is 4.73 MJ.L$^{-1}_{EtOH}$.[245] Thus, overall direct and indirect CO_2 emissions would be expressed by:

$CO_{2\ grid}$ is the equivalent CO_2 emission factor for electricity generation, accounting for grid losses. In Europe, according to the European Environmental Agency the average emission factor is roughly 400 g.kWh^{-1}, but considering the grid losses of 6%, when 1 kWh is requested by the factory, in reality, it will request 1.06 kWh to the power plants, meaning the $CO_{2\ grid}$ factor would be 424 g.kWh$^{-1}_{final}$, instead (i.e. $CO_{2\ grid}$=424). $EF_{natural\ gas}$ is the emission factor for natural gas, considered as 55 g CO_2/MJ,[191] ρ is the ethanol density, and yield is the amount of ethanol

produced from 1 L of juice fermentation. From laboratory-scale data, the amount of JAT necessary to produce 1 L of juice is $0.21x10^{-3}$ / 0.15 = 1.4 kg.

3.2.8. Overall system and system comparators: energy and emission analysis

The overall system of transforming JAT to ethanol (Figure 6) is computed by summing direct energy inputs, and, direct and indirect CO_2eq emissions. The scope of this analysis encompasses cultivation, preparation, fermentation, and distillation (cradle-to-gate). 1 L_{EtOH} or 1 MJ_{EtOH} is used as FU.

Furthermore, this industrial scale-up scenario (Figure 6) is then compared against a reference fossil system, considering gasoline with a LHV of 43.2 MJ/kg and a density of 0.745 $kg.L^{-1}$ (1 $L_{gasoline}$ = FU)[246], and also with bioethanol from sugarbeet and sugarcane refinery.

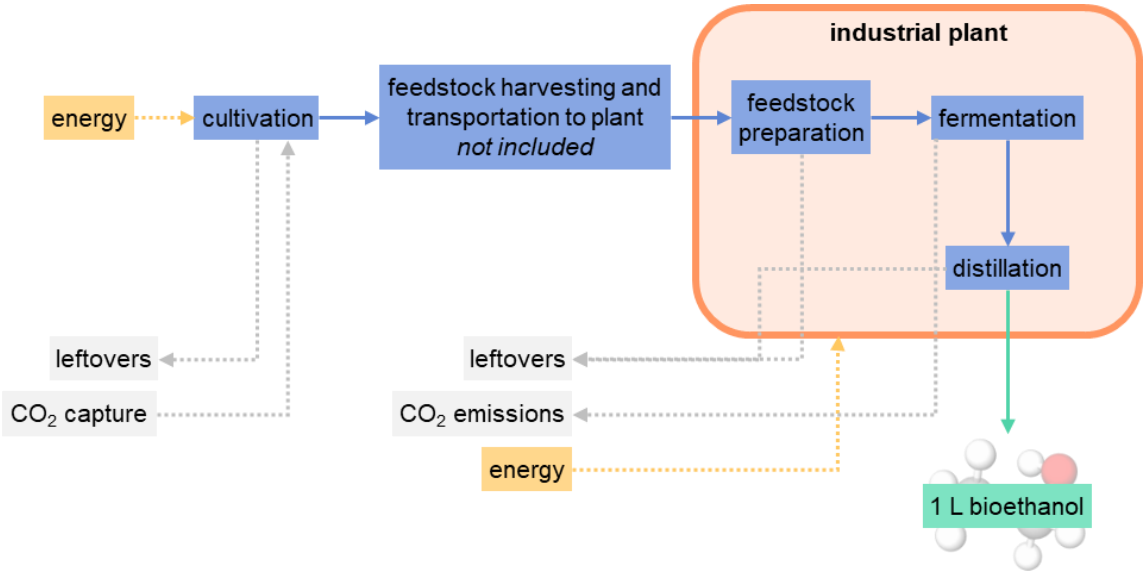

Figure 6. Inventory boundaries, in this work, for JA feedstock to bioethanol industrial technology (orange box on the right).

Ethanol as a product that works as a substitute for gasoline implies the need of comparing the JAT-to-ethanol system with the production of the same amount of MJ but with gasoline (1 L_{EtOH} has 27 MJ/kg x 0.789 $kg.L^{-1}$ = 21.3 MJ and the same energy is achieved with 0.66 $L_{gasoline}$, according to their lower heating values). Likewise, when looking for how much ethanol is equivalent to 1 L of gasoline, the factor is 43.2 / 27 x 0.745 / 0.789 = 1.51, i.e. 1 $L_{gasoline}$ is energetically equivalent to burning 1.51 L_{EtOH}.

Indeed, when different systems are compared, the same criteria for allocation or system expansion should be followed. So, for an accurate comparison, if for bioethanol it is

not used co-product credit, this assumption must be respected for the gasoline reference system. In addition, if LUC is considered for biomass cultivation and processing, then LUC of the crude oil extraction field and of the refinery land would also have to be in account. Usually, fossil fuel published values have allocation/system expansion assumptions and the effort in this work is to do some reverse engineering to obtain unbiased values. For example, a RED directive value could only be compared with a reference system that also used energy allocation, no LUC, and no biogenic emissions.

Oil refineries produce a number of different products simultaneously from a single feedstock (gasoline and co-products). Whereas the total amount of energy (and other resources) used by refineries is well documented, there is no simple, non-controversial way to allocate energy and emissions to a specific product. For gasoline, allocation in refinery[247] could be mass-based, energy-based or market value-based. According to Wang et al.[247] , in terms of mass, gasoline is 46% of the products of a refinery. In terms of energy, it accounts for 53.3% of all refinery products energy content (based on LHV) and 58.75% when considering market value basis. Without any allocation, the process of refining consumes 2947.9 kJ/0.62 $L_{gasoline}$ and is responsible for emitting 611 g CO_2eq/$L_{gasoline}$.

The Joint Research Center (JRC) report[202] considers systems expansion rather than allocation, where co-products have credits because they avoid the fossil production of some products. Alternative fuels can only be reasonably expected to supply about 10 to 20% of the road fuel demand. Therefore, the issue is how much can be saved by not producing the marginal 10% or 20% gasoline of the 2010-2020 expected demand (“marginal substitution approach”). In fact, in current EU methodology, in order to estimate the savings from substituting conventional fuels, the subject is what primary energy and GHG can be saved by using less conventional fuels rather than how much energy/GHG emissions are associated with the overall fuel processing.

In this work, a comparison between 100% gasoline reference system and 100% bioethanol system (100% *versus* 100%) is proposed, instead of the marginal reduction of gasoline production approach. In this context, data from the JRC report[246] presented for gasoline, ethanol from sugarbeet and ethanol from sugarcane was used for calculations and further comparison with energy/GHG emissions of JAT ethanol.

Gasoline, identified as “COG1” in the report,[246] states 0.07 $MJ/MJ_{gasoline}$ for crude oil production and 0.08 $MJ/MJ_{gasoline}$ for crude refining, which corresponds to an overall value of 0.15 $MJ/MJ_{gasoline}$ for marginal crude oil transformation (i.e. the 10 to 20% that will be avoided). In reality, 100% gasoline system corresponds to a total energy value of 0.15/0.2 or 0.75 $MJ/MJ_{gasoline}$ or 24 $MJ.L^{-1}_{gasoline}$. Similarly, for CO_2eq emissions, 10 to 20% crude production and refining comprise 11.6 $g.MJ^{-1}_{gasoline}$. So, in fact, without allocation (or without the marginal approach up to 20% replacement), the real GHG emissions are 11.6/0.2 $g.MJ^{-1}_{gasoline}$ or 1867

$g.L^{-1}_{gasoline}$. The values of JRC report[202] are higher than those of Wang et al.,[247] because it includes crude oil production and the latter only refining process. In L_{EtOH} equivalent (i.e. for the same energy released based on LHV) the total values of the system (11%) are: 15 $MJ.L^{-1}_{EtOH}$eq and 1154 $gCO_2.L^{-1}_{EtOH}$eq.

In the JRC report,[246] ethanol from sugarbeet identified as "SBET1a", pulp used as animal feed and slops not used, states 0.12 MJ/MJ_{EtOH} for cultivation and 1.25 MJ/MJ_{EtOH} for processing without credit for pulp. This is equivalent to a total of 28.9 $MJ.L^{-1}_{EtOH}$. Regarding CO_2eq emissions, these are 18.1 $g.MJ^{-1}_{EtOH}$ for cultivation (56% of those due to N-fertilizer) and 16.8 $g.MJ^{-1}_{EtOH}$ for processing. This is equivalent to 735 $g.L^{-1}_{EtOH}$.

For sugarcane, identified as "SCET1", it states 0.09 MJ/MJ_{EtOH} for cultivation and 1.88 MJ/MJ_{EtOH} for processing, without bagasse credits.[246] This is equivalent to a total of 41.5 $MJ.L^{-1}_{EtOH}$. Regarding CO_2eq emissions, these are 17.9 $g.MJ^{-1}_{EtOH}$ for cultivation (47% of those due to N-fertilizer) and 16.8 $g.MJ^{-1}_{EtOH}$ for processing. This is equivalent to 731 $g.L^{-1}_{EtOH}$.

3.3. Results and Discussion

3.3.1. JAT production and LUC

From the small plantation of JA carried out in a poor forest soil, with no water/fertilizers/pesticides supply, 48 kg of JAT were harvested, corresponding to a yield of 40,000 kg/ha. This yield is about one half of the maximal JAT crop yield reported (up to 90 $ton.ha^{-1}$).[228,237] This lower yield is due to the poor cultivation conditions.

Figure 7 shows the environmental conditions (rainfall water accumulation, air temperature) during JA cultivation period (214 days) in a non-arable land in a village at Castelo Branco region (Portugal). Castelo Branco region is known for its hot summers, almost without rain, and cold winters. During April, when JA was planted, there was an abundant rainfall (65.7 mm) that could favor the plant germination; however, in the following months (from 1 May to 31 August) there were only a few rainy days (total precipitation = 68.9 mm), with just 4 days of heavy rainfall and no rain in August. Thus, during the hot summer (21 June to 20 September) most of the rainwater was assembled in September (66.5 mm, 1 to 20 September). Indeed, the growth of JA in these environmental conditions shows its high resistance to drought. From overall precipitation of 356.2 mm, 38% of rainwater was assembled from April to August, and the other 62% within the last two months (221.6 mm in September and October). The heavy rainfall occurred in the last two months before the JAT harvest probably favored the tubers development/ maturation permitting to attain a crop yield of 40 t $JAton.ha^{-1}$.

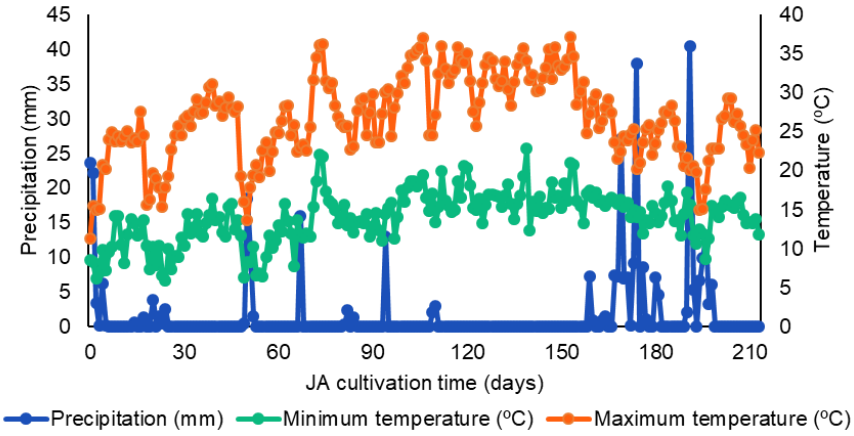

Figure 7. Data on the amount of rain and air temperatures (maximum/minimum) during the JA cultivation period (214 days, from April to October), in Castelo Branco region (Portugal).

In fact, the crop yield may be improved through the use of water irrigation and/or fertilizers (e.g. nitrogen), as already demonstrated by agronomic studies carried on JA plants.[237,238] However, water-intensive crops are not sustainable to be used as energy crops for industrial bioethanol production because water is a scarce resource.

In this study, DLUC impact on carbon stock and biomass carbon uptake, by JA cultivation on forest soil, was estimated considering data from the literature[237] on effect of N-fertilizer addition on JAT productivity. For LUC impact calculations (Equation 1 and Equation 2, in the Materials and Methods), based on default values of land use provided by IPCC)[231], a deforestation scenario to cope with an annual 10 ML ethanol productivity is considered. So, in Figure 8, the final LUC emission factor (t CO_2/ha) as a function of biomass (JAT) productivity (t biomass/ha) and the N-fertilizer effect on JAT productivity are depicted. LUC decreases linearly with biomass productivity increase due to an enhancement in the carbon uptake ability per ha. To observe N-fertilizer influence on CO_2eq emissions, a factor of 2.6 kg CO_2eq/kg N[248] was used and then this effect was added on LUC (minimum/maximum values; see light-green and dark-green lines in Figure 8). These CO_2eq emissions are smaller than those due to potential LUC effects (light-blue and dark-blue lines in Figure 8). Thus, in terms of global warming, N-fertilizer quantities up to 150 kg/ha are negligible compared to LUC effects.

3.3.1.1. BIOETHANOL PRODUCTION: CBP *VERSUS* SSF

About 700 mL of juice per kg of JAT were obtained, after 3 sequential grindings. JAJ, with about 130 g.L^{-1} of total hydrolysable fermentable sugars (inulin and glucose/fructose), was kept as -20 ºC until further use. Ethanol production from JAJ was performed by two strategies: in a CBP and in a SSF approach. The results of the batch fermentations with JAJ are presented in Figure 9 and Figure 10, and Table 6 are summarized the most important metabolic parameters.

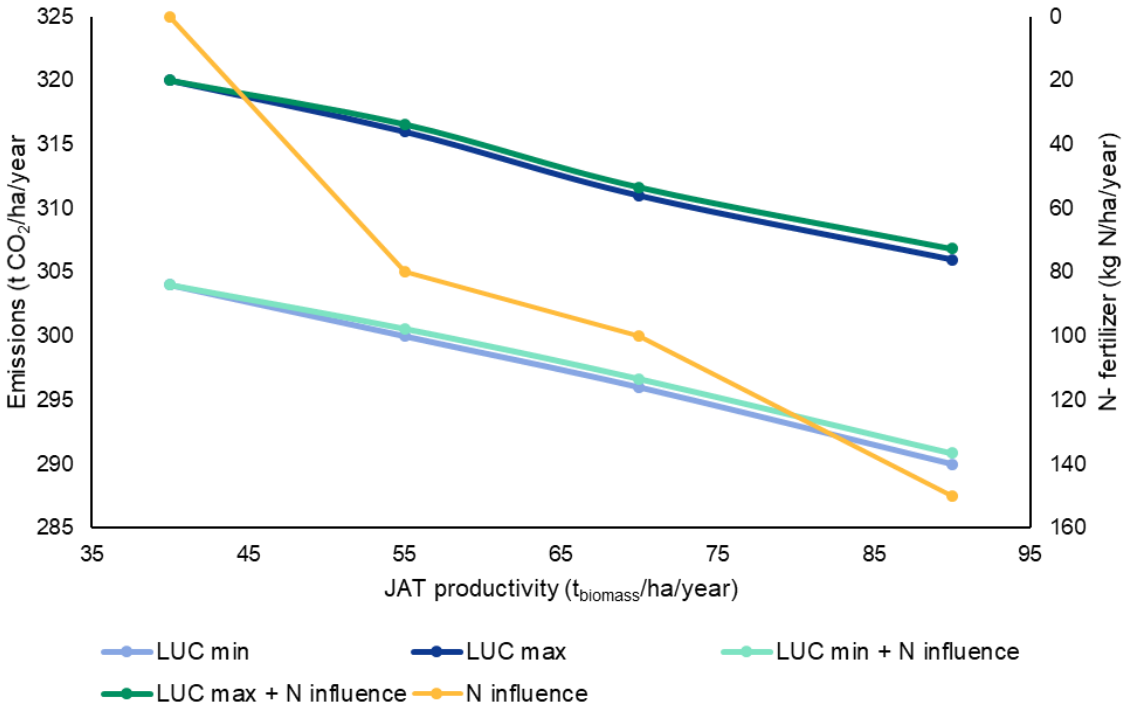

Figure 8. LUC and [LUC + N-fertilizer] influence on CO2 equivalents emissions based on JAT productivity. The raw data (yellow) showing the effect of N-fertilizer addition (80, 100 and 150 kg/ha) on JAT productivity were collected from Baldini et al.;[237] LUC impact calculations were carried out for the depicted productivities using IPCC AFOLU guideline;[249] and a factor of 2.6 kg CO2/kg N[235] was used for [LUC + N-fertilizer] influence estimations. (min: minimum; max: maximum; N: nitrogen)

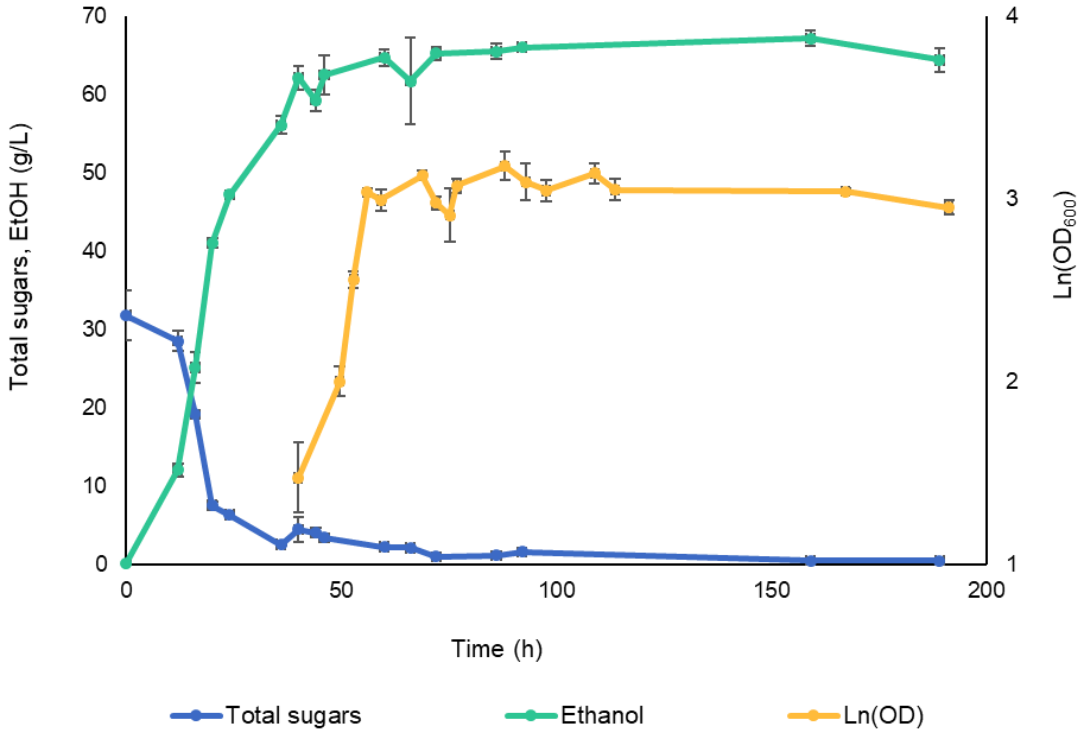

Figure 9. Yeast growth, sugar consumption and ethanol production time-course profiles during batch fermentation of JAJ (130 $g.L^{-1}$ total hydrolyzable sugars), as sole carbon and nutrients source, by Z. bailii strain Talf1 in a CBP for ethanol (Reprinted from Paixão et al.[250])

Fermentative medium	Strain	Enzyme addition	Time (h^{-1})[c]	Total sugars consumption ($g.L^{-1}$)[d]	μ_{max} (h^{-1})	Max. EtOH productivity ($g.L^{-1}.h^{-1}$)	Max. EtOH ($g.L^{-1}$)[e]
JAJ[a]	*Z. bailii* Talf1	-	60	128	0.13	3.62	65.0
	S. cerevisiae CCMI 885	inulinases[b]	60	125	0.11	2.40	62.8

Note: The results are mean values of three replicates (n = 3). Max: maximum.
[a] JAJ as the only carbon and nutrients source (≈130 $g.L^{-1}$ total hydrolyzable sugars).
[b] *Z. bailii* strain Talf1 crude enzymatic extract.
[c] Minimal fermentation time at which the sugars are fully consumed (≈0) and ethanol production is maximal/near maximal.
[d] Total sugars consumption = $[sugars]_{initial} - [residual\ sugars]_t$, t = 60 h.
[e] The ethanol produced is indicated in $g.L^{-1}$ (concentration units), but this value can be converted in grams or liters [65 $g.L^{-1}$ → 65 g x 150 mL / 1000 mL = 9.75 g ethanol, which corresponds to about 0.012 L ethanol (9.75 g/0.789 g/mL = 12.36 mL; ethanol density = 789 $kg.L^{-1}$)].

Table 6. Metabolic parameters from JAJ fermentations towards bioethanol production.

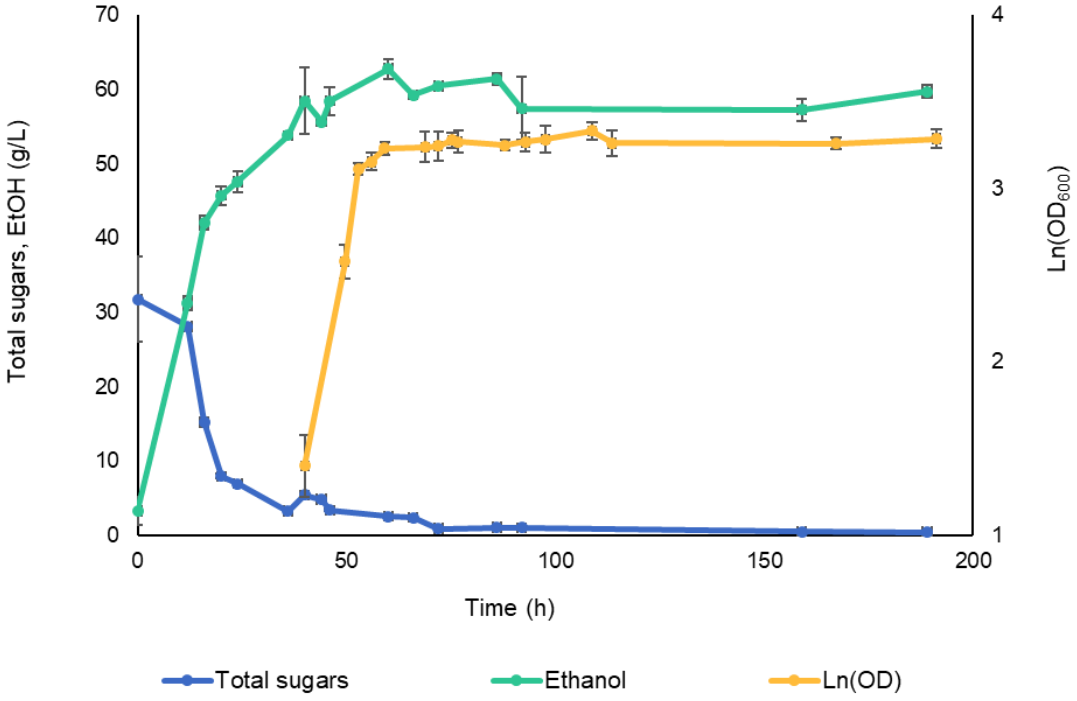

Figure 10. Yeast growth, sugar consumption, and ethanol production time-course profiles during batch fermentation of JAJ (130 $g.L^{-1}$ total hydrolyzable sugars), as sole carbon and nutrients source, by *S. cerevisiae* CCMI 885, supplemented with 5% (v/v) of Talf1 enzymatic extract, in a SSF process for ethanol.

The CBP was carried out with the inulinase-producing and ethanologenic yeast *Z. bailii* Talf1. Figure 9 shows the growth, sugar consumption and ethanol production profiles of this strain during the batch fermentation of JAJ, as only carbon and nutrients source. The *Z. bailii* strain Talf1, able to produce all its necessary enzymes, presented a specific growth rate (μ_{max}) of 0.13 h^{-1} and maximum ethanol productivity of 3.62 $g.L^{-1}.h^{-1}$, achieving 65 $g.L^{-1}$ of ethanol directly from the pure JAJ within 60 h (Table 6). So, for a consumption of 128 $g.L^{-1}$ total sugars an ethanol yield of 65 / 128 ≈ 0.51 g/g of sugars was attained, which corresponds to the

theoretical ethanol yield.[251] In fact, JAJ as a complex feedstock has other carbon sources than sugars (e.g. proteins, vitamins), not quantified, that can also be used for microbial growth and/or as inducers of the metabolism that may contribute for the higher yield.

The SSF process for bioethanol production from pure JAJ (Figure 10) was carried out using 5% (v/v) Talf1 enzymatic extract and the *S. cerevisiae* CCMI 885 as the ethanologenic yeast. The quantity of enzyme added to the SSF process was sufficient for preventing the occurrence of a sugar limitation since from the first day of the fermentation there was an accumulation of sugars in the broth ready to be used. So, in the presence of Talf1 inulinases, the *S. cerevisiae* presented a specific growth rate of 0.11 h^{-1} and could achieve 62.8 $g.L^{-1}$ of ethanol within 60 h (Table 6). In these conditions, the maximum ethanol productivity was 2.40 $g.L^{-1}.h^{-1}$ and the ethanol yield was 0.50 g/g of sugars consumed, which corresponds to 98% of theoretical ethanol yield.

Indeed, depending on the fermentation strategy, SSF or CBP, a bioethanol yield of 57 to 59 $L_{EtOH}/t_{biomass}$ will be expected, respectively, corresponding to 228 to 2360 L_{EtOH}/ha, in native forest land, without irrigation or fertilizer addition. These promising results highlight the great potential of the yeast *Z. bailii* strain Talf1 or its enzymatic crude extract (inulinases) to be used for further optimization, accounting the increase of the initial fermentable sugars (e.g. adding JAT flour in a fed-batch approach)[228,240] up to a concentration that may rise the produced bioethanol to maximum levels reached so far (155 $g.L^{-1}$),[226] and scale-up towards their future industrial application to produce bioethanol from inulin or inulin-rich materials either in a CBP or SSF process.

To envisage a hypothetical industrial scenario, the most energy-efficient process and the one that achieved a higher maximum ethanol productivity was chosen. Thus, additionally to the fermentation metabolic parameters (Table 6) an analysis of the overall direct energy consumption was performed for each process, and the corresponding inventories are presented in Table 7 and Table 8.

Whenever possible, to calculate the direct energy consumption by each equipment used in the bioprocesses (vacuum pump; orbital incubator; centrifuge), *in situ* measurements were carried out, as described in Section 3.2. Regarding these energy measurements, performed at laboratory-scale, each equipment showed different current intensity patterns over time as shown in Figure 11. This evolution over time allowed to measure actual energy consumption by each equipment (Equation 3 was used in these calculations).

	Process	Item	Energy (MJ)	Energy per FU ($MJ.L^{-1}_{EtOH}$)
Inputs	JAJ (150 mL, from 0.21 kg JA)	JA tubers	n.a.	n.a.
		juice extraction	8.40×10^{-2}	6.80×10^{0}
		sterilization (autoclave)	2.60×10^{-2}	2.10×10^{-2}
	CBP *Z. bailii* seed (10%)	medium sterilization (autoclave)	5.40×10^{0}	4.37×10^{2}
		10% YMB	5.43×10^{-1}	4.39×10^{1}
		10% sterilization (V.P.)	6.14×10^{-7}	4.97×10^{-5}
		10% incubation w/agit. (O.I.)	1.80×10^{-5}	1.46×10^{-3}
		10% centrifugation	5.40×10^{-8}	4.37×10^{-6}
	CBP fermentation	incubation wo/agit. (O.I.)	4.42×10^{-4}	3.57×10^{-2}
	TOTAL		6.05×10^{0}	4.90×10^{2}
Outputs	Bioethanol (maximum productivity)		65 $g.L^{-1}$ ($3.62\ g.L^{-1}.h^{-1}$)	
			9.75 g	
			2.63×10^{-1} MJ[a]	

Notes: O.I.: orbital incubator; w/agit.: incubation with agitation; wo/agit.: incubation without agitation; V.P.: vacuum pump.

[a] For the produced bioethanol, the energy conversion (MJ) was calculated based on its lower heating value ($LHV_{EtOH} \approx 27$ MJ/kg).

Table 7. Energy consumption inventory for the process of bioethanol production through CBP, using *Z. bailii* Talf1.

	Processes	Item	Energy (MJ)	Energy per FU ($MJ.L^{-1}_{EtOH}$)
Inputs	JAJ (150 mL, from 0.21 kg JA)	JA tubers	n.a.	n.a.
		juice extraction	8.40×10^{-2}	7.04×10^{0}
		sterilization (autoclave)	2.60×10^{-2}	2.18×10^{0}
	SSF inulinases production, separation and sterilization (5%)	medium sterilization (autoclave)	5.40×10^{0}	452×10^{2}
		10% *Z. bailii* seed[a]	5.43×10^{-2}	4.55×10^{0}
		5% (YMB + 25% v/v JAJ)	2.71×10^{-1}	2.27×10^{1}
		5% sterilization (V.P.)	3.07×10^{-7}	2.57×10^{-5}
		5% incubation	9.01×10^{-6}	7.55×10^{-4}
		5% centrifugation	2.70×10^{-8}	2.26×10^{-5}
		5% sterilization (V.P.)	3.07×10^{-7}	2.57×10^{-5}
	SSF *S. cerevisiae* seed (10%)	medium sterilization (autoclave)	5.40×10^{0}	4.52×10^{2}
		10% YMB	5.43×10^{-1}	4.55×10^{1}
		10% sterilization	6.14×10^{-7}	5.14×10^{-5}
		10% incubation w/agit. (O.I.)	1.80×10^{-5}	1.51×10^{-3}
		10% centrifugation	5.40×10^{-8}	4.52×10^{-6}
	SSF fermentation	incubation wo/agit (O.I.)	4.42×10^{-4}	3.70×10^{-2}
	TOTAL		6.38×10^{0}	5.34×10^{2}
Outputs	Bioethanol (maximum productivity)		62.8 $g.L^{-1}$ ($2.40\ g.L^{-1}.h^{-1}$)	
			9.42 g	
			2.54×10^{-1} MJ[b]	

Notes: O.I.: orbital incubator; w/agit.: incubation with agitation; wo/agit.: incubation without agitation; V.P.: vacuum pump.

[a] Calculated from values presented in Table 7.

[b] See Table 7.

Table 8. Energy consumption inventory for the process of bioethanol production through SSF, using the Talf1 inulinases and the *S. cerevisiae* CCMI 885 as the ethanologenic yeast.

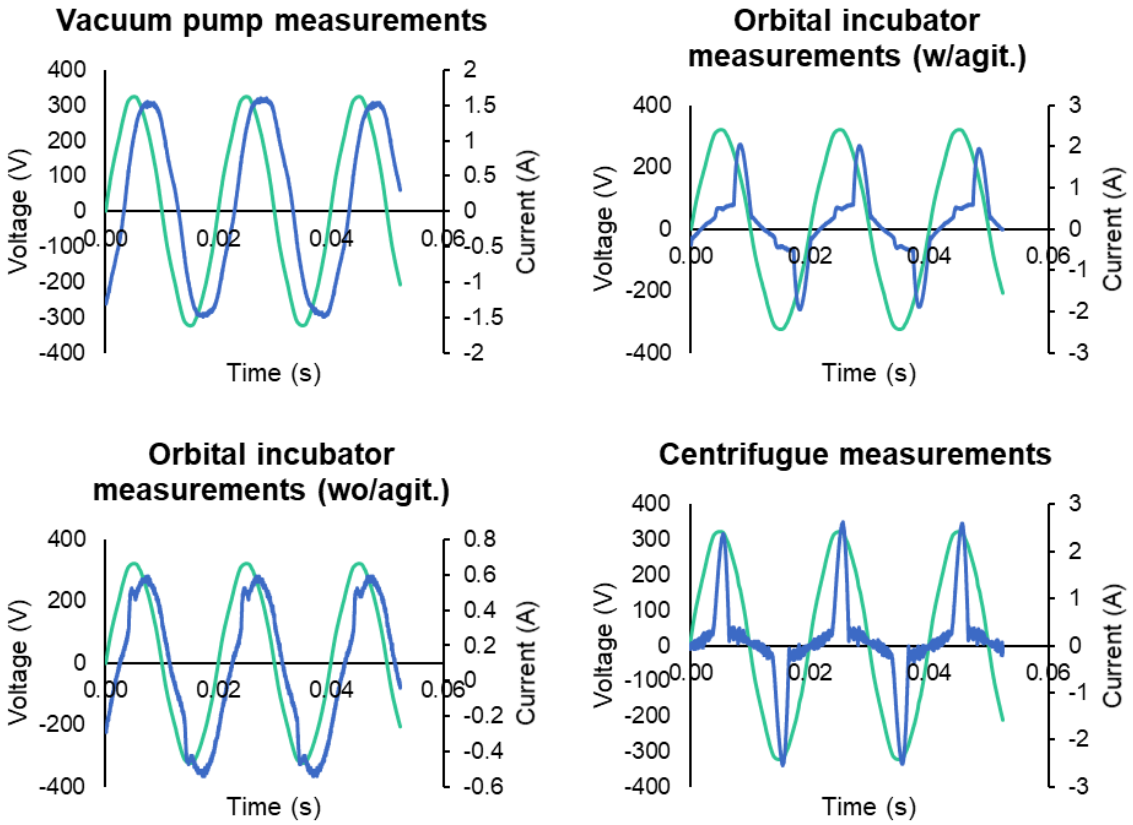

Figure 11. Graphical representation of voltage and current measurements for different equipment, acquired by a portable oscilloscope. (Green: voltage; blue: current)

The comparison of these real measurements with the corresponding estimations based on equipment maximum power and working hours, the usually applied method, point out to remarkable differences in the final energetic value calculated, as depicted in Table 9. Indeed, experimental energy measurements are highly recommended for accurate data since maximum power of the equipment and working hours are not enough information and can lead to misleading results ($E_{expected}/E_{measured}$ up to a factor of 10,000) and consequently to lower energy efficiency values due to overestimated energy consumptions.

Preparation step/fermentation process	Measured energy (MJ)[a]	Expected energy (MJ)[b]	$E_{expected}/E_{measured}$
CBP incubation without agitation	4.42×10^{-4}	4.05×10^{0}	9163
SSF incubation with agitation	9.01×10^{-6}	5.40×10^{-2}	5993
SSF centrifugation	2.70×10^{-8}	2.70×10^{-4}	10000
CBP	6.05×10^{0}	1.02×10^{1}	2
SSF	6.38×10^{0}	10.6×10^{1}	2

[a] Measured energy = $E_{measured}$, energy measured using a portable oscilloscope.
[b] Expected energy = $E_{expected}$ = maximum power x working hours.

Table 9. Energy consumption by equipment used at laboratory-scale assays: expected values (estimates based on calculations using equipment's maximum power and working hours data) *versus* measured values (based on real-time oscilloscope measurements).

However, in this particular work, the errors would be constant for the overall energetic calculations within both bioprocesses, not affecting significantly the final net difference between the total energetic values spent by each process.

Based on the overall energy consumption inventory for each studied process (Table 7 and Table 8), the bioprocess with higher energy efficiency can be selected. For the CBP, the energy required to produce 1 L of ethanol (6.05 MJ per 9.75 g ethanol ≡ 488 M//L_{EtOH}) is lower than for the SFF strategy (6.38 MJ per 9.42 g ethanol ≡ 536 MJ.L^{-1}_{EtOH}), so the most efficient ethanol bioprocess in terms of energy consumption is the CBP. This strategy was the one that also achieved higher ethanol yield (≈0.51 g/g) and maximum productivity (3.62 g.L^{-1}.h^{-1}, Table 6). Therefore, CBP was selected for further industrial scale-up scenario evaluation.

3.3.1.2. CBP SCALE-UP SCENARIO

Scale-up to production levels of 10 ML ethanol per year, would naturally imply a high area of land-use and agricultural machinery, e.g. tractors, consuming diesel fuel. Taking potato agriculture as an example, from Ecoinvent 3.0 database, this would mean a diesel consumption of 27 L/ha, which represents direct CO_2eq emissions of 71 kg/ha.

Despite the difference that could be encountered between the flask ethanol production rate and that from a large bioreactor, with an impeller (agitator blades), in this study it is assumed that the rate is kept. However, the impeller rotational speed is a crucial element that will greatly influence the overall energy consumption in the industrial scale-up fermentation process. In fact, the rotational speed may be optimized for maximum ethanol production. Considering three possible rotational speeds, namely 200 rpm, 350 rpm and 500 rpm, and a range of production efficiencies of 0.0048 to 0.0014 L_{EtOH}/LWV/h (LWV = lab-scale working volume), or 1.08 to 3.62 g.L^{-1}.h^{-1}, Figure 12 was displayed for the evaluation of the different electricity requirements towards the best CBP from JAJ to bioethanol scale-up scenario. Herein, three scenarios (Sc) are considered: Sc A) using the lab-scale average productivity (1.08 g.L^{-1}.h^{-1}) to obtain an annual production of 10 ML bioethanol; Sc B) using the lab-scale maximum productivity (3.62 g.L^{-1}.h^{-1}) without change in the bioreactors (same size/number) but producing more ethanol per year (≈33 ML), accounting for enough biomass available; and Sc C) using the lab-scale maximum productivity rate (3.62 g.L^{-1}.h^{-1}) and resized bioreactors [e.g. lower tank reactors (<Dt and/or <Ht and consequently lower Da) or decreasing the number of reactors within the industrial plant] for an annual production of 10 ML ethanol. From these industrial scenarios, Sc C is the most economical in terms of net energy consumption, independently of the rotational speed considered (Figure 12). In fact, the energy requirements [calculations based on Equation 9 and Equation 10] are dependent on rotational speed [Equation 8] and diameter of the impellers [Equation 7and Equation 8], as well as on ethanol

productivity ($g.L^{-1}.h^{-1}$). So, using industrial bioreactors as that depicted in Figure 5, if the actual ethanol production increases (≈33 ML EtOH, Sc B) then to reach the stipulated annual output (10 ML) the size/number of the bioreactors can be reduced, implying net energy savings associated to mechanical rotation (Sc C: 0.36 kWh/L_{EtOH} = 1.30 $MJ.L^{-1}_{EtOH}$, Figure 12). However, in terms of industrial plant process efficiency, the production of more ethanol/year (>3-fold more) without any "resizing" of the bioreactors has advantages, if the necessary amount of biomass is assured. In this context, Sc B using 200 rpm as the impeller rotational speed (Figure 12) seems to be the most adequate industrial scenario, although accounting for net energy consumption of 0.81 kWh/L_{EtOH} (2.92 $MJ.L^{-1}_{EtOH}$). Moreover, for each scenario, using the power regression model (R^2 = 1) fitted to the energy data points (see equations Figure 12), it is possible to make further extrapolations for the energy requirements using lower impeller rotational speeds (e.g. 100 rpm), but a good homogenization must be assured to guarantee the achievement of the ethanol production required in industrial plant.

Thus, accounting an industrial scale-up process of the lab-scale CBP from JAT to ethanol, considering scenario B (3.62 $g.L^{-1}.h^{-1}$ as maximal ethanol productivity; no resizing (Dt = Ht = 7.2 m); 200 to 500 rpm - Figure 12). Figure 13 shows the overall direct energy consumption ($MJ.L^{-1}_{EtOH}$), discriminating the energy associated to different steps of the process (cultivation, juice extraction, fermentation and distillation steps). From these results, once again, it is clear the great influence of the impeller rotational speed on electrical energy consumption, highlighting the 200 rpm as the most adequate for a more energy-efficient fermentation. So, for a scale-up scenario considering a bioreactor with an impeller rotational speed of 200 rpm the distillation may be the most energy-consuming step.

Distillation needs heat while juice extraction and fermentation need electricity. However, the electricity and heat demands could be covered by local electricity production, in similarity to the sugarcane industry where the lignin fraction of the feedstock is used to provide electricity and heat needed by the facility. Indeed, the lignin from JA stalks/foliage/tuber peels can be used for electricity and heating power, considering whole JA as a platform for biorefinery. But, to avoid too much beneficial assumptions for the side of JAT this eventual hypothesis of self-energy consumption will be deterred from any CBP scale-up scenario.

Besides the actual energy needs for an industrial scenario considering an ethanol production rate of 3.62 $g.L^{-1}.h^{-1}$ and an impeller rotational speed of 200 rpm (Sc B at 200 rpm; Figure 12 and Figure 13), the respective direct and indirect CO_2eq emissions are depicted in Figure 14. The goal of the six sub-scenarios (Sc 1 to Sc 6) categorized in Figure 14 is to observe the effect of LUC, N-fertilizer, agriculture machinery, direct electricity consumption and direct natural gas consumption (for the distillation heat generation), and biogenic emissions for the overall CO_2eq emissions. Emissions ranging from about 679 to 1945 g CO_2eq/L_{EtOH} may be observed.

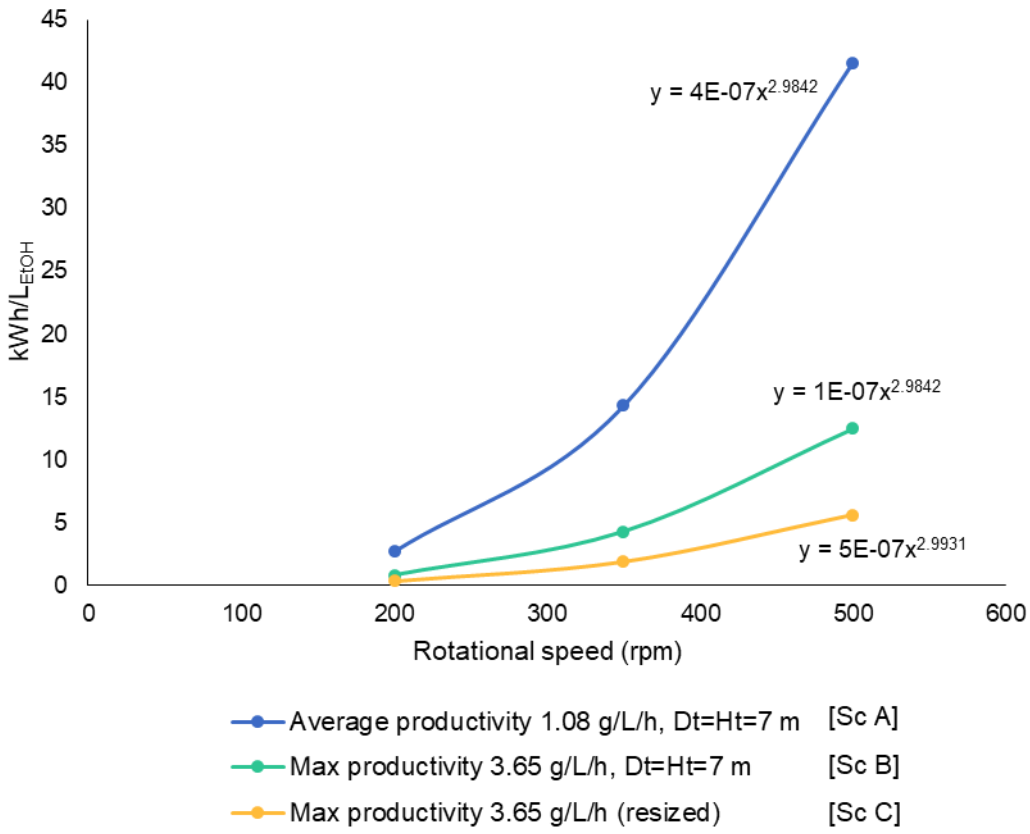

Figure 12. Energy requirements for scale-up from flask to industrial bioreactors (DT = Ht ≈7 m). Annual energy demand versus working speed (200 to 500 rpm), for three industrial scenarios (Sc A, Sc B, and Sc C), considering an ethanol productivity of 1.08 or 3.62 g.L^{-1}.h^{-1}. (Max: maximum)

Figure 13. Energy consumption per liter of produced ethanol for different impeller rotational speeds (200, 350 and 500 rpm), considering the various CBP steps within JAT to bioethanol industrial process outlined accounting for 3.62 g.L^{-1}.h^{-1} as ethanol productivity (Sc B in Figure 12).

Biogenic emissions and N-fertilizer associated emissions have a quite high effect. The N-fertilizer effect could be observed by comparing Sc 1 and Sc 2. With no-LUC, the N-fertilizer in a concentration of 150 kg/ha added ≈276 g CO_2eq/L_{EtOH}. This value contrast with that observed prior in Figure 8 when LUC was accounted in the emissions analysis, where the N-fertilizer had a negligible contribution to the overall emissions. Similarly, from Figure 14, the effect of the biogenic emissions could be observed by comparing Sc 4 and Sc 1 (+750 g CO_2eq/L_{EtOH}), and the LUC effect may be observed by comparing Sc 5 and Sc 4 (+133 g CO_2eq/L_{EtOH}), demonstrating their significant contribution for the overall direct emissions.

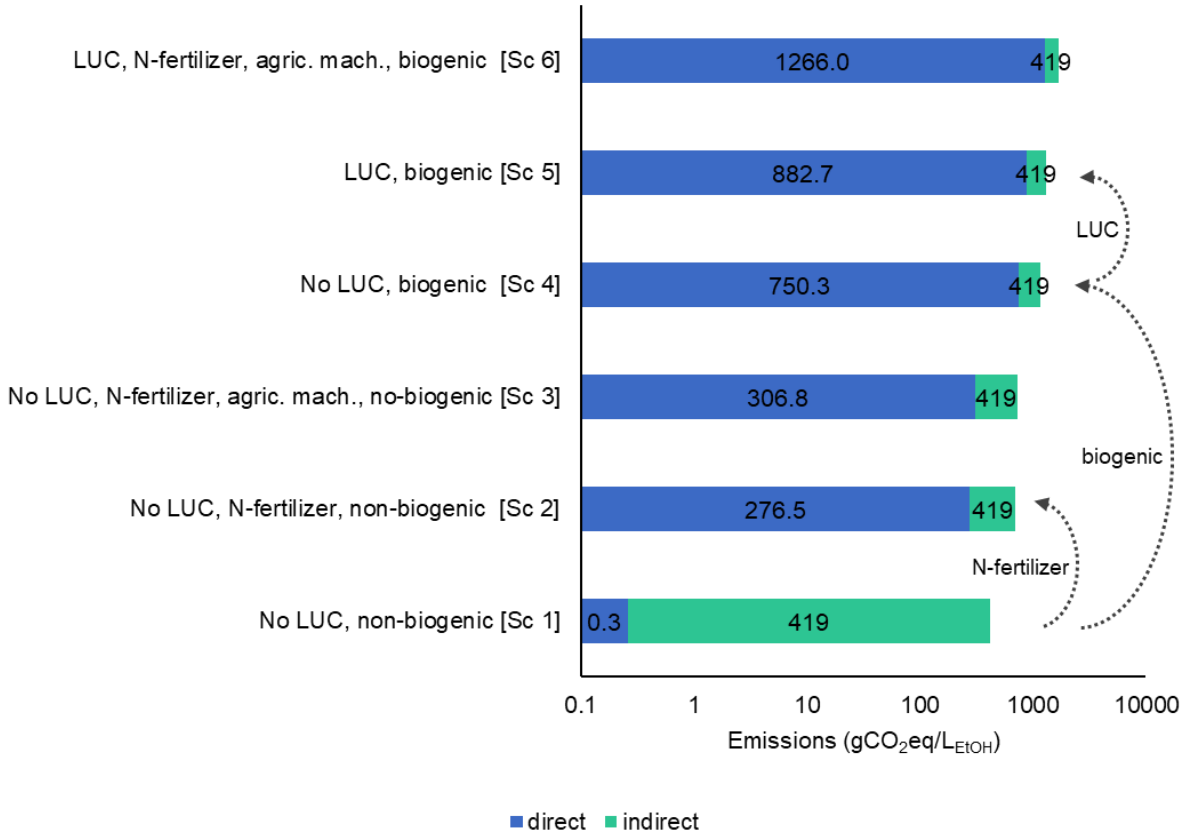

Figure 14. CO2eq emissions per liter of produced ethanol, considering six different sub-scenarios (Sc 1 to Sc 6) for the industrial process with an ethanol production rate of 3.62 $g.L^{-1}.h^{-1}$ and a bioreactor rotational speed of 200 rpm. The dotted arrows highlight the effect of the factors: N-fertilizer addition; biogenic emissions; and LUC, respectively, on overall carbon footprint. (agri. mach.: agriculture machinery; N-fertilizer: 150 kg/ha)

The RED directive disregards the biogenic emissions, but in this study, it is proven that they could be substantial (750 g CO_2eq/L_{EtOH}). In fact, the biogenic emissions (from fermentation) could increase 3.88-fold the direct carbon emissions (from 260 to 1010 g CO_2eq/L_{EtOH}), corresponding to an increase of more than 2-fold of the total emissions (679 to 1429 g CO_2eq/L_{EtOH}), and have a much higher effect than LUC. In this case, LUC adds only 13% to the direct emissions (1010 to 1143 g CO_2eq/L_{EtOH}), which corresponds to an increase of 9% in the overall emissions (Figure 14, Sc 4 vs Sc 5).

When comparing systems, the same boundaries, criteria for allocation or system expansion should be followed, as well as the same criteria for the accounting of biogenic CO_2 emissions and LUC emissions. In this work, it was intended to compare a JAT to ethanol industrial system with crude oil to gasoline system and with other feedstock to ethanol systems. For an accurate comparison of the industrial simulated scenario with other systems to produce the same amount of energy existing in 1 L of ethanol, no co-products credits, no LUC and no biogenic emissions were considered. This caution was to avoid the benefit of one system over the other.

Therefore, the CO_2eq emissions and the energy consumed for ethanol from JAT, through CBP by *Z. bailii* strain Talf1, were compared to those for other industrial equivalent products, namely: conventional gasoline (COG1), sugarbeet ethanol (SBET1a) and sugarcane ethanol (SCET1),[246] using 1 L of ethanol equivalent produced as FU (Figure 15). As can be seen in Figure 15, the COG1 considering the crude oil extraction and refining process is more energy-intensive and carbon-intensive than the ethanol from JAT industrial scenario using an impeller rotational speed of 200 rpm. SBET1a and SCET1 products (JRC report[246]) seem to have lower CO_2 emissions than COG1 and JAT ethanol at 350 rpm, but higher energy consumption. Nevertheless, if the 200 rpm impeller rotational speed industrial scenario is considered, the JAT to ethanol CBP may improve the energy efficiency and the overall carbon emissions to less than 9 $MJ.L^{-1}_{EtOH}$ and 679 g CO_2/L_{EtOH}, respectively. These values are significantly lower than those reported for the ethanol from the commonly used conventional feedstocks (SBET1a and SCET1, Figure 15), especially the overall carbon footprint value. Productivity data or impeller rotational speed was not found in the JRC report,[246] therefore in the data for SBET1a and SCET1 different microbial productivities in the fermentations as well as rotational speeds may have been considered, which can cause the energy consumption/carbon emissions differences stated.

So, using a fair comparative analysis based on suitable comparators (i.e. considering the same boundaries and criteria (for allocation, co-products, etc.) as those used for the gasoline reference system), the overall energy and CO_2eq emissions values depicted in Figure 15 for JAT ethanol at 200 rpm seem very promising in comparison with the corresponding 100% values for conventional gasoline (15 $MJ.L^{-1}_{EtOH}$eq and 1154 g CO_2/L_{EtOH}eq).

However, the current EU conjecture reflects the “marginal substitution approach”, described in JRC reports,[202,246] and explained in the methods section (see section 3.2.). So, in this context, if the EU expected goal until 2020 horizon is to obtain only 20% of ethanol-blended gasoline the energy/emissions net values to be taken for comparison should be 20% of the depicted ones, i.e. 3 $MJ.L^{-1}_{EtOH}$eq and 231 g CO_2eq/L_{EtOH}eq (values corresponding to 20% of reduction on gasoline production) instead 15 $MJ.L^{-1}_{EtOH}$eq and 1154 g CO_2/L_{EtOH}eq (Figure 15). This approach, of course, would turn gasoline the best option overall any biofuel system

depicted. Hence, for an accurate comparison it is crucial the development of adequate comparators. Otherwise, no biofuel system will ever be competitive over conventional gasoline system. In addition to the obvious influence of the use of unsuitable comparators, calculations based on estimates rather than accurate measures can also have a great influence towards industrial scenario extrapolations. Indeed, as demonstrated for laboratory-scale assays, energy calculations ($E_{measured}$ *versus* $E_{expected}$, Table 9), estimations are usually higher than the actual values, and may further contribute to erroneous comparisons between different systems.

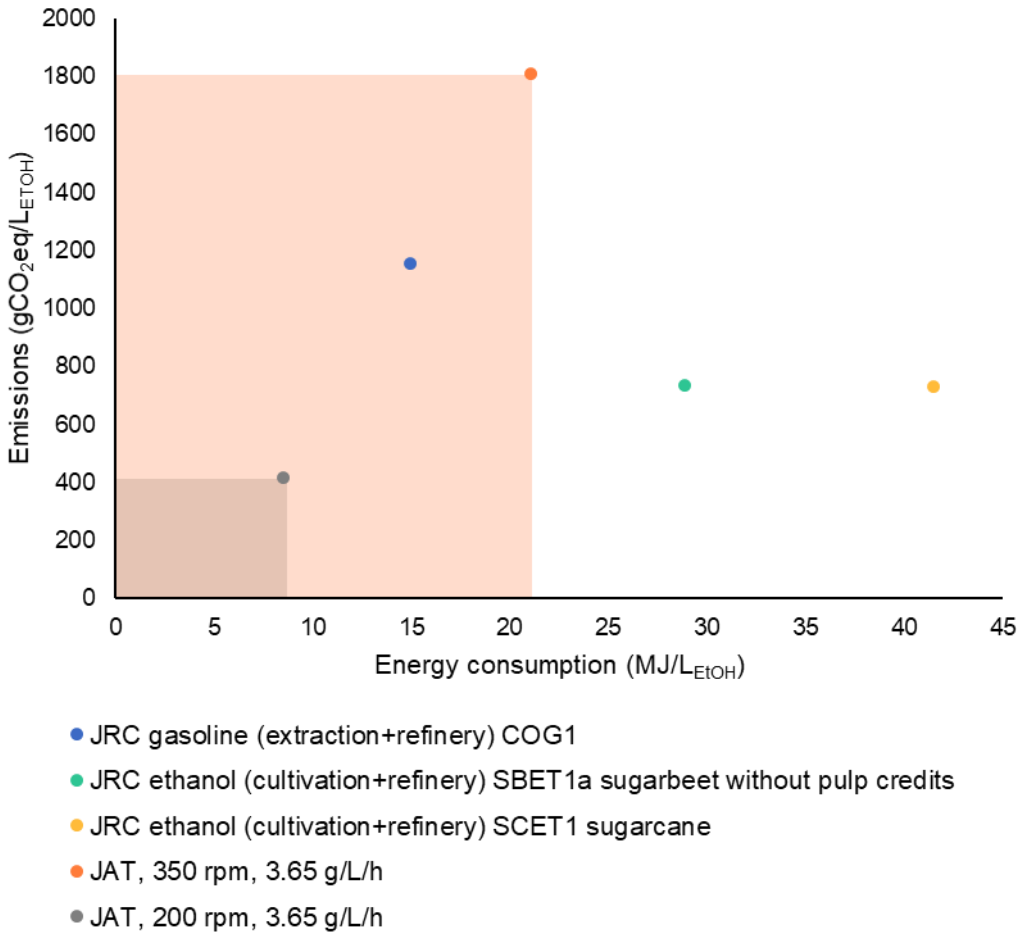

Figure 15. CO2eq emissions per liter of produced ethanol equivalent versus energy consumption per liter of produced ethanol equivalent, for different scenarios. No LUC, no biogenic, no co-product credits were considered (Sc 1 in Figure 14 for JAT ethanol, 200 rpm).

Hence, the comparison of different industrial fuels, gasoline and ethanol, in terms of overall CO_2eq emissions and energy consumption (100% process), highlights the chance for JA feedstock, considering an upscale scenario with bioreactors using a rotational speed of only 200 rpm for an ethanol productivity of 3.62 $g.L^{-1}.h^{-1}$ (Figure 15). Indeed, JA has been recognized as a promising feedstock for bio-economy development due to its numerous

advantages over the conventional food crops (sugarcane, sugar beet, maize, wheat), such as: low cultivation input, high crop yield, wide adaptation to climatic and soil conditions, high resistance to pests and plant diseases, and minimal to zero irrigation/fertilizer requirements. Moreover, this emerging energy crop can itself be exploited as a platform for biorefinery, since besides biofuels (bioethanol) a variety of other bioproducts can be obtained from it, such as functional food (inulin, fructose); bioactive compounds (antifungal, antioxidant and/or anticancer ingredients); chemicals (e.g. lactic acid; furfural); bio-fertilizers; etc.[234,239,252] In fact, the multiple applications of JA feedstock in association with a low LUC, if the cultivation in non-arable soils with no irrigation water/fertilizers is considered, turns this crop into an attractive sustainable energy crop for further research worldwide.

Furthermore, an interesting indicator of geospatial land-use is how much land we need to produce 1 MJ of energy. It is quite interesting to observe the trade-off between culture changes in productivity with the use of fertilizers and the increment in CO_2eq emissions due to N_2O. In the case of JA feedstock, considering a scale-up scenario towards 10 ML production capacity of bioethanol/ annually and no fertilizers about 4237 ha of land requirements are needed. But, with the use of 100 kg N/ha, the land requirements could be reduced almost by half without much influence on carbon footprint due to N_2O emissions. Moreover, if we consider the bioreactors' land occupation (7.2 m diameter, 4 bioreactors (see Table 5) → $4 \times \pi \times (7.2/2)^2$ < 1 ha) it is much less than the crop geospatial land requirements of 2119 ha. If one looks to the area occupied by a crude oil refinery and its energy output, ≈900 million MJ/ha are obtained; but in this work's industrial case scenario it gets 4 orders of magnitude less (10,000,000 L x 0.789 $kg.L^{-1}$ x 27 MJ/kg/2119 ha). This means that for the same energy output per ha, the JA productivity and/or the ethanol yield must raise a lot. However, it is important to state that in this study a forest soil (nonarable land) was used and no water was added during the entire cultivation period, which means that the only water present was the one from rainfalls.

However, before scale-up, optimization assays are still necessary to get the bioethanol production by *Z. bailii* strain Talf1 at maximum yields (up to 155 $g.L^{-1}$ of ethanol, which implies about 1 L of ethanol per 1.54 kg of fermentable JAT inulin or 648 mL_{EtOH}/kg_{JAT} inulin; i.e. ≈136 L_{EtOH}/t_{JAT} biomass considering that only up to 21% of fresh JAT biomass is inulin).[234] Then, further use of whole JA plant (stalks, foliage, flowers, and tubers),[221,239,252] within a biorefinery concept, in the optimized bioprocess towards a cost-effective bioethanol fermentation should also be outlined. In fact, to design an energy biorefinery model from JA aiming to get a low-carbon solution, besides bioethanol and bio-based high added-value chemicals, the potential of using the crop residues (e.g. JA stalks; JA fermentation byproducts), as bio-fertilizers towards soil sustainability or animal feed/forage, should be evaluated towards a near zero-waste bioenergy system.

3.4. CONCLUSIONS

In this study, the JA was cultivated without neither irrigation nor fertilizers in a native forest land at the north of Portugal climate conditions. In these poor conditions, a productivity of 40 t JAton.ha^{-1} was achieved, corresponding to a juice yield of about 28,000 L/ha (150 mL/0.21 kg JAT).

Regarding the JAJ fermentation process directly to bioethanol, two routes based on *Z. bailii* strain Talf1/Talf1 inulinases were considered: CBP versus SSF. The difference in these two strategies is the use of a sole biocatalyst (CBP strain) or a combination of biocatalysts (SSF: inulinases + ethanologenic strain) for inulin hydrolysis and further sugar fermentation to ethanol. The best yield and less energy-consuming process was CBP with a maximum observed productivity of 3.62 g.L^{-1}.h^{-1}. In addition, this work highlighted the importance of direct energy consumption measurements as opposed to estimations based on equipment specifications and working hours, since misleading information can be obtained ($E_{expected}/E_{measured}$ up to a factor of 10,000).

Laboratory to industrial extrapolation is a risky approach to make but permits to outline a scenario that assumes the same hourly lab-scale productivity and a possible range of fermenter impeller rotational speeds. For a productivity of 3.62 g.L^{-1}.h^{-1}, an ethanol yield of about 59 $L_{EtOH}/t_{biomass}$ can be attained from JAT. So, considering this maximal productivity for the scale-up, the outlined CBP industrial scenario using a rotational speed of 200 rpm was the most challenge approach towards ethanol production, in terms of overall energy consumption and carbon footprint. Indeed, for this industrial process, overall carbon emissions ranging from 679 to 1945 g CO_2eq/L_{EtOH} could be observed when the influence of different factors (e.g. LUC, biogenic, N-fertilizer, agriculture machinery) was considered. These results indicated that biogenic emissions (750 g CO_2eq/L_{EtOH}) are much higher than the emissions due to LUC (133 g CO_2eq/L_{EtOH}) or N-fertilizer effect (276 g CO_2eq/L_{EtOH}).

In addition, it is quite interesting to observe the trade-off between culture changes in productivity with the use of fertilizers and the increment in CO_2eq emissions due to N_2O. In the case of JA, considering a scale-up scenario towards 10 ML production capacity of bioethanol/annually and no fertilizers about 4237 ha of land requirements are needed. But, with the use of 100 kg N/ha the land requirements could be reduced by about 50% without much influence on carbon footprint due to N_2O emissions.

Moreover, considering an industrial scenario with no LUC, no biogenic and no co-products credits, the comparison of the overall energy consumption and CO_2eq emissions (100% process) from JAT ethanol CBP (9 MJ.L$^{-1}_{EtOH}$; 679 g CO_2/L_{EtOH}) with sugarcane/sugarbeet ethanol process (29/42 MJ.L$^{-1}_{EtOH}$ for SBET/SCET; 731/735 g CO_2/ L_{EtOH} for SCET/SBET, respectively) and with gasoline refinery (15 MJ.L$^{-1}_{EtOH}$eq; 1154g CO_2/L_{EtOH}eq),

highlights the JA as a promising sustainable alternative feedstock to be a focus of research for ethanol fuel for gasoline blends (E20 to E100).

SECTION 4

GLOBAL WARMING POTENTIAL OF BIOMASS-TO-ETHANOL: REVIEW AND SENSITIVITY ANALYSIS THROUGH A CASE STUDY

4. GLOBAL WARMING POTENTIAL OF BIOMASS-TO-ETHANOL: REVIEW AND SENSITIVITY ANALYSIS THROUGH A CASE STUDY

The work in this section is included in the published manuscript:

Rui Pacheco and Carla Silva, 2019, *Global Warming Potential of biomass-to-ethanol: review and sensitivity analysis through a case study*. Energies. DOI: 10.3390/en12132535

The publication comes as a follow-up to the work presented in the previous section. The disparity of results and lack of information on global warming effects of fermentation products and industrial scenarios led to a need to assess the influence of presumptions when considering the impacts of bioethanol production. The author, Rui Pacheco, contributed to this work by assembly of inventory, impact assessment (Global Warming Potential), and test on time horizon, assessment report and countries' electricity mix influence on results. Also by assessing differences between conventional and dynamic Life Cycle Assessment, and simulating scenarios considering genetic modification of yeast strains.

4.1. INTRODUCTION

Energy-related CO_2 emissions, mostly due to fuel burning, were of 29 gigatons (Gt) in 2007, 34 Gt in 2011, and are expected to increase to 40 Gt until 2030,[253] contributing to the potential increase of global average temperatures of about 6 ºC, even including the global production of 120 million tons of oil equivalent of biofuels.[254,255] Recent updates on the RED increase the target of consumption of renewable energy by 2030 from 27 to 32%, with a limitation of feedstocks such as used cooking oil and animal fats but setting a minimum target for advanced biofuels use of 3.5% by 2030.[256]

Ethanol (EtOH), particularly ethanol obtained from biotechnological processes, presents itself as an interesting alternative, replacing part of the oil-derived liquid fuels.[255] In fact, it has already been stated that the potential market for bioethanol is estimated to be of about 45 exajoules by the year 2050.[257] With almost no technical changes on vehicles' engines, percentages of 5-10% ethanol-gasoline blends can be used.[255,258] Ethanol has a high octane number and a reduced tendency to create knocking in spark-ignition engines. It also allows for low-temperature combustion, due to its oxygen content, contributing to the reduction of carbon monoxide (CO) and nitrous oxides (NOx) emissions.[258] In Europe, ethanol is blended with gasoline fuel in 5 or 10% volume (E5 or E10). In the U.N. the blend is 15% in volume (E15), and there are also pumps that provide gasoline fuel blended with 85% ethanol (E85) for flex-fuel vehicles. In Brazil, the conventional gasoline has 27% ethanol blended and there are

pumps that offer 100% ethanol (E100) due to the growing market of flex-fuel vehicles. Table 10 shows the main differences between gasoline and ethanol as its potential substitute.

	Gasoline	Ethanol
Feedstock	crude oil	corn, sugarcane, vegetable waste
Gasoline equivalent	100%	73 to 83%
Density	0.725 $kg.L^{-1}$	0.785 $kg.L^{-1}$
Energy content (LHV)	≈31.2 to 32.3 $MJ.L^{-1}$	≈21.2 $MJ.L^{-1}$
Energy content (HHV)	≈33.4 to 34.6 $MJ.L^{-1}$	≈23.4 $MJ.L^{-1}$
Emissions	2.49 $kgCO_2/L$	1.51 $kgCO_2/L$
	77.82 gCO_2/MJ	71.68 gCO_2/MJ

Table 10. Gasoline versus ethanol.[191,259] (LHV: lower heating value; HHV: higher heating value)

Brazil and the US produce around 89% of global bioethanol, mainly from sugarcane and corn respectively, while Europe and China use cereals.[255] Although promising as a substitute for gasoline, ethanol using maize or sugarcane as raw material constitutes about 40 to 70% of the production cost,[260,261] and the production of 1G ethanol directly competes with land use for food agriculture.[255] For large-scale production of fuel ethanol, the use of cheaper and more abundant substrates is desirable. Lignocellulosic biomass (such as crop residues, hardwood, softwood, cellulose wastes, herbaceous biomass, and municipal solid wastes, being 2G types of biomass) is considered an attractive feedstock, due to its availability, low cost and for reducing competition with food (but not necessarily with feed).[262,263] However, the current production cost for 2G ethanol production from lignocellulose is still too high, which is one of the major reasons why this kind of production has not yet made its breakthrough.[264]

First-generation ethanol originated from bioenergy crops and lignocellulosic biomass can potentially become a renewable fuel in place of transportation fuels, such as gasoline.[261] Used to increase the oxygen content of gasoline, allowing for better oxidation of hydrocarbons and a reduction of the volume of emissions to the atmosphere, primarily of aromatic compounds and CO. Additionally, CO_2 emissions from fuel burn are in part compensated by CO_2 absorption from the crops from which the ethanol is produced.[265]

Biomass production as feedstock for biofuels is expected to increase greatly, with biofuels contributing 10 to 20% of the primary energy supply by 2050. However, this prediction assumes that there will not be water shortage or food agriculture yields will increase (partly due to genetically modified crops).[257] Also, the supply of bioethanol can be constricted by the availability (or lack of) of arable land, due to competition with food production, which can drive increases in the price of ethanol and agricultural foods.[257]

Lignocellulosic materials have been suggested as feedstock of interest to substitute bioenergy crops, but fermentation by common yeast needs pre-treatments that often lead to the release of inhibitors and sugars that are not easily processed by the microorganisms, often resulting in low ethanol yields.[64,266] JA has the advantages of 2G feedstock (non-competition

with food/energy crops) without their difficulty to be fermented and produce ethanol in economically viable amount.

To address sustainability questions raised by the choice of alternatives for full fossil-based fuels, LCA comes as a useful tool. LCA studies compile and evaluate the material and energy flows, and potential environmental impact of these along the life cycle of a product, ideally from the extraction of the needed raw materials, through the production, use, and disposal of the product and possible auxiliary materials and equipment. The assessment considers all attributes of the natural environment, human health, and resources.[267–270]

Depending on the broadness of the study and selected boundaries, LCA can cover aspects such as global warming potential, fossil resource depletion, acidification, and toxicity aspects, among others, making it an interesting tool for quantification of environmental impacts of a given product system.[271]

LCA studies on bioethanol production are prolific and present a lot of results with different focuses, including GHG emissions (measured in unit of mass of CO_2 equivalent), energy consumption, land use, water footprint, economic viability. Multiple variables are shown for different perspectives. Some studies focus on the production of ethanol,[261] from a global warming, land use and energy balance perspective, using sugarcane and corn, and bagasse as feedstock, respectively. Others include water input or focus on GHG emissions using switchgrass and corn stover as feedstock.[272,273] In addition, some present results on the cultivation and processing of maize, sugarbeet, sugarcane, and wheat for the production of bioethanol.[21] The study presented in Section 3 compares, in an industrial scenario, ethanol produced by JA with gasoline, including the influence of direct LUC, biogenic emissions from fermentation and crop CO_2 uptake, and fermenter agitation speed. JA is a perennial tuberous plant that is tolerant to drought, high concentrations of salts and is highly resistant to frost and plant diseases. It can grow in marginal lands and does not require fertilization of the soil or competes for arable lands with food crops.[212,221–224] However, as in the case of the reviewed studies, the emissions are assumed to occur in the first year of the factory operation (conventional LCA (cLCA) and the reality is that emissions are produced throughout the factory lifetime (dynamic LCA (dLCA)).

Table 11 presents a general review on LCA studies for bioethanol yield and CO_2eq emissions, considering different feedstock and countries. Different studies include different processes on the production chain with different assumptions which, alongside countries' specific electricity mix, contribute to diverse values that are often difficult to compare with each other.

Study	Country	Feedstock	Genera-tion	CO_2eq emissions ($kg.L^{-1}_{EtOH}$)	Ethanol yield ($L_{EtOH}/kg_{feedstock}$)	Included processes
272	India (only CO_2)	sugarcane bagasse (e)	2G	3.88	0.30	bagasse transportation; ethanol production; reformulated gasoline use (includes biogenic CO_2)
		sugarcane bagasse (da)	2G	5.55	0.24	
273	Canada	switchgrass	2G	0.49	0.33	biomass production; ethanol production; ethanol transportation and distribution; use
		corn stover	2G	0.33	0.34	
261	Colombia	corn	1G	n.a.	0.45	pre-treatment; hydrolysis; fermentation; separation; dehydration; wastewater treatment
		sugarcane	1G	n.a.	0.08	
274	Brazil	sugarcane (2002)	1G	0.39 (h) 0.40 (a)	0.09	sugarcane production; processing; etanol production
		sugarcane (2005/6)	1G	0.42 (h) 0.44 (a)	0.09	
		sugarcane (2020 scenario)	1G	0.33 (h) 0.35 (a)	0.09	
18	Brazil	sugarcane	1G	0.45	0.07	sugarcane production; harvesting; transportation; processing; ethanol production; distribution
21	France	sugarbeet	1G	0.87	0.075	sugarbeet production;transportation; ethanol production; distribution; ethanol disposal
275	India	sugarcane	1G	2.45	0.25	sugarcane production; sugarcane processing to sugar; sugarcane processing to ethanol
202	Brazil	sugarcane	1G	0.35	n.a.	sugarcane production + local transport; ethanol production (without surplus energy credits)
276	Europe	sugarbeet	1G	0.8	0.11	cultivation plus ethanol production, energy allocation
		wheat		0.52-1.45	0.37	
		corn		0.88	0.38	
		sugarcane		0.32	0.09	
		sugarbeet		1.12	0.11	cultivation plus ethanol production, no allocation
		wheat		0.85-2.42	0.37	
		corn		1.61	0.38	
204	US	corn year 2000	1G	1.29	n.a.	cultivation plus ethanol production
		corn year 2015		1.04		
190	Portugal	Jerusalem artichoke	1G	0.42	0.057	cultivation; ethanol production (juice extraction; processing; fermentation; distillation)
		JA with biogenic CO_2		1.43		n.a.
		JA with direct land use change		1.56		n.a.

Table 11. GHG emissions for the production of bioethanol and yield from different feedstock. (n.a.: not available; e: enzymatic process; da: dilute acid process; h: hydrous ethanol; a: anhydrous ethanol)

These reviewed studies also reflect different co-product allocation procedures and crediting. For example, the BIOGRACE database for Europe presents values with energy allocation for the inputs and outputs, and system expansion for crediting electricity production from co-products, with and without allocation. We observe a variation in results of 180% considering only crediting variations and up to 83% for allocation variations (deviation from the minimum). For the biogenic accounting influence, we get up to 240% deviation from the minimum.[190,276] For land use, the same deviation is up to 271%. According to the reviewed information, we can observe the high range of results due to different methodological approaches and geographical regions/electricity mixes: for the yield 0.07 to 0.45 $L_{EtOH}/kg_{feedstock}$ and for the GHG emissions 0.31 to 5.55 $kgCO_2/L_{EtOH}$.

This study aims to evaluate the differences in global warming potential (GWP) metric (CO_2eq) results if a dLCA is considered instead of cLCA; the influence of the factory geographical placement by means of different electricity mixes; the difference in considering updated IPCC (100-years time horizon) CO_2 equivalency factor for CH_4 and N_2O; the difference in considering a 20- instead of 100-year time horizon; and the influence of considering different impact category methods for the same GWP metric, e.g., TRACI 2005 2.1, IPCC GWP 20 years 1.01, ILCD 2011 Midpoint+ 1.07, IMPACT 2002+ 2.12, EDIP 2003 1.05, and CML 2001.

The case study is an industrial-scale scenario (cradle-to-gate) of bioethanol production using JA as feedstock,[190] which served as the basis for the sensitivity analysis. The results obtained from the different influences were analyzed and discussed, while also considering modifications in the final ethanol yield so we can argue what factors are the most influential. Finally, guidelines to include in future LCA studies are sketched.

4.2. Materials and Methods

In the brief literature review (Table 11), there were several identified geographical locations using different electricity mixes (e.g., Brazil, India, Colombia, Canada, France, and Portugal), different methodological choices (e.g., with or without LUC; with or without biogenic emissions; with or without allocation) that result in a huge range of CO_2 eq/L_{EtOH} values. The goal of this study is to investigate further, and evaluate the impact of different impact assessment methodologies, different IPCC assessment report CO_2 equivalency factors, different time horizon years, lifetime of the ethanol production factory and conventional *versus* dynamic approach in GWP estimates, to finally conclude which parameters are the most influential on the results.

The case study was described in the previous chapter (and published article). The non-biogenic, no LUC and no allocation scenario was used as a reference to observe the other issues on CO_2eq results.

Data from all the laboratory-scale processes (juice extraction, juice sterilization and fermentation) was gathered for the modelling of an industrial scenario, in a 20,000 L fermentation tank, agitated by paddles at 200 rpm., considering the maximum ethanol productivity of 3.62 $g.L^{-1}.h^{-1}$ (corresponding to a yield of 0.06 $L_{EtOH}/kg_{feedstock}$).

4.2.1. LIFE CYCLE ASSESSMENT

Functional Unit

The functional unit was defined as one liter of produced ethanol (1 $L_{EtOH} \approx L_{EtOH}$).

System Boundaries

Considering the most approachable and relevant steps or the process and scale of design, key stages of the ethanol production from JA were selected (Figure 16): land preparation, cultivation of JA tubers, processing of harvested products, fermentation for the production of ethanol, distillation and respective energy inputs and outputs.

Life Cycle Inventory

The Ecoinvent 3.3 (2016)[277] library was used for the LCA study. The Simapro software's library was used for the electricity mix of Portugal (Table 12) and values not directly measured or calculated by the authors. The CO_2eq intensity for this electricity mix is gCO_2eq/kWh.

Input	Value (kWh)
geothermal	0.008
hard coal	0.445
hydro	0.306
wind	0.241
compensation for grid losses	0.308
emissions (g CO_2/kWh)	295

Table 12. Electricity mix for Portugal (2016) for the production of 1 kWh.

Inventory data was considered for a scenario of a 20,000 L industrial fermenter (Table 13). Common industrial use of this kind of equipment was contemplated. Electricity consumption for an agitator paddle working at 200 rpm was considered. A CO_2 absorption of 1.390 $kgCO_2/kg_{JA\ tubers}$ was considered. The lack of irrigation for cultivation is highlighted, as JA can get its water inputs from common rainwater (as opposed to other ethanol feedstock).[190]

Process	Item	Value
land preparation	area	1,000 ha
	diesel	0.023 L
cultivation	water	0.000 L
	JA tubers yield	39,069.700 kg
processing	electricity	1207.000 kWh
	JA juice yield	27,906.930 kg
fermentation	electricity	7035.118 kWh
	EtOH yield	2299.050 L
distillation	natural gas	0.179 L

Table 13. Inventory for the considered processes of bioethanol production from JA.

Impact Assessment

Considering the scope of this work and the impact category to be analyzed, the following LCA methods were used to assess GWP, as they produced results in mass of CO_2eq for a given product assembly process: TRACI 2005 2.1, IPCC GWP 20 years 1.01, ILCD 2011 Midpoint+ 1.07, IMPACT 2002+ 2.12, EDIP 2003 1.05, and CML 2001 2.05 20 years, 100 years and 500 years.

Global warming is considered to be the warming that can be caused by increased emission of GHG from human activities. GWP is used to calculate the potency of GHG (CO_2, CH_4, N_2O) relative to 1 kg of CO_2 at time zero.[278]

No allocation or system expansion was performed.

Time Horizon Influence

The IPCC recommends the use of a 100-year time horizon to see the amount of CO_2 that has the same radiative force 100 years from time zero. However, 20 years or 500 years will also be used. For example, the impact assessment methodologies CML 2001 have these possible time horizons.

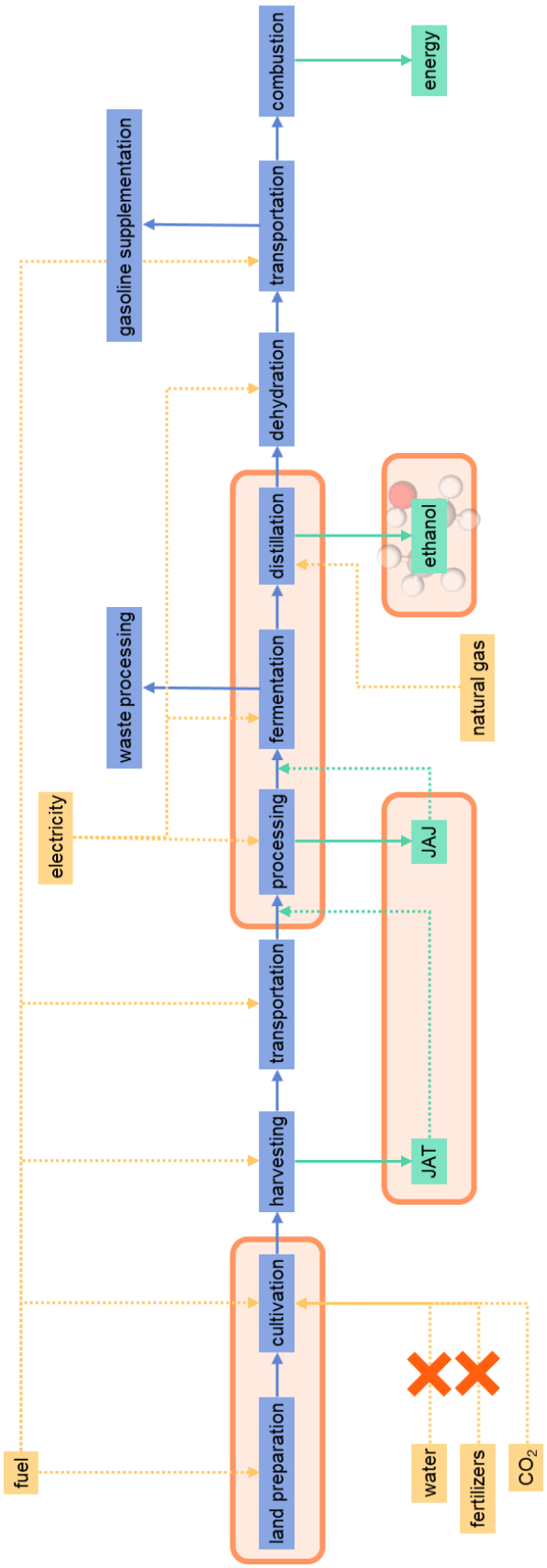

Figure 16. Process chain for the production of bioethanol from JA. Blue boxes: main processes; yellow boxes: inputs; green boxes: outputs. All processes were considered to emit CO2eq. System boundaries include the orange highlighted processes and respective inputs and outputs. (JAT: JA tubers; JAJ: JA juice)

Dynamic LCA *versus* Conventional LCA

In cLCA, as in the case of the reviewed studies, the emissions are assumed to occur in the first year of the factory operation (between 0 and 1 year, see Figure 17), and the reality is that emissions are produced throughout the factory lifetime (dLCA, constant pulse emissions throughout the lifetime).

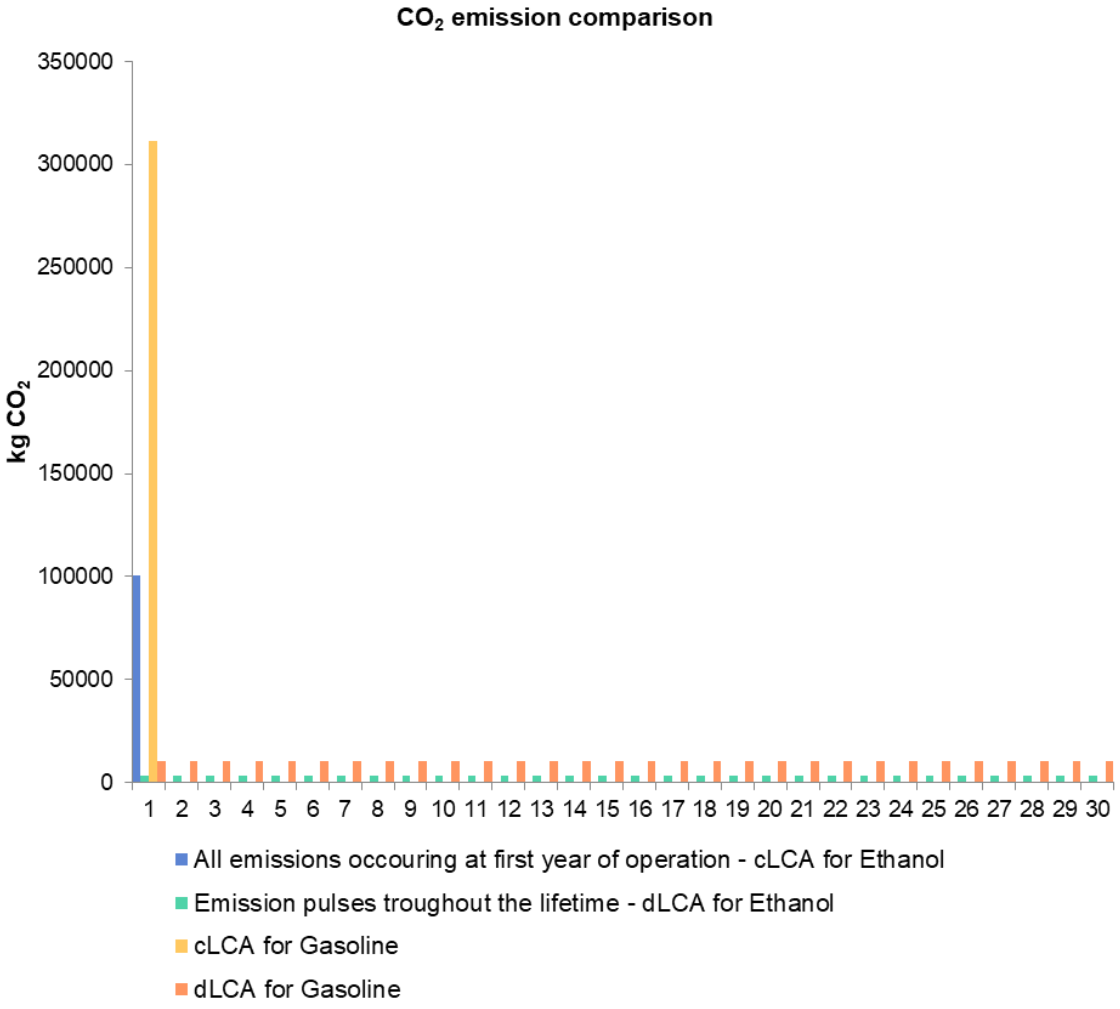

Figure 17. Example of the difference in conventional CO_2 emissions and actual emissions during a factory lifetime. Comparison between the presented case study for ethanol[190] and gasoline, without allocation, in a cradle-to-gate approach[202] for a 30-year operation lifetime.

Each emission pulse follows a lifetime decay due to natural mechanisms throughout time and, therefore, its global warming potential also changes throughout time.

The Bern carbon cycle was used to model the emission pulses decay:[279]

Equation 12.

$$C_{CO_2}(t) = a0 + \sum_{i=1}^{a} a_i e^{-\frac{t}{\tau i}}$$

where a0 = 0.217, a1 = 0.259, a2 = 0.338, a3 = 0.186, τ1 = 172.9, τ2 = 18.51, τ3 = 1.186.

For CH_4 and N_2O:

Equation 13.

$$C_{CO_2} \; or \; N_2O(t) = e^{-\frac{t}{\tau i}}$$

where $\tau CH_4 = 12$, $\tau N_2O = 114$.

The radiative forcing (RF_x) in $W.m^{-2}.kg^{-1}$ of emission is computed for an atmospheric background CO_2 of 389 ppm: 177×10^{-15} $W.m^{-2}.kg^{-1}$ CO_2; 1.1682×10^{-13} $W.m^{-2}.kg^{-1}$ CH_4 and 3.54×10^{-13} $W.m^{-2}.kg^{-1}$ N_2O.[280]

A pulse emission (E(t)) has an instantaneous radiative forcing RF given by:

Equation 14.

$$RF(t)\left[\frac{W}{m^2}\right] = E(t) \times RF_x$$

or, considering all emission pulses since time zero, the cumulative radiative forcing, or absolute global warming potential (AGWP) is as follows.

Equation 15.

$$AGWP(t)[W/m^2] = \int_{t0}^{t} RF_x(t)C_x(t)dt$$

where *x* stands for the emissions of CO_2, CH_4, and N_2O.

The AGWP divided by the AGWP of 1 kg CO_2 pulse at time zero stands for the GWP, in a certain time horizon (TH), in CO_2eq, is given by:

Equation 16.

$$GWP(TH) = \frac{\int_x \int_{t0}^{t} RF_x(t)C_x(t)dt}{\int_{t0}^{t} RF_{CO_2}.C_x(t)dt}$$

An open-source Excel spreadsheet with the above-described model from CIRAIG[281] was used for our calculations.

A scenario of a fermenter producing 10 000 L of bioethanol per year was considered, over a life time of 30 years or 50 years. For the fermenter lifetime, a constant emission per year as GHG emissions were: 3353.733 kg CO_2/year, 7.826 kg CH_4/year, and 0.113 kg N_2O/year.

4.3. RESULTS AND DISCUSSION

Table 14 shows the emissions of CO_2eq per liter of bioethanol produced by the fermentation process described above, as calculated by different LCA impact assessment methodologies, and compares them with the standard gasoline and ethanol production indicated by JRC.[202]

		Value (kg CO_2eq/L_{EtOH})							
	Method	T	IP	IL	IM	E	C20	C100	C500
	Time horizon	100	20	100	100	100	20	100	500
	Country	U.S.	n.a.	n.a.	Switzerland	Denmark	Netherlands		
Processes	**Ethanol from JA (this work)**								
	Land preparation and cultivation	0.0456	0.0469	0.0456	0.1390	0.1430	0.0466	0.0458	0.0452
	Processing	0.1130	0.1300	0.1130	0.1120	0.1160	0.1230	0.1130	0.1080
	Fermentation	0.2010	0.2310	0.2010	0.1190	0.2070	0.2190	0.2000	0.1920
	Distillation	$2.56x10^{-8}$	$4.90x10^{-8}$	$2.56x10^{-8}$	$1.88x10^{-8}$	$2.56x10^{-8}$	$4.01x10^{-8}$	$2.48x10^{-8}$	$1.85x10^{-8}$
	Total	0.3600	0.4800	0.3600	0.4490	0.4660	0.3890	0.3590	0.3450
	Gasoline[275,282]	1.154							
	Ethanol from sugarcane[275,282]	0.75							

Table 14. Inventory for the considered processes of bioethanol production from JA. LCA methods legend – T: TRACI 2005 2.1; IP: IPCC GWP 20 years 1.01; IL: ILCD 2011 Midpoint+ 1.07; IM: IMPACT 2002+ 2.12; E: EDIP 2003 1.05; C20/100/500: CML 2001 2.05 20/100/500 years. Gasoline and ethanol from sugarcane values are correspondent to cradle-to-gate approach and do not include allocation or transportation.

In all cases, ethanol production using JA appears to have slightly lower emissions than "standard" 1G ethanol from sugarcane. The presented value from JRC[202] considers sugarcane and ethanol production (and excludes transportation), but these two main processes do not discriminate sub-processes such as harvesting or distillation, which might lead to different emission results. Conflicting or disparate results in LCA studies are often attributed to the use of functional units, system boundaries, and/or methods as well as the lack of information and data that is published.[281] In fact even when considering the same processes, the different selected methods ended up giving different emission results. As shown in Table 14, some methods have different time horizons and were made in different countries, and one might infer that that could have an influence on the genesis of the method.

The different values for the same metric GWP, 100 years time horizon, in CO_2eq, have to do with different considerations, for example, the impact assessment method IM considers CO emission oxidation to CO_2 and E, in addition to CO oxidation, considers NOx conversion to N_2O, and therefore has higher CO_2eq values (difference for minimum up to 30%). The influence of the time horizon can be observed in the C impact assessment methodology, producing a range of 0.345 to 0.389 kgCO_2eq/L_{EtOH} (difference for minimum up to 13%).

Figure 18 addresses the factory placement issue, different locations/countries mean different electricity mixes. Different countries, with different political and economic landscapes as well as different natural and technological resources, have different ways of generating and managing energy. Using the TRACI method (which, along with ILCD, resulted in the lowest GHG emission value) for impact calculation, the process was kept the same, but electricity input was changed according to the electricity mix of different countries (from the Ecoinvent 3.3 library), affecting the "processing" and "fermentation" steps of the production chain (according to the electricity needs of these states, see Table 13). Portugal ranked third among the other three countries selected for this example. The differences can be mostly attributed to the use of renewable and nuclear energies. These types of energies are considered to be of null or low CO_2eq emissions, and their use in France and Brazil is evidently higher than in Portugal and India, which for their part have a higher reliance on coal and oil.[253]

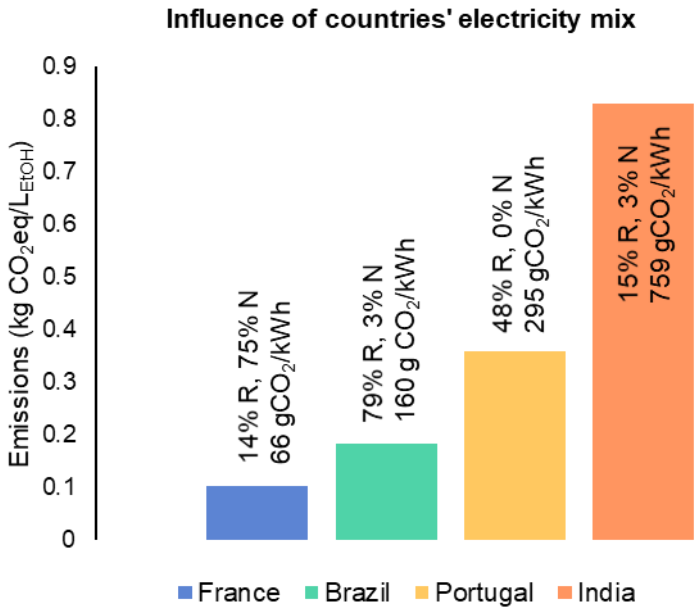

Figure 18. Comparison of emissions for the production of 1 LEtOH, considering electricity mixes from different countries and CO2 intensity (CO2/kWh). (R: renewable energies; N: nuclear energy)[283,284]

As can be seen, the electricity mix alone can cause a range of results from 0.1 to 0.85 kg CO_2eq/L_{EtOH} (deviation from minimum up to 750%).

The chronological time at which the study is carried out can also be considered, as they might use different IPCC assessment reports (AR) for GWP relative to CO_2eq values. For example, the carbon footprint for the 100-year time horizon for methane was, as in the IPCC's AR2 (1996), 21, but in AR5 (2013) it rose to 28, meaning that the release of 1 kg of this gas went from being equivalent to 21 to 28 kg CO_2, in 17 years.[285] However, when GHG values from different ARs are considered for the production of ethanol studied in this work, the differences are not so relevant (Figure 19).

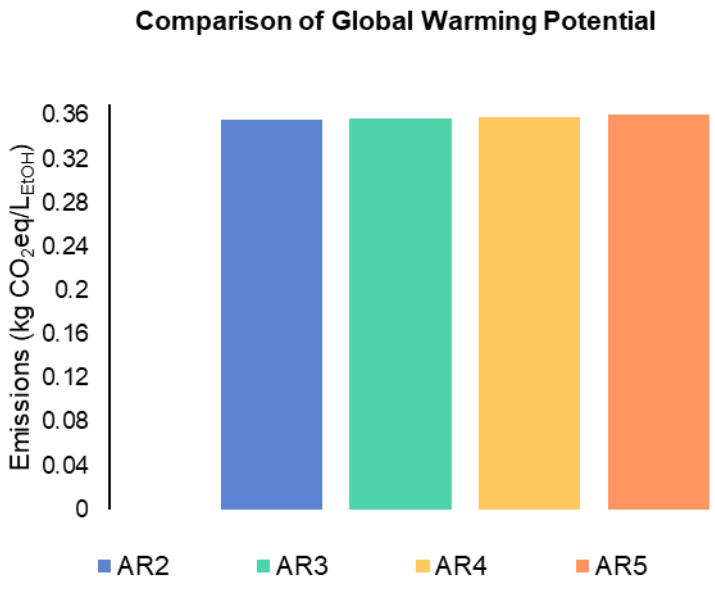

Figure 19. Comparison of GWP, using different values for CO2eq, considered in the references IPCC Ars, for 100 years.

As can be seen, the use of different IPCC assessment reports does not have a significant impact on CO_2eq values (deviation from the minimum less than 3%). This is due to the fact that GHG emissions from fossil origin considered in the inventory of the work are mainly composed of CO_2, in a much higher percentage than methane or nitrous oxides. As 1 kg CO_2 is always 1 kg CO_2eq, the time at which the study was made is not relevant, and the same can eventually be applied to comparisons with other studies. This type of result is indicative of the difficulty to compare different studies and correlate year-zero emissions to long term GHG impact and interested readers and decision-makers must be aware of the temporal evolution of assumptions. Nevertheless, time can still be taken into account in LCA studies. Emissions are usually considered in a single aggregate emission. There is a lack of information about emissions from processes during the time at which they occur, and this type of study relies on restricted steady-state models, but this decreases accuracy and comes as a great limitation. Considering the instantaneous release of a large amount of GHG does not have the same impact as the release of the same amount at a small rate over several years.[286] Figure 20 (A) shows differences of impacts relative to 1 kg CO_2 at time zero, considering instantaneous (solid line) and dynamic (dotted line). The dynamic impact, at the 30-year mark, is of -42.2% compared to the instantaneous scenario, softening to a value of -10.9% at 100 years. Figure 20 (B) shows that the instantaneous impact, at the same mark, is 33.0% when considering emissions over 30 years *versus* all the emissions being theoretically released in the first year, converging to a 4.1% at 100 years. The difference between these two time horizons is quite relevant and is an example of the relevance and need for carefully analyzing

temporal factors in industry scenarios. In LCA studies, not considering the temporal profile of the subject can lead to an underestimation of impacts of GHG emissions, which can result in different decision-making processes.

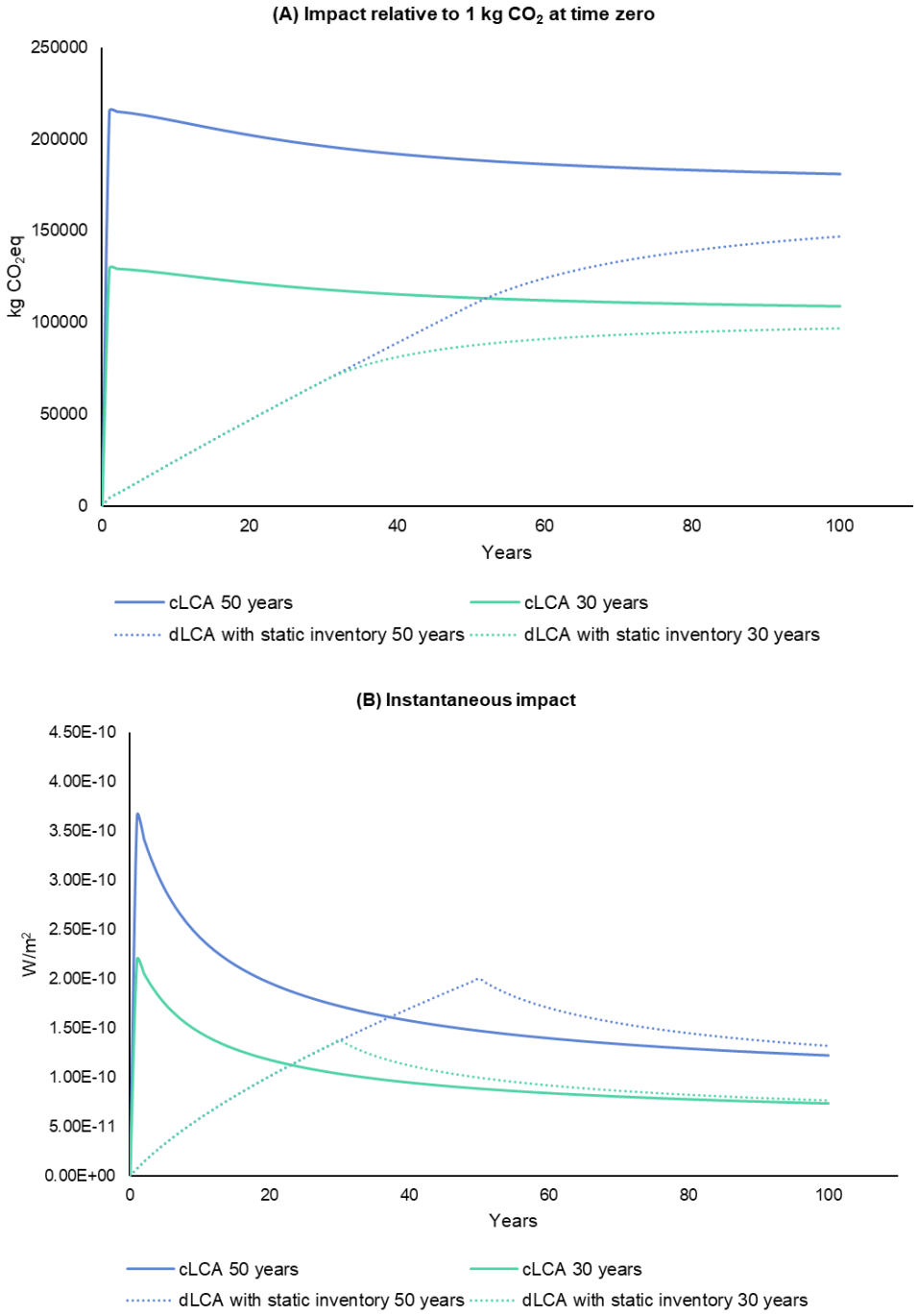

Figure 20. (A) Comparison of GW impacts relative to 1 kg of CO2 at time zero, between conventional and dynamic scenarios of GHG emissions (see Equation 16). (B) Comparison of GW instantaneous impacts between conventional and dynamic scenarios of GGF emissions (see Equation 15). Values considered for a scenario of an industrial facility working for 30 and 50 years.

The lifetime of the factory has also an influence on the CO_2eq results, the longer the lifetime, the higher the impacts. The 20 years' difference between cLCA and dLCA is 61% and for the 100 years difference between dLCA and cLCA is of -10%. Therefore, the deviation from the minimum value at 20 years is up to 159% and 12% for 100 years. The same values for 50 years of operation are 332% and 23%, respectively. The cLCA having always the higher values, which means researchers are systematically overestimating the carbon footprints.

Figure 21 shows the influence of future productivity improvements due to genetic modifications.[287–291] Results obtained under Simapro software analysis using the TRACI method were similar to ones obtained previously,[190] no biogenic, no land use, no allocation and cradle-to-gate borders with no transportation.

As can be seen, future productivity improvements can cause a range from 0.05 up to almost 0.45 kg CO_2eq/L_{EtOH}, with a possible reduction of emissions of around 800%.

Finally, besides calculating the carbon footprint of a biomass-to-bioethanol pathway, we also compare this with an equivalent fossil biomass-to-gasoline pathway, with the same assumptions (in CO_2eq/L_{EtOH}eq, based on LHV ratio). Of course, a refinery is a multiproduct system, and our case study is a single product factory, so the comparison is unfair from the beginning. Nevertheless, a comparison of systems on cLCA *versus* dLCA view is depicted in Figure 22.

For impact relative to 1 kg CO_2 at time zero of the ethanol equivalent scenario, at 20- and 100-year time horizons, a difference of -77% and -18% can be seen between dLCA and cLCA, respectively. As for instantaneous impact, the same time horizons present differences of -46% and 8%. As previously noted, high cLCA values can lead to an overestimation of impacts; however, the biomass-to-ethanol approach is clearly less impactful than oil-to-ethanol.

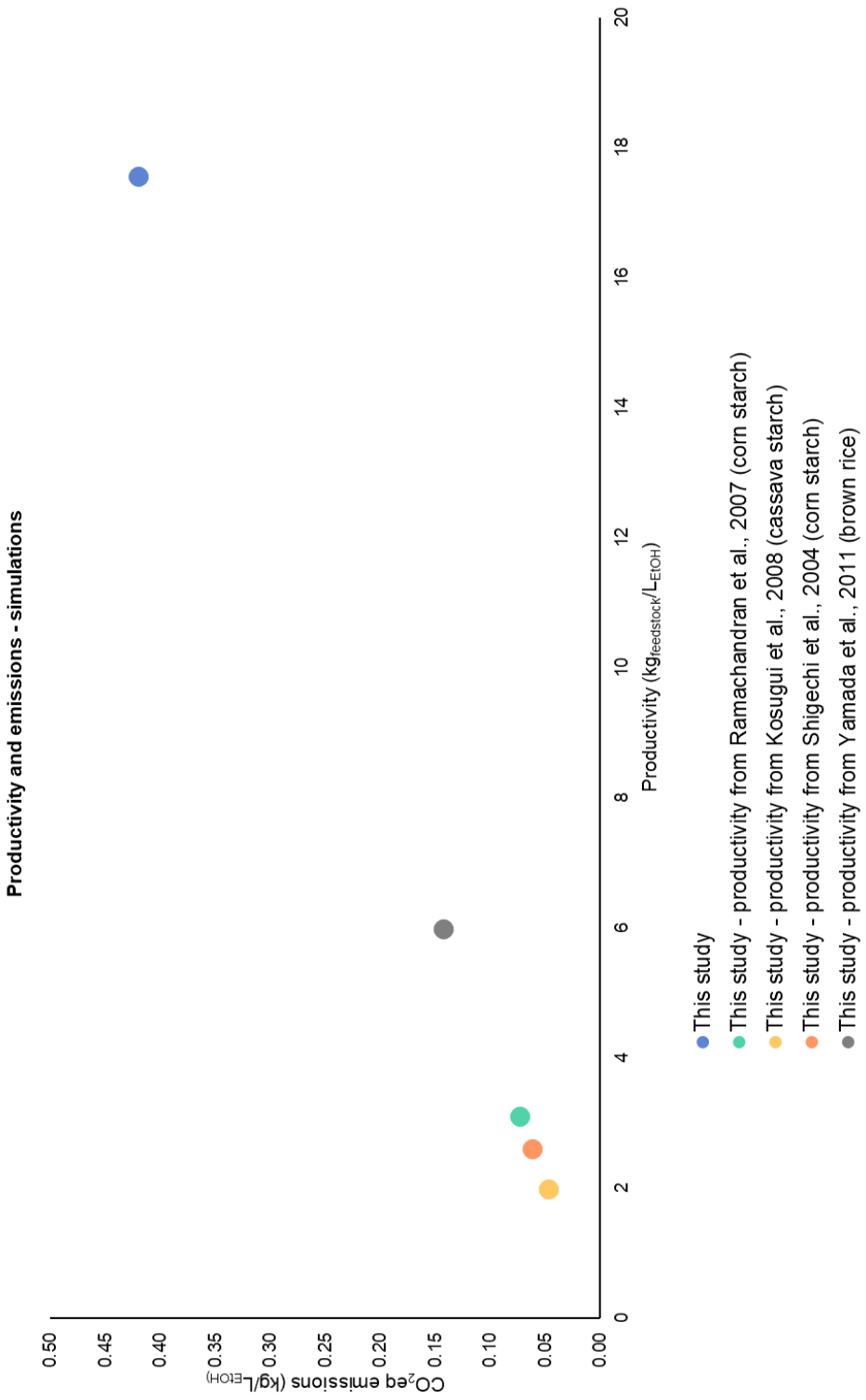

Figure 21. Relationship between productivity and emissions from different simulated scenarios, considering possible genetic modification of yeast strains. Results for the present study only include TRACI values (see Table 14)

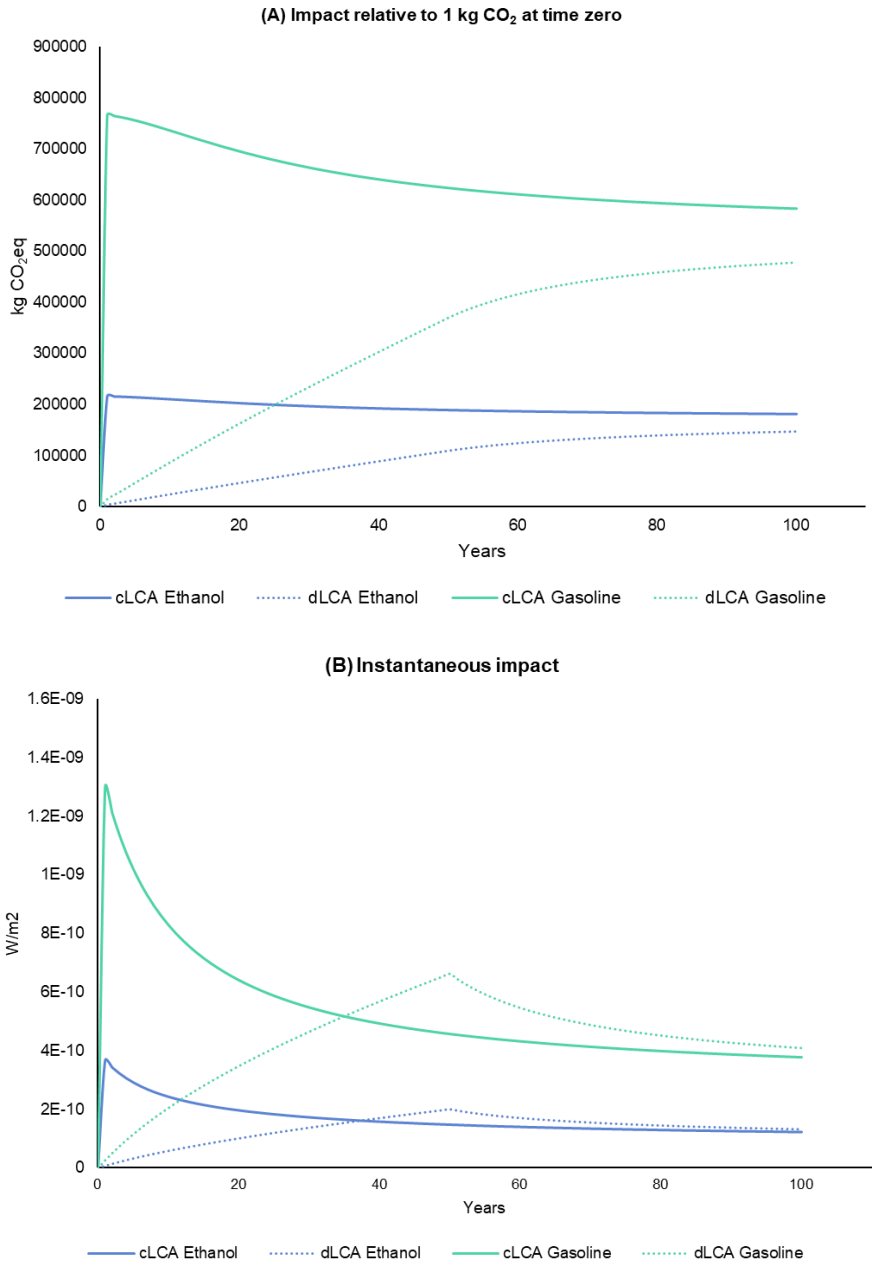

Figure 22. Relationship between biomass-to-ethanol and crude oil-to-ethanol equivalent (gasoline). (A) Comparison of GW impacts relative to 1 kg of CO2 at time zero between conventional and dynamic scenarios of GHG emissions (see Equation 16). (B) Comparison of GW instantaneous impacts between conventional and dynamic scenarios of GHG emissions (see Equation 15).

4.4. CONCLUSIONS

This work aimed to quantify the impacts of methodological choices on CO_2eq emissions of a biomass-to-ethanol pathway so future studies include as much information as possible. From the non-extensive literature review we found, in terms of deviations from minimum values the following influences:

Allocation up to 83%, land use up to 271%, and biogenic up to 240%.

From this work, it was found that the same deviations were as follows, considering the different topics analyzed:

Impact assessment methods up to 30%, location of the factory (countries' electricity mix) up to 750%, IPCC assessment reports up to 3%, time horizon (20 or 100 years) up to 13%, factory operational lifetime up to 108%, and productivity up to 800%.

Therefore, to make an informed decision, it is suggested that every carbon footprint study should have, besides the uncertainty due to inventory data, a sensitivity analysis for other parameters, mainly those that are foreseen to have a higher impact based on this study: factory location (or future electricity mix projections, or own local electricity production), land use issues, no allocation and allocation, and dynamic evaluation. It is also argued that if the carbon footprint of the pathway is not the aim, the comparison with other systems should be made by using the same premises, i.e., the same methodological options. The comparison between the biomass-to-ethanol and oil-to-ethanol equivalent approach clearly showed the higher impacts of the later, with differences up to 222% between the cLCA of both approaches, and 209% for dLCA, at 100 years, easily distinguishing the most environmentally friendly scenario. Of course, a refinery is a multiproduct system and this case study a single product factory, so the comparison is unfair.

It is very important for the decision-maker to have the awareness that a carbon footprint is not a hard number and always has a huge range attached to a mean value. The higher the operational factory time, the higher the differences between conventional and dynamic GWP metric.

SECTION 5

INFLUENCE OF TEMPERATURE, CARBON SOURCE AND GENETIC BACKGROUND ON BIOETHANOL PRODUCTION BY *S. cerevisiae*

5. INFLUENCE OF TEMPERATURE, CARBON SOURCE AND GENETIC BACKGROUND IN BIOETHANOL PRODUCTION BY *S. CEREVISIAE*

5.1. INTRODUCTION

The improvement of fermentation processes, either for the production of 1G or 2G bioethanol, needs to address multiple factors that can limit yeast's performance and eventually impede the implementation of new and/or improved industrial production chains.

Most of the yeast strains used in industrial fermentation grow at a temperature range of 25 ºC to 37 ºC, and temperature control is often needed to cool down the system, as the metabolic activities are exothermic. If such cooling is not performed, the accumulated heat can become a stress on yeasts.[53] While fermentation productivity also depends on factors such as the biomass used as substrate, performance of the fermenting yeast, and available process technologies, it has been recommended a focus of efforts on identifying thermostable, ethanol-resistant yeast strains, preferably tolerant to temperatures that are optimal for saccharification (a type of fermentation described in Section 3). Tolerance to high temperatures can, therefore, lead to several cost reductions.[53] In laboratory-scale reactions, the temperature is controlled by incubators that need energy to work. Assessing the influence of temperature in different strains in different scenarios can service prospects of scale-up scenarios, as a viable ratio between energy spent to maintain a given temperature and energy produced in the form of ethanol is to be achieved. Additionally, the increase of the concentration of ethanol in the fermentation broth, at least up to a threshold level, is essential to keep the costs and energy expenses related to ethanol distillation at acceptable levels.[292]

The viability of the fermentation will also depend on the capacity of yeast to consume different types of monosaccharides. The interest and need for integration of 1G and 2G biorefineries was discussed in Section 2. Fermentation of 1G biomass and 2G lignocellulosic biomass hydrolysates requires the efficient consumption of xylose and high tolerance to acetic acid of industrially interesting yeast species, such as *S. cerevisiae*.[293] Unfortunately, *S. cerevisiae* cannot naturally catabolize pentoses but its robustness towards acetic acid at low pH is a very interesting trait that can be further improved by genetic and environmental manipulation.[68,69,294] Additionally, *S. cerevisiae* preference for glucose as carbon source, due to carbon catabolic regulation in a Crabtree positive yeast, and the incapacity to naturally catabolize xylose results in a reduced ethanol productivity and maximum production.[149,295] Nevertheless, in yeast strains capable of consuming xylose (genetically engineered strains), the presence of other substrates in the medium, such as other sugars or ethanol, has shown to improve xylose consumption.[296] There is, therefore, a need to assess growth and fermentation profiles of pentose consuming yeasts, regarding the sugar consumption rate, and

effects of the accumulated ethanol, especially regarding other important factors in fermentation processes, such as temperature. As mentioned in Section 1, conjugated with high temperatures, the increase of ethanol concentration in the fermentation medium reduces fermentation performance affecting its economic viability.

If, for once, xylose consumption is important for total harnessing of the carbon content of lignocellulosic feedstock, acetic acid resistance is of high value not only due to the natural presence of inhibitory concentrations of acid in 2G feedstock, but also given the fact that acetic acid diminishes the chances of medium contamination with bacteria and bacterial growth during fermentation.[297] Acetic acid is also produced as a byproduct of alcoholic fermentation by *S. cerevisiae*, and an increased concentration in the medium, coupled with high concentrations of ethanol, supra-optimal temperatures and the presence of other possible toxic chemicals present in the lignocellulosic hydrolysates and metabolites produced can lead to low ethanol productivity and even to fermentation arrest.[298]

Acetic acid tolerance in yeast has a polygenic basis,[299] nevertheless, the *HAA1* gene, encoding a recently described transcription factor, activates a large majority (≈80%) of *S. cerevisiae* genes that are determinants of tolerance to acetic acid. Therefore, Haa1 has a key role in the remodeling of genomic expression program of yeast, in response to acetic acid stress,[300] and the increased expression of *HAA1* leads to the increase of yeast tolerance to this weak acid.[87,297,300]

A mutated *HAA1* (*HAA1**; c.1517 G > A in both copies of the gene) leads to increased tolerance to acetic acid stress, when inserted into an industrial yeast strain for 2G ethanol production.[299] Strain GSE16-T18_HAA1* is equivalent to the xylose metabolizing yeast strain *S. cerevisiae* GSE16-T18 containing a mutation in both copies of the *HAA1* gene. The strain GSE16-T18 was obtained from backcrossing of strain GS1.11-26 with a segregant of Ethanol Red. The first is a result of an integration of a “xylose pathway cassette” into the *PYK2* allele of the Ethanol Red strain, as this gene encodes a dispensable glucose-repressed second isoform of pyruvate kinase, only expressed in non-fermentable carbon sources. The cassette contained genes encoding a codon-optimized xylose isomerase from *Clostridium phytofermentans*,[125] a codon-optimized yeast xylulokinase Xks1, a yeast pentose/hexose transporter Hxt7,[71] and the enzymes of the non-oxidative part of the PPP, transketolase, transaldolase, ribulose-5-phosphate 3-epimerase, and ribose-5-phosphate ketol-isomerase. The obtained strain was then subjected to mutagenesis, genome shuffling and several rounds of evolutionary adaptation for xylose fermentation with serial transfers in medium with 40 $g.L^{-1}_{xylose}$.[24]

The strain GSE16-T18_HAA1*, was originated from a cross between GSE16-T18 and a haploid segregant of *S. cerevisiae* strain JT22689, containing the mutated *HAA1* gene.[24] The glucose utilization and specific growth rate in aerobiosis of this strain are inefficient, compared

with its parent strain GS1.11-26, but is capable of consuming xylose, while also displaying increased tolerance to inhibitors, such as acetic acid.[266] The mutation in the *HAA1** gene has shown to greatly increase tolerance to acetic acid, significantly reducing the duration of the lag phase, and enhancing the fermentation rate, when compared with the strain without the *HAA1** mutation, in the presence of inhibitory concentrations of acetic acid.[299]

If acetic acid is one of the most important toxic chemicals to handle 2G fermentation processes, temperature is one of the most important physical parameters in the bioprocess. The preferred temperature for *Saccharomyces* yeasts is between 25 ºC and 35 ºC, with 35 ºC being in the maximum of the optimal temperature range for growth and fermentation, in the absence of toxic compounds or other stresses.[301] An increase of temperature from sub-optimal to optimal values is known to increase growth rates and metabolism, but strains of the yeast species *S. cerevisiae* cannot grow at 43 ºC, 42 ºC being the maximum temperature for most of the strains, even in the absence of other environmental stresses.[302,303] In 2G fermentation, some processes, such as SSF (referred on Section 3), need to compromise between optimal fermentation temperatures (30-35 ºC, depending on the concentration of ethanol produced and the tolerance of the yeast strain; for lignocellulosic hydrolysates, the presence of acetic acid and other inhibitory compounds additionally reduces the optimal temperature for fermentation) and functional saccharification temperatures (55 ºC).[304,305] Also, fermentation in tropical countries need to address inhibitory temperatures, and thermotolerant yeasts can reduce operational costs.[53,306]

In this section, the ethanol productivity and maximum concentration produced by different *S. cerevisiae* strains, under different relevant conditions, was tested. The effect of ethanol concentrations and process temperature was examined, as well as the use of different concentrations of the most important carbon sources present in lignocellulosic biomass: glucose and xylose. In particular, the influence that the expression of the mentioned *HAA1** gene has in the fermentative process performance was also tested at increasing process temperatures (30ºC-41ºC) high gravity fermentation medium lacking growth inhibitors, in particular acetic acid, since the effect of Haa1 in acetic acid- or formic acid- stressed fermentations is well documented in previous works.[86,293] The effect of *HAA1** expression in yeast thermotolerace was tested and this phenotype reported for the fisrt time. For this, the construction of a plasmid expression vector with a positive marker containing the cloned *HAA1** gene was performed, and the resulting construct was used for transformation in yeast strains of interest and the performance of the recombinant strains compared in different fermentation conditions.

5.2. MATERIALS AND METHODS

5.2.1. YEAST STRAINS

The *S. cerevisiae* strains, described in Table 15, were used for fermentation assays. Before the works, the strains were stored at -80 ºC.

Strain	Genetic highlights	Main characteristics	Source
BY4741	*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	laboratory strain; auxotrophic	EUROSCARF collection
Ethanol Red	n/a (industrial strain)	commercial strain; prototrophic; high EtOH yield and tolerance; thermotolerant	Lesaffre Advanced Fermentation
GSE16-T18_HAA1*	GSE16-T18 with c.1517 G > A mutation in both copies of *HAA1*	prototrophic; ferments xylose; increased tolerance to acetic acid, when compared to GSE16-T18	VIB-KU Leuven Center for Microbiology[299]

Table 15. *S. cerevisiae* strains used in the assays.

5.2.2. PLASMID VECTORS AND RECOMBINANT PLASMID CONSTRUCTION

The mutated *HAA1* gene (*HAA1**) from *S. cerevisiae* strain GSE16-T18_HAA1* was amplified through polymerase chain reaction (PCR), with custom primers including a recombinant tail with a *Sal*I restriction enzyme recognition site (Table 16). The pGREG506 vector from the DRAG & DROP collection,[307] with a previously cloned *S. cerevisiae HAA1* promoter,[308] was digested with *Sal*I enzyme, to obtain a linearized fragment for homologous recombination with the amplified *HAA1** gene. The transformation was performed following the MP Biomedical™ Alkali-Cation™ Yeast Transformation Kit. The process induces the uptake of plasmid DNA by intact yeast cells. Figure 23 generically summarizes the process.

The successfulness of the construction was confirmed by Sanger sequencing, and subsequent transformant selection was made by selective cultivation in G418 (geneticin) antimicrobial supplemented YPD plates. The vector carries a gene marker for G418 resistance selection in yeast, avoiding the need for auxotrophic markers in the host strain, which is advantageous for the transformation of several commercial prototrophic strains.[309]

Primer name	Sequence
HAA1_*Sal*I_FW (forward)	CAATACCAGTACTGGTGCATCCTGCTCCTTAAGTTAGAGTTCT CTGTTGTCACTCTACCA
HAA1_*Sal*I_RV (reverse)	ATGTAAGCGTGACATAACTAATTACATGACTCGAGAGAGAAGC AAGAGACGAAAAGC

Table 16. Primer sequence for PCR amplification of the mutated *HAA1* gene.

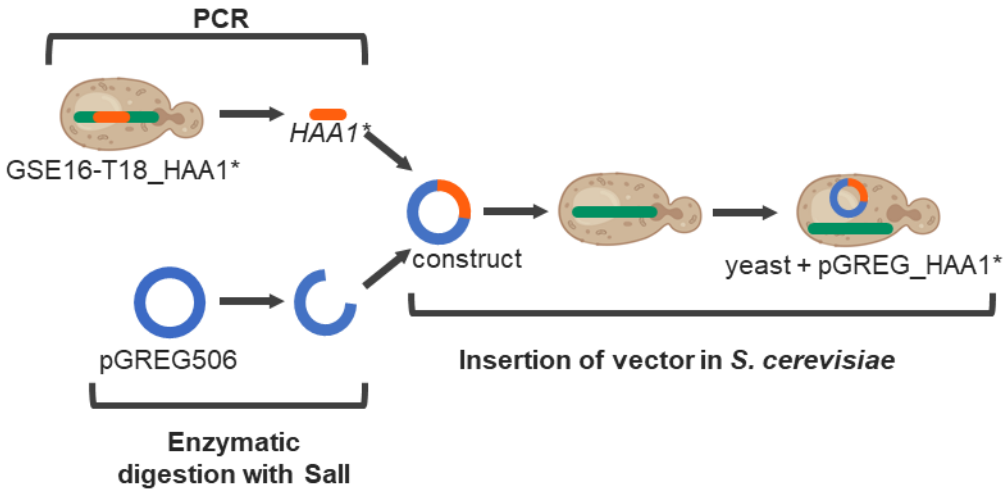

Figure 23. Obtaining a yeast strain containing a constructed plasmid expressing the *HAA1** gene. (green line: simplified 16-chromosomes-yeast genome; orange line: *HAA1** gene; blue circle: pGREG506 plasmid vector)

5.2.3. GROWTH AND FERMENTATION ASSAYS

Yeast cell cultivation was performed in 50 mL YPD liquid medium, containing 10 $g.L^{-1}$ of yeast extract, 20 $g.L^{-1}$ of peptone and variable amounts of glucose and or xylose (see Table 17). Glucose/xylose ratios were considered to be simulated contents of sugars in lignocellulosic substrates.[293,310] Flasks were topped by a rubber stopper with a cotton covered needle, allowing for the release of gases from the fermentation. The assays were performed with orbital agitation at 250 rpm. The starting optical density at 600 nm was standardized at 0.5 and inoculates were prepared with exponential cells. The assays were reproduced at separate times, under the same conditions, and the outcomes were similar; the presented growth and fermentation results in Section 5.3 represent the average of the obtained measurements.

At adequate time points, culture samples were collected to assess growth, by $OD_{600\ nm}$ measurement. Samples of culture supernatants were obtained for glucose, xylose and ethanol quantification by High-Performance Liquid Chromatography (HPLC). The analysis was performed on an Aminex HPX-87 H Ion Exchange Chromatography column, eluted at 65 ºC with 0.005 M H_2SO_4, at a flow rate of 0.6 mL/min for 30 minutes, using a refractive-index detector. In these conditions, glucose had a retention time of 8.3 minutes, xylose of 9 minutes and ethanol of 18.7 minutes. Reproducibility and linearity were tested, and appropriate calibration curves were used for concentration determination.

Throughout this work, several assays were performed, to assess ethanol productivity, glucose and/or xylose consumption, and effects of temperature, as follows:

Assay	Carbon source ($g.L^{-1}$)	Temperature (ºC)
I	glucose 200	30, 35, 40
II	glucose 37 + xylose 37 xylose 40	30, 35, 40
III	glucose 100 + xylose 80 glucose 100 + xylose 37 xylose 40	30
IV	xylose 40	30
V	glucose 20	30, 35, 41
VI	glucose 300	30, 35, 41

Table 17. Fermentation assays, considering different carbon sources and temperatures.

5.2.4. PHENOTYPE SCORING OF YEAST STRAINS FOR THERMOTOLERANCE

For phenotype scoring of tolerance to high temperatures, selected strains were inoculated in liquid YPD medium and grown over-night. Cell suspensions of 4 µL, at an optical density at 600 nm of 1.0, were spotted onto YPD agar plates, as well as dilutions of the initial cell suspension (1:5, 1:10, 1:20, 1:40), and plates were incubated at 30 ºC, 35 ºC, and 41 ºC for 48 hours. At 24 hours and 48 hours, the plates were photographed to register growth phenotype for the different strains. Three independent growth assays were done, and the results obtained were consistent.

5.3. RESULTS AND DISCUSSION

A selection of the relevant results obtained is presented, following the assays mentioned above.

5.3.1. TEMPERATURE EFFECTS ON YEAST GROWTH AND ETHANOL PRODUCTION IN GLUCOSE MEDIUM

The following temperatures were tested in different fermentation assays: 30 ºC, 35 ºC and 40 ºC, accounting for sub-optimal, optimal and supra-optimal temperatures, respectively for *S. cerevisiae* growth and fermentation, in the absence of environmental stresses.[50] Concerning the two yeast strains tested, the industrial strain Ethanol Red has a higher tolerance to ethanol than the laboratory strain BY4741.[311]

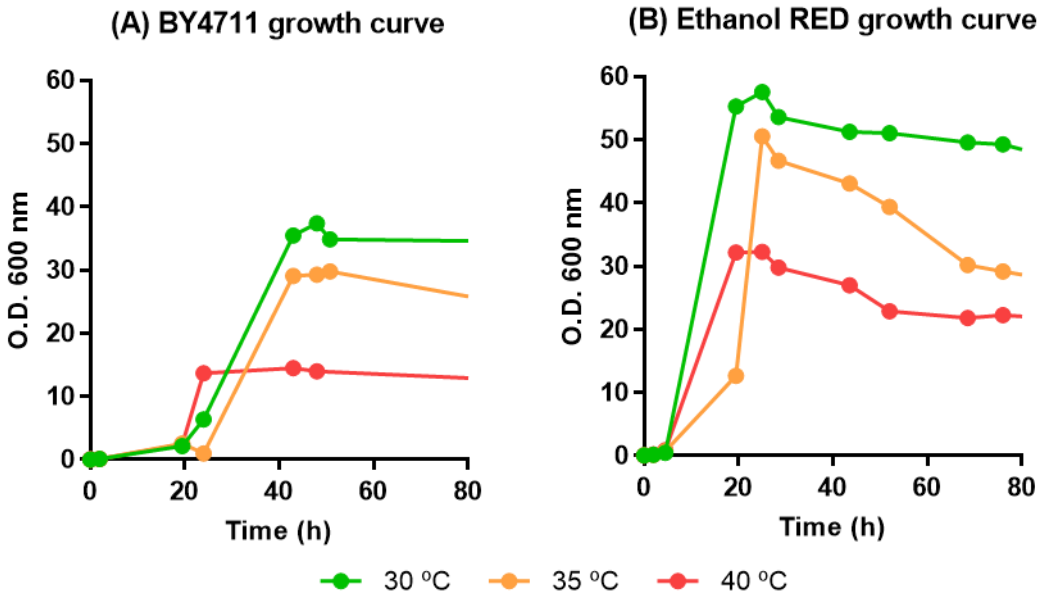

Figure 24. Growth curves of strains (A) BY4741 and (B) Ethanol Red, at 30 ºC (green), 35 ºC (orange) and 40 ºC (red), in a 200 $g.L^{-1}_{glucose}$ medium. The experimental data shown is the average of the values obtained from two independent fermentations that provided similar results

The growth in a medium with 200 $g.L^{-1}$ of glucose as carbon source of strains BY4741 and Ethanol Red exhibited essentially the same growth profiles for the three temperatures tested, although Ethanol Red reached a higher biomass concentration (based on culture $OD_{600\ nm}$) and produced ethanol concentration (Figure 24 and Figure 25) (Assay I). An increase in temperature from 30 ºC to 40 ºC leads to a decrease of the biomass and ethanol produced and an increase in the amount of consumed sugar, consistent with the deleterious conjugated effect of the ethanol accumulated during fermentation and the process temperature, suggesting that growth stopped prematurely, especially at 40 ºC, for both strains. The effects of temperature on fermentation (Figure 25) were not as drastic as observed for growth (Figure 24), which is already described and related to the fact that cells unable to grow are still capable

of glucose fermentation. For this reason, all the sugar concentration present in the medium was exhausted and the final concentration of ethanol produced reached similar levels for the three temperatures, even though more slowly at 40 ºC in the case of the laboratory strain. At 40 ºC, the industrial high ethanol tolerant strain Ethanol Red reached a maximum ethanol concentration of 9.6% v/v, after 24 hours of culture, when glucose was totally depleted, while the laboratory strain BY4741, at the same temperature, reaches a maximal concentration of 10.2% v/v of ethanol, after 68 hours of fermentation and glucose exhaustion (Figure 25). However, at lower temperatures, (30 and 35 ºC), after 30 hours of fermentation, the process was over for both strains, and all glucose present was consumed and a maximum of ≈10.7% v/v of ethanol was produced by BY4741 or Ethanol Red.

These results suggest that the tolerance of the industrial strain to the conjugated effect of the produced ethanol and supra-optimal temperatures is higher when compared with the laboratory strain, confirming indications from the literature.[301] The observed difference can likely be higher if higher concentrations of glucose are used since those may lead to the accumulation of more deleterious ethanol concentrations.

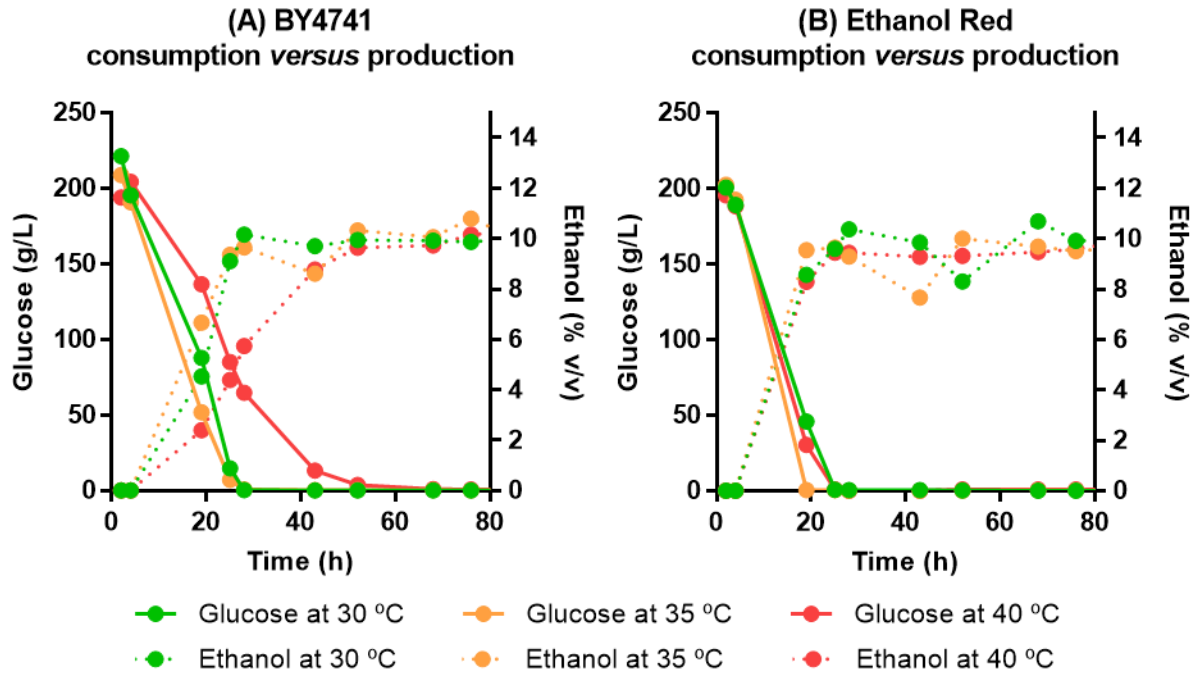

Figure 25. Glucose consumption (solid line) and ethanol production (dotted line) of (A) BY4741 and (B) Ethanol Red, at 30 ºC (green), 35 ºC (orange) and 40 ºC (red), in a 200 $g.L^{-1}_{glucose}$ medium. The experimental data shown is the average of the values obtained from two independent fermentations that provided similar results.

5.3.2. TEMPERATURE EFFECTS IN RECOMBINANT YEAST GROWTH AND ETHANOL PRODUCTION IN XYLOSE AND GLUCOSE + XYLOSE MEDIA

Temperature is an important process condition for the performance of the fermentation of lignocellulosic substrates. In the next experiences performed, yeast cells were inoculated in a medium containing glucose and xylose at the average concentrations present in lignocellulosic hydrolysates (37 $g.L^{-1}$ glucose and 37 $g.L^{-1}$ xylose) (Figure 26). Additionally, the yeast fermentation of a medium with xylose (40 $g.L^{-1}$) as sole carbon source was also prepared, to assess the influence of the presence/absence of glucose in the fermentation profile at different temperatures (Assay II). An increase in temperature from 30 to 40 ºC had a negative effect in strain GSE16-18_HAA1* (T18_HAA1*) growth. This strain is reported[293,312] to be of interest for fermentation in the presence of two of the major 2G fermentation constraints or requirements: presence of toxic concentrations of acetic acid and the need of xylose consumption. Strain T18_HAA1* is therefore of interest for the assessment of temperature influence in lignocellulosic fermentations. Growth at 40 ºC leads to a similar final biomass concentration, as assessed by culture optical density, in both media with glucose+xylose or only xylose, as carbon sources, but the maximum specific growth rate in the mixed sugar medium (with glucose and xylose) was higher than when xylose was the single sugar. At 30 ºC and 35 ºC, growth in the medium with only xylose as carbon source had a longer exponential phase than in the mixed medium, and reached a higher final biomass concentration (based on culture optical density) (Figure 26).

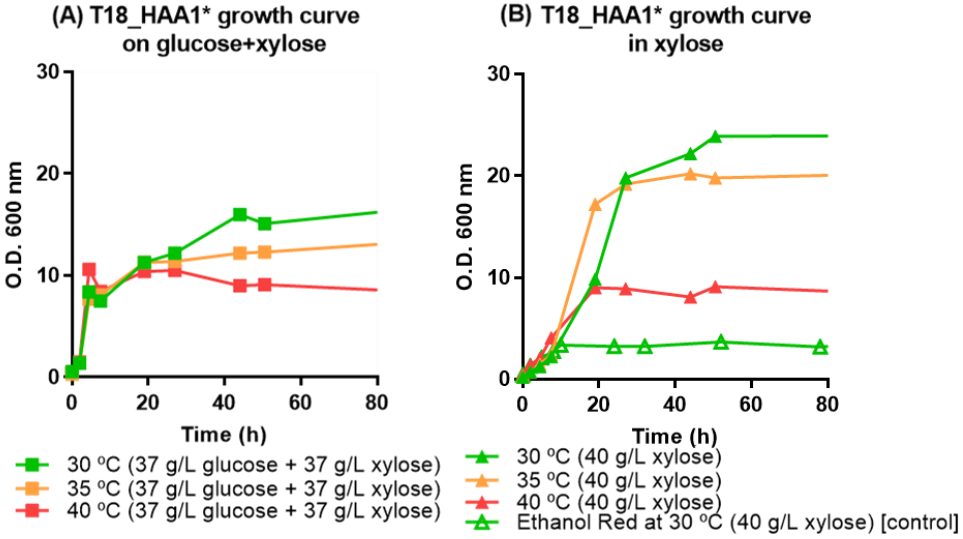

Figure 26. Growth curves of strain T18_HAA1, at 30 ºC (green), 35 ºC (orange) and 40 ºC (red), in 37+37 $g.L^{-1}_{glucose+xylose}$ medium (A, ■), and 40 $g.L^{-1}_{xylose}$ medium (B, ▲Δ). Ethanol Red (Δ) is used as control for growth in xylose sole carbon source. The experimental data shown is the average of the values obtained from two independent fermentations that provided similar results.

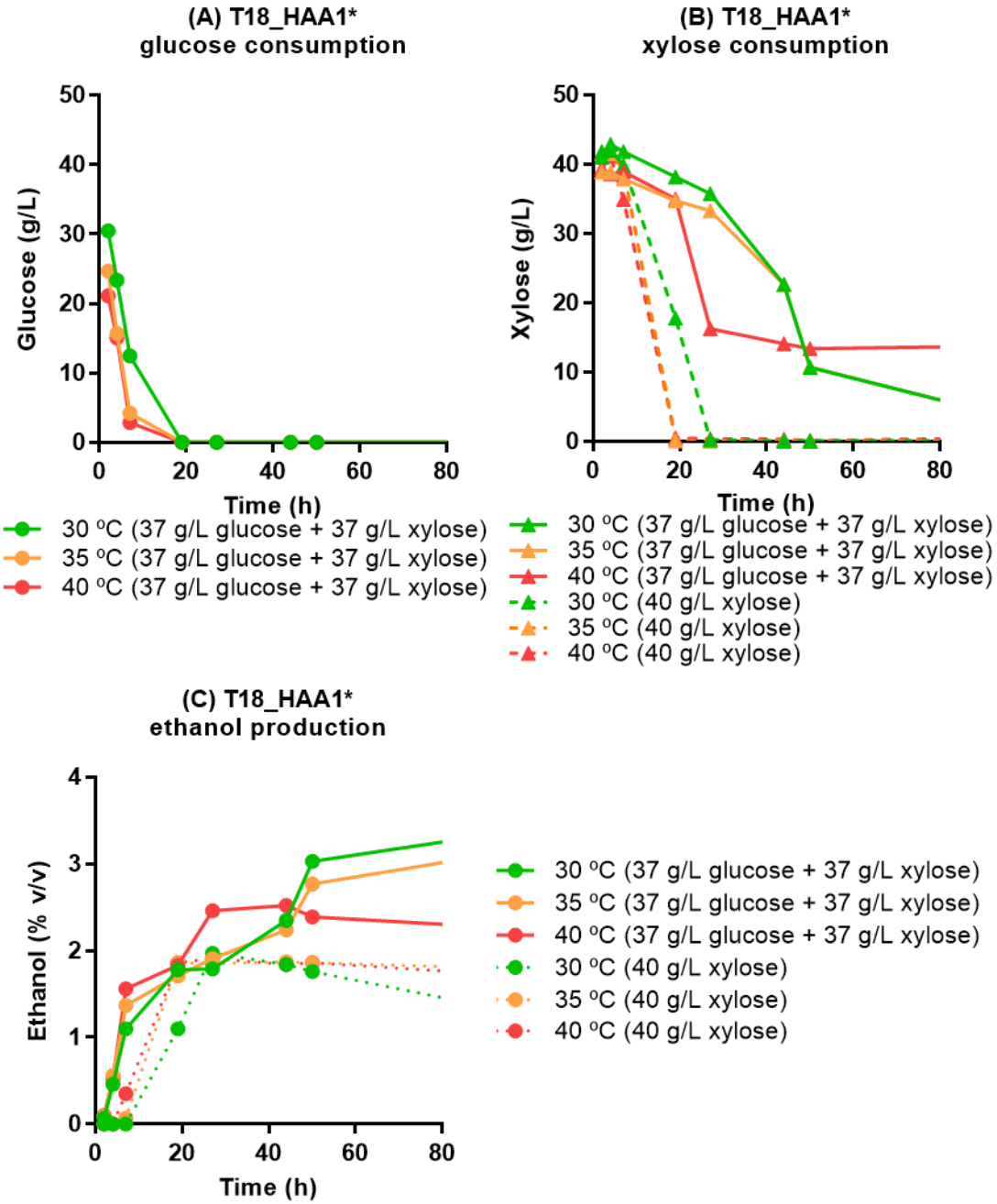

Figure 27.Glucose (A) and xylose (B) consumption, and ethanol production (C) of T18_HAA1*, at 30 ºC (green), 35 ºC (orange) and 40 ºC (red), in media with 37+37 $g.L^{-1}_{glucose+xylose}$ and 40 $g.L^{-1}_{xylose}$. The experimental data shown is the average of the values obtained from two independent fermentations that provided similar results.

In the mixed medium (37 $g.L^{-1}$ glucose + 37 $g.L^{-1}$ xylose), at the time of total depletion of glucose (19h hours), the following yield values were calculated[313]: 1.48 $mol_{EtOH}/mol_{glucose}$ (30 ºC), 1.41 $mol_{EtOH}/mol_{glucose}$ (35 ºC), and 1.53 $mol_{EtOH}/mol_{glucose}$ (40 ºC), 74.0%, 71.5% and 76.5% of the maximum theoretical ethanol yield from glucose (2 $mol_{EtOH}/mol_{glucose}$), respectively. In the medium with xylose as single carbon source, ethanol production yields per mass of xylose were: 1.39 mol_{EtOH}/mol_{xylose} (30 ºC) and 1.33 mol_{EtOH}/mol_{xylose} (35 ºC and 40 ºC), 83.0% and 79.6% of the maximum theoretical ethanol yield from xylose (1.67 mol_{EtOH}/mol_{xylose}),

respectively. The apparent higher ethanol yield in xylose compared with the mixture of sugars is probably related with the higher and more inhibitory concentration of ethanol produced from the mixture, due to the higher total concentration of carbon sources, when compared with the initial concentration of xylose used. A higher inhibitory effect of ethanol results in decreased production yields due to increased dissipation of energy for detoxification mechanisms. Due to yeast's preference for glucose, the fermentation of the mixed sugars starts with the catabolization of glucose with a higher maximum specific growth and fermentation rates, followed by the catabolization of xylose at a lower specific growth and fermentation rates reaching a final concentration of ethanol of ≈7% v/v (Figure 27). The growth profiles show a diauxic growth typical of the sequential use of the two sugars and the concentration of ethanol produced is expected to inhibit growth and fermentation.

The increase of process temperature interferes with the uptake and/or metabolism of xylose and glucose (Figure 27 B). At 40 ºC and 35 ºC, glucose (in the mixed sugar fermentation) and xylose (when as the single sugar, at 40 $g.L^{-1}$) consumption is quicker than at 30 ºC (Figure 27, B). However, the concentration of xylose when in the mixed sugar medium was not fully consumed at any of the temperatures after 80 hours of culture, while at 30 ºC and 35 ºC xylose was exhausted after 150 hours of culture (results not shown). At 40 ºC, xylose fermentation stopped prematurely, and its concentration remained ≈12 $g.L^{-1}$ until the end of the experience. In this experiment, the toxicity caused by the accumulation of the produced ethanol, by itself, is not likely since its concentration was below 4% v/v, and at 30 ºC and 35 ºC it is not enough to prematurely stop the fermentation of glucose. Nevertheless, the conjugated stressing effect of the ethanol and the supra-optimal temperature (40 ºC) affected the yeast's capacity of xylose fermentation. Consequently, in the medium with glucose and xylose, the increase of temperature to 40 ºC implicated a decrease in maximum ethanol production from the metabolism of xylose.

In the media with xylose as the only carbon source (Figure 27 B, dashed lines), the xylose consumption rate is similar at 35 ºC and 40 ºC, but it is slower at 30 ºC, even though all the sugar is depleted by 27 hours of culture. By that time, ethanol productivity reached a maximum of ≈2% v/v at all temperatures.

As discussed before and clearly suggested by the above-described results, industrial-sized fermenters often require the cooling of the fermentation medium to allow an economically viable ethanol yield. Cooling is considered to be the major energy-consuming step in industrial fermentations, implying increased costs and increased associated GHG emissions (both directly, by the cooling equipment, and indirectly, by the energy production operations).[314,315] Hence, the thermotolerance of *S. cerevisiae* strains is of major importance, since an increased resistance to high temperatures directly leads to the reduction of economic costs and environmental impacts of the production chain. For these cases, LCA studies of simulated

scaled-up scenarios would be of interest, to infer on the GWP impact of cooling processes, given different tolerance levels to high temperatures, both on 1G and 2G fermentations. Also, Fermentation processes at high temperatures also result in higher saccharification efficiency, for which thermotolerant strains come of interest for the improvement of processes.[55]

To the best of my knowledge, this is the first time that the influence of temperature on T18_HAA1* strain growth and performance in mixed glucose/xylose medium was studied.

Temperature is a controllable factor in fermentation, but the efficacy of 2G ethanol production is also dependent on the ability of strains to metabolize non-glucose sugars present in lignocellulosic hydrolysates.

For control purposes and to assess the magnitude of differences caused by different concentrations of sugars in the medium, T18_HAA1* growth and fermentation was compared with the laboratory strain BY4741 and the commercial strain Ethanol Red (Figure 28, A), in a medium with xylose as the only sugar, at a concentration similar to that commonly present in several lignocellulosic substrates (40 $g.L^{-1}$)[293,310] (Assay IV). As expected, the lack of the expression of xylose catabolic pathways (described in Section 1) impedes BY4741 and Ethanol Red strains to grow using this carbon source, and only T18_HAA1* shows significant growth (Assay III).

Regarding the fermentation of different combinations of glucose and xylose concentrations (Figure 29, B and C), the effect of catabolic repression implicated that the xylose present was only used after glucose concentration in the fermentation medium became limited. In the two concentration settings, glucose was totally consumed after 24 hours of culture, but only for the setting 100 $g.L^{-1}$ glucose + 37 $g.L^{-1}$ xylose (C) xylose was fully consumed, after 150 hours of culture (results not shown in Figure 29). However, only ≈58% of xylose was consumed in the setting 100 $g.L^{-1}$ glucose + 80 $g.L^{-1}$ xylose (B), after 284 hours of fermentation (results not shown in Figure 29). In Figure 29 C, it is possible to see that xylose consumption leads to a higher maximum value of ethanol in the supernatants, peaking at 6.0% v/v at 24 hours (dotted line), when compared with Figure 29 B, where xylose concentration has only a small decrease. Still, after attaining a maximum ethanol concentration, the volume of ethanol in the supernatants in 100 $g.L^{-1}$ glucose + 37 $g.L^{-1}$ xylose medium began to decrease, presumably due to ethanol utilization as carbon source after complete depletion of glucose and xylose, since ethanol concentration decreased to ≈4.00% v/v at 284 hours of culture (not shown in the figure). Interestingly, the ethanol production rate is lower in the medium with 100+80 $g.L^{-1}_{glucose+xylose}$, despite the higher total concentration of sugar in the medium. To the best of my knowledge, this is the first report on the sugar consumption profile (glucose and

xylose) of T18_HAA1*, with different monosaccharide ratios, considering the two most common carbon sources in lignocellulosic substrates.

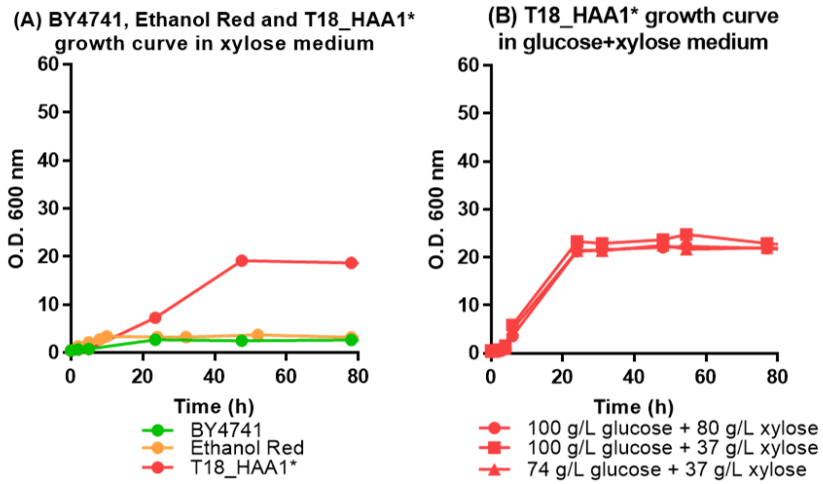

Figure 28. Growth curves of strains BY4741 (green), Ethanol Red (orange) and T18_HAA1* (red), at 30 ºC, (A) in media containing xylose as sole carbon source (40 $g.L^{-1}$), and (B) different concentrations of glucose and xylose (100+80 [●], 100+37 [■], and 74+37 [▲] $g.L^{-1}_{glucose+xylose}$). The experimental data shown is the average of the values obtained from two independent fermentations that provided similar results.

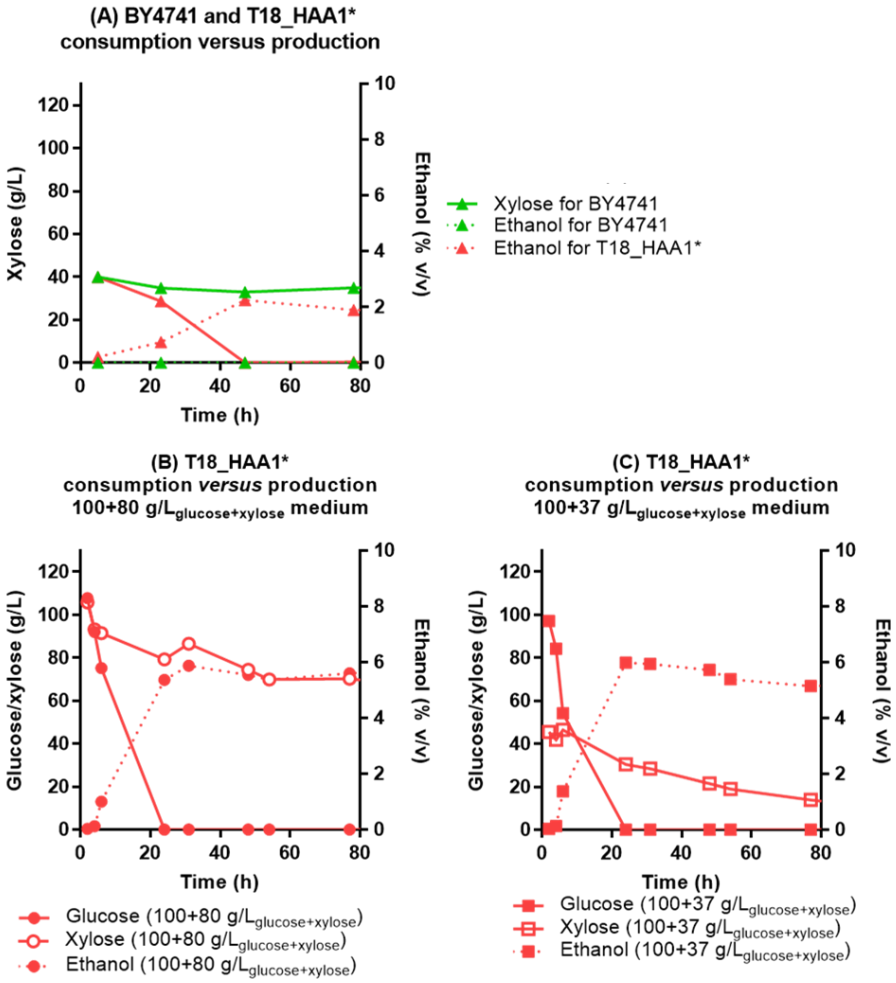

Figure 29. (A) Xylose consumption (solid line) and ethanol production (dotted line) of strains BY4741 (green) and T18_HAA1* (red), in a medium with xylose as sole carbon source (40 $g.L^{-1}$). (B) (C) Glucose (solid line, ●■▲) and xylose (solid line, ○□Δ) consumption, and ethanol production (dotted line, ●■▲) of strain T18_HAA1* in different concentration mixes of glucose and xylose. The experimental data shown is the average of the values obtained from two independent fermentations that provided similar results.

5.3.3. INFLUENCE OF *HAA1** IN THE TOLERANCE TO HIGH TEMPERATURES

5.3.3.1. EFFECT OF *HAA1** EXPRESSION IN YEAST THERMOTOLERANCE

It was found that the expression of the gene *HAA1* with a single mutation leads to a higher improvement of yeast robustness towards acetic acid, compared with the original gene sequence. A comparison between the native *HAA1* gene of Ethanol Red, and the mutated *HAA1** gene of T18_HAA1* show 11 single nucleotide variations in the sequences (total sequence size: 2085) (Table 18).

Location in the sequence (bp)	Nucleotide variation (*HAA1* of Ethanol Red > *HAA1** of T18_HAA1*)
617	A > C
644	T > C
1016	T > A
1150	T > G
1151	T > C
1330	T > C
1333	C > A
1454	T > C
1576	G > A
1862	A > G
2045	C > T

Table 18. Position of nucleotide variations in the *HAA1** gene of strain T18_HAA1*, compared with the *HAA1* gene of strain Ethanol Red.

All the detected nucleotide variations were located in the corresponding sequence of the transactivation domain of Haa1. The two variations located at 1330 bp and 1862 bp in the *HAA1** sequence have previously been reported (F440Y and S622F, respectively).[87,316] It has been suggested that domains in the C-terminal region of Haa1 may be involved in the regulation of the protein's activity, but additional work is needed to fully comprehend the influence of the referred variations and how they interfere with the effect of the gene in yeast tolerance to external stresses.[87]

However, it has been reported that the gene can also be linked to resistance to inhibitors other than weak acids, such as cycloheximide and miconazole.[312,317,318] The high temperatures required for fermentation processes, such as simultaneous saccharification, can also disturb yeast's protein stability, cytoskeleton structures, and cell membrane, leading to dysfunction of proteins and metabolic imbalances or loss of metabolic activity, as well as defects in the maturation of transfer RNA.[319–321] In this work, the effect of *HAA1** expression in yeast thermotolerance was examined. For this, the *HAA1** gene sequence was amplified through PCR and cloned into the pGREG506 plasmid. This plasmid has the positive selection marker kanMX, which confers to the transformed yeast resistance to geneticin, allowing the selection of transformed prototrophic industrial and laboratory strains, as well as, the

auxotrophic laboratory strains. Additionally, this marker also has a lower impact in yeast growth, when compared with auxotrophic markers.[322] Hence, through homologous recombination, a construct was obtained containing the cloned *HAA1** gene. This construct was made with the intent of studying the effect of the expression of a mutated *HAA1** gene in yeast strains, namely industrial prototrophic strains with high ethanol productivity, such as Ethanol Red, in media with high concentrations of glucose and to assess the actual influence of the mutated gene in yeast tolerance to supraoptimal temperatures and other environmental stresses.

In this section of the work, the influence of the previously described mutated *HAA1** gene on tolerance to high temperatures was assessed in assays of phenotype scoring (Assay V). The Ethanol Red strain was transformed with the pGREG cloning vector, and this vector with the *HAA1** gene inserted and growth of the transformed strains was compared at different temperatures by spot assays (Figure 30).

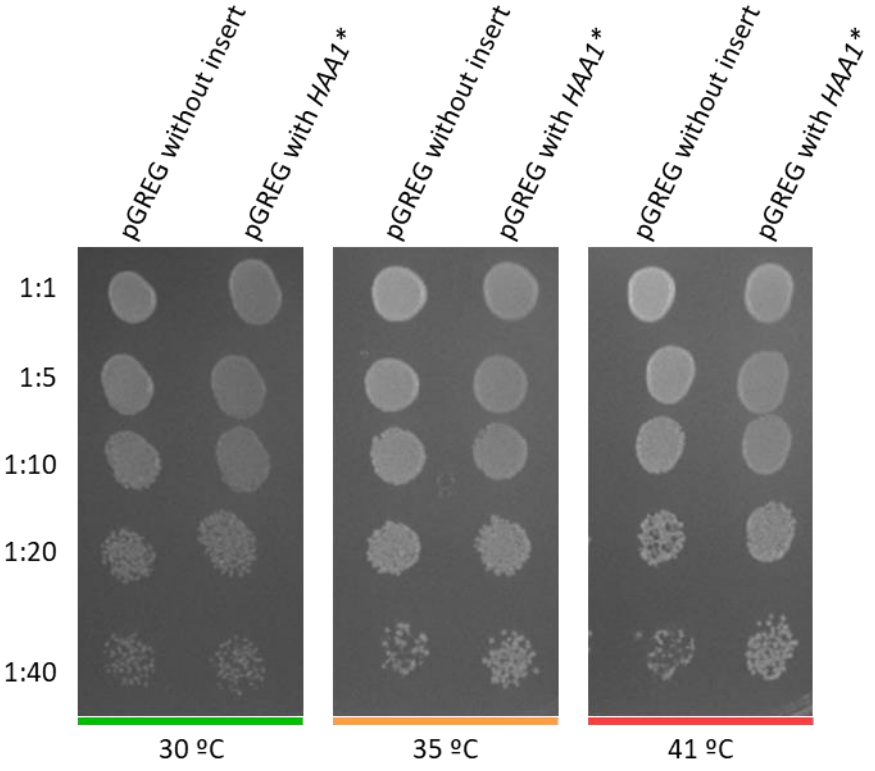

Figure 30. Influence of *HAA1** expression in the growth phenotype of Ethanol Red at different temperatures. Ethanol Red transformed with a pGREG cloning, and pGREG vector with the *HAA1** insert were compared, after 24 hours. [Dilution factors on the left, from 1:1 to 1:40; Initial $O.D._{600\ nm}$ of 1.0] This experience was repeated three times with the same results.

The expression of *HAA1** at 35 ºC (slightly above the range of optimal temperatures described for most of the *S. cerevisiae* strains[306] and especially at 41 ºC (clearly a supraoptimal growth temperature) increased yeast growth performance (Figure 30). The stress caused by elevated temperatures is known to increase the production of reactive oxygen

species (ROS).[323] These are neutralized by both non-enzymatic and enzymatic processes in the yeast cell, the latter demanding NADPH as a source of reduction equivalents.[324] To compensate NADPH oxidation, there is an increased influx through PPP and the acetic acid pathway, leading to an increased acetic acid production, which in turn negatively affects the growth of strains and the production rate of ethanol.[320,325,326] Ethanol Red is a highly ethanol tolerant and robust strain but it is sensitive to acetic acid,[312] so the expression of *HAA1** (beyond the native *HAA1* of the strain) might have a role in resisting the effects of high temperatures in the cell, preventing the damages caused by oxidative stress due to the presence of weak acids. Additionally, *HAA1* transcriptionally regulates cell wall and plasma membrane proteins.[327,328] An increased expression of the gene can, therefore, counteract the negative effects of high temperatures in the stability of the referred proteins. Previous works have suggested that the expression of *HAA1* (alongside with *PRS3*) has a role in the integrity of yeast cell wall.[328]

The effect of *HAA1** expression in Ethanol Red thermotolerance is a new and very important phenotype. Together with the marked effect in acetic acid tolerance, points this gene as an essential determinant of robustness during lignocellulosic hydrolysates fermentation.

5.3.3.2. EFFECT OF *HAA1** EXPRESSION IN HIGH GRAVITY FERMENTATIONS AT DIFFERENT TEMPERATURES

Fermentation assays to examine the effect of *HAA1** expression during high gravity fermentations at different temperatures were performed with the transformed strains were performed, in liquid YPD medium, with 300 $g.L^{-1}$ of glucose as carbon source, at the three selected temperatures (Figure 31) (Assay VI).

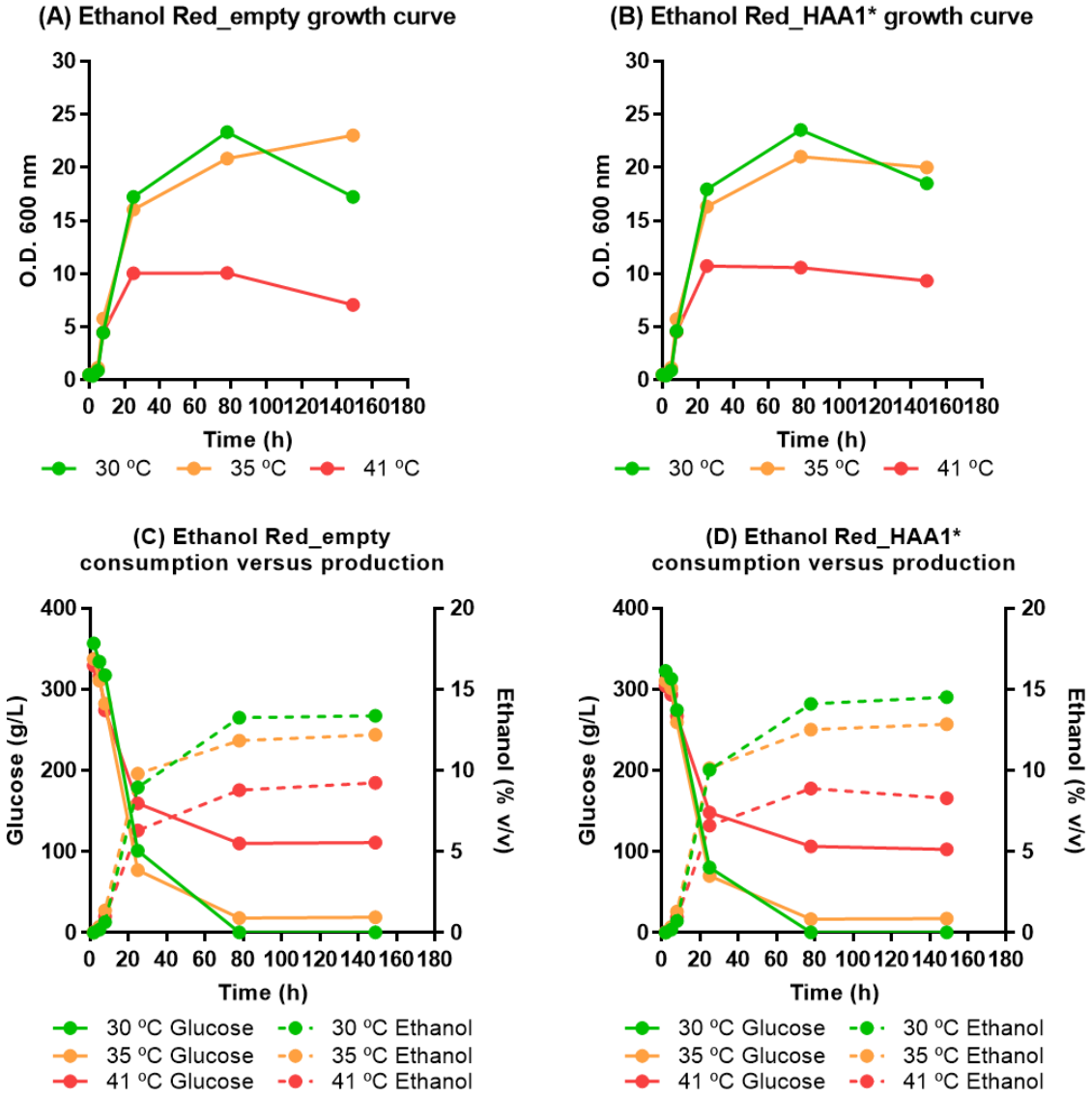

Figure 31. Growth curves of Ethanol Red transformed with the pGREG plasmid (Ethanol Red_empty) (A) and Ethanol Red transformed with the pGREG plasmid containing the *HAA1** gene (Ethanol Red_HAA1*) (B). Glucose consumption (solid lines) and ethanol production (dashed lines) of Ethanol Red_empty (C) and Ethanol Red_HAA1* (D). Fermentations performed at 30 ºC (green), 35 ºC (orange) and 41 ºC (red), in YPD liquid media containing 300 $g.L^{-1}$ of glucose as sole carbon source. The experimental data shown is the average of the values obtained from two independent fermentations that provided similar results.

At the three temperatures tested, the effect of *HAA1** expression was found to be slightly positive concerning the specific growth/production/consumption rates and the maximum biomass and ethanol concentrations produced and glucose concentration consumed, even at 30 ºC and 35 ºC. Regarding the expected higher effect at the highest temperature tested (41 ºC), this effect is not clear. It is intriguing why *HAA1** expression from a recombinant plasmid had such positive effects considering the metabolic burden expected from the recombinant protein expression. Since Ethanol Red is highly ethanol tolerant and that *HAA1* expression does not increase ethanol tolerance, it was considered the hypothesis of the *HAA1** expression being alleviating the inhibitory effect of the accumulation of acetic acid in the fermentation medium as the result of metabolism.

The concentration of acetic acid in the supernatants, at 25 hours of culture, was measured (Table 19).

Temperature (ºC)	**Acetic acid concentration (mM)**	
	Ethanol Red_empty	Ethanol Red_HAA1*
30	27.5	31.1
35	30.9	28.3
41	27.3	30.4

Table 19. Acetic acid production during fermentation by Ethanol Red transformed with the pGREG plasmid, and Ethanol Red transformed with the pGREG plasmid with the *HAA1** gene insert. Measurements made at 25 hours of culture.

Concentrations of acetic acid in the medium higher than 20 mM for as short as ≈3 hours can lead to the activation of a programmed cell death pathway in *S. cerevisiae*.[329] Ethanol Red in particular, has shown to be sensitive to acetic acid and have an increased latency phase after exposure.[330] In this case, the expression of *HAA1** clearly improves the fermentative performance of the yeast at 30 ºC, even though there is a higher concentration of acetic acid in the medium (at 25 hours of culture), Ethanol Red_HAA1* has an increased production of ethanol, when compared with Ethanol Red_empty, despite the similar glucose consumption profile between strains. The influence of *HAA1** expression is also noticeable at 41 ºC, as both strains reach a maximum ethanol production of ≈9.7%, but Ethanol Red_HAA1* reaches that value before Ethanol Red_empty, and from that point begins to use the produced ethanol as a carbon source, regardless of the higher concentration of acetic acid in the medium (30.4 mM for Ethanol Red_HAA1* *versus* 27.3 mM for Ethanol Red_empty). The high temperature, as previously reported,[331] seems to affect the consumption of glucose (which is almost totally depleted at 30 ºC and 35 ºC), but the increased tolerance conferred by the presence of *HAA1** allows the yeast cells to continue to metabolize the carbon sources present in the medium and increase their biomass. At 30 ºC and 35 ºC, the growth curves of both strains are relatively

similar, but at 41 ºC, Ethanol Red_*HAA1** appears to have increased biomass than the strain without the mutated gene.

In future studies, quantification of secondary products such as acetic acid, succinic acid, glycerol and trehalose[332] should be performed to assess possible deviations of carbon use to non-fermentative pathways. Additionally, expression of heat shock *HSP31* gene, which is regulated by the *HAA1* gene,[333] should also be assessed to further confirm the influence of the latter in tolerance to high temperatures.

In addition, *HAA1** may have a positive effect in osmotic stress, resulting from high-glucose concentrations. This effect was not tested yet, but is an important phenotype to be assessed in future experiences.

5.4. CONCLUSIONS

Temperature, type of carbon source, carbon source concentration, chemical stresses and genetic background are some of the factors that can affect yeast growth and fermentation capacity.

Regarding the effects of temperature, the commercial strain Ethanol Red appeared to be less negatively influenced by increased temperatures (ranging from 30 ºC to 40 ºC) than the laboratory strain BY4741, concerning glucose consumption and ethanol production. However, the different yeast performance cannot be exclusively associated to temperature because Ethanol Red is much more tolerant to ethanol than BY4741 and, consequently, to the conjugated effect of ethanol-temperature during fermentation. Temperature is an important factor in the fermentation of lignocellulosic substrates and its influence was also tested using the xylose-catabolizing strain T18_HAA1*, where fermentation at the supraoptimal temperature of 40 ºC resulted in a decreased production of ethanol, compared to 30 ºC and 35 ºC, due to a diminished rate of metabolization of xylose after depletion of glucose and accumulation of ethanol. Assessing and counteracting high temperature influence on 2G fermentation is important not only for ethanol productivity, but also because the quite common need of cooling processes, which are energy consuming (and, therefore, directly and indirectly, GHG emitting). Thermotolerance of *S. cerevisiae* strains is a very important trait, as it might be a preponderant factor in decreasing the environmental impact of fermentation processes at industrial scales, besides contributing to the economic viability of bioethanol-producing processes.

Regarding the ability of fermentation of sugars normally present in lignocellulosic substrates it was possible to confirm the efficacy of the engineered *S. cerevisiae* strain T18_HAA1* in xylose consumption. Consumption of xylose is only happening after glucose

depletion in the medium, however, only for the setting of mixed sugar medium of 100 $g.L^{-1}$ of glucose and 37 $g.L^{-1}$ of xylose (compared with the scenarios of 100 $g.L^{-1}$ glucose + 80 $g.L^{-1}$ xylose and 74 $g.L^{-1}$ glucose + 37 $g.L^{-1}$ xylose) the total consumption of the pentose was observed, resulting in ethanol production from all the sugar present.

In Ethanol Red, a highly ethanol tolerant strain, a slightly increased tolerance to high fermentation temperatures (41 ºC) was attributed to the expression of the *HAA1** mutated gene (through a constructed plasmid). At 41 ºC, the inhibition of glucose consumption, typical effect of elevated temperatures, was surpassed and the ethanol produced by Ethanol Red_HAA1* was used as a carbon source, resulting in an increased biomass production. Additionally, for the first time, the effect of expressing the *HAA1** mutated gene was demonstrated in the phenotype of the Ethanol Red yeast strain.

The industrial strain Ethanol Red with a constructed vector containing the *HAA1** showed a slightly increased tolerance to high temperatures, allowing the yeast to surpass the glucose consumption inhibition, and allowing the use of ethanol as a carbon source, resulting in increased biomass. For the first time, a thermotolerance phenotype was demonstrated in Ethanol Red expressing an extra *HAA1* gene.

This section presents experiments that highlight topics of interest for the general improvement of fermentation processes, regarding yeast strains' natural or induced robustness towards the use of lignocellulosic material as a basis for ethanol production, as well as towards external fermentation constrains, such as high temperatures. Further studies must be done, considering other settings. As the general aim of ethanol production to be used as a fuel is to reduce GHG emissions associated with fuel burn, impact studies such as the one presented in Section 4 should be done, to infer on the real sustainability of the improvements, facing scaling-up propensities.

SECTION 6

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

6. General Discussion, Conclusions, and Future Perspectives

This thesis tried to embrace two topics that are of relevance considering the steps needed to be taken to decrease emissions of GHG to the atmosphere and therefore, accomplish political and industrial objectives in order to reduce global warming: analysis of the impact of ethanol production processes, and improvement of methods for ethanol production. The use of alternative feedstock for fermentation is a central point in upgrading processes, either by using less environmentally impactful 1G substrates or improving effectiveness of 2G fermentation.

In Section 2, the integration of 1G and 2G ethanol production processes are pointed out as being favorable both at environmental and economic levels. The integration of the two types of biorefineries shows decreases in CO_2eq emissions up to 60%. Additionally, engineering of yeast is highlighted as of relevance for the increasing of ethanol productivity.

The performed works have shown that non-mainstream fermentation feedstock can be of industrial interest for the production of bioethanol, both in terms of land competition with food cultures, and in terms of fertilizer and water requirements, which by themselves present economic and environmental drawbacks. Also, scale-up modeling shows the importance of considering the transition between laboratory studies to industrial implementations, when factors such as land-use change and biogenic CO_2 can have great impacts in final GHG emissions, up to 133 g CO_2eq/L_{EtOH} and 750 g CO_2eq/L_{EtOH}, respectively (Section 3).However, works on the environmental impacts of ethanol production processes, that are often used as basis for decision making both by companies and lawmakers, should be carefully done. LCA analysis ought to take into account multiple possible variations caused not only by the selected impact assessment methodologies, but also by variables such as countries' electricity production mix, lifetime of the industrial plant, and considered steps of the production chain. Section 4 shows and concludes that big differences in the results of LCA works can be caused by small changes in the considered inputs for the study, and this is only related to Global Warming Potential. Variations in factors such as countries' electricity mix can lead to variations in GWP results up to 750%, operational lifetime of the factory up to 108% and considerations on different productivities from yeast strains can lead to differences in final emissions per product up to 800%. Environmental impacts can also be studied considering Water Footprint, Energy Demand, Ecosystem Damage, and Human Health, to name a few. Future efforts must be made to encompass as much impact information as possible while considering the great need of uniformization of analysis between processes.

If, on one side, trying alternative 1G feedstock for fermentation can be a way of reducing environmental impacts of the production of ethanol, another is the improvement of yeast strains for more viable use of lignocellulosic (2G) biomass. Factors such as temperature, type and amount of sugar content, and presence of inhibitor chemicals, should be taken into account. Section 5 highlights the influence of temperature on *S. cerevisiae* strains, considering not only ethanol productivity profiles but also taking into account that on an industrial scale, thermotolerance can be of great importance in order to reduce the environmental (and economic) impact of the factory. Strains resistant to high temperatures allow the reduction of the use of cooling systems, which are energy consuming (in some cases, ≈24MJ.L^{-1}_{EtOH}[315]). In these cases, a good three-point ratio between ethanol production versus energy consumption versus CO_2eq emissions/emission reduction must be achieved. Additionally, consumption of as much biomass sugar content is desirable, due to the obvious advantages of increased productivities and reducing of waste. It was shown that an increased temperature led to a diminishing of cell biomass production by *S. cerevisiae* strains BY4741 and Ethanol Red. Growth and fermentation in mixed media with different concentrations of glucose and xylose was also tested, as a simulation of lignocellulosic hydrolysate, with a xylose-consuming yeast strains, GSE16-T18_HAA1*. The assays showed that, even though the strain is capable of xylose uptake, it only begins to consume the pentose after the depletion of glucose, but at a higher rate in the mixed medium 100+37 g.$L^{-1}_{glucose+xylose}$ (compared to 100+80 g.$L^{-1}_{glucose+xylose}$). Ethanol production rate is also slower in the 100+80 g.$L^{-1}_{glucose+xylose}$ setting, given the osmotic stress caused by the higher total sugar concentration.

It was also reported that the specific mutation present in the *HAA1* gene leads to an increased tolerance to acetic acid (when compared with the non-mutated gene), another pivotal factor influencing 2G ethanol production, in the GSE16-T18_HAA1* strain. The *HAA1** gene was cloned into a plasmid vector to be inserted into *S. cerevisiae strains* commonly used for fermentation research studies or in prototrophic industrial strains through the selection for the geneticin resistance marker gene. The influence of *HAA1** in the thermotolerance of Ethanol Red was assessed, for the first time, in strains grown at 41 ºC. The expression of the gene also led of a slightly better ethanol fermentation performance, leading to increased amounts of produced ethanol at 30 ºC and 35 ºC, and an increased general robustness of the strain when grown at 41 ºC, compared with an Ethanol Red strain lacking the mutated *HAA1** gene.

Of undeniable interest, would be the simultaneous construction of genetically manipulated yeasts, or the selection of strains *S. cerevisiae* or of non-conventional yeasts of particular interest, for improved ethanol production from agro-forest-industrial residues and LCA analysis, to give from the beginning an insightful overlook on the large-scale implications of genetic improvements. Yeast genetic background, and consequent phenotypic expression,

can be of great interest on a laboratory scale, but the ultimate exploitation of such improvements in yeast robustness is always essential and for industrial scale, where small biological changes in *S. cerevisiae* can have great impacts on the whole process, mainly regarding the impacts of ethanol production chains in the environment, especially compared with standard fuels, such as gasoline and ethanol from common 1G biomass.

THESIS PUBLICATIONS

THESIS PUBLICATIONS

By chronological order:

Title: Evaluation of Jerusalem artichoke as a sustainable energy crop to bioethanol: energy and CO_2eq emissions modeling for an industrial scenario
Year: 2018
Authors: Susana Paixão, Luís Alves, Rui Pacheco, Carla Silva
Journal: Energy
Type of publication: research article
DOI: 10.1016/j.energy.2018.02.145
Author's contribution: assessment of the system inventory, processing of the scale-up model, accounting for biogenic CO_2 emissions, and final energy and emissions analysis.

Title: Global Warming Potential of biomass-to-ethanol: review and sensitivity analysis through a case study
Year: 2019
Authors: Rui Pacheco, Carla Silva
Journal: Energies
Type of publication: research article
DOI: 10.3390/en12132535
Author's contribution: assembly of inventory, impact assessment (Global Warming Potential), and test on time horizon, assessment report and countries' electricity mix influence on results, assessment of the differences between conventional and dynamic Life Cycle Assessment, and simulation of scenarios considering genetic modification of yeast strains.

Title: Sustainability of sugarcane for energy purposes
Year: 2019
Authors: Carla Silva, Rui Pacheco, Danilo Arcentales, Fernando Santos
Book: Sugarcane biorefinery, technology and perspectives
Type of publication: book chapter
ISBN: 9780128142363
Author's contribution: review on 1G and 2G ethanol, emissions and energy flows of processes, GHG emissions related to ethanol production and input/output inventory

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