UNIVERSIDADE DE SÃO PAULO
CENTRO DE ENERGIA NUCLEAR NA AGRICULTRA

RUBENS PEREZ CALEGARI

Ethanol production from the fermentation of sterilized wort by electron beam followed by methane production from its vinasse

Piracicaba
2021

RUBENS PEREZ CALEGARI

Ethanol production from the fermentation of sterilized wort by electron beam followed by methane production from its vinasse

Revised version according to Resolution CoPGr 6018 from 2011

Thesis presented to Center for Nuclear Energy in Agriculture of the University of São Paulo as a requisite to the Doctoral Degree in Sciences

Concentration Area: Nuclear Energy in Agriculture and Environment

Advisor: Prof. Dr. Valter Arthur
Co-advisor: Prof. Dr. Antonio Sampaio Baptista

Piracicaba

2021

Dados Internacionais de Catalogação na Publicação (CIP)

Seção Técnica de Biblioteca - CENA/USP

Calegari, Rubens Perez

Produção de etanol a partir da fermentação de mosto esterilizado com feixe de elétrons seguida da produção de metano com o uso da vinhaça gerada / Ethanol production from the fermentation of sterilized wort by electron beam followed by methane production from its vinasse / Rubens Perez Calegari; orientador Valter Arthur. - - Versão revisada de acordo com a Resolução CoPGr 6018 de 2011. - - Piracicaba, 2021.

150 p.

Tese (Tese – Programa de Pós-Graduação em Ciências. Área de concentração: Energia Nuclear na Agricultura e no Ambiente) – Centro de Energia Nuclear na Agricultura da Universidade de São Paulo, 2017.

1. Acelerador de elétrons 2. Biocombustíveis 3. Biogás 4. Cana-de-açúcar 5. Digestão anaeróbica 6. Elementos traço 7. Etanol 8. Fermentação alcoólica 9. Metano 10. Suplementação de metais I. Título.

CDU (663.252.4 : 628.349)

Elaborada por:
Marilia Ribeiro Garcia Henyei
CRB-8/3631
Resolução CFB Nº 184 de 29 de setembro de 2017

To all the people who had their lives cut short due to complications from the COVID-19 pandemic.

ACKNOWLEDGEMENTS

Carry out the doctorate, and in particular, the execution of this thesis was a challenging journey for me as a person and as a professional. This thesis was conceived in 51 months and each page was created with the help of several people to whom I will be forever grateful for. Unfortunately, I do not have a good memory, and there are too many names to remember. If I did not mention you, I apologize. Thank you very much, everyone!

To god and my spiritual guides, for the protection and guidance during this life journey.

To my parents Valdomiro and Isolete, for unconditional love and support. Thank you for believing in me and encouraging me to do my best always!

To my sister Renata, a future biologist and unconditional animal lover. I am so proud of you!

To Luis Guilherme for all the support, patience, and companionship during this very important period of my life.

To my advisors Prof. Dr. Valter Arthur and Prof. Dr. Antonio Sampaio Baptista for the support and professional advice. Thank you for all the opportunities and for believing in my potential. You have helped me become the professional I am today!

To Center for Nuclear Energy in Agriculture of the University of São Paulo (CENA-USP) and for the Graduate Program of Science for the opportunity to pursue a doctorate and improve my knowledge.

To the Graduate Office of CENA-USP, in special Cleide, Gilson, Magali, and Marcos for all the support during my PhD journey.

To the Library Office of CENA-USP, in special Marilia for the support with the thesis deposit.

To GTSBio Group from ESALQ-USP, in special Eric, Ana and Layna, thank you for your help in the experiments. I am also grateful to my work colleagues and great friends Manuella, Gabriela, Bianca, Laysa, Tamires, Pietro, Matheus, Eduardo, Maria Carolina, Marcelo, Gustavo, and Victor for the nice discussions and good moments that we shared in the lab.

To the sucroenergetic plants Iracema (São Martinho SA – Iracemápolis, SP, Brazil) and Granelli (Charqueada, SP), and the biogas plant Tekniska Verken (Linköping AB – Linköping, Sweden) for the donated material used in the experiments.

To the technicians Rose, Sylvino, and Pedro of the Department of Agri-food Industry Food and Nutrition of ESALQ-USP for the support in the laboratory.

To my Sandwich Ph.D. supervisor Prof. Dr. Alex Enrich Prast, thank you very much for welcoming me as best as possible at Linköping University and Sweden. I am also grateful for your friendship and advice.

To the members of the Biogas group of Linköping University for the valuable discussions, scientific contributions, and opportunities to share our results in the Biogas Research Center meetings. I would especially like to thank Luka, Sepehr, Annika, Mette, Betina, Eva-Maria, Jan, Baitong, and Prince for direct or indirect collaboration in this thesis. My gratitude also extends to the Research Engineers Mårten, Ingrid, Sussane, and Duc from the Environmental Change lab (Tema M) of Linköping University for all the help in the biogas experiments and analyzes.

To my friends that showed me the best of Sweden Luka, Milda, Gregg, Laura, Thuane, Betina, Johan, Marina, Alex, Angela, Victor, Baitong, and Prince. Tack så mycket for all the fikas, trips, frisbee golf games, barbecues, and ice skating! You will always be in my memory and in my Swedish flag!

To the friendships I made in Piracicaba during the master's and doctoral degrees Bruno, Igor, Flávio, Murilo, and Marcos, which provided me with unforgettable moments and reminded me that there is life outside the university environment! Also, a special thank you to the old friends from Francisco Beltrão, who even with the distance remained close during these years Roger, Adriana, Eva, Maria Angélica, Juliano, Dieicon, and Jean.

To all the professionals who are fighting the COVID-19 pandemic. Thank you very much health professionals, vaccine researchers, and all the people directly and indirectly engaged in combat this virus!

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [Grants number 202165/2019-1 and 140184/2019-8]. This work was also supported by the Swedish Energy Agency through the Biogas Research Center (BRC) in Linköping [Grant number: 35624-2].

" We can only see a short distance ahead, but we can see plenty there that needs to be done"

Alan Turning

ABSTRACT

RUBENS, R. P. **Ethanol production from the fermentation of sterilized wort by electron beam followed by methane production from its vinasse**. 2021. 150 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2021.

Microbial contamination present in the ethanol fermentation process negatively affects ethanol efficiency and productivity. Currently, to control bacterial contamination, the sucroernegetic industries carry out the acidic treatment of yeast, however, this causes damage to the fermentation, as it promotes stress to the yeast, allows the presence of bacteria and the replacement of the selected yeast during fermentation. Besides, acid treatment is responsible for high levels of sulfate in vinasse, which, when digested, generates H_2S, a toxic compound which interferes negatively in the methanogenesis process. For these reasons, the present work proposes the production of ethanol from wort sterilized by electron beam as a substitute for the acid treatment, and anaerobic digestion of the vinasse from the process. The present study was conducted in three stages. First, tests of wort sterilization by electron beam were performed. Second, the fermentation of the irradiated worts was carried out. Third, the anaerobic digestion (AD) of vinasse and the anaerobic co-digestion (AC) of vinasse with filter cake and sugarcane straw were evaluated. Contingencies did not allow the generation of sufficient vinasse for conducting the third stage; therefore, it was decided to use sulfate-rich vinasse from the industry, and to test its AD and AC with the addition of the trace elements (TE) Fe, Ni, Co, Se, Mo, and Zn, in order to circumvent the problems caused by the high sulfate concentration. In the first stage, only the radiation dose of 80 kGy was able to sterilize the wort, however, 40 kGy was sufficient to inactivate >99.99% of the contaminating microorganisms. In addition, there was no sugar inversion and no formation of the inhibitory compounds' flavonoid, furfural, and 5-HMF. In the fermentation of the irradiated worts the dose of 20 kGy promoted the highest ethanol yield among the tested worts (0, 10, 20, and 40 kGy), showing the possibility of using electron beam for the treatment of wort, which allows decrease in losses caused by microbial contamination, in addition, allows increase in fermentation yield and productivity. In the AD and AC experiments, iron played an important role in decreasing the H_2S production. In addition, the other metals made it possible to increase the methane yield. The vinasse AD and TE reactor (R2) reached a yield between 222.29-411.39 $NmlCH_4\ g^{-1}$ VS, and the H_2S in biogas remained below 500 ppm. The control reactor that did not received TE (R1) reached 36,000 ppm of H_2S and failed due to acidification. However, after pH correction and TE addition, it

was possible to re-establish AD in R1. In the AC reactors R3 and R4 after the addition of all TE, a yield between 54.61-109.14 $NmlCH_4\ g^{-1}$ VS was observed in R3, and between 54.15-110.21 $NmlCH_4\ g^{-1}$ VS in R4. Moreover, the H_2S concentration remained below 186.42 ppm in R3 and below 70.74 in R4. In general, anaerobic co-digestion with the addition of metals maintained greater stability over the 238 days of experiment, with low accumulation of volatile fatty acids and H_2S.

Keywords: Alcoholic fermentation. Anaerobic co-digestion. Biofuel. Biogas. Electron accelerator. Sugarcane.

RESUMO

CALEGARI, R. P. **Produção de etanol a partir da fermentação de mosto esterilizado com feixe de elétrons seguida da produção de metano com o uso da vinhaça gerada**. 2021. 150 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2021.

A contaminação microbiana presente no processo de fermentação alcoólica afeta negativamente a eficiência e a produtividade de etanol. Atualmente, para controlar a contaminação bacteriana, as indústrias sucroenergéticas realizam o tratamento ácido do fermento, porém, este causa prejuízos para a fermentação, pois causa estresse ao fermento, permite a presença de bactérias e a substituição da levedura selecionada durante a fermentação. Além disso, o tratamento ácido é responsável por altos teores de sulfato na vinhaça, que ao ser digerida, gera H_2S, composto tóxico e que interfere negativamente no processo de metanogênese. Por esses motivos, o presente trabalho propõe a produção de etanol a partir de mosto esterilizado por feixe de elétrons em substituição ao tratamento ácido e a biodigestão anaeróbia da vinhaça proveniente do processo. O presente estudo foi conduzido em três etapas, na primeira foram realizados testes de esterilização com feixe de elétrons, na segunda a fermentação de mosto irradiado e na terceira etapa foi avaliada a digestão anaeróbia (DA) de vinhaça e a codigestão anaeróbia (CA) de vinhaça com torta de filtro e palha de cana-de-açúcar. Imprevistos não permitiram a geração de volume de vinhaça suficiente para a condução da terceira etapa, portanto, optou-se por utilizar vinhaça rica em sulfato proveniente da indústria, e testar sua DA e CA com adição dos elementos traço (ET) Fe, Ni, Co, Se, Mo e Zn, com objetivo de contornar os problemas causados pela alta concentração de sulfato. Na primeira fase apenas a dose de radiação de 80kGy foi capaz de esterilizar o mosto, no entanto, 40 kGy foi suficiente para inativar >99.99% dos micro-organismos contaminantes. Além disso, não houve inversão de açúcares nem formação dos compostos inibitórios flavonoides, furfural e 5-HMF. Na fermentação do mosto irradiado, a dose de 20 kGy promoveu o maior rendimento alcóolico entre os mostos testados (0,10, 20 e 40 kGy), evidenciando a possibilidade do uso do feixe de elétrons para o tratamento do mosto, o que possibilita redução nas perdas causadas pela contaminação microbiana, além de permitir aumento no rendimento e produtividade fermentativa. Nos ensaios de DA e CA o ferro desempenhou um papel importante na diminuição da produção de H_2S, ademais, os outros metais possibilitaram aumentar o rendimento de metano. O reator de DA de vinhaça e ET (R2) atingiu rendimento entre 222,29-411,39 $NmLCH_4g^{-1}VS$, e o H_2S no biogás permaneceu abaixo de 500 ppm.

Já o reator controle que não recebeu ET (R1) chegou a 36,000ppm de H_2S e apresentou falha devido acidificação. No entanto, após a correção do pH e adição de ET foi possível restabelecer a DA em R1. Nos reatores de CA R3 e R4 após a adição de todos os ET observou-se rendimentos entre 54,61-109,14 $NmLCH_4g^{-1}VS$ em R3 e entre 54,15-110,21 $NmLCH_4g^{-1}VS$ em R4. Além disso, a concentração de H_2S manteve-se abaixo de 186,42 ppm em R3 e abaixo de 70,74 em R4. De forma geral, a codigestão anaeróbia com adição dos metais manteve maior estabilidade ao longo dos 238 dias de ensaio, com baixo acúmulo de ácidos graxos voláteis e H_2S.

Palavras-chave: Fermentação alcoólica. Codigestão anaeróbia. Biocombustível. Biogás. Acelerador de elétrons. Cana-de-açúcar.

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

5-HMF 5-hydroxymetylfurfural

AC Anaerobic co-digestion

AD Anaerobic digestion

ANEEL National Electricity Agency (Agência Nacional de Energia Elétrica, ANEEL in Portuguese acronyms)

ANOVA Analysis of variance

AnSBBR Anaerobic sequencing bath biofilm reactor

BMP Biochemical methane potential

CBC Central Bank of Brazil

CCEE Brazilian Wholesale Market Operator (Câmara de Comercialização de Energia Elétrica, CCEE in Portuguese acronyms)

CFU Colony forming Unit

COD Chemical oxygen demand

CSTR Continuos stirred-tank reactor

CTR Technological Radiation Center (Centro de Tecnologia das Radiações in Portuguese acronyms)

CV Coeficient of variation

DDG Distillers dried grain

E-beam electron beam

EPS Extracellular polymeric substances

ESALQ Luiz de Queiroz College of Agriculture (Escola Superior de Agricultura “Luiz de Queiroz” in Portuguese acronyms)

GC-FID Gas chromatography -Flame ionization detector

GHG	Greenhouse gases
HRT	Hydraulic retention time
ICP-MS	Inductively coupled plasma mass spectrometry
IPEN	Nuclear and Energy Research Institute (Instituto de Pesquisas Energéticas e Nucleares in Portuguese acronyms)
IR	Ionizing radiation
LCFA	Long-chain fatty acids
LoQ	Limit of quantification
nd	not detected
OHPA	Obligate hydrogen-producing acetogens
OLR	Organic loading rate
PCA	Plate count agar
pDB	propionate degrading bacteria
ppm	parts per million
RenovaBio	Brazilian National Biofuel Policy
RPM	Revolutions per minute
SAL	Sterilization Assurance Level
SEM	Scanning electron microscope
SRB	Sulfate-reducing bacteria
TE	Trace elements
TEMA-M	Department of Thematic Studies
TRS	Total reducing sugars
TS	Total solids

UASB	Upflow anaerobic sludge blanket reactor
VFA	Volatile fatty acids
VS	Volatile solids

LIST OF SYMBOLS

A	Ampere
$AlCl_3$	aluminium chloride
ATM	atmospheric pressure
˚Brix	degrees Brix
Ca^{2+}	calcium cation
$CaCO_3$	calcium carbonate
Cd	cadmium
CH_4	methane
CH_3CO_2H	acetic acid
Cl^-	chloride anion
Co	cobalt
Co^{60}	radioactive isotope of cobalt of the mass number 60
CO_2	carbon dioxide
$CoCL_2.6H_2O$	cobalt chroride hexahydrate
C :N	carbon-to-nitrogen ration
Cu	copper
D_{10}	required dose to destroy 90% of the population or 1 log
e^-	electron
e_{aq}	solvated electron
Fe	iron
$FeCl_3$	iron chloride
$FeS_{(s)}$	iron sulfide (solid)
$FeS_{(aq)}$	iron sulfide (aqueous)
Fl^-	Fluoride anion
g	g-force

h	hour
H^+	hydrogen radical
H	hydrogen atom
H_2	molecular hydrogen
Ha	hectare
HNO_3	nitric acid
H_2O_2	hydrogen peroxide
H_2O^+	oxoniumyl
HO_2	superoxide radical
H_2S	hydrogen sulfide
J	Joule
K^+	potassium cation
kGy	kilogray
km	kilometer
kWh	kilowatt-hour
Li	lithium
Li^+	lithium cation
Log	logarithm
M	molar concentration
m^3	cubic meter
MeV	megaelectronvolt
Mg	Megagram
Mg	magnesium
mg	milligram
Min	minutes
ml	milliliter
mM	millimolar

Mo	molybdenum
MWh	megawatt-hour
N_2	nitrogen
Na^+	sodium cation
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
$Na_2SeO_3.5H_2O$	sodium selenite pentahydrate
NH_4^+	ammonium cation
Ni	nickel
$NiCl_2.6H_2O$	Nickel chloride hexahydrate
NmL	Normal milliliter
N_2O	nitrous oxide
NO_3^-	nitrate anion
NaH_2PO_4	sodium phosphate monobasic
$NaMoO_4.2H_2O$	sodium molybdate dihydrate
NaOH	sodium hydroxide
nm	nanometer
Nm^3/ year	normal cubic meter per year
OH^-	hydroxyl radical
pH	power of hydrogen
$PO4^{3-}$	phosphate anion
S	sulfur
Se	selenium
S :F	Sulfur to iron ratio
SO_4^{2-}	sulfate ion
US$	United States dollar
V	Volt

Zn	zinc
ZnCl2	zinc chloride
W	tungsten
W-L	Wood-Ljungdahl pathway
γ	Gamma radiation
μg	microgram
μl	microliter
μm	micron
%w/w	weight per weight
%v/v	volume per volume
%w/v	weight per volume
˚C	Celsius degree
Δt	Processing time

SUMMARY

1. INTRODUCTION

With the intention of reducing greenhouse gases and increase environmental, economic, and social sustainability, the new Brazil's National Biofuels Policy (RenovaBio Program) was created, instituted by decree Law 13.576/2017 (BRASIL, 2017), which aim to decarbonization of transport activities by stimulating the production of various biofuels, such as ethanol, biodiesel and biogas.

Brazilian ethanol is obtained mostly from sugarcane by fermentation, using the yeast *Saccharomyces cerevisiae*. The efficiency of this biological agent in transforming the sugar from the sugarcane juice or molasses into alcohol depends a lot on the quality of the wort to be fermented. Typically, this substrate is not sterilized before being used, which allows a large number of contaminants to enter into the process, which negatively affects alcoholic fermentation efficiency and productivity (STUPIELLO; HORII, 1981; BONATELLI; IENCZAK; LABATE, 2019).

The sterilization of the wort could provide reduction of costs and losses from contamination, such as the use of antibiotics, defoamers, acid treatment of the yeast cream, and consumption of other substances (NOLASCO JUNIOR, 2010). Besides, a sterile wort may allow the use of selected yeast strains during the entire harvest period.

It could also reduce the yeast recycling time in the industrial unit, which increases productivity over time. Another interesting aspect is that it allows having a wine with similar microbiological characteristics during the harvest, which makes it easier it obtain special alcohols, such as neutral alcohol. This means the possibility to optimize the process, reduce costs and increase productivity. It should be noted that some technologies, which today are not usual due to the lack of stability of the process, can become viable if working with sterile wort and aseptic conditions (NOLASCO JUNIOR, 2005).

There are several methods that can be used for the sterilization of the wort, the substrate for alcoholic fermentation. However, in industrial plants such as the ethanol industry, where the volume of wort processed is huge, the traditional methods like conventional heat and chemical agents can be very costly. On the other hand, this industry has an energy surplus (CERVI et al., 2019), which can help to circumvent this problem, or rather,

is self-sufficient in energy, and part of this energy can be used to generate a source to sterilize the wort, such as electron beam, a type of ionizing radiation.

Some studies have demonstrated the efficiency of the electron beam application in disinfection and sterilization of medium such as industrial wastewater. It has been found that a dose of 1kGy allows the elimination of approximately 90% of the bacteria and 7 kGy could promote sterilization of the medium (SKOWRON et al., 2013). In addition to the microbial control, other applications of ionizing radiation have also been successfully studied, such as the reduction of anionic surfactants, thereby facilitating the organic matter fraction degradation of treated wastewater and helping the water recovery (MORAES et al., 2004; NASIR et al., 2010).

The possibility of expanding the sucroenergetic industry, coupled with a demand that gradually increases, arises along with the concern of the generated wastes destination, since these wastes have a high pollution potential to the environment (CHRISTOFOLETTI et al., 2013). However, with the current available technologies these residues can no longer be a nuisance for the industry and become by-products used to new products generation (FREITAS et al., 2019).

Vinasse, the main residue from ethanol production, is a liquid effluent rich in organic matter, mineral nutrients, and water. Due to that, it is possible the use of vinasse for biogas production through anaerobic digestion (PARSAEE; KIANI DEH KIANI; KARIMI, 2019).

The anaerobic digestion of vinasse for biogas production has been shown to be an interesting process because it promotes the vinasse organic load reduction, production of biogas rich in methane, and the waste of this process (digestate) can be used as a plant fertilizer (LAMONICA, 2006; PARSAEE; KIANI DEH KIANI; KARIMI, 2019). Besides, the production of biogas (or biomethane after purification and upgrading) is of great importance for the RenovaBio Program (KLEIN et al., 2019).

Nevertheless, the presence of sulfate in the substrate used for anaerobic digestion may be responsible for inhibition of the process, especially the production of methane by methanogenic archaea (CHEN; CHENG; CREAMER, 2008) and increase the hydrogen sulfide (H_2S) production by sulfate-reducing bacteria (SRB), which is a highly corrosive, toxic and unpleasant odor gas (MUYZER; STAMS, 2008).

The high concentration of sulfate in the vinasse (between 500 and 3000 mg SO_4^{-2} l^{-1}) is attributed to the residual sulfuric acid from the acid treatment of the yeast cream in the fermentation process (LENS et al., 1998). If this sulfate concentration could be reduced, the anaerobic digestion of the vinasse would occur more efficiently with greater production of biogas rich in methane.

Another alternative to avoid the problems caused by sulfate presence in anaerobic digestion is the co-digestion with other residues and the addition of metals, such as Fe, Ni, Co, Mo, Se, and Zn in the form of trace elements. The co-digestion with other organic residues of great importance for the sucroenergetic industry, such as sugarcane straw and filter cake (also known as cake mud or filter mud) can provide greater balance of nutrients and dilution of methanogenic inhibitors present in the vinasse such as sulfate, providing a bioprocess with greater stability (MATA-ALVAREZ, 2002; SARKER et al. 2019; SATOTO et al., 2010).

Furthermore, the addition of trace elements may provide a boost in the process through correction of the deficiency of micronutrients required by the anaerobic process (ZITOMER; JOHNSON; SPEECE, 2008). The iron, for example, can precipitate sulfur, preventing the inhibition of methanogenesis by sulfate-reducing bacteria and the formation of H_2S in biogas (SHAKERI YEKTA et al., 2017). In addition, the sulfate precipitation by iron provides better bioavailability of trace elements for the microbial consortium (SPEECE, 2008).

For these reasons, it is believed that the use of electron beam is a good strategy for sterilization of the wort, as it allows it to be installed on the ethanol production line and can use part of the surplus energy of the sucroenergetic industry. Thus, resulting in greater efficiency in the ethanol fermentation process and, consequently, in the anaerobic digestion of the vinasse.

1.1.Objectives

1.1.1. General objective

This thesis aims to obtain better quality wort after electron beam application. It is expected less sugar losses due to the action of contaminating agents, greater fermentative efficiency, obtain a vinasse with lower sulfate content, possible conversion of vinasse into biogas with lower hydrogen sulfide (H_2S) concentration, and, thus, reduce production costs, increase productivity and the production of ethanol and biogas.

1.1.2. Specific objectives

As this work was carried out to study alcoholic fermentation and anaerobic digestion, the specific objectives were separated for each process.

Alcoholic fermentation

i) Evaluate the efficiency of wort sterilization with electron beam.

ii) Evaluate the yield and productivity of alcoholic fermentation in wort after electron beam sterilization.

Anaerobic digestion

i) Evaluate the organic matter reduction of the vinasse and its co-digestion with sugarcane straw and filter cake.

ii) Evaluate the methane yield between processes.

iii) Evaluate the production and toxicity of H_2S between treatments.

iv) Evaluate the supplementation of the substrate with iron and the trace elements Ni, Co, Se, Mo, Zn and their impact on anaerobic digestion/co-digestion.

1.2.Changes in the original scope of the thesis

The Thesis Project was initially composed of three phases. The first phase consisted of testing on a bench scale the most appropriate irradiation dose to sterilize the wort and not compromise its quality for alcoholic fermentation. The second planned phase would be to carry out the sterilization and fermentation test on a pilot scale. The third and final phase would consist of evaluating the process of anaerobic digestion and biogas production from the vinasse generated in the second phase. However, a series of unforeseen events occurred during the project, which made us change some strategies so that we could complete the study. The contingencies and decision making are described below.

The first phase took place as planned, however, the second phase, which was scheduled to be carried out at the pilot ethanol production plant located in the Sugar and Alcohol Sector of ESALQ/USP could not be carried out. There were delays in the installations of the pilot plant

(which was not yet ready at the beginning of this project). In addition, there was a need to sterilize a large volume of wort to be able to conduct fermentation in 0.5 m^3 fermentation tanks and thus, generate a large volume of vinasse sufficient for conducting anaerobic digestion tests in reactors.

The first alternative considered was to sterilize the must near the pilot plant by hiring a service provider that has a mobile unit with an electron accelerator attached to a truck. Hiring this service would facilitate the experiments logistics, since the truck could be attached to the pilot plant during the experiments. However, contracting this service was not possible due to the unavailability of the service provider.

The second alternative was to sterilize the wort at IPEN/USP, where the first phase of the project was conducted. However, a series of adaptations would be necessary in the electron accelerator of this unit, since it works in a batch way and the process would have to be carried out continuously for possible sterilization of a large volume of wort.

Another obstacle was the logistics for conducting these activities, as IPEN is located in the city of São Paulo and ESALQ's pilot plant is located in Piracicaba, a distance of 155 km between the units. What would need more human resources to carry out.

So, as an alternative for completing the studies, it was decided to use vinasse from a sucroenergetic industry of the region. This industry, like the others, does not sterilize the wort and does the acid treatment of the yeast cream, which consequently generates a vinasse with a high concentration of sulfate.

To overcome the problems resulting from the high concentration of sulphate in the vinasse, the alternative thought was to add iron (Fe) to the substrate of the reactors for the sulfur (S) precipitation and its problems in anaerobic digestion minimized, based on recommendations of Shakeri Yekta (2014) and Speece (2008). It was also decided to study the addition of other metals in the form of trace elements (Cobalt, Nickel, Selenium, Zinc and, Molybdenum) towards optimization of the anaerobic digestion process, based on Gustavsson (2012) and Hendriks et al. (2018) recommendations.

In addition, it was decided to study the anaerobic codigestion of vinasse with other residues from the sucroenergetic industry (sugarcane straw and filter cake), to further minimize the effects of the presence of S in the vinasse and obtain greater production and biogas productivity in the industry. For this, the experiment was carried out in 4 anaerobic reactors of

the CSTR type (R1, R2, R3 and R4). Reactor R1 used only vinasse as a substrate, reactor R2 received vinasse with addition of Fe and trace elements, reactor R3 used vinasse, sugarcane straw and filter cake as substrate and reactor R4 received the same substrate as R3 but with addition of Fe and trace elements.

It should be noted that this alternative for the thesis tests was only possible because this experiment was carried out at the University of Linköping during the sandwich doctoral period (September 2019 to August 2020).

In March 2020 the covid-19 pandemic spread worldwide and reached Sweden. Restrictions have been imposed by the government and the University of Linköping to prevent the spread of the virus. Despite that, the Biogas Group, and the Department of Thematic Studies (TEMA-M) allowed and prioritized the continuation of this study during the pandemic following social distance protocol. However, a fire in the laboratory on June 10, 2020, prevented the continuation of the anaerobic digestion studies, which ended. These experiments were carried out for 238 days.

References

BRASIL. Lei No 13.576, de 26 de Dezembro de 2017. Dispõe sobre a Política Nacional de Biocombustíveis (RenovaBio) e dá outras providências. **Diário Oficial da União**, Brasília, DF, 27 dez. 2017. Seção 1, p. 4. Available at: <http://www.planalto.gov.br/ccivil_03/_ato2015-2018/2017/lei/l13576.htm>.

BONATELLI, M. L.; IENCZAK, J. L.; LABATE, C. A. Sugarcane must fed-batch fermentation by *Saccharomyces cerevisiae*: impact of sterilized and non-sterilized sugarcane must. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, v. 112, n. 8, p. 1177–1187, 2019.

CERVI, W. et al. Bioelectricity potential from ecologically available sugarcane straw in Brazil : A spatially explicit assessment. **Biomass and Bioenergy**, v. 122, p. 391–399, 2019. Available at: <https://doi.org/10.1016/j.biombioe.2019.02.001>.

CHEN, Y.; CHENG, J. J.; CREAMER, K. S. Inhibition of anaerobic digestion process: A review. **Bioresource Technology**, v. 99, n. 10, p. 4044–4064, 2008.

CHRISTOFOLETTI, C. A. et al. Sugarcane vinasse: Environmental implications of its use. **Waste Management**, v. 33, n. 12, p. 2752–2761, 2013. Available at: <http://dx.doi.org/10.1016/j.wasman.2013.09.005>.

FREITAS, F. F. et al. The Brazilian market of distributed biogas generation: Overview, technological development and case study. **Renewable and Sustainable Energy Reviews**, v. 101, p. 146–157, 2019. Available at: <https://doi.org/10.1016/j.rser.2018.11.007>.

GUSTAVSSON, J. **Cobalt and nickel bioavailability for biogas formation**. Linköping, Sweden: Linköping University, Department of Thematic Studies, Water and Environmental Studies, 2012. 64 p. (Linköping Studies in Arts and Science, n. 549).

HENDRIKS, A. T. W. M.; VAN LIER, J. B.; DE KREUK, M. K. Growth media in anaerobic fermentative processes: The underestimated potential of thermophilic fermentation and anaerobic digestion. **Biotechnology Advances**, v. 36, n. 1, p. 1–13, 2018. Available at: <https://doi.org/10.1016/j.biotechadv.2017.08.004>.

KLEIN, B. C. et al. Low carbon biofuels and the New Brazilian National Biofuel Policy (RenovaBio): A case study for sugarcane mills and integrated sugarcane-microalgae biorefineries. **Renewable and Sustainable Energy Reviews**, v. 115, art. 109365, 2019. Available at: <https://doi.org/10.1016/j.rser.2019.109365>.

LAMONICA, H. M. Potencial de geração de excedentes de energia elétrica com o biogás produzido a partir da biodigestão da vinhaça na indústria sucro-alcooleira brasileira. In: Encontro de Energia no Meio Rural 6., 2006, Campinas. **Proceedings online**. Available at: <http://www.proceedings.scielo.br/scielo.php?script=sci_arttext&pid=MSC0000000022006000200027&lng=en&nrm=abn>.

LENS, P. N. L. et al. Biotechnological treatment of sulfate-rich wastewaters. **Critical Reviews in Environmental Science and Technology**, v. 28, n. 1, p. 41–88, 1998. Available at: <https://www.tandfonline.com/doi/abs/10.1080/10643389891254160>.

MATA-ALVAREZ, J. **The biomethanization of the organic fraction of municipal solid waste**. London: IWA publishing, 2002. 336 p.

MORAES, M. C. F. et al. Whole acute toxicity removal from industrial and domestic effluents treated by electron beam radiation: Emphasis on anionic surfactants. **Radiation Physics and Chemistry**, v. 71, n. 1–2, p. 463–465, 2004.

MUYZER, G.; STAMS, A. J. M. The ecology and biotechnology of sulphate-reducing bacteria. **Nature Reviews Microbiology**, v. 6, n. 6, p. 441–454, 2008. Available at: <https://www.nature.com/articles/nrmicro1892>.

NASIR, N. M. et al. Decomposition and biodegradability enhancement of textile wastewater using a combination of electron beam irradiation and activated sludge process. **Water Science and Technology**, v. 62, n. 1, p. 42–47, 2010. Available at: <http://iwaponline.com/wst/article-pdf/62/1/42/446174/42.pdf>.

NOLASCO JUNIOR, J. **Desenvolvimento de processo térmico otimizado para mosto de caldo de cana na fermentação alcoólica**. 2005. 181 p. Dissertação (Mestrado em Ciências de Alimentos) – Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, 2005.

NOLASCO JUNIOR, J. **Eficiência de processo térmico para mostos a base de caldo de cana e melaço na produção de bioetanol.** 2010. Tese (Doutorado em Ciência de Alimentos) - Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, 2010.

PARSAEE, M.; KIANI DEH KIANI, M.; KARIMI, K. A review of biogas production from sugarcane vinasse. **Biomass and Bioenergy**, v. 122, p. 117–125, 2019. Available at: <https://doi.org/10.1016/j.biombioe.2019.01.034>.

SARKER, S. et al. A review of the role of critical parameters in the design and operation of biogas production plants. **Applied Sciences**, v. 9, n. 9, art. 1915, 2019. https://doi.org/10.3390/app9091915.

SATOTO N. E.; GALLERT, C.; WINTER, J. Co-Digestion of Press Water and Food Waste in a Biowaste Digester for Improvement of Biogas Production. **Bioresource Technology**, v. 101, n. 18, p. 6987-6993, 2010. Available at: <https://doi.org/10.1016/j.biortech.2010.03.123>.

SHAKERI YEKTA, S. **Chemical speciation of sulfur and metals in biogas reactors**: implications for cobalt and nickel bio-uptake processes. Linköping, Sweden: Linköping University, Department of Thematic Studies, Environmental Change, 2014. (Linköping Studies in Arts and Science, n. 637).

SHAKERI YEKTA, S. et al. Importance of sulfide interaction with iron as regulator of the microbial community in biogas reactors and its effect on methanogenesis, volatile fatty acids turnover, and syntrophic long-chain fatty acids degradation. **Journal of Bioscience and Bioengineering**, v. 123, n. 5, p. 597–605, 2017. Available at: <http://dx.doi.org/10.1016/j.jbiosc.2016.12.003>.

SKOWRON, K. et al. Radiation hygienization of cattle and swine slurry with high energy electron beam. **Radiation Physics and Chemistry**, v. 87, p. 88–96, 2013.

SPEECE, R. E. **Anaerobic biotechnology and odor/corrosion control for municipalities and industries**. Nashville: Archae Press, 2008.

STUPIELLO, J. P.; HORII, J. Considerações sobre tratamentos de caldo de cana para fermentação alcoólica. **Álcool e Açúcar**, n. 3, p. 40–46, 1981.

ZITOMER, D. H.; JOHNSON, C. C.; SPEECE, R. E. Metal stimulation and municipal digester thermophilic/mesophilic activity. **Journal of Environmental Engineering**, v. 134, n. 1, p. 42–47, 2008. Available at: <https://ascelibrary.org/doi/abs/10.1061/%28ASCE%290733-9372%282008%29134%3A1%2842%29>.

2. LITERATURE REVIEW

2.1. Alcoholic fermentation and bacterial contamination

Currently, Brazil is the world's largest producer of sugarcane, having this great importance for world agribusiness. The harvest estimate for 2020/21 in Brazil is 665.1 million tons, which indicates an increase of 3.5% in relation to the previous one (CONAB, 2020).

Among the most diverse by-products derived from sugarcane, sugar and ethanol stand out economically. Besides, the sugarcane industry, also known as the sucroenergetic industry, stands out in the biofuels sector, as it is responsible for the generation of renewable fuel that its production does not affect the ozone layer (CONAB, 2020).

The commercial production of ethanol in Brazil for fuel purposes occurs through the biological pathway, which starts with sugar-rich raw material, sugarcane juice or sugarcane molasses, which is subjected to the fermentation process and then results in alcohol, the main product enzymatic activity of *Saccharomyces cerevisiae* yeast (LEÃO, 2002).

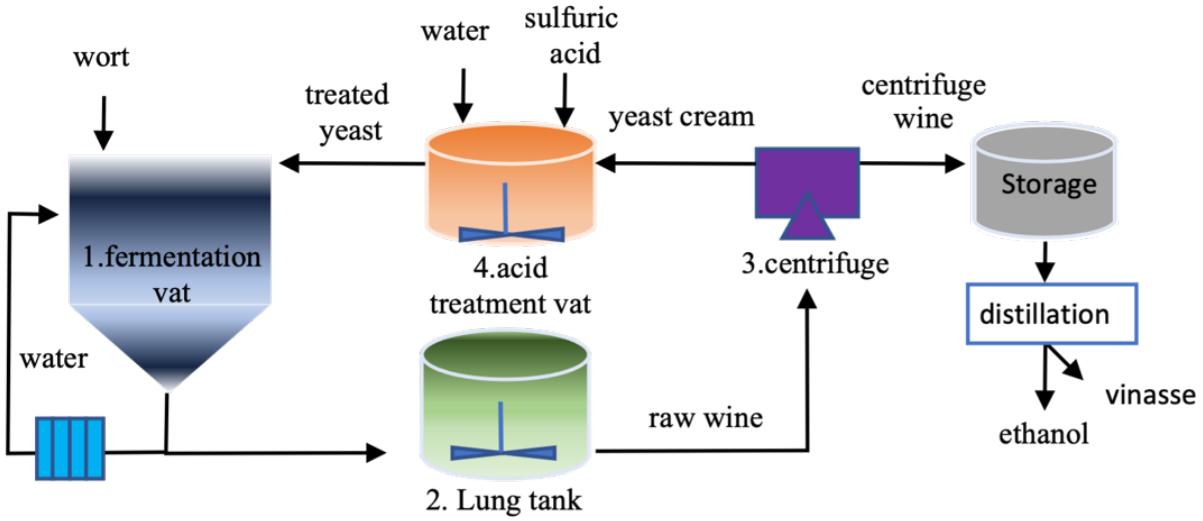
The efficiency of this biological agent in transforming the sugarcane juice into alcohol depends a lot on the quality of the wort to be fermented. Usually, this substrate is not sterilized before being used, which allows a large number of bacterial contaminants to enter the process, which negatively affects efficiency and productivity (STUPIELLO; HORII, 1981).

The most important damages caused by bacterial contamination are the degradation of sucrose, formation of lactic and acetic acids that cause loss of sugars (FREDERICK, 1994; OLIVA NETO; YOKOYA, 1997), the lysis of yeasts (RIBÉREAU-GAYON et al., 2006), and the increase in flocculation, which consequently can reduce the yeast viability (CHERUBIN, 2003; RAVNÖ; PURCHASE, 2005).

In alcoholic fermentation, gram-positive bacteria are predominant with the genera *Bacillus* and *Lactobacillus* being the most frequent. Besides the production of acids that can inhibit the alcoholic fermentation, the bacterial contamination can form polysaccharides, which increase the viscosity of the broth (AMORIM; OLIVEIRA; CAMPOS, 1981; GRAVES et al., 2006).

To control bacterial contamination, the ethanol plants carry out the acid treatment of yeast at the end of each fermentation cycle. The yeast cells are centrifuged and washed with sulfuric acid and then returned to the fermenter tank to start a new fermentation cycle (Figure 2.1). This process, named Melle-Boinot, is repeated throughout the harvest, which lasts about 200 days a year. However, wild yeast strains quickly contaminate the yeast cream and after a few cycles, only wild strains survive acid treatment (BROWN et al., 2013).

Figure 2.1 - Melle-Boinot process scheme. Font: Adapted from Nunes and Finzer (2019)



The acid treatment is responsible for several stresses in yeast cells, such as high osmotic pressure and low pH. As a response to osmotic stress and bacterial contamination, the yeast produces a greater amount of glycerol, thereby reducing the fermentation yield (BASSO; BASSO; ROCHA, 2011).

Thereafter, part of the sulfuric acid used in this treatment is loaded into the fermentation vat, being present throughout the fermentation process and presenting itself residually in the effluent of the wine distillation process, vinasse (BROWN et al., 2013).

Another common way to control bacterial contamination is the use of antibiotics, but bacterial resistance to antibiotics has been a limiting factor in the efficiency of controlling contamination (MUTHAIYAN; LIMAYEM; RICKE, 2011).

In this case, the use of sterilized wort to produce alcohol may allow the use of selected yeast strains, with desirable and stable characteristics throughout the entire harvest. In addition, it is possible to reduce costs by eliminating and/or reducing the use of antibiotics, defoamers,

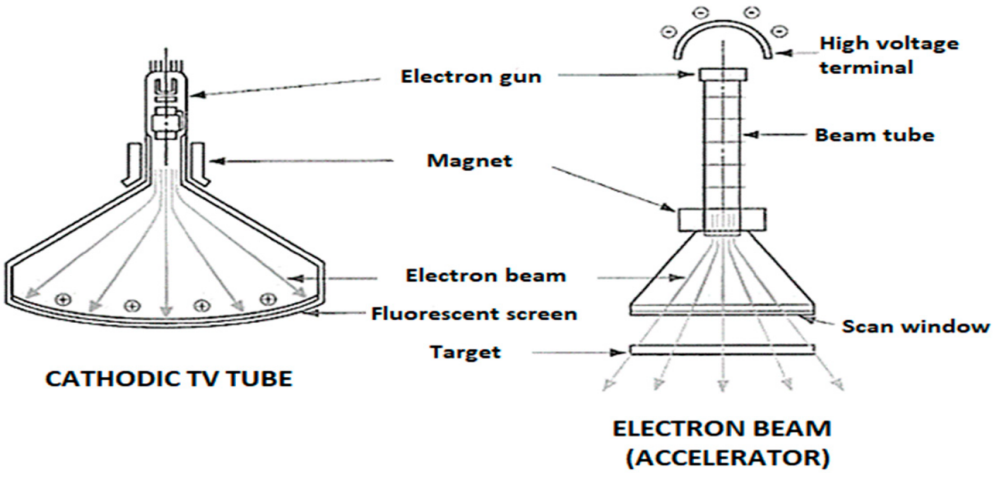
acid yeast treatment, and consumption of other inputs. It is also possible to reduce the yeast recycling time in the industrial unit, which allows increasing productivity over time and optimize the process, reducing costs and increasing productivity. It should also be noted that some technologies, which today are not usual, due to the lack of stability in the production process, can become viable if working in aseptic conditions (BRAZZACH, 1970; NOLASCO JUNIOR, 2005).

Several methods can be used to sterilize the wort to produce alcohol. However, in the sugar and alcohol industry, the volume of processed juice is very large, so that traditional methods, such as conventional heating or the use of chemical agents become very expensive. On the other hand, this industry has an energy surplus, which can help to circumvent this problem, or better, it is self-sufficient in energy, and part of this energy can be used to generate a source that allows sterilizing the wort.

2.1.1. Electron beam

The electron beam is a type of ionizing radiation, which is produced in electron accelerators. These can be defined as systems where a high voltage potential is established between a cathode and an anode in a vacuum tube. The cathode emits a beam of electrons called cathode rays or electronic beams, which follow the same principle as the television tube, where the big difference is that the latter uses approximately 25000 volts of energy, while the accelerator uses the order of millions of volts (CAPODAGLIO, 2020). The operating scheme of this equipment can be illustrated in Figure 2.2.

Figure 2.2 - Cathodic tv tube (left) and electron beam accelerator (right). Font: Capodaglio (2020).



2.1.2. Chemistry of the radiation

Gamma radiation (γ) from radioactive isotopes, electrons, and X-rays generated in accelerators, is known as ionizing radiation (IR), which interact with the products and as a result of collisions with orbital electrons that constitute these products, lose energy to these electrons, which are excited by acquiring higher levels of energy or are expelled completely from their orbits colliding with other orbital electrons (MOLINS, 2001; RELA, 2003).

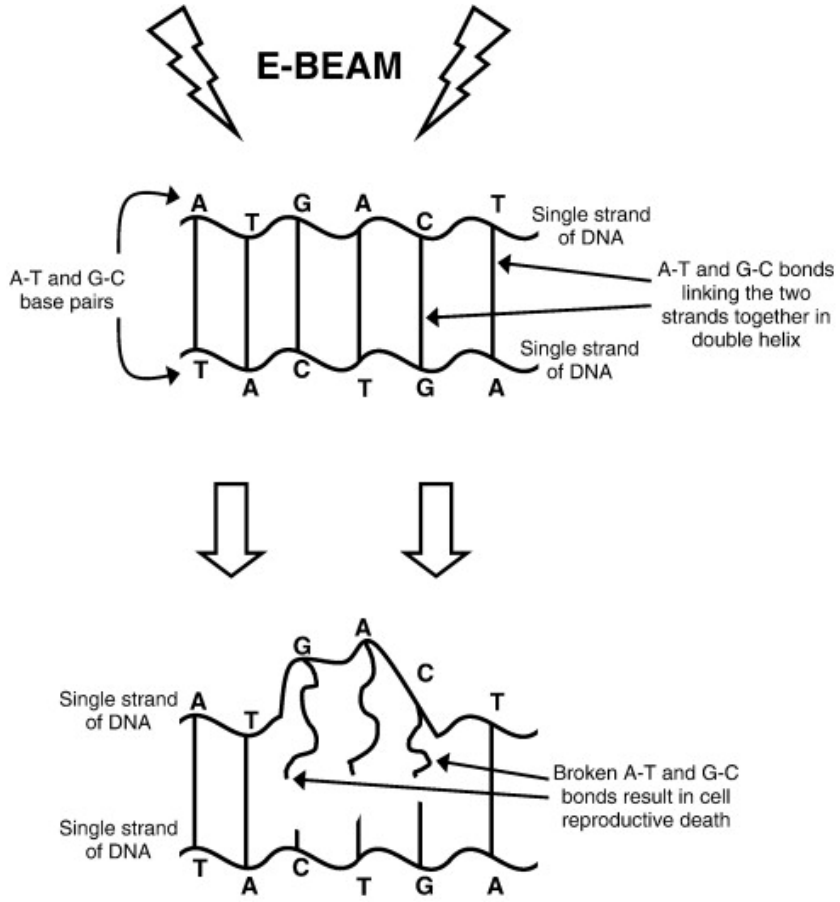
The products of ionization ions, secondary electrons, atoms, and excited molecules subsequently lose their energies to the environment that surrounds them and revert to stable states of free radicals (MILLER, 2006).

The energy from ionizing radiation when interacting with matter is absorbed by the medium and redistributed between the atoms and molecules that constitute it, producing, among other species, the highly reactive free radicals H^+ and OH^- (RELA, 2003).

The interaction of ionizing radiation with matter can be direct or indirect. Direct action is considered as the interaction of matter with the target molecule, while indirect action occurs when the target molecule is affected by some chemical species formed after the interaction of radiation with water (CAPODAGLIO, 2020).

At first, the effects are simple. An energy photon or an electron hits the cell's genetic material and causes DNA damage. The lesion can break just a single DNA helix, or, if the DNA orientation is appropriate, the energy or electron can break the DNA double helix. Injury to the simple helix may not be lethal and may result in mutations. However, a large number of lesions in the simple helix of DNA can exceed the repair capacity of the microbial cell, which results in cell death (Figure 2.3) (TAHERGORABI; MATAK; JACZYNSKI, 2012; LUNG et al., 2015). The effect of IR on microorganisms, such as bacteria, fungi, viruses, algae, and protozoa can be lethal due to the direct and indirect effects of radiation (FELLOWS, 2018; CAPODAGLIO, 2020).

Figure 2.3 - Direct effect of inactivation of micro-organisms by e-beam targeting the cell's genetic material. Font: Tahergorabi; Matak; Jaczynski (2012).



The absorption of ionizing radiation causes chemical changes in the cellular components of microorganisms, which can have consequences for the cell's activity. Among all the effects that are involved in the action of radiation on cells, the change in their DNA is considered the most important because affects the vital functions of cells, including reproduction (URBAIN, 2012). As a consequence of the changes induced by radiation, cell death can occur, which is usually associated with the loss of the cell's ability to reproduce (MOLINS, 2001).

The chemical changes undergone by irradiated organic material are induced by the direct action of macro and micronutrients present in the material, or an indirect action caused by reactive intermediate formed by radiolysis of water (TROJANOWICZ; BOJANOWSKA-CZAJKA; CAPODAGLIO, 2017).

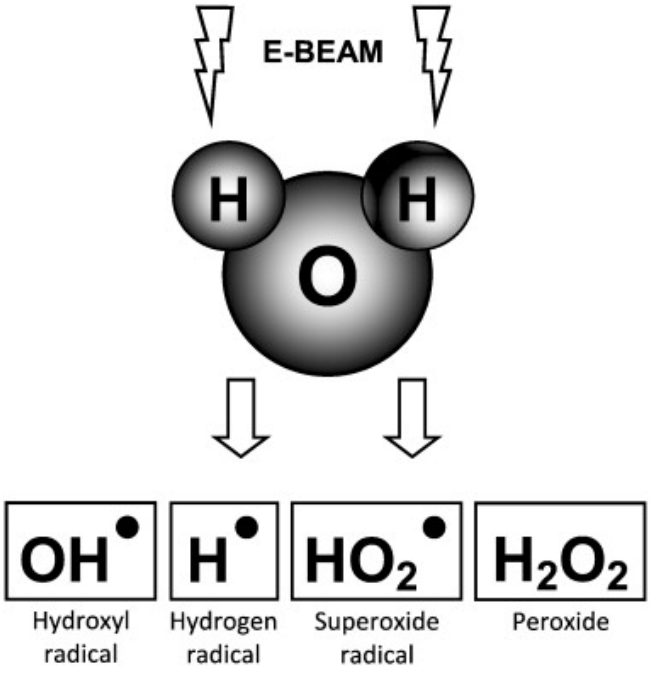
In a medium with a high-water content (≥ 60%), ionizing radiation is absorbed by the compound in higher concentration, that is, water, which can cause the formation of radicals (OH), solvated electrons (e_{aq}), hydrogen atoms (H), molecular hydrogen (H_2) and hydrogen

peroxide (H_2O_2). these radicals interact with the components of the medium, an interaction called secondary or indirect (ARENA, 1971; TAHERGORABI; MATAK; JACZYNSKI, 2012).

Ionizing radiation from γ rays or electron beams is capable of breaking chemical bonds when they are absorbed by materials. Ionization products can be electrically charged (ions) or neutral (free of radicals). Then, the ionization products can promote reactions that cause changes in the irradiated material (radiolysis). These are reactions that destroy micro-organisms, insects, and parasites during the irradiation (MILLER, 2006; FELLOWS, 2018).

The interactions of ionizing radiation with molecules adjacent to the genetic material are more complex. The chemistry of water radiation is well known. The radiation causes the loss of an electron in the water molecule, producing H_2O^+ and e^-. These products react with other water molecules to produce numerous compounds, including hydrogen, hydroxyl radical, molecular hydrogen, oxygen, and hydrogen peroxide. Among these, hydroxyl radicals (OH^-) and hydrogen peroxides (H_2O_2) are the most reactive components of these interactions (Figure 2.4) (TAHERGORABI; MATAK; JACZYNSKI, 2012; TROJANOWICZ; BOJANOWSKA-CZAJKA; CAPODAGLIO, 2017).

Figure 2.4 - Free radicals' formation from water radiolysis. Font: Tahergorabi; Matak; Jaczynski (2012).



In addition to the effect on genetic material, radiation has several effects on other cellular components. Applying radiation to the cells can interact directly or indirectly with components of the cells, such as membranes, enzymes, and plasmids. These interactions can be potentially lethal for the cell (TAHERGORABI; MATAK; JACZYNSKI, 2012).

Bacterial spores are generally more resistant to any process of inactivation than vegetative cells. Higher doses of ionizing radiation are needed to inactivate spores than vegetative bacteria cells. According to Urbain (2012), the lethal doses for vegetative cells are between 0.5 to 10 kGy and for bacterial spores, the dose must be between 10 to 50 kGy.

2.1.3. Wort sterilization by irradiation

IR has already been successfully tested for the control of microbial agents in sugar solutions by several authors (ACOSTA; LODOS, 1982; ZELLER; OLIVEIRA; ZAGO, 1984; ALCARDE; MARCOS; WALDER, 2001; ALCARDE; WALDER; HORII, 2003; PODADERA, 2007; URBAIN, 2012; GRABOWSKI, 2015; SUÁREZ-LEPE et al., 2015; LIMA et al., 2016).

The minimum dose (γ-radiation) for the sterilization of sugarcane syrup was described as 12.67 kGy by (ZELLER; OLIVEIRA; ZAGO, 1984). Besides, the dose of 10 kGy was the most effective to preserve the syrup for 105 days whereas the final total reducing sugars (TRS) concentration was 14% higher than the control treatment.

Gamma irradiation applied to sugarcane molasses contaminated with *Bacillus subtilis* has also shown to be efficient in reducing 99% of the contamination with doses of 5 to 10 kGy (URBAIN, 2012).

Alcarde, Marcos and Walder (2001) described that doses of 2 to 10 kGy (γ-radiation) applied to sugarcane wort were able to increase the ethanol yield and the fermentation efficiency. The increase was report as similar as the treatment with chemical agents.

According to Acosta and Lodos (1982) and Alcarde et al. (2001), gamma radiation can be used for the treatment of wort aiming the alcoholic fermentation. However, the industrial application of gamma radiation is limited by the difficulty of installing and using a source containing radioactive elements in an ethanol plant. Besides, the use of a different IR source, like e-beam is more favorable since the system can be turned on/off whenever it needs and can

be installed in the production line due to its simplicity (ALCARDE; MARCOS; WALDER, 2001; RELA, 2003; SILINDIR; ÖZER, 2009; JAIN et al., 2015).

2.2. Anaerobic digestion and biogas production

The possibility of expanding the sucroenergetic industry, coupled with a demand that gradually increases, arises along with the concern of which destination the waste generated will have, since these residues have a high potential for polluting the environment (CHRISTOFOLETTI et al., 2013). With the current technologies available, these residues can cease to be a nuisance for the industry and become by-products used to generate new products and wealth for the country (FREITAS et al., 2019).

Besides, with the intention of reducing greenhouse gases and increasing environmental, economic, and social sustainability, the new National Biofuels Policy (RenovaBio Program) was created, instituted by Decree Law 13.576 / 2017 2017 (BRASIL, 2017) which aims to decarbonize transport activities by stimulating the production and use of various biofuels, such as ethanol, biodiesel, and biogas.

The production of biogas (or biomethane after purification and refinement) is of great importance for the RenovaBio Program, especially biogas from waste from the sucroenergetic industry, such as vinasse, the main waste resulting from production of ethanol (KLEIN et al., 2019).

Vinasse is an industrial effluent rich in organic matter, minerals, and water. For each liter of ethanol produced, between 10 and 13 liters of vinasse are (PARSAEE; KIANI DEH KIANI; KARIMI, 2019).(PARSAEE; KIANI DEH KIANI; KARIMI, 2019) In addition, vinasse also has high polluting potential, which can cause eutrophication of aquatic systems, soil acidification and greenhouse gases (GHG) emissions due to its high concentration of organic matter (CHRISTOFOLETTI et al., 2013).

Due to the high concentration of organic matter and mineral nutrients present in vinasse, it is possible to use it for the production of biogas through anaerobic digestion (AD) (PARSAEE; KIANI DEH KIANI; KARIMI, 2019).

In addition to vinasse, the sugarcane industry generates other organic residues of great importance and with potential of use as a substrate for biogas production, among them stand out the filter cake and part of the sugarcane straw.

The filter cake is a solid and fibrous residue from the purification of the sugarcane juice after clarification and filtration. This residue represents 2.8-4.5% (w/w) of sugarcane processed in an industry and is mainly composed of lignocellulosic material. In general, the filter cake is used as fertilizer in sugarcane fields after composting process (LÓPEZ GONZÁLEZ; PEREDA REYES; ROMERO ROMERO, 2017).

Bagasse is also an important lignocellulosic residue in this industry; however, it is already used in electricity cogeneration through thermal conversion, and therefore may be restricted in terms of its use to produce biogas (JANKE et al., 2015). However, sugarcane straw, which has a similar chemical composition to bagasse, can be used as a substrate to produce biogas, as it has a low incentive for use in electricity cogeneration and is generally left in its entirety in the cane fields after harvesting as a form of soil protection and nutrition.

Straw has great agronomic and environmental importance for sugarcane plantations, however, part of the straw can be removed for bioenergy production purpose (CERVI et al., 2019).

Carvalho et al. (2017) suggest that 7 Mg ha^{-1} of straw is the maximum amount needed to be left in the soil to ensure environmental and agronomic benefits, taking into account that the average of a cane field is 8- 30 Mg ha^{-1} of straw, the rest could be used as substrate for anaerobic digestion.

AD is a technological form of converting biomass through a syntrophic association of bacteria and archaea in the absence of oxygen (BATSTONE; VIRDIS, 2014) and has been considered a promising way of treating organic waste, such as agro-industrial waste, waste urban solids, industrial effluents, domestic sewage, among others.

The methane production through AD of vinasse has been shown to be an interesting process, as it is responsible for the reduction of organic matter, production of methane-rich biogas, and the resulting effluent of the system can still be used as a fertilizer, as it still contains mineral nutrients (PARSAEE; KIANI DEH KIANI; KARIMI, 2019). However, vinasse AD with other residues, also known as anaerobic co-digestion (AC) is still a new and relatively unexplored topic.

AC may be responsible for biogas productivity increase (SARKER et al., 2019) since the use of different substrates in a reactor can optimize the nutrients balance, such as C:N ratio, phosphorus and sulfur concentrations. This can contribute to buffering capacity improve and better pH control of the reactor, in addition to biogas production increase (MATA-ALVAREZ, 2005; NAYONO; GALLERT; WINTER, 2010).

The fermentation process for the biogas production takes place in different stages. In each phase a different group of microorganism acts, being the anaerobic treatment of the effluents more advantageous in comparison to the aerobic treatment, as it requires less energy and space (ERSAHIN et al., 2011). The process is carried out in for stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

The first stage is hydrolysis, in which complex organic compounds are degraded into basic monomers by hydrolytic enzymes (CORTEZ et al., 2007). Subsequently, these smaller compounds are oxidized by bacteria yielding alcohols and volatile organic acids, such as acetic and propionic acids, constituting the second state, the acidogenesis. The products of acidogenesis will serve as the substrate for the next stages of AD, which are acetogenesis and methanogenesis, consecutively (MOSEY, 1982; RITTMANN; MCCARTY, 2012).

Acetogenesis is the stage in which volatile organic acids and alcohols are converted by acetogenic microorganisms into acetic acid, hydrogen, and carbon dioxide (ANGELIDAKI et al., 2011). Which are used by methanogenic archaea in the last stage, methanogenesis. In this phase, the products of acetogenesis are converted into methane (CH_4) and carbon dioxide (CO_2). This phase is the slowest and controls the conversion rates of the process (CANHOS; VAZOLLER, 1999).

However, the hydrolysis process in the first stage of AD is not always efficient, which can cause low efficiency of organic matter removal due to large amounts of organic matter that does not degrade in the process effluent (digestate), and, consequently, less biogas production (CALEGARI, 2017; SILVERIO, 2017). In addition, if the effluent from this process with a high organic load if used as a biofertilizer it can promote the emission of GHG into the atmosphere, such as N_2O, CO_2, H_2S, and CH_4 (DE OLIVEIRA et al., 2013; LOPES et al., 2017).

To address the issue related to the digestates that are not fully degraded, studies have shown that digestated from AD or AC have the potential to produce more biogas, these studies propose that post-treatment and recirculation of the digestate in an effective way

to reduce GHG emissions, in addition to maximizing the biogas yield of the substrate (MENARDO; GIOELLI; BALSARI, 2011).

2.3. H_2S in biogas

During anaerobic digestion, the components of biogas produced vary depending on the type of substrate used, however, the main components are mainly methane (CH_4), carbon dioxide (CO_2), and hydrogen sulfide (H_2S). Hydrogen sulfide is mostly formed through the reduction of sulfate and the degradation of organic compounds especially amino acids and proteins concentrated with sulfur (DÍAZ; RAMOS; FDZ-POLANCO, 2015).

High concentrations of H_2S can be detrimental to the AD process; it impacts biogas production by disturbing the methanogenesis stage and cause inhibition to the trace elements needed for the digestion process. Low organic loads can also result in sulfide toxicity (PARSAEE; KIANI DEH KIANI; KARIMI, 2019).

In the anaerobic digestion of the vinasse, H_2S is one of the main pollutants. This is because vinasse contains high concentration of sulfate due to the acid treatment of the yeast with sulfuric acid. Usually, the range of sulfate in the vinasse is between 500 and 3000 mg $SO_4^{2-}L^{-1}$ (LENS et al., 1998).

Hydrogen sulfide is an end product from Sulfate-Reducing Bacteria (SRB). The SRB plays a key role in hydrogen consumption during the AD. In relation to methanogenic archaea, SRB's affinity constant for hydrogen and propionate is estimated to be about five times larger. Besides, the most popular SRB strains grow at 25 and 35 ˚C, however, there are a few thermophilic strains that can work efficiently above 60 ˚C (SPEECE, 2008).

Usually, SRB are described in two categories: non-acetate-utilizing group and complete substrate oxidation group. The first one is not able to utilize acetate and grow slightly faster at 3 to 4 hours doubling time. Acetate is its end product of incomplete oxidation of some organic acids such as propionate, lactate, butyrate and long-chain fatty acids (LCFA) in the presence of sulfate (REIS et al., 1992). Hydrogenase enzymes are used in many SRB strains, allowing them to consume H_2. The incomplete conversion of propionate to acetate and H_2 is an important role played by SRB (OMIL et al., 1998).

The second group of SRB requires more than 20 hours doubling time (WIDDEL, 1988). Besides slow acetate utilization by this group of SRB, acetate seems to be important. However, it has no impact on the rate of sulfide formation in biofilms due to its kinect disadvantages (TANAKA; HVITVED-JACOBSEN; HORIE, 2000). Also, some bacteria in this group can oxidize lactate and use hydrogen as electron donor (SPEECE, 2008).

Propionate and H_2 are the most common electron donors for SRB. Lactate, pyruvate, butyrate, LCFA, glycerol, ethanol, and some dicarboxylic acids like succinate can also be used by SRB. Some SRB species may use malate, fumarate, aromatic compounds, and some amino acids (BRYSCH et al., 1987).

The negative impacts caused by H_2S in AD process has resulted in scientific research bringing out techniques to eliminate its effect. Some of these techniques involves purification of the biogas with physical methods such as membrane separation and active carbon process and chemical processes such as the addition of NaOH. There are also suggestions of biological methods. Though there has been much focus on the negative effects caused by sulfide, other reports suggest sulfide are essential for microbial growth (DÍAZ et al., 2015; SHAKERI YEKTA et al., 2017). The removal of H_2S is also importance for health and safety reasons since it is highly toxic, corrosive, and have unpleasant odor (MUYZER; STAMS, 2008).

2.4.Iron and trace elements supplementation for biogas production

Sometimes trace elements are not present in ideal concentrations or in bioavailable forms that microorganisms can use (ZITOMER et al., 2008). When not bioavailable iron and other micronutrients (metals) like cobalt, nickel, selenium, molybdenum, and zinc can be added to many nutrient-limited anaerobic digesters, resulting in increases on the rate of biogas generation. These and other nutrients are needed for anaerobic digestion microorganisms to grow properly (SPEECE et al., 1983; SPEECE, 1988; GUSTAVSSON et al., 2011; HENDRIKS et al., 2018; MAMIMIN et al., 2019).

The critical challenge in operating an anaerobic reactor is knowing how much VFA can be tolerated without causing a significantly pH drop. However, the lack of metals (trace elements) can contribute to the VFA accumulation in the process. Elevated acetate is often caused by iron bioavailability deficiency. Elevated propionate, on the other hand, is frequently

associated with the lack of proximity of the microbial consortia of the propionate degraders and the H_2-utilizers, along with the lack of inorganic cations (SPEECE, 2008).

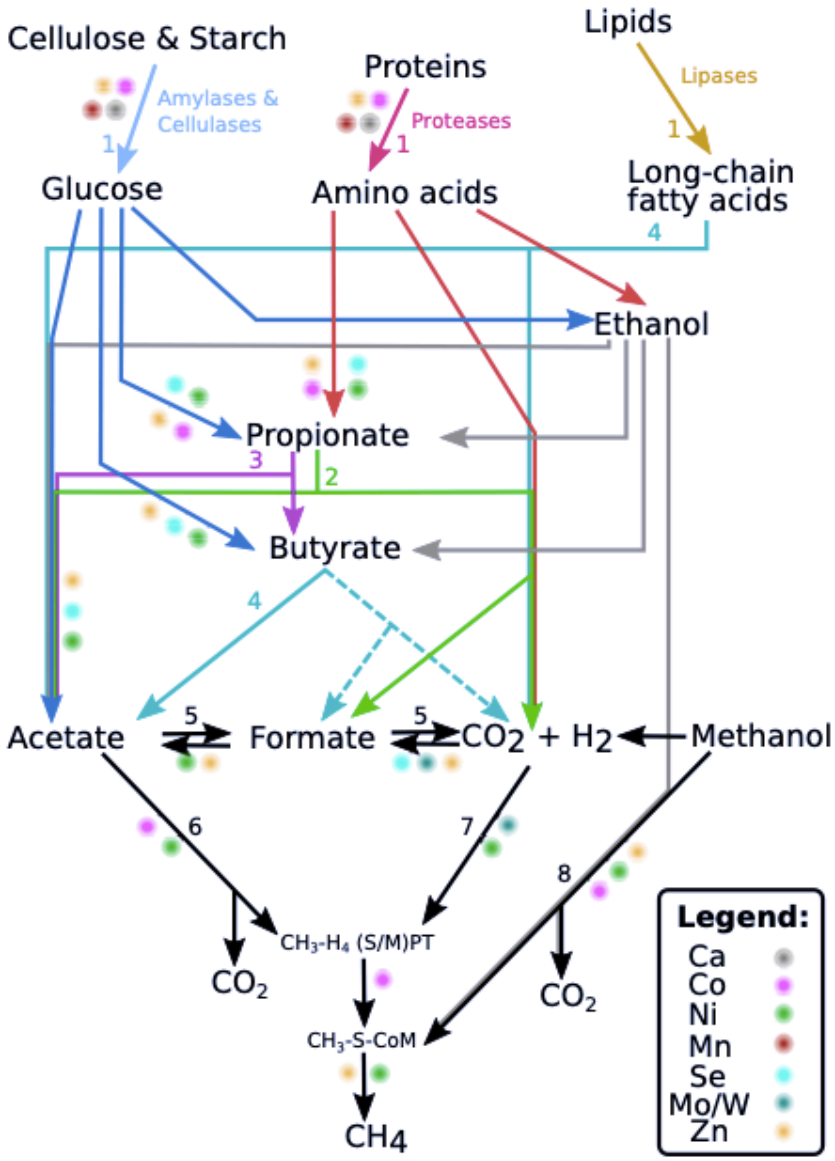
Another critical challenge in AD processes is to overcome high H_2S production and its inhibition to the methanogenesis. Since under sulfide-dominated conditions, the chemical speciation of the trace elements is driven by H_2S and the metal sulfide precipitates formation, which limits the supply and bioavailability of trace metals for AD consortia (SHAKERI YEKTA, 2014). Iron is an important metal for controlling H_2S in AD systems and can precipitate $FeS_{(s)}$ in order to reduce the effects of sulfide presence (LENS et al., 1998).

The availability of metal-binding ligands, which are involved in chemical reactions such as (co) precipitation, dissolution, aqueous complex forming, adsorption, and desorption, determines the presence of metal species in biogas reactors. Inorganic ligands such as carbonate, phosphate, and sulfide are known to regulate the chemical speciation of trace metals under anaerobic conditions (CALLANDER; BARFORD, 1983; SHAKERI YEKTA et al., 2017) Furthermore, the oxidation of organic matter generates organic molecules with high metal-binding affinities, like amino, carboxylic, thiol and alcohol groups (SMITH; BELL; KRAMER, 2002; SHAKERI YEKTA et al., 2012).

The trace elements are required in small quantities (micronutrients) by microorganisms, but they are essential for the cells functioning since these metals work as cofactors for some of the essential enzymes involved in the anaerobic digestion (Figure 2.5) (ŠAFARIČ, 2019).

During AD processes, a lack of micronutrients may result in lower biogas production rate and process instability (SPEECE, 1988). Moreover, the addition of these metals such as Fe, Ni, Co, Se, W, and Mo have been shown to improve the conversion rates of acetate, propionate, H_2 and CO_2. (MOESTEDT et al., 2016; HENDRIKS; VAN LIER; DE KREUK, 2018).

Figure 2.5 - Trace elements requirements for each AD step Font: Šafarič (2019)



2.5.Generation and use of biogas in the Brazilian Sucroenergetic sector

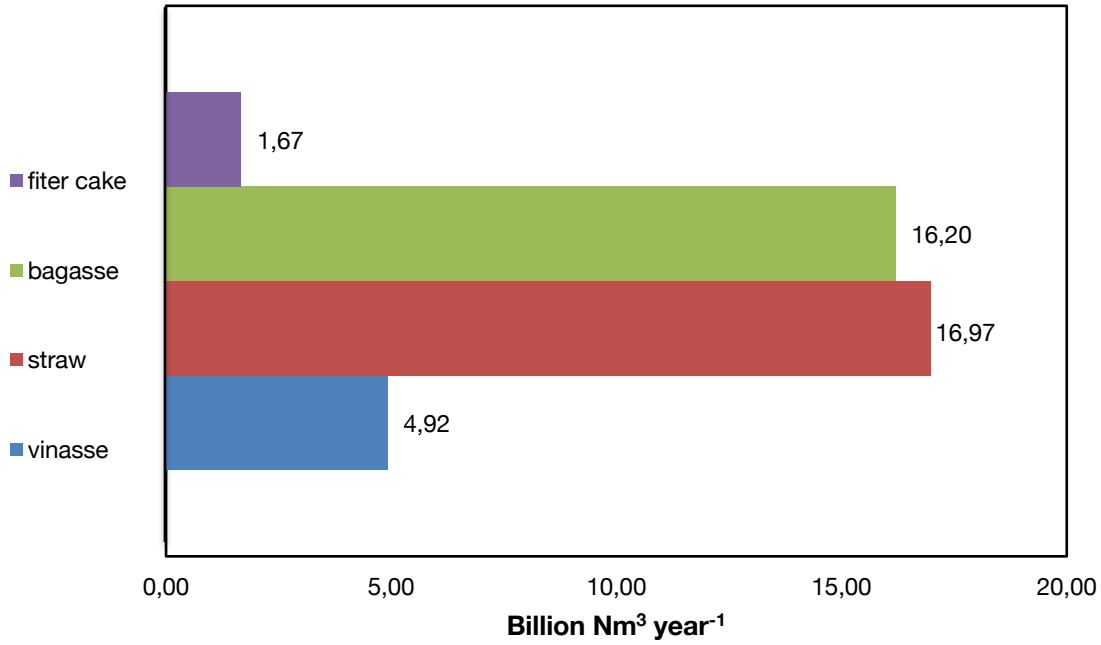
The biogas generated from the AC of residues from the sucroenergetic industry can be used in different forms of energy generation. One of the possibilities is the addition of biogas in natural gas grid, as this grid is close to the sugarcane industries, especially in the São Paulo State. A procedure which is already successfully done in other countries, such as Sweden and Denmark (LANTZ; BÖRJESSON, 2014; ARYAL; KVIST, 2018).

Other attractive option is the replacement of the diesel used by the trucks fleet in the sucroenergetic sector with biogas. This would make it possible to reduce the costs of transportation of vinasse and the need to purchase fossil fuels (ABIOGÁS, 2020).

According to Abiogás (2020), the Brazilian potential for electric energy from biogas in the sucroenergetic sector is estimated at 85.15 thousand GWh / year, with the country's total potential of 173,762. 41 GWh coming from the sucroenergetic, agribusiness, and sanitation sectors. The estimated biomethane production potential in the country is 44.7 billion m^3, with the sucroenergetic sector responsible to produce 21.06 billion m^3.

In the sugarcane industry straw stands out with the greatest potential for biogas generation, with 16.97 billion Nm^3/year, followed by bagasse with 16.20 Nm^3/year, stillage with 4.92 Nm^3/year, and filter cake with 1.67 Nm^3/year (Figure 2.6).

Figure 2.6 - Biogas potential for each residue from sucroenergetic industry in Brazil in 2019/20 harvest. Font: ABiogás (2020)



Thus, the biogas production from the residues of the sugarcane industry has proved to be an attractive technique from the technical-economic and environmental perspective. Which meets the country’s interest in an increasingly clean and sustainable energy matrix.

References

ACOSTA, S.; LODOS, J. The action of gamma-radiation on sugar-cane as a new sterilization method. **Sugar y Azucar**, v. 77, n. 9, p. 55-59, 1982.

ALCARDE, A. R.; MARCOS, J.; WALDER, M. Comparison Between Gamma Radiation and Kamoran HJ in the Decontamination of Kamoran H Sugarcane Must. **Journal of Food Processing and Preservation**, v. 25, p. 137–147, 2001.

ALCARDE, A. R.; WALDER, J. M. M.; HORII, J. Fermentation of irradiated sugarcane must. **Scientia Agricola**, v. 60, n. 4, p. 677–681, 2003.

AMORIM, H. V.; OLIVEIRA, A. J.; CAMPOS, H. Infecção, problema sério na produção de álcool. In: CONGRESSO NACIONAL DA SOCIEDADE DOS TÉCNICOS AÇUCAREIROS DO BRASIL, 2., 1981, Rio de Janeiro. **Anais...** Piracicaba: STAB, 1981. v.1, p.158-168.

ANGELIDAKI, I. et al. Biomethanation and its potential. **Methods in Enzymology**, v. 494, p. 327-351, 2011.

ARENA, V. **Ionizing radiation and life**: An introduction to radiation biology and biological radiotracer methods. 1. ed. St. Louis: Mosby, 1971.

ARYAL, N.; KVIST, T. Alternative of Biogas Injection into the Danish Gas Grid System-A Study from Demand Perspective. **Chemengineering**, v. 2, n. 43, p. 1–11, 2018. Available at: <www.mdpi.com/journal/chemengineering>.

ASSOCIAÇÃO BRASILEIRA DO BIOGÁS E DO BIOMETANO - ABIOGÁS. **Potencial brasileiro de biometano**. São Paulo, 2020. 28 p. Available at: <https://abiogas.org.br/wp-content/uploads/2020/11/NOTA-TECNICA_POTENCIAL_ABIOGAS.pdf/>. Accessed at: 18 mar. 2019.

BASSO, L. C.; BASSO, T. O.; ROCHA, S. N. Ethanol Production in Brazil: The Industrial Process and Its Impact on Yeast Fermentation. In: BERNARDES, M. A. S. (Ed.). **Biofuel production -** Recent developments and prospects. Rijeka: InTech, 2011. p. 85–100.

BATSTONE, D. J.; VIRDIS, B. The role of anaerobic digestion in the emerging energy economy. **Current Opinion in Biotechnology**, v. 27, p. 142-149, 2014.

BRAZZACH, M. L. **Emprego de desinfetantes químicos em fermentação alcoólica de mosto de melaço de cana**. 1970. 40 p. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 1970.

BROWN, N. A. et al. Transcriptional profiling of Brazilian *Saccharomyces cerevisiae* strains selected for semi-continuous fermentation of sugarcane must. **FEMS Yeast Research**, v. 13, n. 3, p. 277–290, 2013.

BRYSCH, K. et al. Lithoautotrophic growth of sulfate-reducing bacteria, and description of *Desulfobacterium autotrophicum* gen. nov., sp. nov. **Archives of Microbiology**, v. 148, n. 4, p. 264–274, 1987. Available at: <https://link.springer.com/article/10.1007/BF00456703>.

CALEGARI, R. P. **Produção de biogás a partir de vinhaça concentrada**. 2017. 81 p. Dissertação (Mestrado em Microbiologia Agrícola) - Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, 2017.

CALLANDER, I. J.; BARFORD, J. P. Precipitation, chelation, and the availability of metals as nutrients in anaerobic digestion. II. Applications. **Biotechnology and Bioengineering**, v. 25, n. 8, p. 1959–1972, 1983. Available at: <https://onlinelibrary.wiley.com/doi/full/10.1002/bit.260250806>.

CANHOS, V. P.; VAZOLLER, R. F. Microrganismos e vírus. In: JOLY, C. A.; BICUDO, C. E. M. (Org.). **Biodiversidade do Estado de São Paulo, Brasil**: síntese do conhecimento ao final do século XX. São Paulo: FAPESP, 1999.

CAPODAGLIO, A. G. Critical perspective on advanced treatment processes for water and wastewater: AOPs, ARPs, and AORPs. **Applied Sciences**, v. 10, n. 13, art. 4549, 2020.

CARVALHO, J. L. N. et al. Agronomic and environmental implications of sugarcane straw removal: a major review. **GCB Bioenergy**, v. 9, n. 7, p. 1181–1195, 2017. Available at: <https://onlinelibrary.wiley.com/doi/full/10.1111/gcbb.12410>.

CERVI, W. et al. Bioelectricity potential from ecologically available sugarcane straw in Brazil: A spatially explicit assessment. **Biomass and Bioenergy**, v. 122, p. 391–399, 2019. Available at: <https://doi.org/10.1016/j.biombioe.2019.02.001>.

CHERUBIN, R. A. **Efeitos da viabilidade da levedura e da contaminação bacteriana na fermentação alcoólica**. 2003. 124 p. Tese (Doutorado em Microbiologia Agrícola) - Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, 2003.

CHRISTOFOLETTI, C. A. et al. Sugarcane vinasse: Environmental implications of its use. **Waste Management**, v. 33, n. 12, p. 2752–2761, 2013. Available at: <http://dx.doi.org/10.1016/j.wasman.2013.09.005>.

COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB. **Acompanhamento da safra brasileira de cana-de-açúcar - Safra 2019/20**. Terceiro levantamento. Brasília, DF, 2020. Available at: <https://www.conab.gov.br/info-agro/safras/cana/boletim-da-safra-de-cana-de-acucar>.

CORTEZ, L. A. B. et al. Biodigestão de efluentes. **Biomassa para energia**. Campinas: Editora da UNICAMP, 2007. cap. 15, p. 493–529.

DE OLIVEIRA, B. G. et al. Soil greenhouse gas fluxes from vinasse application in Brazilian sugarcane areas. **Geoderma**, v. 200–201, p. 77–84, 2013.

DÍAZ, I.; RAMOS, I.; FDZ-POLANCO, M. Economic analysis of microaerobic removal of H_2S from biogas in full-scale sludge digesters. **Bioresource Technology**, v. 192, p. 280–286, 2015. Available at: <http://dx.doi.org/10.1016/j.biortech.2015.05.048>.

ERSAHIN, M. E. et al. Anaerobic treatment of industrial effluents: an overview of applications. In: EINSCHLAG, F. S. G. (Ed.). **Waste water-treatment and reutilization**. London: IntechOpen, 2011. Chap. 1, p. 3-28.

FELLOWS, P. J. **Tecnologia do Processamento de Alimentos**: Princípios e Prática. São Paulo: Artmed, 2018.

FREDERICK, M. K. B. **Estudo genético, fisiológico e molecular de *Lactobacillus fermentum* envolvidos na floculação de leveduras**. 1994. 56 p. Dissertação (Mestrado em Engenharia de Alimentos) - Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, 1994.

FREITAS, F. F. et al. The Brazilian market of distributed biogas generation: Overview, technological development and case study. **Renewable and Sustainable Energy Reviews**, v. 101, p. 146–157, 2019. Available at: <https://doi.org/10.1016/j.rser.2018.11.007>.

GRABOWSKI, C. **The Impact of Electron Beam Pretreatment on the Fermentation of Wood-based Sugars**. 2015. 63 p. Honors These - Department of Chemistry, SUNY College of Environmental Science and Forestry, Syracuse, NY, 2015. Available at: <https://digitalcommons.esf.edu/honors/63>.

GRAVES, T. et al. Effect of pH and lactic or acetic acid on ethanol productivity by Saccharomyces cerevisiae in corn mash. **Journal of Industrial Microbiology and Biotechnology**, v. 33, n. 6, p. 469–474, 2006. Available at: <https://academic.oup.com/jimb/article/33/6/469/5993019>.

GUSTAVSSON, J.; SVENSSON, B. H.; KARLSSON, A. The feasibility of trace element supplementation for stable operation of wheat stillage-fed biogas tank reactors. **Water Science and Technology**, v. 64, n. 2, p. 320–325, 2011. Available at: <https://pubmed.ncbi.nlm.nih.gov/22097002/>.

HENDRIKS, A. T. W. M.; VAN LIER, J. B.; DE KREUK, M. K. Growth media in anaerobic fermentative processes: The underestimated potential of thermophilic fermentation and anaerobic digestion. **Biotechnology Advance**s, v. 36, n. 1, p. 1–13, 2018. Available at: <https://doi.org/10.1016/j.biotechadv.2017.08.004>.

JAIN, R. et al. Influence of selenium on metallothionein gene expression and physiological characteristics of sugarcane plants. **Plant Growth Regulation**, v. 77, n. 2, p. 109–115, 2015. Available at: <http://dx.doi.org/10.1007/s10725-015-0042-1>.

JANKE, L. et al. Biogas Production from Sugarcane Waste: Assessment on Kinetic Challenges for Process Designing. **International Journal of Molecular Sciences**, v. 16, n. 9, p. 20685–20703, 2015.

KLEIN, B. C. et al. Low carbon biofuels and the New Brazilian National Biofuel Policy (RenovaBio): A case study for sugarcane mills and integrated sugarcane-microalgae biorefineries. **Renewable and Sustainable Energy Reviews**, v. 115, art. 109365, 2019. Available at: <https://doi.org/10.1016/j.rser.2019.109365>.

LANTZ, M.; BÖRJESSON, P. Greenhouse gas and energy assessment of the biogas from co-digestion injected into the natural gas grid: A Swedish case-study including effects on soil properties. **Renewable Energy**, v. 71, p. 387–395, 2014.

LEÃO, R. M. **Alcool**: energia verde. São Paulo: Iqual Editora, 2002.

LENS, P. N. L. et al. Biotechnological treatment of sulfate-rich wastewaters. **Critical Reviews in Environmental Science and Technology**, v. 28, n. 1, p. 41–88, 1998. Available at: <https://www.tandfonline.com/doi/abs/10.1080/10643389891254160>.

LIMA, R. B. et al. Sucrose and color profiles in sugarcane (*Saccharum* sp.) juice analyzed by UFLC-ELSD and Synapt High-Definition Mass Spectrometry during radiation treatment. **Radiation Physics and Chemistry**, v. 121, p. 99–105, 2016. Available at: <http://dx.doi.org/10.1016/j.radphyschem.2015.12.022>.

LOPES, I. M. et al. Emissions of N_2O in Soils under Sugarcane Cultivation in the Atlantic Forest biome: Effect of Harvesting Systems and Vinasse Fertilization. **Revista Virtual de Química**, v. 9, n. 5, p. 1930–1943, 2017.

LÓPEZ GONZÁLEZ, L. M.; PEREDA REYES, I.; ROMERO ROMERO, O. Anaerobic co-digestion of sugarcane press mud with vinasse on methane yield. **Waste Management**, v. 68, p. 139–145, 2017.

LUNG, H. M. et al. Microbial decontamination of food by electron beam irradiation. **Trends in Food Science and Technology**, v. 44, n. 1, p. 66–78, 2015. Available at: <http://dx.doi.org/10.1016/j.tifs.2015.03.005>.

MAMIMIN, C. et al. Trace metals supplementation enhanced microbiota and biohythane production by two-stage thermophilic fermentation. **International Journal of Hydrogen Energy**, v. 44, n. 6, p. 3325–3338, 2019.

MATA-ALVAREZ, J. **The biomethanization of the organic fraction of municipal solid waste.** London: IWA publishing, 2005.

MENARDO, S.; GIOELLI, F.; BALSARI, P. The methane yield of digestate: Effect of organic loading rate, hydraulic retention time, and plant feeding. **Bioresource Technology**, v. 102, n. 3, p. 2348–2351, 2011.

MILLER, R. B. **Electronic irradiation of foods**: an introduction to the technology. Heidelberg: Springer Science, 2006.

MOESTEDT, J. et al. Effects of trace element addition on process stability during anaerobic co-digestion of OFMSW and slaughterhouse waste. **Waste Management**, v. 47, p. 11–20, 2016. Available at:< https://doi.org/10.1016/j.wasman.2015.03.007>.

MOLINS, R. A. **Food Irradiation**: Principles and Applications. Hoboken: John Wiley & Sons, 2001.

MOSEY, F. E. New developments in the anaerobic treatment of industrial wastes. **Water Pollution Control**, v. 81, n. 4, p. 540–552, 1982.

MUTHAIYAN, A.; LIMAYEM, A.; RICKE, S. C. Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations. **Progress in Energy and Combustion Science**, v. 37, n. 3, p. 351–370, 2011. Available at: <http://dx.doi.org/10.1016/j.pecs.2010.06.005>.

MUYZER, G.; STAMS, A. J. M. The ecology and biotechnology of sulphate-reducing bacteria. **Nature Reviews Microbiology**, v. 6, n. 6, p. 441–454, 2008. Available at: <https://www.nature.com/articles/nrmicro1892>.

NAYONO, S. E.; GALLERT, C.; WINTER, J. Co-digestion of press water and food waste in a biowaste digester for improvement of biogas production. **Bioresource Technology**, v. 101, n. 18, p. 6987–6993, 2010.

NOLASCO JUNIOR, J. **Desenvolvimento de processo térmico otimizado para mosto de caldo de cana na fermentação alcoólica**. 2005. 181 p. Dissertação (Mestrado em Ciências de Alimentos) – Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, 2005.

NUNES, T. S.; FINZER, J. R. D. Calculation of the Yield of an Alcoholic Fermentation. **International Journal of Advanced Engineering Research and Science**, v. 6, n. 6, p. 572–574, 2019. Available at: <https://ijaers.com/detail/calculation-of-the-yield-of-an-alcoholic-fermentation/>.

OLIVA NETO, P. de; YOKOYA, F. Effects of nutritional factors on growth of Lactobacillus fermentum mixed with Saccharomyces cerevisiae in alcoholic fermentation. **Revista de Microbiologia**, v. 28, n. 1, p. 25–31, 1997.

OMIL, F. et al. Long-Term Competition between Sulfate Reducing and Methanogenic Bacteria in UASB Reactors Treating Volatile Fatty Acids. **Biotechnology and Bioengineering,** v. 57, n. 6, p. 676-685, 1998.

PARSAEE, M.; KIANI DEH KIANI, M.; KARIMI, K. A review of biogas production from sugarcane vinasse. **Biomass and Bioenergy**, v. 122, p. 117–125, 2019. Available at: <https://doi.org/10.1016/j.biombioe.2019.01.034>.

PODADERA, P. **Estudo das propriedades do açúcar líquido invertido processado com radiação gama e feixe de elétrons**. 2007. 99 p. Tese (Doutorado em Tecnologia Nuclear - Aplicações) - Instituto de Pesquisas Energéticas e Nucleares, Universidade de São Paulo, São Paulo, 2007.

RAVNÖ, A. B.; PURCHASE, B. S. Dealing with dextran in the South African sugar industry. In: Annual Congress of South African Sugar Technologists' Association, 79., 2005, Kwa-Shukela, Mount Edgecombe, South Africa. **Proceedings…** Mount Edgecombe, South Africa: South African Sugar Technologists' Association, 2005. p. 28-47.

REIS, M. A. M. et al. Effect of hydrogen sulfide on growth of sulfate reducing bacteria. **Biotechnology and Bioengine**ering, v. 40, n. 5, p. 593–600, 1992. Available at: <https://onlinelibrary.wiley.com/doi/full/10.1002/bit.260400506>. Acesso em: 21 maio. 2021.

RELA, P. R. **Desenvolvimento de dispositivo de Irradiação para tratamento de efluentes industriais com feixe de elétrons**. 2003. Tese (Doutorado em Tecnologia Nuclear - Aplicações) - Instituto de Pesquisas Energéticas e Nucleares, Universidade de Universidade de São Paulo, São Paulo, 2003.

RIBÉREAU-GAYON, P. et al. **Handbook of enology**. v. 1: The microbiology of wine and vinifications. Hoboken: John Wiley & Sons, 2006.

RITTMANN, B. E.; MCCARTY, P. L. **Environmental biotechnology**: principles and applications. New York: McGraw-Hill Education, 2012.

ŠAFARIČ, L. **Anaerobic Digester Fluid Rheology and Process Efficiency: Interactions of Substrate Composition, Trace Element Availability, and Microbial Activity**. 2019. Thesis (Doctor in Environmental Science) - Department of Thematic Studies, Linköping University, Linköping, 2019.

SARKER, S. et al. A review of the role of critical parameters in the design and operation of biogas production plants. **Applied Sciences**, v. 9, n. 9, art. 1915, 2019. https://doi.org/10.3390/app9091915.

SHAKERI YEKTA, S. et al. Characterization of dissolved organic matter in full scale continuous stirred tank biogas reactors using ultrahigh resolution mass spectrometry: A qualitative overview. **Environmental Science and Technology**, v. 46, n. 22, p. 12711–12719, 2012. Available at: <https://pubs.acs.org/sharingguidelines>.

SHAKERI YEKTA, S. **Chemical speciation of sulfur and metals in biogas reactors**: implications for cobalt and nickel bio-uptake processes. 2014. Thesis (Doctor in Environmental Science) - Department of Thematic Studies, Linköping University, Linköping, 2014.

SHAKERI YEKTA, S. et al. Importance of sulfide interaction with iron as regulator of the microbial community in biogas reactors and its effect on methanogenesis, volatile fatty acids turnover, and syntrophic long-chain fatty acids degradation. **Journal of Bioscience and Bioengineering**, v. 123, n. 5, p. 597–605, 2017. Available at: <http://dx.doi.org/10.1016/j.jbiosc.2016.12.003>.

SILINDIR, M.; ÖZER, A. Y. Sterilization methods and the comparison of E-beam sterilization with gamma radiation sterilization. **Fabad Journal of Pharmaceutical Sciences**, v. 34, n. 1, p. 43–53, 2009.

SILVERIO, M. S. **Digestão anaeróbia de vinhaça 2G para produção de biogás**. 2017. 80 p. Dissertação (Mestrado em Microbiologia Agrícola) – Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, 2017.

SMITH, D. S.; BELL, R. A.; KRAMER, J. R. Metal speciation in natural waters with emphasis on reduced sulfur groups as strong metal binding sites. **Comparative Biochemistry and Physiology - C Toxicology and Pharmacology**. v. 133, n. 1-2, p. 65-74, 2002.

SPEECE, R. E. A survey of municipal anaerobic sludge digesters and diagnostic activity assays. **Water Research**, v. 22, n. 3, p. 365–372, 1988.

SPEECE, R. E. **Anaerobic biotechnology and odor/corrosion control for municipalities and industries.** Nashville: Archae Press, 2008.

SPEECE, R. E.; PARKIN, G. F.; GALLAGHER, D. Nickel stimulation of anaerobic digestion. **Water Research**, v. 17, n. 6, p. 677–683, 1983.

STUPIELLO, J. P.; HORII, J. Considerações sobre tratamentos de caldo de cana para fermentação alcoólica. **Álcool e Açúcar**, n. 3, p. 40–46, 1981.

SUÁREZ-LEPE, J. A. et al. Electron Beam Irradiation of Wine Grapes: Effect on Microbial Populations, Phenol Extraction and Wine Quality. **Food and Bioprocess Technology**, v. 8, n. 9, p. 1845–1853, 2015.

TAHERGORABI, R.; MATAK, K. E.; JACZYNSKI, J. Application of electron beam to inactivate Salmonella in food: Recent developments. **Food Research International**, v. 45, n. 2, p. 685–694, 2012. Available at: <http://dx.doi.org/10.1016/j.foodres.2011.02.003>.

TANAKA, N.; HVITVED-JACOBSEN, T.; HORIE, T. Transformations of Carbon and Sulfur Wastewater Components Under Aerobic-Anaerobic Transient Conditions in Sewer Systems. **Water Environment Research**, v. 72, n. 6, p. 651–664, 2000. Available at: <https://onlinelibrary.wiley.com/doi/full/10.2175/106143000X138265>.

TROJANOWICZ, M.; BOJANOWSKA-CZAJKA, A.; CAPODAGLIO, A. G. Can radiation chemistry supply a highly efficient AO (R) P process for organics removal from drinking and waste water? A review. **Environmental Science and Pollution Research**, v. 24, n. 25, p. 20187–20208, 2017.

URBAIN, W. **Food irradiation**. Amstedam: Elsevier, 2012.

WIDDEL, F. Microbiology and ecology of sulfate-and sulfur-reducing bacteria. In: ZEHNDER, A. J. B. (Ed.). **Biology of anaerobic microorganisms**. New York: John Wiley and Sons, 1988. p. 469–585.

ZELLER, A. L. V; OLIVEIRA, A. J.; ZAGO, E. A. Conservação de xarope de cana-de-açúcar pelo emprego da radiação gama. **STAB-Açúcar, Álcool e Subprodutos**, v. 2, p. 29–36, 1984.

ZITOMER, D. H.; JOHNSON, C. C.; SPEECE, R. E. Metal Stimulation and Municipal Digester Thermophilic/Mesophilic Activity. **Journal of Environmental Engineering**, v. 134, n. 1, p. 42–47, 2008. Available at: <https://ascelibrary.org/doi/abs/10.1061/%28ASCE%290733-9372%282008%29134%3A1%2842%29>.

3. Sterilization of wort from sugarcane molasses by electron beam for bioethanol production

Abstract

The presence of microbial contamination in the wort during the fermentation process results in damages of billions of dollars per year all around the world and promotes the industry dependence of chemicals and antibiotics to control the contamination. For these reasons, this study aimed to use the electron beam to sterilize wort from sugarcane molasses and investigate its bioethanol fermentation. Five treatments (T0 – T4) were carried out using ionizing doses of radiation through the electron accelerator: (0, 10, 20, 40, and 80 kGy) and a positive control (T5-steam by autoclave) were performed. It was evaluated total mesophiles, total bacteria, sugars, phenolics, flavonoids, 5-hidroximetylfurfural, and Furfural. After the irradiation process, it was conducted an alcoholic fermentation assay using baker's yeast *Saccharomyces cerevisiae*. It was not observed inversion of sugars and formation of the inhibitory by-products flavonoids, furfural, and 5-hidroximetylfurfural, with exception of the phenolic compounds. Only T4 and T5 were able to sterilize the wort. However, T3 was capable of inactivation of >99.99% of the microorganisms. In the fermentation T2 promoted the best ethanol yield and productivity among the irradiated treatments, evidencing the possibility of electron beam use in the wort treatment prior to fermentation which may allow a reduction in losses caused by microbial contamination, besides the possibility of promoting fermentation yield and productivity increase.

Keywords: biofuel. electron accelerator. microbial contamination. Radappertization. sucro-energetic industry.

3.1. Introduction

The worldwide demand for pollution reduction and uses of renewable energy has increased in recent years. At the same time, the need to make the best use of natural resources for highly efficient and sustainable biofuels. In this sense, the production of ethanol in Brazil is mainly based on the fermentation of sugarcane using the mesophilic yeast *Saccharomyces cerevisiae* (PONCE et al., 2016). The development of strategies to increase the efficiency and productivity of this microorganism is of great importance.

The efficiency of this biological agent in turning the sugar from the sugarcane juice or molasses into alcohol depends very much on the quality of the wort to be fermented. In industrial production, usually, this substrate is not sterilized before the fermentation process, which allows the entry of a large number of microbial contaminants in the process, which negatively affects efficiency and productivity (AMORIM et al., 2011; LOPES et al., 2016).

Among the main losses caused by microbial contamination, we can cite formation of acids, increased flocculation, and reduction of yeast viability. The population of contaminating bacteria in fermentation can reach levels higher than 10^7 cells ml^{-1}, which can lead to a significant reduction in alcoholic yield, up to 55% of the expected theoretical value (AMORIM; OLIVEIRA; CAMPOS, 1981). Moreover, a concentration of 10^8 cells ml^{-1} may be responsible for the drop in production of 10 to 30 thousand liters of ethanol in a distillery with a production capacity of one million liters per day (AMORIM et al., 2011).

Commonly, the Brazilian sucro-energetic industries perform the acid treatment of yeast cream (Melle-Boinot process), which requires a large volume of sulfuric acid to reduce bacterial contamination (BASSO et al., 2008; COSTA; CERRI; CECCATO-ANTONINI, 2018; DA SILVA-NETO et al., 2020). This process is repeated countless times throughout the harvest, which last about 200 days a year (BROWN et al., 2013). However, it is not completely efficient (CECCATO-ANTONINI, 2018), may select resistant bacteria and, consequently promote the osmotic stress of the yeasts, and as a response, the yeasts produce greater amounts of glycerol, thereby reducing the yield of the fermentation (BASSO; BASSO; ROCHA, 2011). Furthermore, wild yeast strains rapidly contaminate the fermentation and, after a few cycles, only the wild strains survive the acid treatment (BROWN et al., 2013).

Another common way to control bacterial contamination in distilleries is the use of antibiotics, but drug resistance has been a limiting factor in the contamination control efficiency (MUTHAIYAN; LIMAYEM; RICKE, 2011).

In this case, the use of sterilized wort for ethanol production may allow the use of selected yeast strains, with desirable and stable characteristics throughout the harvest season. Besides, it is possible to reduce costs by eliminating or reducing the use of antibiotics, anti-foaming agents, acid treatment of yeast, and consumption of other inputs. It is also possible to reduce the time of recycling the yeasts in the industrial unit, which increases productivity over time. This means that could optimize the process, reduce costs and increase productivity (NOLASCO JUNIOR, 2010).

It should also be noted that some technologies, which are not usual today due to the lack of stability of the fermentation process to ethanol production may become viable if working under aseptic conditions.

There are several methods that can be used for the sterilization of the wort, the substrate for alcoholic fermentation. However, in industrial plants such as the ethanol industry, where the volume of wort processed is huge, the traditional methods like conventional heat and chemical agents can be very costly. On the other hand, this industry has an energy surplus (CERVI et al., 2019), which can help to circumvent this problem, or rather, is self-sufficient in energy, and part of this energy can be used to generate a source to sterilize the wort.

In this way, Electron beam (e-beam), a type of Ionizing radiation (IR) is a quite effective technology for microorganisms inactivation and generally depends on the dose of radiation applied, where the logarithmic number of microorganisms decreases linearly with increasing dose (SAMPA et al., 2007).

In addition, the doses used for sterilization do not produce radioisotopes or radioactive waste, demonstrates benefit over other irradiators such as Cobalt 60 (gamma irradiation), so its residue is the products derived from the radiolysis of water, such as water, hydrogen, and oxygen (KOCHETKOV; KUDRJASHOV; CHLENOV, 1979; SCHWARZ, 1981).

These radicals interact with the molecules and produce oxidation, reduction, dissociation, and degradation. Besides that, the IR can promote water radiolysis and, consequently, the destruction of microorganisms, insects, and other parasites (MOLINS, 2001).

It should be noted that the main target of IR is the cells' genetic material DNA and/or RNA. The lesions promoted in the single DNA helix can result in mutation, but a large number of injuries may exceed the repair capacity of the microbial cell resulting in cell death (STOCKWELL et al., 2017). Other damages in crucial components of the cell such as proteins and lipids can be promoted by IR (NIE et al., 2012).

Withal, e-beam is a very safe method, it is cold, has a high Sterility Assurance Level (SAL), needs a short exposure time and the control parameter of the method is the dose applied. Furthermore, it is an on-off technology that operates with electric power and has a much higher dosing rate than other radiation technologies, such as gamma (γ) and X-rays (SILINDIR; ÖZER, 2009). One limitation of the technology is that it has low penetrating ability in materials (LUNG et al., 2015). But the sugarcane wort can be irradiated in a thin liquid layer at controlled flow (ALCARDE; MARCOS; WALDER, 2001).

E-beam is an emerging technology that has been applied to industrial purposes, such as pharmaceutical and medical packing sterilization, food disinfection and sterilization (radappertization), and also to the treatment of water and municipal and industrial wastewater (KURILOVA et al., 2015; LUNG et al., 2015).

For these reasons, it is believed that the use of e-beam is a good strategy for the radappertization of the wort (commercial sterilization) because it allows it to be installed in the production line and to use part of the surplus energy of the industrial plant. Carrying in this way, greater efficiency in the processes of alcoholic fermentation.

Therefore, this study aimed to evaluate the application of the e-beam to control the contamination in wort from sugarcane molasses for alcoholic fermentation and to investigate the yield and productivity of alcoholic fermentation from wort treated with e-beam ionizing radiation.

3.2. Material and Methods

3.2.1. Material

The molasses of sugarcane used for the preparation of the wort to the alcoholic fermentation were obtained from a factory in Piracicaba city, São Paulo State, Brazil (22º 43 '31 "S, 47º38' 57" W), followed by physicochemical characterization and stored in a freezer (- 20 °C).

3.2.2. Wort clarification

The sugarcane molasses used for the preparation of the wort underwent the clarification process following Braga (2006) recommendations with the addition of 2,5 g of NaH_2PO_4 l^{-1} in boiled molasses. After the addition of the reagent, the molasses was autoclaved and held for 48 hours for further separation of the supernatant from the sedimented material.

At the end of the clarification step, the molasses with an initial concentration of 70 ˚Brix 9628.75 g L^{-1} of total reducing sugars) was diluted with distilled water until the final concentration of 16,7 ˚Brix (150 g l^{-1} of total reducing sugars) was obtained.

3.2.3. Preparation of the contaminating inoculum and inoculation

For the preparation of the contaminant inoculum, a sample of 10 grams of soil from several points in a cane field was collected, to simulate the groups of contaminating microorganisms, normally, found in the process of alcoholic fermentation in sugarcane mills. This sample was mixed with 90 ml of the clarified molasses, filtered using quantitative filter paper N.640, 125 mm (Hellma®), and placed in a 250 ml Erlenmeyer flask. This Erlenmeyer was maintained at 30 °C, under stirring at 100 RPM, for 24 hours, using a shaker model Minitrons Infors®.

After 24 hours, the inoculum suspension reached $2.01x10^{12}$ CFU ml^{-1} (Colony Forming Unit) of total mesophile and $1.32x10^{12}$ CFU ml^{-1} of total bacteria. Then, the inoculum was used to contaminate the wort. The final concentration in the wort was $1x10^{7}$ CFU ml^{-1} of total mesophile.

3.2.4. Treatments

The wort utilized in the investigation was submitted to six treatments. The first treatment, the negative control – without elimination of contaminants microorganisms (T0); four treatments using different ionization radiation doses from electron beam source: 10 kGy (T1); 20kGy (T2); 40 kGy (T3); 80kGy (T4) and one treatment using steam sterilization – as the positive control (T5), which will be detailed below.

3.2.4.1.Electron beam treatments

The irradiation treatments were performed in the Technological Radiation Center (CTR) of Energy and Nuclear Research Institute (IPEN – CNEN/SP). The samples were irradiated by the Electron Beam Accelerator JOB 188 (Dynamitron®).

The batch irradiation process was chosen, and the wort was added to borosilicate rectangular glass vessels (Pyrex®) and packed with plastic film 0.1 mm. Each vessel received 300 ml of wort obtained from clarified molasses, which corresponds to a height of 4 mm of the sample. For each batch, 4 vessels were irradiated.

The Electron Accelerator was set to energy, width, and current of the electron beam of $2{,}4x10^{-13}$ J (1,5 MeV), 0,112 m, and $5{,}61x10^{-3}$ A, respectively. The tray speed was 0.112 m s^{-1}, proportional to a dose of 5 kGy per run.

3.2.4.2.Steam treatment

The T5 treatment was performed by autoclaving the clarified molasses at 121 ˚C, 1 ATM, for 20 minutes in a vertical autoclave (Phoenix®).

3.2.5. Evaluation of the contamination control

The evaluation of the growth of total bacteria and total mesophiles was performed by the logarithmic variation in the number of CFU: Log (CFU ml^{-1} +1). It was added 1 to the CFU because of the treatments that presented 0 CFU due to the result of Log 0 is an undefined value.

The D_{10} (Required dose to destroy 90% of the population or 1 log) for total bacteria and total mesophile were calculated in kGy according to Equation 3.1, where N_0 is the initial CFU ml^{-1} and N_{final} is the CFU ml^{-1} after irradiation.

$$D_{10} = \frac{applyed\ dose}{\left(Log_{10}\ N_0\ - Log_{10} N_{final}\right)} \quad (3.1)$$

The efficiency control of the microorganisms was calculated according to Equation 3.2.

$$Efficiency\ of control\ (\%) = \left(\frac{N_0 - N_{final}}{N_0}\right) x100 \quad (3.2)$$

3.2.5.1.Chemical and microbiological analyses

After the treatments, the materials underwent chemical and microbiological analyses described below.

3.2.5.2. Total mesophile and total bacteria

For the enumeration of the microbial contamination levels, the total mesophile and total bacteria were measured by taken aseptically a sample of 1ml of the wort and serial diluted with 9 ml of saline solution (0.85% NaCl in distilled water).

After the serial dilution, the samples were pour plated in Plate Count Agar (PCA) in order to determine the total mesophile and, in PCA with 10 mg l^{-1} to determine the total bacteria. All plating was performed in triplicate with incubation at 30 ˚C for 48 hours.

3.2.5.3. Sugars, glycerol, and manitol

Glycerol, mannitol, and the sugars sucrose, glucose, and fructose were measured by ion chromatography, following the method described by the manufacturer (EITH et al., 2006).

It was used the ion chromatograph 930 Compact IC Metrohm® equipped with the column 1 Metrosep Carb 150 / 4.0, at 35 ˚C with eluent solution of 200 mM sodium hydroxide and the flow was set to 0.5 ml min^{-1}. The wort samples were diluted 200 times and the wine samples were diluted 50 times with ultrapure water. After that, the samples were filtered with 0.45 μm cellulose acetate filter. All the samples were measured in triplicates and the volume of sample injected was 20 μl.

3.2.5.4. Furfural and 5-hydroxymetylfurfural

Furfural and 5-hydroxymetylfurfural (5-HMF) were measured by gas chromatography with Flame Ionization Detector (FID), following the method 72 described by US Department of Labor Occupational Administration Safety and Health (1988).

It was used the gas chromatograph GC-FID Shimadzu equipped with the column ZB-5 Phenomenex® 60 m x 0.32 mm x 1,00μm. The chromatographic conditions were injector temperature 170 ˚C, injection mode split, carrier gas N_2, flow rate 6.0 ml min^{-1} (carrier gas), split ratio 8.0, column temperature 50 ˚C for 5 min, then temperature program to 150 ˚C at 5˚C min^{-1}, detector temperature 200 ˚C.

The wort samples were filtered with 0.45 μm cellulose acetate filter and the injection volume of the sample was 2.0 μl.

3.2.5.5.Total phenolics

The total phenolics of the wort samples were determined according to the Folin-Ciocalteu method according to Julkunentiitto (1985) with modifications. The procedure consisted of adding 1000 µl of the sample (or standard for calibration curve) and 500 µl of Folin Ciocalteu reagent (10% v/v) to a test tube, wait 40 minutes and then add 2500 µl of Na_2CO_3 (20 % w/v). After that, the absorbance was measured at 725nm in a quartz cuvette on the spectrophotometer (UV mini-1240 Shimadzu®).

The standard curve was prepared with 6 points of tannic acid (0, 4, 8, 12, 16, 20, 25 µg ml^{-1}) with 3 repetitions at each point. The samples were diluted with ultrapure water 25 times and measured in triplicate.

3.2.5.6.Total flavonoids

The total flavonoids of the worst samples were measured according to Mabry, Markham and Thomas (2012) with modifications. Thus, 4.3 mL of ethanol (70% v/v), 100 µL of 2% $AlCl_3$ (w v^{-1}; solution in methanol), 100 µL of sodium acetate 1M and 500 µL of the sample (or standard for calibration curve) were added to a test tube and kept at rest for 40 minutes. Then, the absorbance was measured at 415nm in a quartz cuvette on a spectrophotometer (UV mini-1240 Shimadzu®).

The standard curve was prepared with 8 points of rutin solution (0, 1, 2, 4, 8, 16 µg ml^{-1}) with 3 repetitions each point. The samples were diluted with ethanol (70% v/v) 50 times also measured in triplicate.

3.2.6. Fermentation

The worts from all treatments, except T4 (80 kGy), were submitted to the fermentation process. we chose the treatments with the first three doses for the fermentation because we understood that these would meet the necessary conditions to reduce microbial contamination and achieve high productivity and fermentative yield glimpsing the industrial application.

The fermentation process was conducted in 500 ml Erlenmeyer flasks containing 200 ml of wort, w of 16,7˚ Brix, 150 g l^{-1} of total reducing sugars, and 3% of the dry yeast *Saccharomyces cerevisiae* "Fleischmann" (AB Brasil Indústria e Comércio de Alimentos Ltda).

The fermentation process was conducted with 5 replicates (reactors) per treatment, at 30 ˚C, under 100 RPM stirring (Minitron Shaker – Infors HT®). The process was monitored through the losses of CO_2 from the reactors during the fermentation. At the end of the fermentation, yeast cell viability and total bacteria were measured, and the fermented wort was centrifuged at 3738,8 *g* (Thermo Fischer Scientific Sorval ST 40R® centrifuge), at 10 ˚C for 10 minutes. Then, the wine was immediately frozen (-20 ˚C) for further analysis.

The chemical and microbiological analyzes of the wines were: total bacteria, sugars, glycerol, and mannitol, as previously described, also yeast cell viability, yeast cell biomass, and alcohol content, as described below.

3.2.6.1. Yeast cell viability

The yeast cell viability was determined according to Pierce (1970) by the differential staining of living and dead cells using 0.1% methylene blue solution and observation on an optical microscope (Nikon®). This analysis was performed at the beginning and the end of the fermentation.

The samples were prepared with the dilution of 500 µl of the wine 10 times with distilled water. After that, 300 µl of the suspension was transferred to a tube with 300 µl of 0.1% methylene blue solution and homogenized. Then, 10 µl of the suspension was transferred to a Neubauer chamber followed by the observation on a microscope (400x).

3.2.6.2. Yeast cell biomass

The yeast cell biomass was determined by the wet weight according to Zago et al. (1996). For this, the fermented wort was centrifuged at 3738,8 *g* (Thermo Fischer Scientific Sorval ST 40R® centrifuge), at 10 ˚C for 10 minutes. Then, the pellet mass was measured on a semi-analytical scale (BL320H Shimadzu®) and compared with the initial mass of the sample.

3.2.6.3. Alcohol content

The alcohol content of the wine was measured by the distillation of 25 ml of sample in a micro-distiller (MA 012/1 Marconi®) followed by density measurement using a Digital Densimeter (EDM 4000 Schmidt Haensch®) at 20 ± 0.05 ˚C, according to Zago et al. (1996).

The density value of the solution was used to calculate the alcohol concentration of the sample by converting the read density in %m m^{-1} using a conversion table at 20 ˚C/ 20 ˚C.

3.2.6.4. Fermentation yield and productivity

The yield (practical) was calculated based on the volume of ethanol obtained from 100g of sugars supplied in the substrate according to Equation 3.3.

$$Yield = \left(\frac{ml\ of\ final\ ethanol}{g\ of\ sugars\ supplied}\right) x100 \quad (3.3)$$

The productivity was calculated according to Equation 3.4 based on the alcohol content at the end of the fermentation t and the fermentation time. The productivity was expressed in grams of ethanol per hour (g L^{-1} h^{-1}).

$$Productivy = \frac{\text{ethanol concentration in the wine g } l^{-1}}{fermentation\ time(h)} \quad (3.4)$$

3.2.7. Electrical consumption estimation

For the estimation of the energy cost to operate the electron accelerator used in this study, initially, it was calculated the energy consumption in KWh of the electron beam operating with a voltage of 1.5x 10^6 V and electric current of 5.61x10^{-3} A for 1 hour. This is the power required to irradiate a sample with a dose of 5kGy approximately. The total energy consumed by the accelerator peripherals such as cooling system, vacuum system, and compressed air was determined through the nominal values of the power supplied by the equipment manufacturers.

Regarding the cost of energy (US$ / MWh), since the sucroenergetic industry is also an electricity producer, which besides being self-sufficient, is still an exporter of the surplus energy produced, it was considered that the energy consumption needed for the accelerator would no longer be commercialized. Therefore, the value of MWh was equivalent to the average amount that would be paid to the ethanol-producing industry, according to the electric energy commercialization contracts of the Brazilian Electricity Regulatory Agency (ANEEL). For this calculation, it was considered that the amount paid for 1 MWh of the excess energy produced by burning biomass (sugarcane bagasse and wood chips) by ethanol-producing plants in Brazil through public auctions held by ANEEL for energy distributors (CCEE, 2021).

According to the auctions held in March / 2016 and April / 2017 (energy supplied respectively in 2020 and 2021) the average amount paid for each MWh of energy was US$ 42.50 or US$ 0.0425 / kWh (ANEEL, 2021). The dollar rate (US$ 1.00 = R $ 5.53) was consulted on 3/21/2021 on the website of the Central Bank of Brazil (BCB, 2021).

The costs of amortization, maintenance and investment were not considering in this study.

3.2.8. Experimental design and statistical analyzes

The experimental design was entirely randomized with six treatments and five replicates per treatment.

The results were submitted to analysis of variance (ANOVA) by the F test and the averages compared in the Tukey test at the significance level of 5% ($p \leq 0.05$). The statistical analyzes were performed using SISVAR 5.6 software (FERREIRA, 2014).

3.3. Results and Discussion

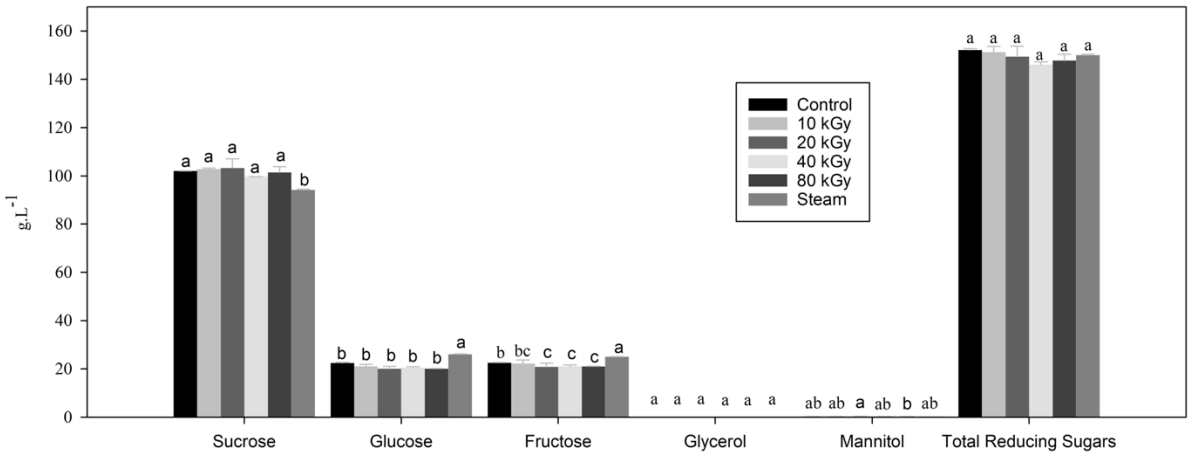
To meet the greenhouse gas emission reduction targets the demand for renewable biofuels has increased. For this, efforts must be made to increase industrial productivity, and for that, better control of microbiological contamination is highly necessary.

The yeast cells reuse during the season countless times can influence the contamination level of the must by bacteria and wild yeasts (LOPES et al., 2016; BREXÓ; SANT'ANA, 2017). The development and predominance of wild strains of yeasts are undesirable for the

process due to the lower productivity, flocculation, foaming, and biofilm formation produced by these microorganisms (BECKNER; IVEY; PHISTER, 2011; DELLA-BIANCA et al., 2013; DELLA-BIANCA; GOMBERT, 2013). These drawbacks increase the use of antifoam, acids, and antibiotics in the industry plant (BREXÓ; SANT'ANA, 2017).

The samples of wort submitted to the treatments T0, T1, T2, T3, T4 and T5 presented 7.00; 3.72; 3.31; 2.41; 0.00 and 0.00 Log (CFU +1) ml^{-1} ($p<0.05$) of total mesophiles, respectively (Fig. 3.1). These results correspond to an efficiency control of the microorganism of 99.94%; 99.97%; >99.99%; >99.99%; 100% and 100% ($p<0.05$) for T1, T2, T3, T4 and T5, respectively.

Figure 3.1 - Sugars concentrations at the start of fermentation. The error bars represent the standard deviation



For total bacteria, T0, T1, T2, T3, T4 and T5 presented 5.00; 2.33; 3.22; 2.33; 0.00 and 0.00 Log (CFU +1) ml^{-1}, respectively. These results correspond to an efficiency of control of 99.99%; 99.98%; >99.99%; 100% and 100% for T1, T2, T3, T4 and T5, respectively.

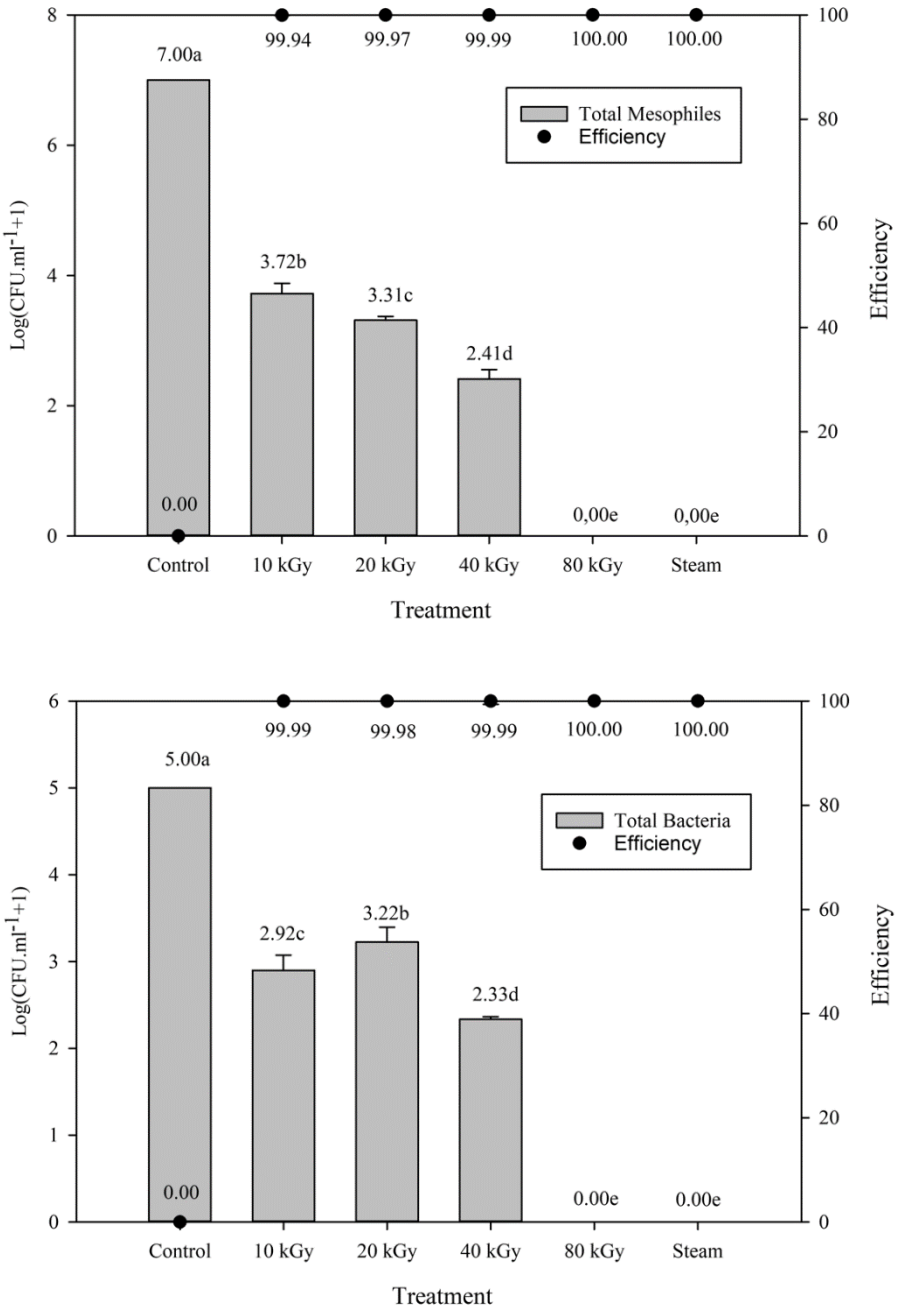
Therefore, the microbial contamination of the wort decreased as the irradiation dose increased, in accordance with Sampa et al. (2007) study.

Only treatments T4 (80 kGy) and T5 (steam - positive control) were able to sterilize the must. However, T3 (40 kGy) was sufficient for 4.59-log reduction of total mesophiles and 2.67-log reduction of total bacteria, corresponding to inactivation of more than 99.99% of the microorganisms present in the wort.

In a study carried out by Nobre et al. (2007) when submitting sugarcane juice to treatment with ionizing radiation (γ - Co^{60}), the dose of 15 kGy was not enough to fully inactivate the *Bacillus subtillis* culture, but achieved a reduction of more than 99.9% for these

bacteria. In the present study, inactivation of total bacteria higher than 99.9% was achieved in the dose of 10 kGy. Similar results were observed for total mesophiles (Fig. 3.2). Furthermore, the study conducted by Nobre et al. (2007) used pure cultures of bacteria while the present study used the total microbiota from a sugarcane field.

Figure 3.2 - Total mesophiles (a) and total bacteria (b) in sugarcane molasses wort after electron beam irradiation treatment in different doses. The error bars represent the standard deviation.



Most of the literature about microbial radioresistance is based on reports of experiments typically involving pure cultures grown under near-optimal conditions (SHURYAK, 2019). In this study, we used the microbiota from the soil of a sugarcane field since it is known that present many microorganisms groups in different levels. In addition, other authors have reported that bacterial contamination in alcoholic fermentation is mostly from the sugarcane field soil (GALLO, 1989; FIGUEIREDO; MACIEL; MARQUES, 2008; COSTA et al., 2015).

A study carried out by Costa et al. (2015) assessing microbial diversity at different stages of sugarcane ethanol production identified 22 archaeal groups, 203 fungi groups, and 355 bacterial groups. The authors also mentioned that the microbial contamination increases through the processes in the ethanol plant and is mostly from the feedstock and soil impurities.

Many microorganisms in soil are organic matter decomposers and also opportunistic plant/animal pathogens (DIEZMANN; DIETRICH, 2009; SYKES et al., 2014). This way of living requires being able to tolerate and possibly exploit the oxidizing compounds used as a defense mechanism by their hosts (HELLER; TUDZYNSKI, 2011) this may justify the high radiotolerance of some soil microorganisms.

Also, some microorganisms can synthesize antioxidant compounds and pigments that aid in radioprotection (KIM et al., 2007), such as vitamin C (MAO; QUE; WANG, 2006), carotenoids (PARVATHY, 1983; JAIN et al., 2015) and flavonoids (MOLINS, 2001; SHURYAK et al., 2017; SHURYAK, 2019). These compounds are commonly found in sugarcane juice (ABBAS et al., 2014) and also present in the sugarcane molasses, the raw material used in this study (Table 1).

The D_{10} (Required dose to destroy 90% of the population) for total mesophiles was 3.06 kGy, whereas for total bacteria it was 4.81 kGy. Bacteria (prokaryotic) are more radioresistant than other microorganisms such as fungi and viruses, so it is justified that the total bacteria D_{10} is higher than the total mesophiles. In addition, the values found are in accordance with the literature, which states that fungi and bacterial spores present D_{10} values between 1 and 10 kGy (CONFALONIERI; SOMMER, 2011; JUNG; LIM; BAHN, 2017; SHURYAK et al., 2017).

Other studies report radioresistant microorganisms like fungi that present chronic and acute radioresistance (D_{10} from 0.1 to 6.5 kGy) (SHURYAK et al., 2017); bacteria, such as *Deinococcus radiodurans* capable of withstanding high doses of radiation (D_{10} of 16 kGy)

(OMELCHENKO et al., 2005) and ability to reconstruction the functional genome (CONFALONIERI; SOMMER, 2011); and also archaea, such as *Thermococcus gammatolerans* sp. nov., which was isolated after exposure of 30 kGy (γ- radiation) (JOLIVET et al., 2003).

Moreover, *Lactobacillus plantarum*, one of the major contaminants of alcoholic fermentation (DONG; LIN; LI, 2015; DELLIAS et al., 2018), is described as a chronic and acute radioresistant microorganism (DALY et al., 2004; SHURYAK et al., 2017).

Most bacterial contaminants are found in the *Lactobacillus* genera (BONATELLI et al., 2017), especially lactic acid bacteria (LAB), like *L. plantarum*, which are responsible for reducing yeast cell viability due to the competition for nutrients and the production of toxic compounds, such as lactic and acetic acids during the fermentation (NARENDRANATH et al., 1997; COSTA et al., 2008).

In general, ethanol plants use antibiotics in order to control bacterial contamination. However, in some cases, does not prevent Lactobacilli infections recurrence, since these microorganisms can form biofilm, which is tolerant to the high concentration of the antibiotics and cleaning (DELLIAS et al., 2018; SAUNDERS et al., 2019).

The large-scale use of antibiotics can induce bacterial resistance (CARVALHO et al., 2020). Also, antibiotic residues such as virginiamycin can be found in distillers dried grain (DDG), from bioethanol fermentation of corn, which is utilized for animal feed (BISCHOFF; ZHANG; RICH, 2016). Regarding sugarcane bioethanol, there is a concern about antibiotic resistance in microorganisms that may be discharged into the environment through fertigation using vinasse, the liquid waste obtained from the distillation of the wine (MENDONÇA et al., 2016). Furthermore, the presence of antibiotics in the vinasse can negatively affect its anaerobic digestion for the production of biogas through the inhibition of acetogenic bacteria and methanogenic archaea (SANZ; RODRÍGUEZ; AMILS, 1996) and reduce the potential to use vinasse to produce other products.

Therefore, a more efficient disinfection process is needed, such as ionizing radiation (IR). However, the use of IR may promote the formation of inhibitors by-products from sugar degradation (MOLINS, 2001).

In our study, it was not observed formation and alteration in the concentration of the inhibitors flavonoids, furfural, and 5-HMF ($p>0.05$) in any condition of treatment evaluated (**Erro! Fonte de referência não encontrada.**). Such compounds are generally produced from sugar degradation, especially in thermal conditions (MOLINS, 2001; EGGLESTON; AMORIM, 2006; CHI et al., 2019). However, it did not occur in this study, including in the steam treatment (T5).

Table 3.1 - Chemical determinations of inhibitory by-products in the sugarcane molasses wort after treatments

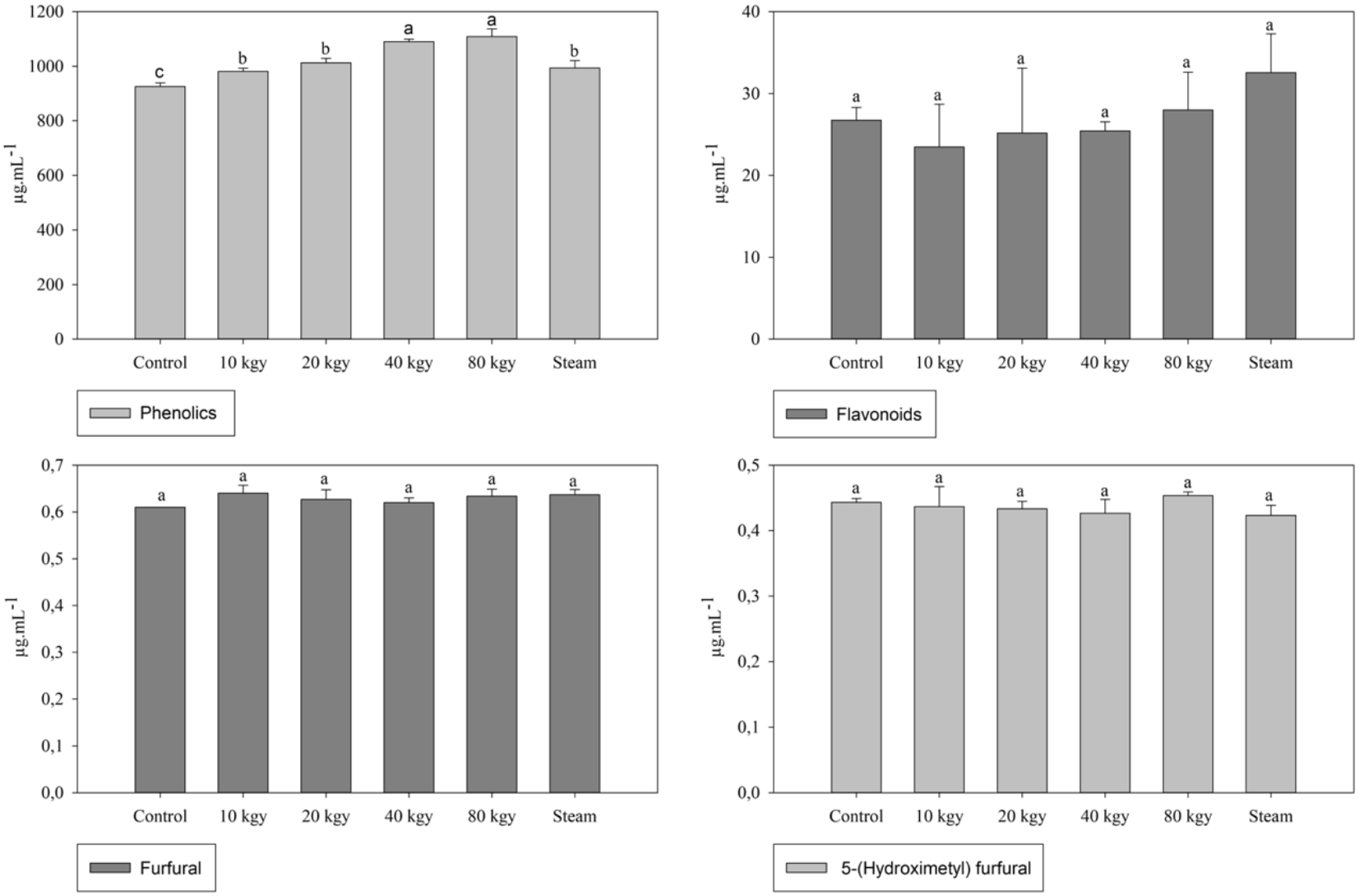
Treatment	Flavonoids	Phenolics	Furfural	5-HMF
		$\mu g\ ml^{-1}$		
T0 - Control	26.74 ± 1.57[a]	925.27 ± 13.72[c]	0.61± 0.00 [a]	0.44 ± 0.00a
T1 - 10 kGy	23.47 ± 5.22[a]	980.68 ± 11.74[b]	0.64 ± 0.02 [a]	0.44 ± 0.02[a]
T2 - 20 kGy	25.18 ± 7.92[a]	1012.64 ± 16.20[b]	0.62 ± 0.02 [a]	0.44 ± 0.02[a]
T3 - 40 kGy	25.44 ± 1.11[a]	1089.38 ±9.94[a]	0.62 ± 0.01 [a]	0.43 ± 0.01[a]
T4 - 80 kGy	27.99 ± 4.61[a]	1108.79 ± 28.00[a]	0.63 ± 0.01 [a]	0.45 ± 0.01[a]
T5 - Steam	32.55 ± 4.75[a]	993.77 ± 27.33[b]	0.64 ± 0.01[a]	0.42 ± 0.01[a]
C.V.	18.6	1.68	2.22	3.54

Averages of n= 5 ± standard deviation. Superscript equal letters in the same column do not differ statistically by the Tukey test at the 5% level of significance.

The aldehydes like furfural and 5-HMF may inhibit key enzymes intervening in the rate of protein synthesis of the central metabolism of the yeasts affecting negatively the growth and fermentation (CABAÑAS et al., 2019). Because of that, the presence of these compounds is highly unwanted in the fermentation substrate.

On the other hand, it was observed a gradual increase of phenolic compounds levels (6%, 9.4%, 17.8%, and 19.8%, for T1, T2, T3, and T4, respectively) according to the radiation dose applied (Figure 3.3). The steam treatment (T5) presented a significantly lower ($p<0.05$) concentration of phenolic compounds than 40 and 80 kGy, but it was statistically equal to treatments 10 and 20 kGy.

Figure 3.3 - Inhibitor's concentrations in sugarcane molasses wort after treatments. The error bars represent the standard deviation



According to Rasmussen, Sørensen and Meyer (2014), the degradation of carbohydrates, especially D-glucose, D-xylose, and L-arabinose can be related to the production of compounds such as phenolics. These compounds have been considered biocatalyst inhibitors (CHI et al., 2019), and their gradual production according to e-beam dose increase was also observed in Lima et al. (2016) study. But, in our work, the presence of phenolics did not inhibit yeast cell viability and biomass production ($p>0.05$) in any treatment during the fermentation (Table 3).

In a study conducted by Martín et al. (2007) using sugarcane bagasse hydrolysate a concentration of 2100 μg ml^{-1} of phenolic compounds was responsible for the yeast (*S. cerevisiae*) inhibition and consequently, poor fermentability. In the same study using an adapted strain of the same yeast it was observed higher ethanol yield on total sugar after 24 h (0.38 g g^{-1}) than the non-adapted yeast (0.18 g g^{-1}) in wort with 1400 μg ml^{-1} of phenolic compounds. In our study, although the yeast has not been adapted to inhibitory toxins, the concentration of phenolics was below 1089.38 μg ml^{-1} (**Erro! Fonte de referência não encontrada.**) in all fermented treatments, which probably reflected *the S. cerevisiae* tolerance to these compounds.

In addition to the low formation of inhibitors, it was not observed significant inversion of TRS (Total Reducing Sugars) in all treatments ($p>0.05$) (**Erro! Fonte de referência não encontrada.**). Otherwise, Lima et al. (2016) observed significant ($p<0.05$) TRS inversion in sugarcane juice irradiate with 20 kGy e-beam dose.

Table 3.2 - Behavior of sugars, glycerol, and mannitol in sugarcane molasses wort after treatments

	Sucrose	Fructose	Glucose	Glycerol	Mannitol	TRS
Treatment			g l^{-1}			
Control	102.03 ± 0.09^{a}	22.47 ± 0.31^{b}	22.55 ± 0.14^{b}	<LoQ	0.28 ± 0,01^{ab}	152.15 ± 0.52^{a}
10 kGy	102.77 ± 0.47^{a}	21.04 ± 0.89^{bc}	22.24 ± 1.43^{b}	<LoQ	0.28 ± 0,01^{ab}	151.19 ± 2.44^{a}
20 kGy	103.30 ± 3.79^{a}	20.04 ± 1.00^{c}	20.88 ± 1.55^{b}	<LoQ	0.30 ± 0,06^{a}	149.38 ± 4.33^{a}
40 kGy	99.65 ± 0.09^{a}	20.35 ± 0.54^{c}	21.00 ± 0.65^{b}	<LoQ	0.27 ± 0,01^{ab}	145.99 ± 1.19^{a}
80 kGy	101.49 ± 2.30^{a}	20.10 ± 0.11^{c}	21.05 ± 0.13^{b}	<LoQ	0.22 ± 0,02^{b}	147.72 ± 2.65^{a}
Steam	94.19 ± 0.27^{b}	26.05 ± 0.10^{a}	25.08 ± 0.02^{a}	<LoQ	0.27 ± 0,00^{ab}	150.02 ± 0.40^{a}
CV	1.82	2.8	4.09	0	9.02	1.58

<LoQ: lower than Limit of Quantification. Averages of n= 5 ± standard deviation. Superscript equal letters in the same column do not differ statistically by the Tukey test at the 5% level of significance.

The fact that in our study the reduction in the concentration of TRS was not observed is interesting because low sugar degradation is essential in a decontamination method aiming no decrease in the ethanol yield due to the sugars degradation (ALCARDE; MARCOS; WALDER, 2000; ALCARDE; WALDER; HORII, 2003).

Regarding sucrose, it was observed decrease in the steam treatment ($p<0.05$). However, there was no decrease in concentration ($p>0.05$) in irradiated treatments. In a study by Podadera (2007), using electron beam to sterilize inverted sugar syrup, it was observed a significant decrease ($p<0.05$) in sucrose concentrations between the control, 5, 10, and 30 kGy samples. Moreover, glucose and fructose concentrations increased significantly ($p<0.05$). Which indicated the degradation of the disaccharide with the breakdown of the glycosidic bond and formation of the reducing sugars glucose and fructose.

At the end of the fermentation, sucrose was not detected in any treatment; also, the residual sugars glucose and fructose presented low concentrations (<0.05%) in all treatments ($p>0.05$), evidencing efficient consumption of sugars by yeasts or other microorganisms during the fermentation process (**Erro! Fonte de referência não encontrada.**.3).

Table 3.3 - Microbiological and biochemical parameters after the fermentation.

		Produced during the fermentation									
Total bacteria	pH	Glycerol	Mannitol	Sucrose	Glucose	Fructose	Alcohol Content	Yeast biomass	Productivity	Yield	Yeast viability
Log (CFU ml^{-1}+1)				g l^{-1}			m m^{-1}	g ml^{-1}	g l^{-1} h^{-1}	%	%
5.55 ± 0.37^{a}	$4.38 \pm .06^{cb}$	15.37 ± 0.67^{a}	0.41 ± 0.02^{a}	<LoQ	0.26 ± 0.02^{a}	0.09 ± 0.02^{a}	6.82 ± 0.19^{c}	0.14 ± 0.01^{a}	0.85 ± 0.02^{ac}	88 ± 2^{c}	81 ± 2^{a}
2.68 ± 0.40^{b}	4.44 ± 0.11^{b}	15.64 ± 0.25^{a}	0.39 ± 0.01^{ab}	<LoQ	0.25 ± 0.07^{a}	0.12 ± 0.02^{a}	7.10 ± 0.13^{ab}	0.15 ± 0.00^{a}	0.89 ± 0.02^{ab}	92 ± 2^{b}	80 ± 2^{a}
2.25 ± 0.05^{b}	4.27 ± 0.02^{c}	15.83 ± 0.52^{a}	0.37 ± 0.02^{ab}	<LoQ	0.48 ± 0.28^{a}	0.14 ± 0.04^{a}	7.09 ± 0.04^{ab}	0.15 ± 0.00^{a}	0.89 ± 0.01^{ab}	93 ± 1^{ab}	79 ± 2^{a}
2.60 ± 0.49^{b}	4.58 ± 0.05^{a}	15.91 ± 0.31^{a}	0.33 ± 0.03^{b}	<LoQ	0.51 ± 0.44^{a}	0.12 ± 0.01^{a}	6.96 ± 0.13^{bc}	0.16 ± 0.02^{a}	0.87 ± 0.02^{bc}	93 ± 2^{ab}	81 ± 2^{a}
2.20 ± 0.15^{b}	4.65 ± 0.05^{a}	15.65 ± 0.20^{a}	0.35 ± 0.08^{ab}	<LoQ	0.28 ± 0.17^{a}	0.09 ± 0.03^{a}	7.28 ± 0.04^{a}	0.14 ± 0.00^{a}	0.91 ± 0.00^{a}	95 ± 0^{a}	82 ± 2^{a}
10.88	1.43	2.74	10.70	0	7.68	22.22	1.73	5.85	1.73	1.72	2.79

The concentration of glycerol was similar in the wine from all treatments, approximately 15 grams per liters ($p>0.05$). According to Bai, Anderson and Moo-Young (2008) during the fermentation commonly a level about 1% (w v^{-1}; 10g l^{-1}) of glycerol is produced.

The high concentration of glycerol in wine can be an indicator of the yeast response to the adversity. High sugar values lead to high concentrations of glycerol in the must, due to the increase in osmotic pressure (PONCE et al., 2016), as well as the presence of bacterial contamination (LI et al., 2009).

There was bacterial contamination in the wine of all treatments. However, the control treatment presented a higher value ($p<0.05$) of 5.55 log (CFU ml^{-1}+1). The presence of bacteria in all the treatments may be due to the contamination during the experiment sampling and poor asepsis.

As well as the high bacterial contamination, the control treatment (T0) presented a higher concentration of mannitol (0.41g l^{-1}) when compared with other treatments ($p<0.05$). Mannitol is a sensitive indicator of contamination and its presence is an indication of the enzymatic dehydrogenation of fructose carried out exclusively by bacteria (EGGLESTON et al., 2007).

The T3 wine exhibit the lowest concentration of mannitol (0.33 g l^{-1}) when compared with the other treatments ($p<0.05$). This was expected because among the treatments submitted to fermentation T3 presented the greatest contamination control. This result is remarkably interesting because according to Eggleston et al. (2007) high concentrations of mannitol may promote yeast flocculation and reduce the efficiency and the productivity of the fermentation. The authors also described that a concentration around 6 g L^{-1} of mannitol can cause a decrease of 4% in ethanol yield.

The control (T0) showed the lowest yield (88%) of the treatments. It is justified due to high bacterial contamination in wine (5.55 log) and conversion of sugars into metabolites such as glycerol and mannitol (Table 3). The highest fermentation yield was reached in the steam treatment (T5) with 95% ($p<0.05$). Right below, the treatments 20 kGy (T2) and 40 kGy (T3) presented 93% yield ($p>0.05$). Higher than usually fed-batch industrial fermentations with 87% average of fermentation using molasses as raw material (ANDRIETTA; MAUGERI, 1994; VIEGAS; ANDRIETTA; ANDRIETTA, 2002) and also greater than the yield described by

Alcarde et al. (2001) which achieved 90.56% in the fermentation of sugarcane juice treated with 10 kGy (γ radiation).

There are great importance and interest in increasing the yield of industrial fermentation. A yield of 93% could be responsible for a significant increase in ethanol production and, consequently, in the revenue of the industrial plant.

Regarding the ethanol productivity, it decreased with the contamination whereas the control treatment showed the lowest value of 0.85 g $l^{-1}h^{-1}$ (Table 3). The highest productivity has been achieved in steam treatment (0.91 g $l^{-1}h^{-1}$) followed by 10 and 20 kGy treatments with 0.89 g $l^{-1}h^{-1}$ in both ($p>0.05$) and 40 kGy treatment with 0.87 g $l^{-1}h^{-1}$ ($p<0.05$). Thus, the treatment of 20 kGy showed better productivity ($p<0.05$) and the same yield as treatment 40kGy, which requires more energy.

Due to that, the dose of 20 kGy is the most recommended as it requires less energy consumption (discussed below).

It is believed that greater changes in the fermentative behavior of the irradiated wort could have been observed if consecutive fermentative recycles and acid treatment of yeast from the control treatment had been carried out. Since the microbial contamination tends to increase throughout the fermentative recycles during the harvest season, as described in the literature (CECCATO-ANTONINI, 2018).

It is also necessary to study more the application of this technology, as well as the increase of the scale and the economic viability.

In our work, the electrical energy consumption to operate the electron accelerator at full power in one hour of use is approximately 150 kWh. Only the electron beam is responsible for 25% (37.5 kWh) of this total. The cooling system, vacuum system, compressed air, and other devices consumed the remaining 75% (112.5 kWh). In this case, the cost was estimated at US$ 6.43.

For the present case, the electron beam was not used at its maximum power. Due to this, for each hour of use of the accelerator, the electrical energy consumption estimated was 122 kWh, of which 113.58 kWh were consumed by the devices of each system mentioned above. Only 8.42 kWh was consumed by the electron beam. The cost was estimated at US$ 5.23.

Erro! Fonte de referência não encontrada. presents the operating cost, considering only the energy consumption of the electron accelerator for each treatment. Δt is the processing time (or sterilization) of the samples by e-beam. Their values were obtained considering the conveyor speed of 0.112 m s^{-1} and the linear length of two aligned trays equal to 0.40 m.

Table 3.4 - Operating cost relative to the use of electron beam for different doses of radiation applied to sugarcane molasses wort

Treatment	Radiation dose (kGy)	Δt (s)	Cost (US$)
T0	0	0	0
T1	10	11,6	0,644
T2	20	23,2	0,128
T3	40	46,4	0,258
T4	80	92,8	0,515

Therefore, operating at a dose of 20 kGy, the energy consumption by the electron accelerator was estimated at 146.18 kWh (33.68 kWh consumed by the electron beam and the rest by the peripheral equipment. The cost of each hour of operation of the accelerator was estimated at US$ 6.27. Considering that in the ethanol-producing plants in Brazil an average of 450 m^3 of wort is produced per hour, the estimated cost of processing 1 m^3 of this material is US$0.014.

It is important to note that the mills can process 1 m^3 of the wort in a short time. Furthermore, the e-beam technology is very fast, and, in a few seconds, the desired result in microbial control can be achieved. This allows the treatment of large volumes of wort in a short time, which facilitates this process implementation in large industries.

It is also important to mention that apart from sterilization of the wort, to have success in the contaminating control in the ethanol industry it is necessary an adequate system to cleaning fermenters, pipelines, centrifuges, valves, and other compartments used to transport or store wine, yeast cream, and wort.

With that, the use of e-beam for sterilization of the substrate could also make it possible to use more productive yeast strains in the fermentation, such as the thermotolerant strains of

S. cerevisiae described by Pattanakittivorakul et al. (2019), which show extremely high ethanol production at 40 ˚C, in addition to tolerating high gravity fermentation and high concentrations of furfural, 5-HMF and acetic acid.

It should be noted that the e-beam could also be applied in other processes within the industry, as in the pretreatment of biomass for the production of second-generation ethanol (POSTEK et al., 2018). The biomass irradiation can facilitate enzymatic hydrolysis with lower temperatures and minimal formation of inhibitory by-products when compared to conventional pretreatments (SINGH et al., 2016).

3.4. Conclusion

The dose of 80 kGy was sufficient for sterilization of the wort from molasses. In addition, the lower dose tested of 10 kGy were able to reduce more than 99.9% of the microbial contamination present in the substrate. The treatment of 20 kGy showed the best yield and ethanol productivity among the irradiated worts, evidencing the possibility of applying the e-beam in the treatment of wort for fermentation, which may allow reduction in losses caused by microbial contamination, besides the possibility of promoting fermentation yield and productivity increase.

References

ABBAS, S. R. et al. Phenolic profile, antioxidant potential and DNA damage protecting activity of sugarcane (*Saccharum officinarum*). **Food Chemistry**, v. 147, p. 10–16, 2014. Available at: <http://dx.doi.org/10.1016/j.foodchem.2013.09.113>.

AGÊNCIA NACIONAL DE ENERGIA ELÉTRICA - ANEEL. **Resultado de Leilões** - ANEEL: Planilha em Excel. Brasília, DF, 2021. Available at: <https://www.aneel.gov.br/resultados-de-leiloes>. Acessed at: 26 mar. 2021.

ALCARDE, A. R.; MARCOS, J.; WALDER, M. Comparison Between Gamma Radiation and Kamoran HJ in the Decontamination of Kamoran H Sugarcane Must. **Journal of Food Processing and Preservation**, v. 25, p. 137–147, 2001.

ALCARDE, A. R.; WALDER, J. M. M.; HORII, J. Fermentation of irradiated sugarcane must. **Scientia Agricola**, v. 60, n. 4, p. 677–681, 2003.

AMORIM, H. V.; OLIVEIRA, A. J.; CAMPOS, H. Infecção, problema sério na produção de álcool. In: CONGRESSO NACIONAL DA SOCIEDADE DOS TÉCNICOS AÇUCAREIROS DO BRASIL, 2., 1981, Rio de Janeiro. **Anais...** Piracicaba: STAB, 1981. v.1, p.158-168.

AMORIM, H. V. et al. Scientific challenges of bioethanol production in Brazil. **Applied Microbiology and Biotechnology**, v. 91, n. 5, p. 1267–1275, 2011.

ANDRIETTA, S. R.; MAUGERI, F. Optimum Design of a Continuous Fermentation Unit of an Industrial Plant for Alcohol Production. In: GALINDO, E.; RAMÍREZ, O. R. (Ed.). **Advances in Bioprocess Engineering**. Heidelberg: Springer, 1994. p. 47–52.

BAI, F. W.; ANDERSON, W. A.; MOO-YOUNG, M. Ethanol fermentation technologies from sugar and starch feedstocks. **Biotechnology Advances**, v. 26, n. 1, p. 89–105, 2008.

BANCO CENTRAL DO BRASIL – BCB. **Estatísticas Monetárias e de Crédito. Cotação do dólar USA**. Brasília, DF, 2021. Available at: <https://www.bcb.gov.br/>. Acessed at: 24 mar. 2021.

BASSO, L. C. et al. Yeast selection for fuel ethanol production in Brazil. **FEMS Yeast Research**, v. 8, n. 7, p. 1155–1163, 2008.

BASSO, L. C.; BASSO, T. O.; ROCHA, S. n. Ethanol Production in Brazil: The Industrial Process and Its Impact on Yeast Fermentation. In: BERNARDES, M. A. S. (Ed.). **Biofuel production** - Recent developments and prospects. Rijeka: InTech, 2011. p. 85–100.

BECKNER, M.; IVEY, M. L.; PHISTER, T. G. Microbial contamination of fuel ethanol fermentations. **Letters in Applied Microbiology**, v. 53, n. 4, p. 387–394, 2011. Available at: <http://doi.wiley.com/10.1111/j.1472-765X.2011.03124.x>.

BISCHOFF, K. M.; ZHANG, Y.; RICH, J. O. Fate of virginiamycin through the fuel ethanol production process. **World Journal of Microbiology and Biotechnology**, v. 32, n. 5, p. 1–7, 2016. Available at: <https://link.springer.com/article/10.1007/s11274-016-2026-3>. Acessed at: 2 fev. 2021.

BONATELLI, M. L. et al. Characterization of the contaminant bacterial communities in sugarcane first-generation industrial ethanol production. **FEMS Microbiology Letters**, v. 364, n. 17, p. 1–8, 2017.

BRAGA, V. S. **A Influência da Temperatura na Condução de Dois Processos Fermentativos para Produção de Cachaça**. 2006. 90 p. Dissertação (Mestrado em Ciência e Tecnologia de Alimentos) - Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Piracicaba, 2006.

BREXÓ, R. P.; SANT’ANA, A. S. Impact and significance of microbial contamination during fermentation for bioethanol production. **Renewable and Sustainable Energy Reviews**, v. 73, p. 423-434, 2017.

BROWN, N. A. et al. Transcriptional profiling of Brazilian *Saccharomyces cerevisiae* strains selected for semi-continuous fermentation of sugarcane must. **FEMS Yeast Research**, v. 13, n. 3, p. 277–290, 2013.

CABAÑAS, K. T. et al. Selection of *Saccharomyces cerevisiae* isolates for ethanol production in the presence of inhibitors. 3 **Biotech**, v. 9, n. 1, p. 1–11, 2019. Available at: <http://dx.doi.org/10.1007/s13205-018-1541-3>.

CARVALHO, R. S. et al. Interaction between *Saccharomyces cerevisiae* and *Lactobacillus fermentum* during co-culture fermentation. **Biocatalysis and Agricultural Biotechnology**, v. 29, art. 101756, 2020.

CÂMARA DE COMERCIALIZAÇÃO DE ENERGIA ELÉTRICA - CCEE. **Ambiente de contratação regulada**. São Paulo, 2021. Available at: <https://www.ccee.org.br/portal/faces/pages_publico/onde-atuamos/comercializacao?_adf.ctrl-state=19fhn2ghfl_1&_afrLoop=112452198849623#!%40%40%3F_afrLoop%3D112452198849623%26_adf.ctrl-state%3D19fhn2ghfl_5>. Acesso em: 26 mar. 2021.

CECCATO-ANTONINI, S. R. Conventional and nonconventional strategies for controlling bacterial contamination in fuel ethanol fermentations. **World Journal of Microbiology and Biotechnology**, v. 34, n. 6, p. 1–11, 2018. Available at: <http://dx.doi.org/10.1007/s11274-018-2463-2>.

CERVI, W. et al. Bioelectricity potential from ecologically available sugarcane straw in Brazil : A spatially explicit assessment. **Biomass and Bioenergy**, v. 122, p. 391–399, 2019. Available at: <https://doi.org/10.1016/j.biombioe.2019.02.001>.

CHI, Z. et al. Comparison of product distribution, content and fermentability of biomass in a hybrid thermochemical/biological processing platform**. Biomass and Bioenergy**, v. 120, p. 107–116, 2019. Available at: <https://doi.org/10.1016/j.biombioe.2018.11.006>.

CONFALONIERI, F.; SOMMER, S. Bacterial and archaeal resistance to ionizing radiation. **Journal of Physics: Conference Series**, v. 261, n. 1, art. 012005, 2011.

COSTA, M. A. S.; CERRI, B. C.; CECCATO-ANTONINI, S. R. Ethanol addition enhances acid treatment to eliminate Lactobacillus fermentum from the fermentation process for fuel ethanol production. **Letters in Applied Microbiology**, v. 66, n. 1, p. 77–85, 2018.

COSTA, O. Y. A. et al. Microbial diversity in sugarcane ethanol production in a Brazilian distillery using a culture-independent method. **Journal of Industrial Microbiology and Biotechnology**, v. 42, n. 1, p. 73–84, 2015.

COSTA, V. M. et al. Production of acetic acid, ethanol and optical isomers of lactic acid by Lactobacillus strains isolated from industrial ethanol fermentations. **Ciencia e Agrotecnologia**, v. 32, n. 2, p. 503–509, 2008. Available at: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1413-70542008000200025&lng=en&nrm=iso&tlng=pt>.

DA SILVA-NETO, J. M. et al. Can ethanol partially or fully replace sulfuric acid in the acid wash step of bioethanol production to fight contamination by *Lactobacillus fermentum*? **Brazilian Journal of Chemical Engineering**, v. 37, n. 2, p. 323–332, 2020. Available at: <https://doi.org/10.1007/s43153-020-00033-x>.

DALY, M. J. et al. Accumulation of Mn(II) in *Deinicoccus radiodurans* facilitates gamma-radiation resistance. **Science**, v. 306, n. 5698, p. 1025–1028, 2004.

DELLA-BIANCA, B. E. et al. What do we know about the yeast strains from the Brazilian fuel ethanol industry? **Applied Microbiology and Biotechnology**, v. 97, p. 979-991, 2013. Available at: <https://link.springer.com/article/10.1007/s00253-012-4631-x>.

DELLA-BIANCA, B. E.; GOMBERT, A. K. Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. **Antonie van Leeuwenhoek**, **International Journal of General and Molecular Microbiology**, v. 104, n. 6, p. 1083–1095, 2013.

DELLIAS, M. de T. F. et al. Biofilm formation and antimicrobial sensitivity of lactobacilli contaminants from sugarcane-based fuel ethanol fermentation. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, v. 111, n. 9, p. 1631–1644, 2018. Available at: <https://doi.org/10.1007/s10482-018-1050-8>.

DIEZMANN, S.; DIETRICH, F. S. *Saccharomyces cerevisiae*: Population divergence and resistance to oxidative stress in clinical, domesticated and wild isolates. **PLoS ONE**, v. 4, n. 4, e5317, 2009.

DONG, S. J.; LIN, X. H.; LI, H. Regulation of *Lactobacillus plantarum* contamination on the carbohydrate and energy related metabolisms of Saccharomyces cerevisiae during bioethanol fermentation**. International Journal of Biochemistry and Cell Biology**, v. 68, p. 33–41, 2015. Available at: <http://dx.doi.org/10.1016/j.biocel.2015.08.010>.

EGGLESTON, G. et al. Mannitol as a sensitive indicator of sugarcane deterioration and bacterial contamination in fuel alcohol production. Zuckerindustrie**. Sugar Industry**, v. 132, n. 1, p. 33–39, 2007.

EGGLESTON, G.; AMORIM, H. Reason for the chemical destruction of sugars during the processing of sugarcane for raw sugar and fuel alcohol production. **International Sugar Journal**, v. 108, n. 1289, p. 271–282, 2006.

EITH, C. et al. **Practical Ion Chromatography**: An Introduction. Herisau, Switzerland: Metrohm, 2006.

FERREIRA, D. F. Sisvar: a guide for its bootstrap procedures in multiple comparisons**. Ciencia e Agrotecnologia**, v. 38, n. 2, p. 109–112, 2014.

FIGUEIREDO, I. C.; MACIEL, B. F.; MARQUES, M. O. A qualidade da cana-de-açúcar como matéria-prima para produção de álcool. **Nucleus**, v. 5, n. 2, p. 82–92, 2008. Special issue. Available at: <http://dialnet.unirioja.es/servlet/articulo?codigo=4037531>.

GALLO, C. R. **Determinação da microbiota bacteriana de mosto e de dornas na fermentação alcoolica**. 1989. 388 p. Tese (Doutorado em Ciências de Alimentos) – Faculdade de Engenharia de Alimentos, Universidade de Campinas, Campinas, 1989.

HELLER, J.; TUDZYNSKI, P. Reactive Oxygen Species in Phytopathogenic Fungi: Signaling, Development, and Disease. **Annual Review of Phytopathology**, v. 49, n. 1, p. 369–390, 2011.

JAIN, R. et al. Influence of selenium on metallothionein gene expression and physiological characteristics of sugarcane plants. **Plant Growth Regulation**, v. 77, n. 2, p. 109–115, 2015. Available at: <http://dx.doi.org/10.1007/s10725-015-0042-1>.

JOLIVET, E. et al. *Thermococcus gammatolerans* sp. nov., a hyperthermophilic archeon from a deep-sea hydrothermal vent that resists ionizing radiation. **International Journal of Systematic and Evolutionary Microbiology**, v. 53, n. 3, p. 847–851, 2003.

JULKUNENTIITTO, R. Phenolic constituents in the leaves of northern willows - Methods for the analysis of certain phenolics. **Journal of Agricultural and Food Chemistry**, v. 33, n. 2, p. 213–217, 1985.

JUNG, K. W.; LIM, S.; BAHN, Y. S. Microbial radiation-resistance mechanisms. **Journal of Microbiology**, v. 55, n. 7, p. 499–507, 2017.

KIM, D. et al. Effects of gamma irradiation on the radiation-resistant bacteria and polyphenol oxidase activity in fresh kale juice. **Radiation Physics and Chemistry**, v. 76, n. 7, p. 1213–1217, 2007.

KOCHETKOV, N. K.; KUDRJASHOV, L. I.; CHLENOV, M. A**. Radiation chemistry of carbohydrates**. 1. ed. Oxford: Pergamon Press, 1979.

KURILOVA, A. A. et al. Application of Electron Beam for Wastewater Disinfection. **Procedia Chemistry**, v. 15, p. 187–192, 2015. Available at: <http://dx.doi.org/10.1016/j.proche.2015.10.030>.

LI, L. L. et al. The induction of trehalose and glycerol in *Saccharomyces cerevisiae* in response to various stresses. **Biochemical and Biophysical Research Communications**, v. 387, n. 4, p. 778–783, 2009. Available at: <http://dx.doi.org/10.1016/j.bbrc.2009.07.113>.

LIMA, R. B. et al. Sucrose and color profiles in sugarcane (*Saccharum* sp.) juice analyzed by UFLC-ELSD and Synapt High-Definition Mass Spectrometry during radiation treatment. **Radiation Physics and Chemistry**, v. 121, p. 99–105, 2016. Available at: <http://dx.doi.org/10.1016/j.radphyschem.2015.12.022>.

LOPES, M. L. et al. Ethanol production in Brazil: a bridge between science and industry. **Brazilian Journal of Microbiology**, v. 47, p. 64–76, 2016. Available at: <http://dx.doi.org/10.1016/j.bjm.2016.10.003>.

LUNG, H. M. et al. Microbial decontamination of food by electron beam irradiation. **Trends in Food Science and Technology**, v. 44, n. 1, p. 66–78, 2015. Available at: <http://dx.doi.org/10.1016/j.tifs.2015.03.005>.

MABRY, T. J.; MARKHAM, K. R.; THOMAS, M. B. **The systematic identification of flavonoids**. Heidelberg: Springer Science, 2012.

MAO, L.; QUE, F.; WANG, G. Sugar metabolism and involvement of enzymes in sugarcane (Saccharum officinarum L.) stems during storage. **Food Chemistry**, v. 98, n. 2, p. 338–342, 2006.

MARTÍN, C. et al. Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. **Bioresource Technology**, v. 98, n. 9, p. 1767–1773, 2007.

MENDONÇA, A. A. et al. First identification of Tn916-like element in industrial strains of *Lactobacillus vini* that spread the tet-M resistance gene. **FEMS Microbiology Letters**, v. 363, n. 3, art. fnv240, 2016. Available at: <https://academic.oup.com/femsle/article-lookup/doi/10.1093/femsle/fnv240>.

MOLINS, R. A. **Food Irradiation**: Principles and Applications. Hoboken: John Wiley & Sons, 2001.

MUTHAIYAN, A.; LIMAYEM, A.; RICKE, S. C. Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations. **Progress in Energy and Combustion Science**, v. 37, n. 3, p. 351–370, 2011. Available at: <http://dx.doi.org/10.1016/j.pecs.2010.06.005>.

NARENDRANATH, N. V et al. Effects of lactobacilli on yeast-catalyzed ethanol fermentations. **Applied and Environmental Microbiology**, v. 63, n. 11, p. 4158–4163, 1997.

NIE, J. H. et al. Oxidative damage in various tissues of rats exposed to radon. **Journal of Toxicology and Environmental Health - Part A: Current Issues**, v. 75, n. 12, p. 694–699, 2012.

NOBRE, P.; HORII, J.; ALCARDE, R. Associação Com Bactérias Contaminantes Da Fermentação Alcoólica. **Ciência e Tecnologia dos Alimentos**, v. 27, n. 1, p. 20–25, 2007.

NOLASCO JUNIOR, J. **Eficiência de processo térmico para mostos a base de caldo de cana e melaço na produção de bioetanol.** 2010. Tese (Doutorado em Ciência de Alimentos) - Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, 2010.

OMELCHENKO, M. V. et al. Comparative genomics of Thermus thermophilus and Deinococcus radiodurans: Divergent routes of adaptation to thermophily and radiation resistance**. BMC Evolutionary Biology**, v. 5, p. 1–22, 2005.

PARVATHY, K. Bottling of sugarcane juice. In: Annual Report of the Scheme for Studies on Harvest & Postharvest Technology, 1983, Coimbatore, India. **Proceedings…** Coimbatore: ICAR, 1983. p. 13-16.

PATTANAKITTIVORAKUL, S. et al. Selection of thermotolerant *Saccharomyces cerevisiae* for high temperature ethanol production from molasses and increasing ethanol production by strain improvement. **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, v. 112, n. 7, p. 975–990, 2019. Available at: <https://doi.org/10.1007/s10482-019-01230-6>.

PIERCE, J. S. Institute of Brewing: Analysis committee measurement of yeast viability. **Jounal of the Institute of Brewing**, v. 76, p. 442–443, 1970.

PODADERA, P. **Estudo das propriedades do açúcar líquido invertido processado com radiação gama e feixe de elétrons**. 2007. 99 p. Tese (Doutorado em Tecnologia Nuclear - Aplicações) - Instituto de Pesquisas Energéticas e Nucleares, Universidade de São Paulo, São Paulo, 2007.

PONCE, G. H. S. F. et al. Sugarcane molasses fermentation with in situ gas stripping using low and moderate sugar concentrations for ethanol production: Experimental data and modeling. **Biochemical Engineering Journal**, v. 110, p. 152–161, 2016. Available at: <http://dx.doi.org/10.1016/j.bej.2016.02.007>.

POSTEK, M. T. et al. Ionizing radiation processing and its potential in advancing biorefining and nanocellulose composite materials manufacturing. **Radiation Physics and Chemistry**, v. 143, p. 47–52, 2018. Available at: <http://dx.doi.org/10.1016/j.radphyschem.2017.09.015>.

RASMUSSEN, H.; SØRENSEN, H. R.; MEYER, A. S. Formation of degradation compounds from lignocellulosic biomass in the biorefinery: Sugar reaction mechanisms. **Carbohydrate Research**, v. 385, p. 45–57, 2014. Available at: <http://dx.doi.org/10.1016/j.carres.2013.08.029>.

SAMPA, M. H. O. et al. Remediation of polluted waters and wastewater by radiation processing. **Nukleonika**, v. 52, n. 4, p. 137–144, 2007.

SANZ, J. L.; RODRÍGUEZ, N.; AMILS, R. The action of antibiotics on the anaerobic digestion process. **Applied Microbiology and Biotechnology**, v. 46, n. 5–6, p. 587–592, 1996. Available at: <https://link.springer.com/article/10.1007/s002530050865>.

SAUNDERS, L. P. et al. Inhibition of *Lactobacillus* biofilm growth in fuel ethanol fermentations by Bacillus. **Bioresource Technology**, v. 272, p. 156–161, 2019.

SCHWARZ, H. A. Free radicals generated by radiolysis of aqueous solutions. **Journal of Chemical Education**, v. 58, n. 2, art. 101, 1981. Available at: <https://doi.org/10.1021/ed058p101>.

SHURYAK, I. et al. Microbial cells can cooperate to resist high-level chronic ionizing radiation. **PLoS ONE**, v. 12, n. 12, p. 1–24, 2017.

SHURYAK, I. Review of microbial resistance to chronic ionizing radiation exposure under environmental conditions. **Journal of Environmental Radioactivity**, v. 196, p. 50-63, 2019.

SILINDIR, M.; ÖZER, A. Y. Sterilization methods and the comparison of E-beam sterilization with gamma radiation sterilization. **Fabad Journal of Pharmaceutical Sciences**, v. 34, n. 1, p. 43–53, 2009.

SINGH, R. et al. Opportunities for utilization of non-conventional energy sources for biomass pretreatment. **Bioresource Technology**, v. 199, p. 398–407, 2016. Available at: <http://dx.doi.org/10.1016/j.biortech.2015.08.117>.

STOCKWELL, B. R. et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. **Cell**, v. 171, n. 2, p. 273–285, 2017.

SYKES, S. M. et al. Comparative Genomic and Transcriptomic Analysis of Wangiella dermatitidis, A Major Cause of Phaeohyphomycosis and a Model Black Yeast Human Pathogen. **G3: Genes, Genomes, Genetics**, v. 4, n. 4, p. 561–578, 2014.

US DEPARTMENT OF LABOR OCCUPATIONAL ADMINISTRATION SAFETY AND HEALTH. Method 72: **Organic Methods Evaluation Branch**. Salt Lake City: OSHA Analytical Laboratory, 1988.

VIEGAS, M. C.; ANDRIETTA, S. R.; ANDRIETTA, M. G. S. Use of tower reactors for continuous ethanol production. **Brazilian Journal of Chemical Engineering**, v. 19, n. 2, p. 167–173, 2002. Available at: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0104-66322002000200012&lng=en&nrm=iso&tlng=en>.

ZAGO, E. et al. **Métodos analíticos para o controle da produção de álcool e açúcar**. Piracicaba: Fermentec; Fealq; Esalq, 1996. 194 p.

4. Supplementation of trace elements to sulfate-rich substrate and their impact in H_2S formation and methane production

Abstract

In the present work the anaerobic digestion (AD) of vinasse and the anaerobic co-digestion (AC) of vinasse with filter cake and sugarcane straw were investigated. The aim was to evaluate the trace elements (TE) supplementation and their impact on the processes, especially on H_2S formation and methane yield from a sulfate-rich substrate. The study was conducted through a BMP test and a continuous experiment in CSTR reactors. The reactors were supplemented with Fe, Ni, Co, Se, Mo, and Zn, in order to circumvent the problems caused by the high sulfate concentration. Iron played an important role in decreasing the H_2S production. In addition, the other metals made it possible to increase the methane yield of the process. The vinasse AD and TE reactor (R2) reached a yield between 222.29-411.39 Nml $CH_4 g^{-1}$ VS, and the H_2S in biogas remained below 500 ppm after addition of all the TE. The control reactor that did not received TE (R1) reached 36,000 ppm of H_2S and failed due to acidification. However, after pH correction and TE addition, it was possible to re-establish AD in R1with similar methane yield to R2. In the AC reactors R3 and R4 after the addition of all TE, a yield between 54.61-109.14 Nml CH_4 g^{-1} VS was observed in R3, and 54.15-110.21 Nml CH_4 g^{-1} VS between in R4. In addition, the H_2S concentration remained below 186.42 in R3 and below 70.74 ppm in R4. In general, anaerobic co-digestion with the addition of metals maintained greater stability over the 238 days of experiment, with low accumulation of volatile fatty acids and H_2S.

Keywords: biogas. sugarcane vinasse. metals supplementation. iron.

4.1. Introduction

Anaerobic digestion (AD) is a biological process very complex in chemical and microbiological aspects in which organic material is anaerobically degraded by different populations of cooperating microorganisms, generating end products in the gas phase (H_2, CH_4, CO_2), valuable organic compounds in the liquid phase and stabilized biosolids (ERSAHIN et al., 2011; RITTMANN; MCCARTY, 2012).

The efficiency of digestion will depend on specific operating conditions such as pH, temperature, type of substrate used, solids concentration, OLR (Organic Loading Rate), HRT (Hydraulic Retention Time) (CRUZ et al., 2010; FUESS et al., 2017).

The presence of sulfate in the substrate used for anaerobic digestion may be responsible for inhibition of the process, especially the production of methane by methanogenic archaea (CHEN; CHENG; CREAMER, 2008) and increase the hydrogen sulfide (H_2S) production by sulfate-reducing bacteria (SRB), which is a highly corrosive, toxic and unpleasant odor gas (MUYZER; STAMS, 2008).

Vinasse, the main residue from sucroenergetic industry is an effluent rich in organic matter, and sulfate. For each liter of ethanol produced, between 10 and 13 liters of vinasse are generated (PARSAEE; KIANI DEH KIANI; KARIMI, 2019). In addition, vinasse also has high polluting potential, which can cause eutrophication of aquatic systems, soil acidification and greenhouse gases (GHG) emissions due to its high concentration of organic matter (CHRISTOFOLETTI et al., 2013). Due to this, an alternative is the production of biogas through anaerobic digestion (AD) of the vinasse (PARSAEE; KIANI DEH KIANI; KARIMI, 2019).

However, the high sulfate concentration in the vinasse can be a problem to its AD, because promotes high H_2S generation (LENS et al., 1998). High concentrations of H_2S can be detrimental to the AD process, it impacts biogas production by disturbing the methanogenesis stage and cause inhibition to the trace elements needed for the digestion process (PARSAEE; KIANI DEH KIANI; KARIMI, 2019).

An alternative to avoid the problems caused by sulfate presence in anaerobic digestion is the anaerobic co-digestion (AC) with other residues and the addition of metals, such as Fe, Ni, Co, Mo, Se, and Zn in the form of trace elements. The co-digestion with other organic residues of great importance for the sucroenergetic industry, such as sugarcane straw and filter cake can provide greater balance of nutrients and dilution of methanogenic inhibitors present in the vinasse such as sulfate, providing a bioprocess with greater stability (MATA-ALVAREZ, 2005; NAYONO; GALLERT; WINTER, 2010; SARKER et al., 2019).

Furthermore, the addition of trace elements may provide a boost in the process through correction of the deficiency of micronutrients required by the anaerobic process (ZITOMER; JOHNSON; SPEECE, 2008). The iron, for example, can precipitate sulfur, preventing the

inhibition of methanogenesis by SRB and the formation of H_2S in biogas (SHAKERI YEKTA et al., 2017). In addition, the sulfate precipitation by iron provides better bioavailability of trace elements for the microbial consortium (SPEECE, 2008).

After AD, the biogas can be used to generate electricity after a cleaning process to eliminate humidity and H_2S, as well as other impurities depending on the source. Biogas can also be upgraded (through the separation of carbon dioxide) producing the so called biomethane, a renewable gas similar to natural gas that can be used to replace this fossil fuel in transportation sector, injected into natural gas grid or another end uses. Thus, the AD gives the benefits of nutrient recycling, reduce GHG emissions and also convert organic matter into biogas (LEME; SEABRA, 2017).

Therefore, the aims of the present study were to evaluate the AD of vinasse, the AC of vinasse, sugarcane straw and filter cake, also, trace elements supplementation.

4.2. Material and Methods

4.2.1. Feedstock

The materials used as substrate in this study were vinasse, filter cake and sugarcane straw. The vinasse came from the Ethanol Plant of Iracema Industry (São Martinho Group), located in the municipality of Iracemápolis, São Paulo State, Brazil. The vinasse was from a process of ethanol production from molasses of sugarcane and in the industry the vinasse was subjected to the process of concentration in evaporator multiple-effects type. The material was sent to Sweden by plane and kept frozen -20 °C until the experiments were carried out. Before being used in the anaerobic digestion process, the vinasse was diluted in distilled water until obtaining the desired chemical oxygen demand (COD). For this, a linear regression curve was established ($R^2 = 0.995$).

The filter cake came from the Granelli industry plant, located in the municipality of Charqueada, São Paulo State, Brazil. After collecting, the filter cake was dried in an oven with air circulation at 70 °C for 24 hours to remove moisture. Then, it was ground in a 1mm smash mill, packed in vacuum bags, and transported to Sweden. At the time of collection, the filter cake had 74.5% moisture, this moisture was added at the time of the experiments with distilled water.

The straw came from a cane field near the same industry that the filter cake was collected. After being collected, the straw was dried in an oven at 70 °C with air circulation for 24 hours to remove moisture. Then, it was ground in a 1mm smash mill, packed in vacuum bags, and transported to Sweden. At the time of collection, the straw had 5.0% moisture, this moisture was added at the time of the experiments with distilled water.

4.2.2. Inoculum

The inoculum used in the BMP tests and reactors' experiments were collected at Tekniska Verken Åby co-digestion plant located in Linköping, Östergötland, Sweden. The co-digestion plant was in operation of mesophilic co-digestion (38 °C) of organic fractions of municipal solid waste (OFMSW) (40%), grocery waste (35%), slaughterhouse waste (20%) and industrial organic wastes (5%). The treatment plant was working with an organic loading rate (OLR) around 2 kg VS m^{-3} d^{-1} and hydraulic retention time (HRT) of 20 days.

The inoculum was transferred to the CSTR reactors used in the experiment (described below) shortly after collection. For the BMP tests, the inoculum was stored in a 15 L CSTR reactor (*Biogas test plant BTP2 - Umweltleistungen*®) at 37 °C and 100 RPM without feeding for 5 days to reduce the endogenous production of biogas during the BMP test, that is, to reduce the production of biogas from the nutrients that were present in the inoculum according to the recommendations of Angelidaki et al. (2009) and Holliger et al. (2016).

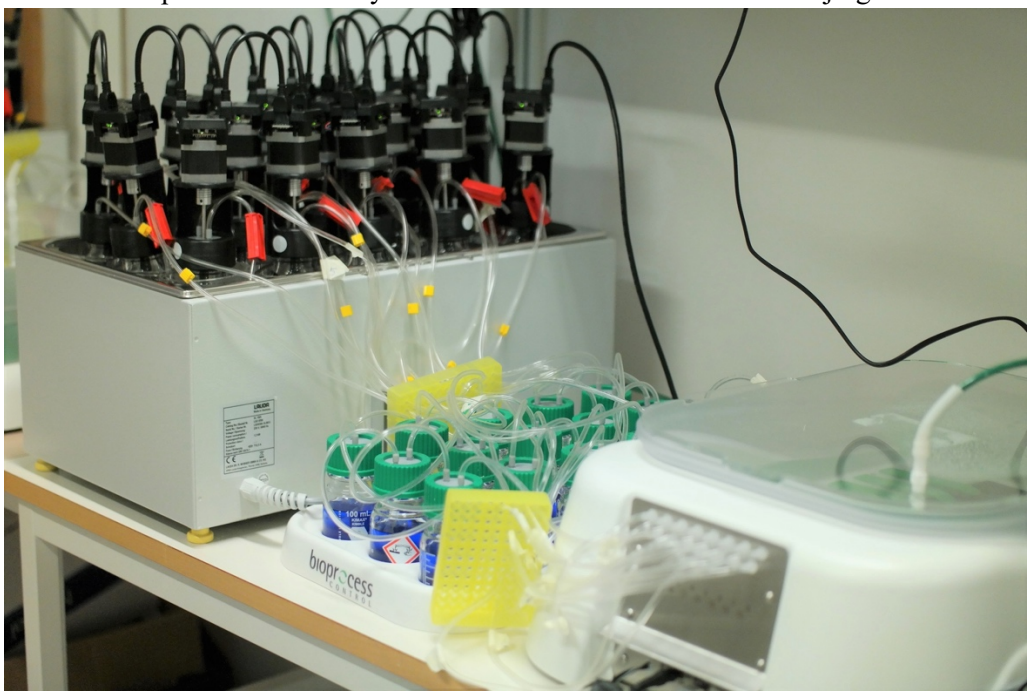
4.2.3. Biochemical Methane Potential tests

The BMP experiments were carried out in triplicates for each treatment and inoculum, according to the procedure described by Holliger et al. (2016). In the tests, 500 ml serum bottles were filled with 100 ml of inoculum and completed with substrate to keep the overall volume at 300 ml. The ratio of inoculum to substrate was 2:1 based in Volatile Solids (VS). To achieve the desired VS concentration of the substrates ultrapure water (milli-Q®) was used.

To set up an anaerobic atmosphere, the bottles were flushed with nitrogen gas and then sealed. Finally, the bottles were put in three water baths sets at a temperature of 37 °C and incubated for 27 days (640 hours) in three AMPTS II systems (Bioprocess Control®, Sweden) with stirring every 15 minutes alternating clockwise and counterclockwise (Figure 4.1). The measurement of the volume of methane generated was continuously evaluated with a reading

every 15 minutes. The BMP test was finished after 3 consecutive days without methane production in all treatments.

Figure 4.1 - Bioprocess Control System. Photo credit: Mette Axelsson Bjerg



The treatments are described in Table 4.1. The treatments with iron received 14g L^{-1} of $FeCl_3$ corresponding to a molar ratio of 1 S:Fe, following Shakeri Yekta et al. (2017) recommendations. Each treatment was performed in triplicate, thus a total of 45 BMP bottles were settle.

Table 4.1 - Biochemical methane potential test treatments description

treatment	description
F	Filter cake 100 %(w/w on VS basis)
F +Fe	Filter cake 100%(w/w on VS basis) + iron
V	Vinasse 100% (w/w on VS basis)
V + Fe	Vinasse 100 % (w/w on VS basis) + iron
S	Straw 100% (w/w on VS basis)
S + Fe	Straw 100% (w/w on VS basis) + iron
VF	Vinasse 97.8% (w/w on VS basis) and Filter cake 2.2% (w/w on VS basis)
VF +Fe	Vinasse 97.8% (w/w on VS basis) and Filter cake 2.2% (w/w on VS basis) + iron
VS	Vinasse 91.8% (w/w on VS basis) and Straw 8.2% (w/w on VS basis)
VS +Fe	Vinasse 91.8% (w/w on VS basis) and Straw 8.2% (w/w on VS basis) + iron
SF	Straw 80% (w/w on VS basis) and filter cake 20% (w/w on VS basis)
SF + Fe	Straw 80% (w/w on VS basis) and filter cake 20% (w/w on VS basis) + iron
VFS	Vinasse 90% (w/w on VS basis), Filter cake 2% (w/w on VS basis), and Straw 8% (w/w on VS basis)
VFS +Fe	Vinasse 90% (w/w on VS basis), Filter cake 2% (w/w on VS basis), and Straw 8% (w/w on VS basis)+ iron

The data provided by the manufacturer's software AMPTS II Bioprocess Control® were methane production values under normal temperature and pressure conditions (N ml CH_4; 0˚C, 1ATM). The methane potential of each treatment was determined by subtracting the methane production of the inoculum.

The data was evaluated by fitting into Gompertz model, in which the biogas generation is assumed to be proportional to the microbial activity (SHAKERI YEKTA et al., 2017). The Gompertz model can be seen in Equation 4.1, where M is total methane production, P is total methane potential, Rm is rate and L is lag phase.

$$M = P * \exp\left(-exp\left(\frac{Rm * e * (L - t)}{P} + 1\right)\right) \quad (4.1)$$

The software SPSS® (IBM, USA) was used to fit the data into Gompertz model and to obtain the statistical parameters mean, standard deviation, total methane production, rate, and lag phase.

The physical-chemical parameters analyzed of the substrates and digestates of the BMP test were: Total Solids (TS), Volatile Solids (VS), Volatile Fatty Acids (VFA), pH, and Chemical Oxygen Demand (COD). The parameters are described below in item 4.2.5.

4.2.4. Continuous experiment in reactors

The study of continuous anaerobic digestion was performed in four CSTR type reactors (Continuous Stirred-Tank Reactor) with total volume of 6 liters and reaction volume of 5 liters each, stirring at 100 RPM for 15 minutes with an interval of 15 minutes, at 37 ˚C, with 20 days HRT and initial Organic Loading Rate (OLR) of 1.125 g VS l^{-1} d^{-1} (2.336 g DQO L^{-1} d^{-1}). The experiment was run for 238 days between 16 October 2019 and 10 June 2020. The reactors were fed daily with 250 ml food portions.

The CSTR reactors (*Biogas test plant BTP2 - Umweltleistungen®, Germany*) used (Figure 4.2) have an automated system of agitation (R100CT, Ingenieurbüro CAT, Germany), temperature control (silicon-heating mat), continuos measurement of pH, volume (Ritter®, Germany), and composition of biogas. The system was equipped with a gas quality analyzer module (CH_4, CO_2, H_2, and H_2S), data logger, and individual SENSOControl touch panel.

Figure 4.2 - CSTR reactors Biogas test plant BTP2-Umweltleistungen® used in the experiment

The parameters analyzed during the experiment were: pH, gas volume, TS, VS, COD, ammonium, VFA, and gas composition. pH and gas volume were measured continuously, TS, VS, COD, and ammonium were measured once a week, VFA were measured at least once a week (when abnormalities were observed the frequency of analysis increased) and the gas composition (CO_2, CH_4, H_2, and H_2S) was measured daily. Detailed description of each parameter can be seen in item 4.2.5.

During days 48-211 in R1 and 48-107 in R2, R3, and R4 the H_2S was measured every second week with Dräger Short-term tubes (CH28201) since the concentration of H_2S was above the limit of quantification of the reactors' analyzer (4000 ppm).

Four reactors were used to conduct the experiment (R1, R2, R3, and R4). R1 and R2 received only vinasse as substrate, R3 and R4 received the co-digestion substrate of vinasse (90% w/w), sugarcane straw (8%; w/w), and filter cake (2%; w/w).

Reactor R1 operated as control treatment and only received iron and trace elements to test its recovery after process failure due to VFA accumulation and prominent pH decrease.

Reactors R2, R3, and R4 operated with addition of iron and trace elements throughout the experiment. The decision to add metals was made after the first observation of VFA accumulation and significant increase of H_2S production.

R3 and R4 operated in similar way, as it was observed that if both did not receive iron and trace elements, there would be a failure of the process, and as there was the objective of continuing to run the reactors after the end of this experiment (to answer other research questions not related to this thesis) it was decided to take all necessary measures to maintain stability in both reactors.

The decisions made during the experiment in reactors R1, R2, R3, and R4 are summarized in the Table 4.2.

Table 4.2 - Overview of the study in reactors R1, R2, R3, and R4

Week	Days	Observation	Reactor
3	20	Start codigestion	R3 and R4
7	45	2 days with 20% OLR + Fe addition	R2, R3, R4
7	47	Fe addition in food portions	R2, R3, R4
8	55-58	Start Ni addition into reactors (4 days)	R2, R3, R4
9	59	Start Ni addition in food portions	R2, R3, R4
13	86	decrease OLR to 25%	R3 and R4
13	86-89	Start Se addition into reactors (4 days)	R4 and R4
13	86	Stop feeding	R1
13	86	Reduced OLR	R2
13	86	Increase Fe concentration	R2, R3, R4
13-14	90-93	Start Se addition into reactor (4 days)	R2, R3, R4
13	90	Start Se addition in food portions	R2, R3, R4
	98	Increase Se concentration into reactors (4 days)	R2, R3, R4
15-16	104-107	Start Co addition into reactors (4 days)	R2, R3, R4
16	111	No feeding. Correction of the reactor's pH to 7.5	R1, R2, R3, R4
18	119-122	Start Mo addition into reactor (4 days)	R2, R3, R4
25	170	Increase OLR	R3 and R4
27	181	Stop feeding +addition of Fe	R2
27	185	Increase OLR	R3 and R4
27	187	Return feeding	R2
28	190-193	Start Zn addition into reactors (4 days)	R2, R3, R4
28	194	Start Zn addition to food portions	R2, R3, R4
29	195	Start pH correction of food portions to 7.5 with NaOH	R1, R2, R3, R4
30	208	Restart feeding	R1
32	222	Increase of OLR	R2
33	226	Increase OLR to 65%	R3, R4
35	238	Interruption and end of the experiment	R1, R2, R3, R4

The OLR varied throughout the experiment to increase the efficiency of the reactor. Decisions on OLR variations depended mainly on the results of the physical-chemical analyzes of the reactor digestate, such as the efficiency of VS reduction, pH, concentration and volume of methane and the presence of inhibitors, such as volatile fatty acids and ammonium. In addition, supplementation of iron and micronutrients (Ni, Se, Mo, and Zn) was performed based on the results of the chemical analysis of the substrate described in Table 4.3.

Table 4.3 - Feedstock characterization. N.d = not detected, <LoQ= lower than limit of quantification

Determination	Vinasse	Straw	Filter cake
TS (%)	2,80 ± 0,01	87,28 ±0,29	63,82 ± 0,21
VS (% of TS)	1,05 ± 0,29	3,06 ± 0,63	1,07 ± 0,27
COD (mg L^{-1})	32331,11±2106,34		
pH	4,92	-	-
Total acidity (mg CH_3CO_2H L^{-1})	1025,07		
Total alkalinity (mg $CaCO_3$ L^{-1})	96,62		
Fl^- (mg L^{-1})	n.d	-	-
Cl^- (mg L^{-1})	12.335,90 ± 54,79	-	-
NO_3^- (mg L^{-1})	n.d	-	-
PO_4^{-3}(mg L^{-1})	<LQ	-	-
SO_4^{-2}(mg L^{-1})	8.405,00 ± 31,42	-	-
Aconitic acid (mg L^{-1})	767,87 ± 16,77	-	-
Succinic acid (mg L^{-1})	1614,07 ± 53,31	-	-
Latic acid (mg L^{-1})	1728,52± 3,38	-	-
Na^+ (mg L^{-1})	376,08 ±0,18	-	-
Li^+ (mg L^{-1})	n.d.	-	-
NH_4^+ (mg L^{-1})	37,13±0,25	-	-
K^+ (mg L^{-1})	8058,22±39,43	-	-
Ca^{2+} (mg L^{-1})	2017,42±43,16	-	-
Acetic acid (mM)	1,6295 ± 0,03	-	-
Propionic acid (mM)	n.d.	-	-
Butyric acid (mM)	n.d.	-	-
Isobutyric acid (mM)	n.d.	-	-
Caproic acid (mM)	n.d.	-	-
Isocaproic acid (mM)	n.d.	-	-
Valeric acid (mM)	n.d.	-	-
Isovaleric acid (mM)	n.d.	-	-
Fe (mg Kg^{-1})	6,59	2153,65	2035,71
Li (mg Kg^{-1})	0,0	1,05	1,60
Cu (mg Kg^{-1})	0,39	3,23	6,93
Zn (mg Kg^{-1})	0,41	15,68	28,17
Mo (mg Kg^{-1})	0,03	0,95	0,18
Cd (mg Kg^{-1})	0,0	0	0,078
Mg (mg Kg^{-1})	186,88	780,9	622,43
Se (mg Kg^{-1})	0,02	0,19	0,05
Co (mg Kg^{-1})	0,04	1,08	0,45
Ni (mg Kg^{-1})	0,07	1,14	1,27
Total carbon (%w w^{-1})	33,24±0,13	42,15±0,34	34,69±0,06
Total nitrogen (%w w^{-1})	3,13±0,01	0,47±0,11	1,14±0,05

The addition of iron started with a Fe/S molar ratio of 0.1 corresponding to 1.4 g l^{-1} of iron, following Shakeri Yekta et al. (2017) recommendations. After observing that the H_2S production and the accumulation of VFA were still high (day 86), it was decided to change the iron concentration to a molar ratio of 1 (14 g L^{-1}). The reagent used was $FeCl_3$.

The concentrations of nickel, selenium, and cobalt were based on Gustavsson et al. (2011) study that reported an optimum concentration of 0.2 mg L^{-1} of Ni, 0.05 mg L^{-1} of Se, and 0.5 mg L^{-1} of Co. The reagents used were $NiCl_2.6H_2O$, $Na_2SeO_3.5H_2O$, and $CoCl_2.6H_2O$.

The selenium concentration was changed to 0.2 mg L^{-1} (day 98) since the propionate concentration of the reactors were still high.

Molybdenum and Zinc supplementation followed Hendriks et al. (2018) recommendations with 10.5 µg $gCOD^{-1}$ of Mo and 29 µg $gCOD^{-1}$ of Zn. The reagents used were $NaMoO_4.2H_2O$ and $ZnCl_2$.

4.2.5. Bioprocess parameters analyzed

The parameters analyzed in the BMP test and in the continuous experiment are detailed below.

Potential of Hydrogen (pH)

The pH was determined according to (SWEDISH STANDARD INSTITUTE, 1998) using a pH electrode (Inolab pH 730, WTW®, Germany).

Chemical Oxygen Demand (COD)

For COD analyzes kits from Hach-Lange cuvette test with measurement ranges of 100-2000 mg L^{-1} O_2 (LCK514) and 100-1000 mg L^{-1} O_2(LCK014) were used according to the manufacturer's instructions.

Ammonium (NH_4^+)

For NH_4^+ analyzes kits from Hach-Lange cuvette test with measurement ranges of 2.0-47 mg L^{-1} NH_4-N (LCK302) and 47-130 mg L^{-1} NH_4-N (LCK303) were used according to the manufacturer's instructions.

Volatile Fatty Acids (VFA)

The VFA concentration was measured in duplicates according to Jonsson and Borén (2002) method. The VFAs measured were acetic, propionic, butyric, isobutyric, capronic, isocoprnic valeric and isovaleric acid. 1 ml of reactor sludge was centrifuged at 12000 RPM for 10 minutes. 400 µl of the supernatant was transferred into an injection vial and then 40 µl

of internal standard (25% formic acid containing 52 mg of crotonic acid) was added. The samples were analyzed in the GC-MS 6890 Series (Hewlett Packard®, USA). 1 µl of the sample was inject (split flow of 30 ml min^{-1}). The capillary column was SGE BP21 (30mx0.32mmx0.25 µm). The temperature program was 80 ˚C (0 min), 175 ˚C (3 ˚C min^{-1}). The gas carrier used was helium at 1.9 ml min^{-1}.

Total Solids and Volatile Solids

Total Solids (TS) and Volatile Solids (VS) were measured according to Baird (2017). Each sample was performed in duplicates. The sample (reactor digestate) was poured into a crucible and then transferred to a 105 °C oven with air circulation (Termaks®) for 20 hours. After cooling, the crucibles were weighted (weight; dried at 105 °C) for TS calculations (Equation 4.2). The crucibles were heated to 550 °C for 2 hours in a muffle furnace (Naber Industrieofenbau®, Bremen) after TS calculation. They were then cooled and weighted for the VS measurement (weight; combusted at 505 °C) (Equation 4.3).

$$TS\ (\%) = \frac{weight\ dried\ at\ 105\ ^{\circ}C - weight\ empty\ crucible}{weight\ wet - weight\ empty\ crucible} * 100 \qquad (4.2)$$

$$VS\ (\%TS) = \frac{weight\ dried\ at\ 105\ ^{\circ}C - weight\ combusted\ at\ 550\ ^{\circ}C}{weight\ dried\ at\ 105\ ^{\circ}C - weight\ empty\ crucible} * 100 \qquad (4.3)$$

Feedstock characterization

The analyzes of TS, VS, COD, NH_4^+, and VFA followed the item 4.2.5 described previously.

Total acidity

The total acidity of the vinasse was determined according to the titration method described by Dilallo and Albertson (1961)**.** 50 ml of the sample was used, and the results were expressed in mg CH_3CO_2H L^{-1}.

Alkalinity

The total alkalinity of the vinasse was measured according to the titration method described by Jenkins et al. (1983). 50 ml of the sample was used, and the results obtained were expressed in mg $CaCO_3$ L^{-1}.

Cations

The cations in the vinasse sodium (Na^+), lithium (Li^+), calcium (Ca^{2+}), and potassium (K^+) were determined by ion chromatography following the method described by Eith et al. (2006). It was used the ion chromatograph Metrohm IC 930 Compact® equipped with the Metrosep C4 250/4.0 column and conductivity detector. The chromatographic conditions were temperature of 30 ˚C, eluent solution of tartaric acid 7.5mM, dipicolinic acid 0.135mM, and ascorbic acid 3mM with 0.9ml min^{-1} flow. The sample was diluted 200 times and filter with 0.45µm cellulose acetate filter. The injected volume of sample was 20 µL.

Anions

The anions in the vinasse fluoride (Fl^-), chloride (Cl^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulfate (SO_4^{2-}) were determined by ion chromatography following the method described by Eith et al. (2006). It was used the ion chromatograph Metrohm IC 930 Compact® equipped with the Metrosep A Supp 5 250/4.0 column and conductivity detector. The chromatographic conditions were temperature of 25 ˚C, eluent solution of sodium carbonate 3.2mM and sodium bicarbonate 1.0mM with 0.7ml min^{-1} flow and solution of sulfuric acid 100mM for the regeneration of the chemical suppressor. The sample was diluted 200 times and filter with 0.45µm cellulose acetate filter. The injected volume of sample was 20 µL.

Organic acids

The organic acids in the vinasse aconitic, succinic, and latic acid were determined by ion chromatography following the method described by Eith et al. (2006). It was used the ion chromatograph Metrohm IC 930 Compact® equipped with the Metrosep OrganicAcid column and conductivity detector. The chromatographic conditions were temperature of 25 ˚C, eluent solution of sulfuric acid 0.5mM and acetone 15% (v/v) with 0.5ml min^{-1} flow and solution of

LiCl 20mM for the regeneration of the chemical suppressor. The sample was diluted 200 times and filter with 0.45µm cellulose acetate filter. The injected volume of sample was 20 µL.

Metals (trace elements)

The metals Fe, Li, Cu, Zn, Mo, Cd, Mg, Se, Co, and Ni were determined by inductively coupled plasma mass spectrometry (ICP-MS) according to the method described by (CHAN et al., 2021). First, 0.3g of the samples (vinasse, sugarcane straw, and filter cake) were digested with 8ml of HNO_3 and 2 ml of H_2O_2 in the microwave Ethos, Milestone ® for 150 min. at 180 ˚C. Standard reference (GBW 08573) and blanks were used in each digestion batch to verify the quality assurance and quality control. After digestion, the samples were cooled at room temperature, filtered, and diluted with ultrapure water (Milli-Q®). For the determination of the metals the ICP-MS PerkinElmer NexION 300D was used.

Total carbon and nitrogen

The total carbon and nitrogen content of the samples were determined by the CHNS/O elemental analyzer EA2400 PerkinElmer® according to the method described by (ODHIAMBO, 2016).

Light optical microscopy analysis

The samples preparation and microscopy analysis were performed at the Sugar and Ethanol Department of Luiz de Queiroz College of Agriculture (LTSBio/ESALQ/USP).

The digestate samples were placed on glass slides and heat-fixed with the aid of a Bunsen burner. They were then stained and observed under an optical microscope (Nikon®). Each sample was stained on three slides with the following combination of reagents: lactophenol cotton blue and ruthenium red; Toluidine blue; phloroglucinol (described below).

Lactophenol blue cotton and ruthenium red

The slides with the fixed samples were covered with lactophenol blue cotton solution (C.I. 42755) for 5 minutes, then washed with distilled water, and covered with the ruthenium red solution 0.2% for 5 minutes (CHAMBERLAIN, 1932). After that, the slides were washed with distilled water, covered with a cover slip and observed in the microscope.

Cotton blue allows staining of chitin and fungal (LECK 1999), while ruthenium red stain pectin (STEELING, 1970) and microorganisms' extracellular structures such as acid polysaccharides and proteins (CHARUK; PIRRAGLIA; REITHMEIER, 1990; WALLER et al., 2004).

Toluidine blue

The slides with the fixed samples were covered with toluidine blue O solution (CI 52040) for 5 minutes, then washed with distilled water, covered with a cover slip and observed in the microscope.

Toluidine blue allows staining of plant cell walls (O'BRIEN; FEDER; MCCULLY, 1964).

Phloroglucinol

The slides with the fixed samples were covered with phloroglucinol solution 0.1% for 5 minutes, then washed with distilled water, covered with a cover slip and observed in the microscope (RUZIN, 1999).

Phloroglucinol is very efficient to stain lignin. The lignin presented a cherry-red color and is easily recognized (SPEER, 1987).

Scanning Electron Microscopy analysis

Sample preparation and analysis were performed at the NAP/MEPA-LFN laboratory at the Luiz de Queiroz College of Agriculture (ESALQ/USP).

The digestate samples were placed on circular coverslips, then placed in a petri dish containing osmium tetroxide, then the petri dish was coated with aluminum foil, stored at rest for 12 hours in a fume hood. Then, the samples were fixed in stubs with double-sided carbon adhesive tape and stored in a desiccator containing silica, in order to dry the samples.

After drying, the samples were subjected to metallization with gold, with the aid of the Bal-Tec® metallizer model SCD-050. Subsequently, the samples were sent for scanning electron microscopy.

Samples were analyzed on the LEO 435 VP scanning electron microscope at variable magnification. The working conditions were 20Kv, measurements when necessary were made directly on the monitor, using the manufacturer's software.

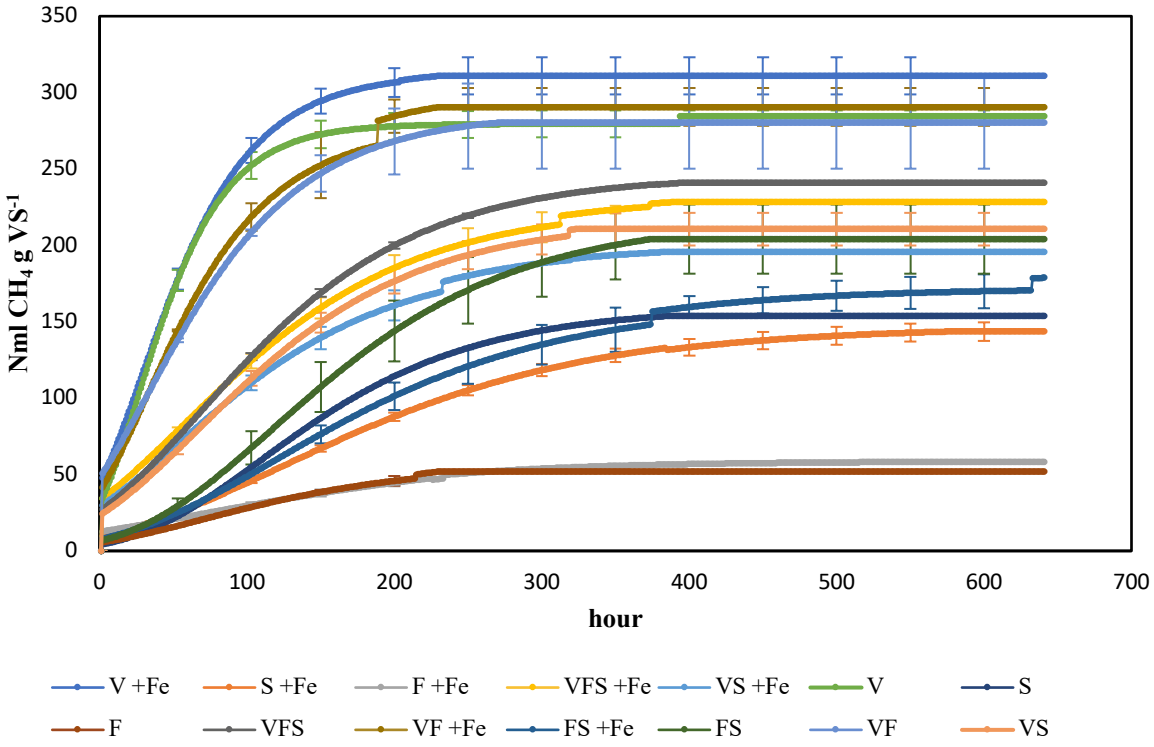
4.3. Results and Discussion

The discussion of the results is separated in the following sections: Biochemical methane potential test, continuous experiment in reactors, and light and scanning electron microscopy of the continuous experiment.

4.3.1. Biochemical Methane Potential tests

Profiles of the methane yield average of the treatments are shown in Figure 4.3. Standard deviations are shown as error bars. The figure represents the methane yield over 27 days (640 hours) of experiment.

Figure 4.3 - Cumulative methane yield of all the treatments over 27 days of BMP assays. The results were fitted to the Gompertz model and were converted to normal conditions (273 K, 1 atm).



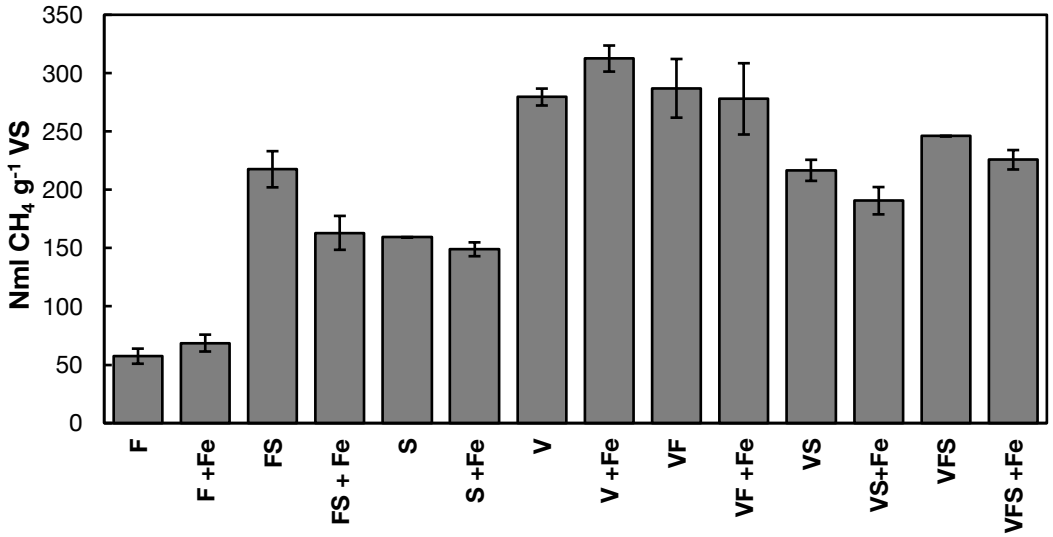
As the substrates were used, the degradation rate increase at first, reached a limit, and then decreased to a minimum, as shown by the slopes of the curves. In the treatments with vinasse and the combination of vinasse and filter cake (V, V+Fe, VF, and VF+Fe) the slopes of the curves were more prominent. However, the treatments with straw, filter cake, and combinations of straw, filter cake, and vinasse (S, S+Fe, F, F+Fe, FS, FS+Fe VS, VS+Fe, VFS, and VFS+Fe) showed lower slopes of the curves, which indicates slower degradation of the substrate. This may be due to the presence of lignocellulosic material in these treatments which needs more time for degradation due to its complexity, since lignin is highly non-degradable, cellulose needs a few weeks for its complete degradation while hemicellulose is degraded in a few days (SARKER et al., 2019). In Table 4.4 and Figure 4.4 the methane yield of each substrate can be seen.

Table 4.4 - Methane yield, lag phase, R-squared, end VFA, and end pH of the substrates investigated.

Substrate	Methane yield (N ml CH4 g^{-1} VS)	Lag phase (days)	R^2 (%)	End VFA	End pH
F	57.37 ± 6.47	13.66 ± 2.3	99.30	n.d.	8.21±0.01
F +Fe	68.61 ± 7.24	70.59 ± 18.06	98.37	n.d.	8.15±0.03
FS	217.60 ± 15.50	22.71 ± 1.59	99.90	n.d.	7.99±0.09
FS +Fe	163.08 ± 14.53	10.74 ± 5.40	99.63	n.d.	7.83±0.07
S	159.35 ± 0	23.43 ± 0	99.99	n.d.	7.90±0.13
S +Fe	149.00 ± 5.94	20.07 ± 0.90	99.80	n.d.	7.93±0.03
V	279.50 ± 7.25	8.67 ± 0.57	93.53	n.d.	8.21±0.01
V +Fe	312.48 ± 11.18	15.80 ±3.57	95.93	n.d.	8.24±0.01
VF	286.96 ± 25.15	26.81 ± 4.16	94.95	n.d.	8.09±0.06
VF +Fe	277.94 ± 30.55	17.30 ± 3.72	97.30	n.d.	8.11±0.04
VS	216.67 ± 9.03	18.27 ± 0.41	99.23	n.d.	8.01±0.08
VS +Fe	190. 63 ± 11.70	31.97 ± 1.97	98.73	n.d.	7.99±0.03
VFS	246.01 ± 0.29	17.70 ± 0.51	99.45	n.d.	7.89±0.09
VFS +Fe	225.73 ± 8.32	32.46 ± 2.40	98.93	n.d.	7.99±0.03

F: Filter cake, F +Fe: Filter cake +iron, FS: Filter cake and Straw, FS +Fe: Filter cake and Straw +iron, S: Straw, S +Fe: Straw + iron, V: Vinasse, V +Fe: Vinasse +iron, VF: Vinasse and filter cake, VF +Fe: Vinasse and filter cake +iron, VS: Vinasse and Straw, VS +Fe: Vinasse and Straw + iron, VFS: Vinasse, Filter cake, and Straw, VFS +Fe: Vinasse, Filter cake, and Straw + iron, n.d.: not detected.

Figure 4.4 - Methane yield of all the substrates. The results were converted to normal conditions (273 K, 1 atm).



The treatments with the monodigestion of filter cake and vinasse showed better performance with the addition of iron (F+Fe: 68.61±7.24 and V+Fe: 312.48±11.18 Nml CH_4 g^{-1} VS) when compared to the treatments without iron (F:57.37±6.47 and V:279.50± 7.25 Nml CH_4 g^{-1} VS). The performance was 16.38% and 10.55% higher for filter cake with iron (F+Fe) and vinasse with iron (V+Fe), respectively.

However, the straw treatment (S:159.35±0 Nml CH_4 g^{-1} VS) and the treatments that received substrate combinations (FS: 217.60±15.50, VF: 286.96±25.15, VS: 216.67±9.03, and VFS: 246.01±0.29 Nml CH_4 g^{-1} VS) presented higher methane yield when compared to the treatments that received iron (FS +Fe:163.08±14.53, VF+Fe: 277.94±30.55, VS+Fe: 190.63±11.70, and VFS+Fe: 225.73±8.32 Nml CH_4 g^{-1} VS).

The best monodigestion treatment was V+Fe with 312.48±11.17 Nml CH_4 g^{-1} VS, while the best substrate combination of co-digestion was VF with 286.96±25.15 Nml CH_4 g^{-1} VS.

Other authors studying the BMP of sugarcane straw found methane yields of 231.1±1 Nml CH_4 g VS^{-1} (JANKE et al., 2017) and 199-252 Nml CH_4 g VS^{-1} (JANKE et al., 2015). When studying the BMP of vinasse, Janke et al. (2015) described methane yields between 246-303 Nml g^{-1}COD, while Leite et al. (2015) described methane yields between 220-269.72 Nml g^{-1} COD, Santos et al. (2019) 328-339 Nml g^{-1} COD, and Janke et al. (2014) methane yield of 647 Nml g^{-1} VS. López-González, Pereda Reyes and Romero Romero (2017) when studying a mixture of vinasse and filter cake (75:25% based on COD) achieved a methane yield of 246 Nml CH4 g^{-1} COD.

In the BMP study carried out by Santos et al. (2019) the methane yields of 3 vinasses were similar. Besides the similarity of the methane yield of the samples, the bioavailability of organic matter and SO_4^{2-} varied between 330 and 2000 mg L^{-1}. The treatment that presented higher SO_4^{2-} concentration in Santos et al. (2019) study was 4 times lower than the vinasse used in the present study (8,405.00 mg L^{-1}), which may have influenced in greater methane yield, especially when compared to the treatment without iron (V) of this work.

Nevertheless, the differences in methodologies of BMP tests, such as the inoculum:substrate ratio, substrate concentration (volatile solids), and the chosen inoculum can affect methane productivity and thus present different results in studies carried out with similar substrates, which makes it challenging the comparison of the results (ANGELIDAKI et al., 2009; HOLLIGER et al., 2016; OHEMENG-NTIAMOAH; DATTA, 2019).

The combination of all the substrates (VFS) presented lower BMP (246.01±0.29 Nml CH_4 g^{-1} VS) than other treatments (V+Fe > VF > V > VF+Fe >VFS). All these treatments that presented higher BMP than VFS also contained vinasse in their composition but had no straw. The higher BMP in these treatments may be due to vinasse having more soluble components in its composition which are easier to degrade. Besides, a study (HUANG, 2020) using the solid

fraction of the digestate of the reactors fed with VFS used in this study (further discussed in item 4.3.2) showed that only the digestate of the reactors presented a methane yield of 144.69±2 Nml CH_4 g^{-1} VS and when applied a physicochemical treatment (autoclave +alkaline) in this digestate the methane yield was 331±12 Nml CH_4 g^{-1} VS (128,76% higher than control). This shows the microorganisms difficulty in degrading the lignocellulosic material in straw and filter cake, a problem that could be overcome with a digestate treatment, known as post-treatment, and anaerobic digestion of this post-treated digestate in a second reactor, thus taking advantage of the remaining organic matter not used in the first reactor and increase the overall methane yield of the process.

The addition of Fe in the vinasse (V+Fe treatment) provided an increase of 10.6% when compared to the treatment without iron (V). This can be justified that although the inoculum used already has iron and trace elements supplementation (speciation and concentration were not disclosed), the concentration of sulfate in the vinasse is much higher than the substrates commonly used in the reactor from which the inoculum was collected (food waste, organic industrial waste, and slaughterhouse waste).

Nonetheless, iron supplementation has shown lower results in other treatments, which may be due to the vinasse dilution with other residues (straw and/or filter cake) and to the fact the inoculum already has iron and trace elements. Straw and filter cake does not have high sulfur concentrations like vinasse, which may allow the iron to not be used, remaining bioavailable in the substrate. Hubbert et al. (1958) and Martinez and Church (1970) indicate that iron concentration between 50-100 mg L^{-1} can start inhibition in mesophilic acidogenic cultures. In the present study, the concentration of iron was 1400mg L^{-1}. However, the inhibition or toxicity of a metal in a biologic process depends on its bioavailability and dissolved concentrations (HENDRIKS; VAN LIER; DE KREUK, 2018). In this case, methanogens are the microorganisms most sensitive to metal toxicity/inhibition (FENG et al., 2010).

In addition, the system used in the experiment (Bioprocess Control) only shows the methane production, as the biogas passes through a NaOH trap to remove CO_2, H_2, and H_2S. Therefore, it was not possible to evaluate these gases, which could contribute to a better understanding of the process.

The VFAs (acetate, propionate, butyrate, isobutyrate, caproate, isocaproate, valerate, and isovalerate) were not detected in any sample of the end of the BMP tests. Also, the pH of the samples at the end of the assays ranged from 7.83 to 8.24. This indicates that possibly there was no inhibition due to the volatile fatty acids accumulation. However, this is a measure from the end of the experiment and may not represent the whole period of the BMP. There may have been VFA inhibition at the beginning of the process, but the microorganisms may somehow circumvent this issue.

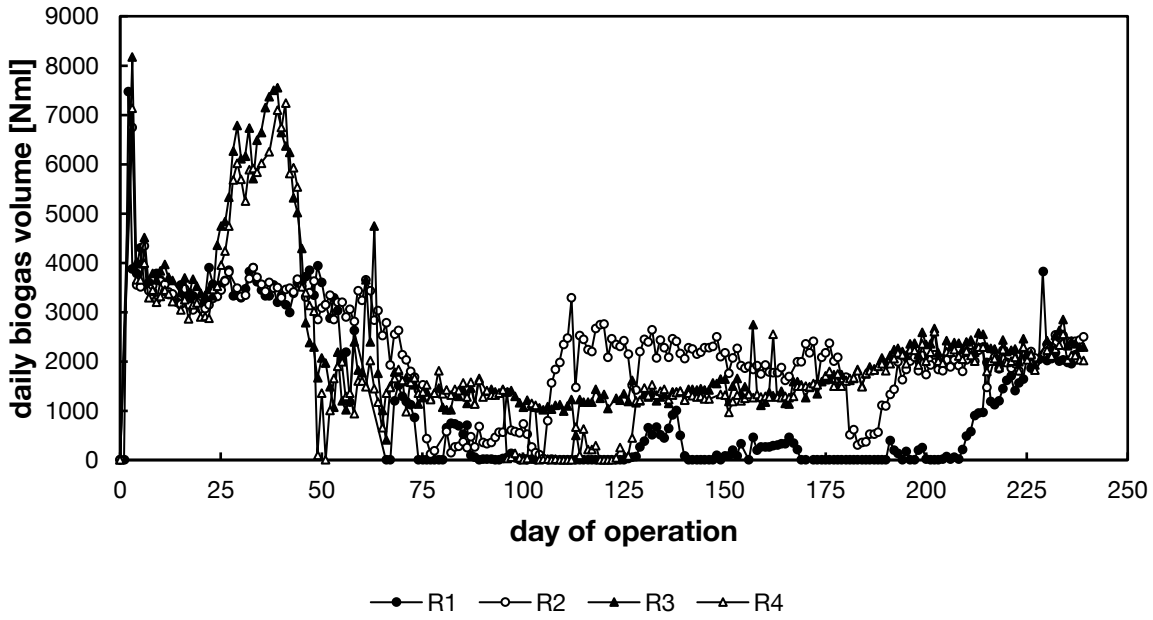
4.3.2. Continuous experiment in reactors

First, an overview of the four reactors will be carried out, then their processes will be described individually.

The four reactors were operated in the same way during the first HRT, the inoculum was conditioned in the reactors for 5 days, after that, the reactors started to be fed with vinasse without any trace element or iron addition.

It can be seen at the beginning of the experiment (Figure 4.5) that there was a drop in the volumetric production of biogas in all the reactors, this is due to the fact that the inoculum from a biogas plant reactor worked with a higher organic load (around 2 g kg m^{-3} d).

Figure 4.5 - Volumetric biogas production of R1, R2, R3, and R4



After 1 HRT, reactors R3 and R4 started to be fed with food portions of co-digestion (vinasse, sugarcane straw and filter cake). During this period there was an abrupt increase in the production of biogas. However, in day 40 lower VS reduction, COD reduction, and pH were observed. Besides that, started the VFA accumulation. Because of this, it was decided to reduce the OLR to 25% in R3 and R4 to avoid reactors failure. Furthermore, the same behavior was observed in R1 and R2 a few days later (day 50). Concomitantly, an increase of H_2S was observed in all reactors. The H_2S concentration was below 50 ppm at day 14, however, at day 23 it was higher than 10,000 ppm in all reactors, which indicated washout of iron and trace elements from the inoculum. Due to that, it was started the iron addition to the reactors in four days, subsequently in the food portions of R2, R3, and R4. The molar ratio of iron:sulfur at this moment was 0.1 and R1 did not received iron since it was the control treatment reactor.

After decrease the OLR in R3 and R4, the volumetric biogas production (Figure 4.5) was similar to the mono-digestion reactor (R2). However, higher fluctuations in biogas production were observed in R2 along with disturbances in the process, such as pH drops and metabolites accumulation such as VFA and H_2. The co-digestion reactors R3 and R4 operated more in a steady state, with lower VFA accumulation, more stable pH, and lower production of CO_2, H_2S, and H_2.

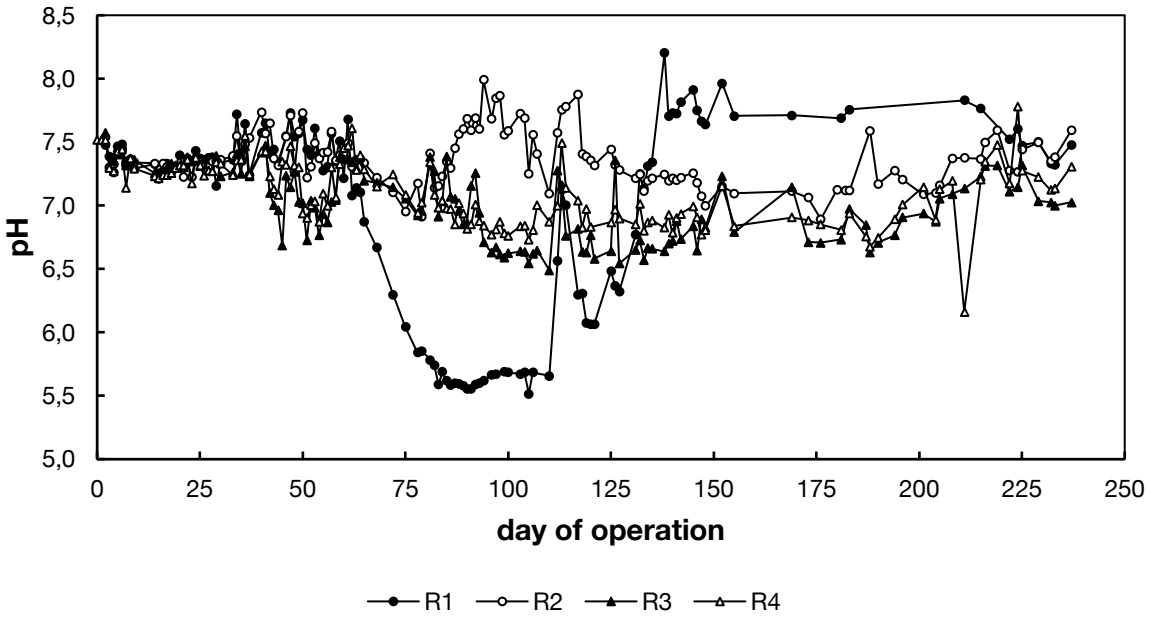
Moreover, as described before, the study conducted by Huang (2020) using the solid fraction of the digestate of R3 and R4 showed a methane yield of 144.69 Nml CH_4 g^{-1} VS in the BMP test and when the digestate was treated (post-treatment) with NaOH and autoclave the methane yield was 331.00 Nml CH_4 g^{-1} VS.

Among with Huang (2020), a second study was conducted by Adiyia (2021). In this study, the co-digestion of the solid fraction of the digestate with glycerol (25% CODg L^{-1}) was evaluated. The glycerol addition to the digestate promoted better biogas production with a methane yield of 318Nml CH_4 g^{-1} COD. The improvement in the biogas production was reported as a consequence of the priming effect of the addition of glycerol, which is easily degradable, and increase of the hydrolysis of the lignocellulosic material in the anaerobic digestion process.

With these studies, it was possible to understand that the remaining organic matter from the co-digestion digestate could be used as substrate in a second reactor and with that, increase the biogas production in the industry. Besides, the better use of organic matter could reduce GHG emissions from the digestate when used as a fertilizer.

Regarding pH, it was observed (Figure 4.6) higher fluctuations in the monodigestion reactors, even with iron and trace elements addition in R2.

Figure 4.6 - pH of R1, R2, R3, and R4 reactors



Furthermore, to keep all reactors in the optimum pH range it was necessary to increase the food portions pH with NaOH (from day 195 until the end of the experiment) since the buffering capacity of the reactors was not able to keep the pH in the optimum range. Besides, the evaluation of the alkalinity of the reactors is necessary, but due to technical problems, it was not possible to assess in this work.

An abrupt drop in the VS reduction and COD reduction can be seen (Figures 4.7 and 4.8) on day 86. This is a response to the reduction in the OLR. Then, can be seen that COD reduction and VS reduction increased gradually after day 100 a response to selenium addition.

Figure 4.7 - Volatile Solids (VS) reduction of R1, R2, R3, and R4. Dashed line between days 90-212 in R1 represents unavailable data due to no reactor feeding.

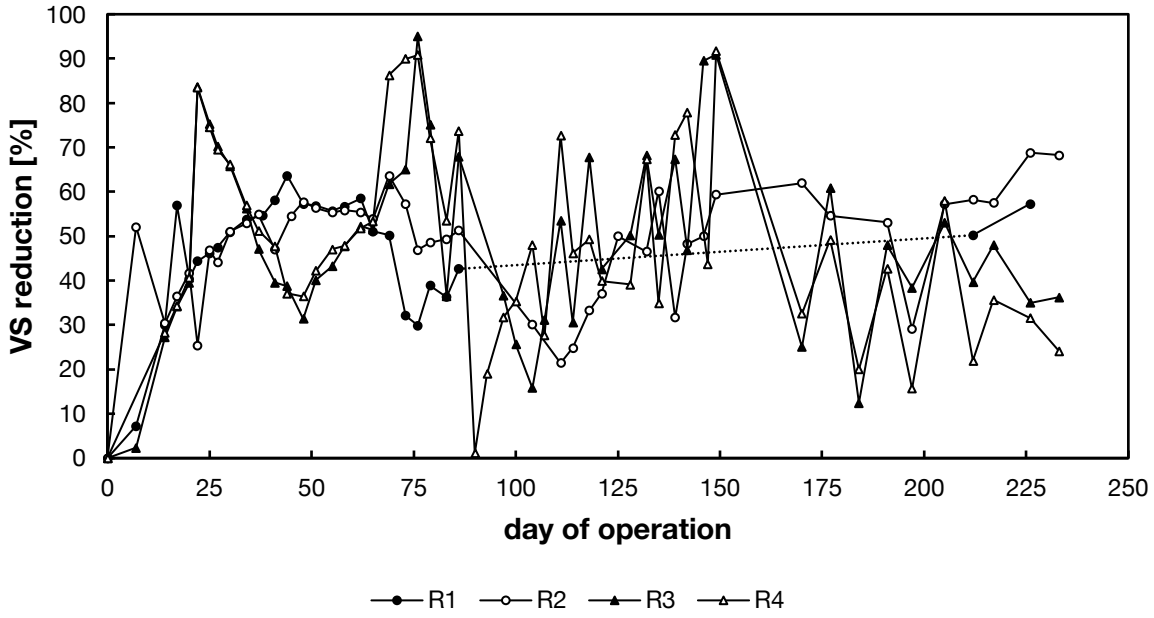
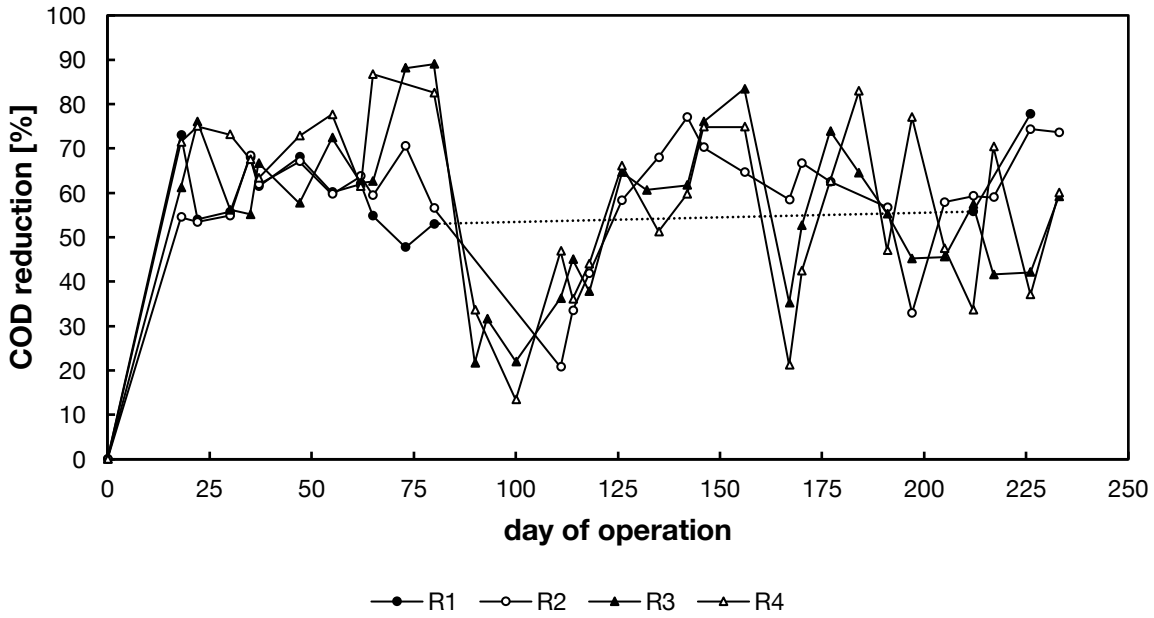


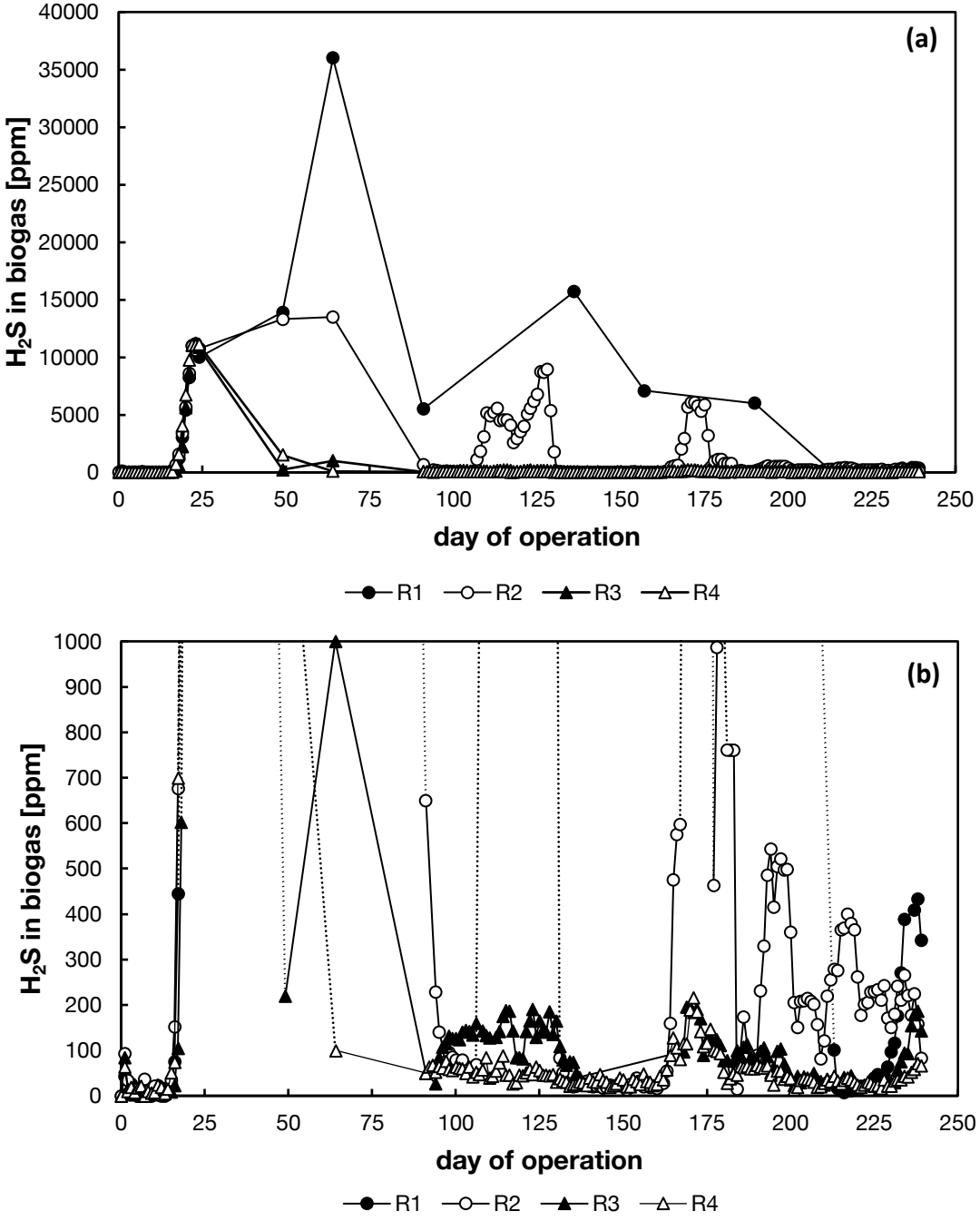
Figure 4.8 - Chemical Oxygen Demand (COD) reduction of R1, R2, R3, and R4. Dashed line between days 90-212 in R1 represents unavailable data due to no reactor feeding.



The expressive increase in the production of H_2S occurred shortly after the first HRT (Figure 4.9). After starting the iron addition, R3 and R4 had a quick response and dropped H_2S production. However, R2 had a prolonged response with a mild decrease in H_2S production. Moreover, R2 had oscillations in H_2S production even with iron addition.

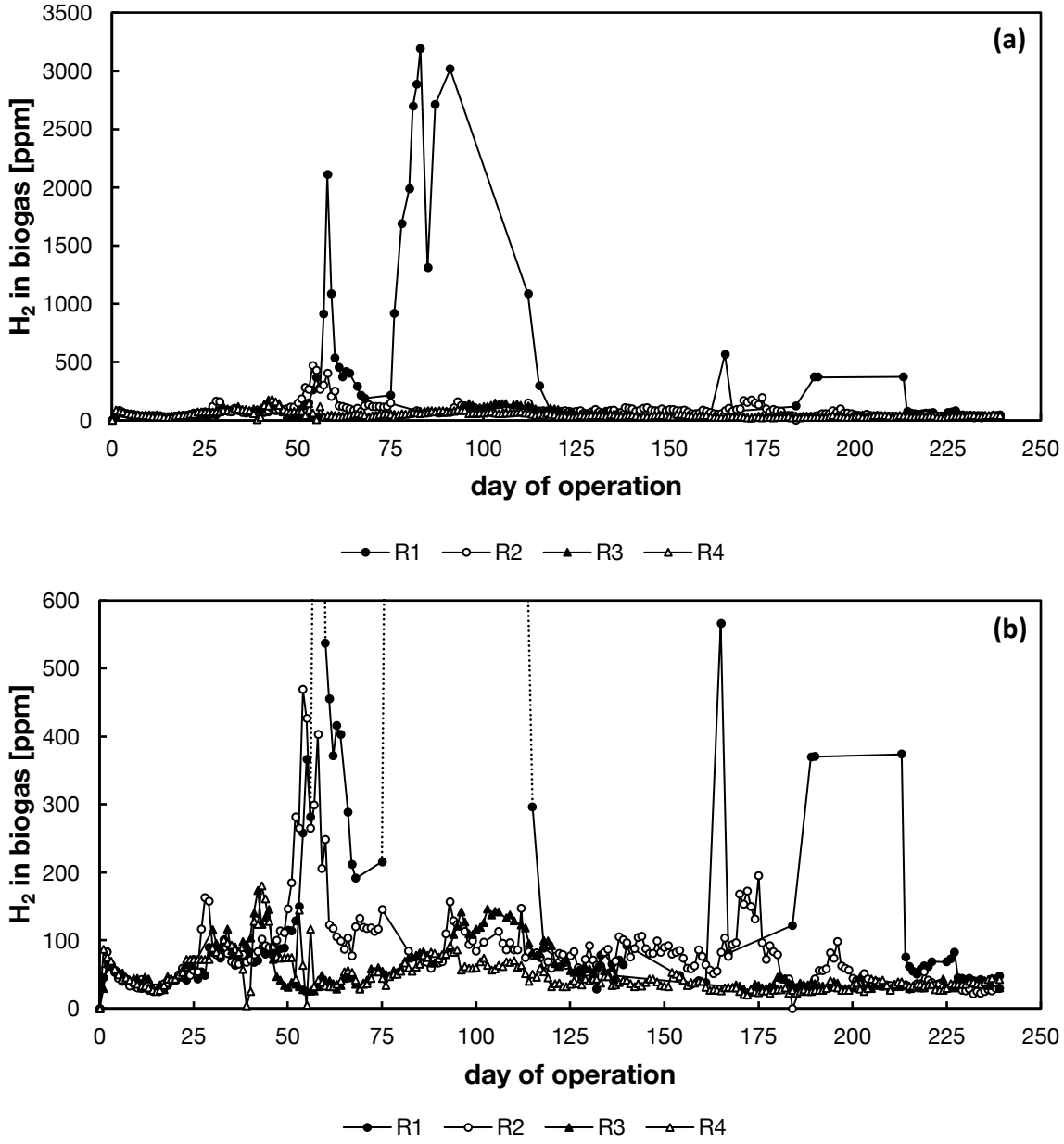
In week 25 (day 168-174) accidentally the iron and trace elements were not added to the food portions. R2 quickly responded with increased H_2S, H_2, acetate and propionate. Yet, the co-digestion reactors showed stability during this period, with low metabolites accumulation.

Figure 4.9- Hydrogen sulfide (H_2S) concentration in biogas of reactors R1, R2, R3, and R4 - (a) 0 to 4000 ppm range, (b) 0 to 500 ppm range. Dashed lines in (b) represent concentration above 1000 ppm.



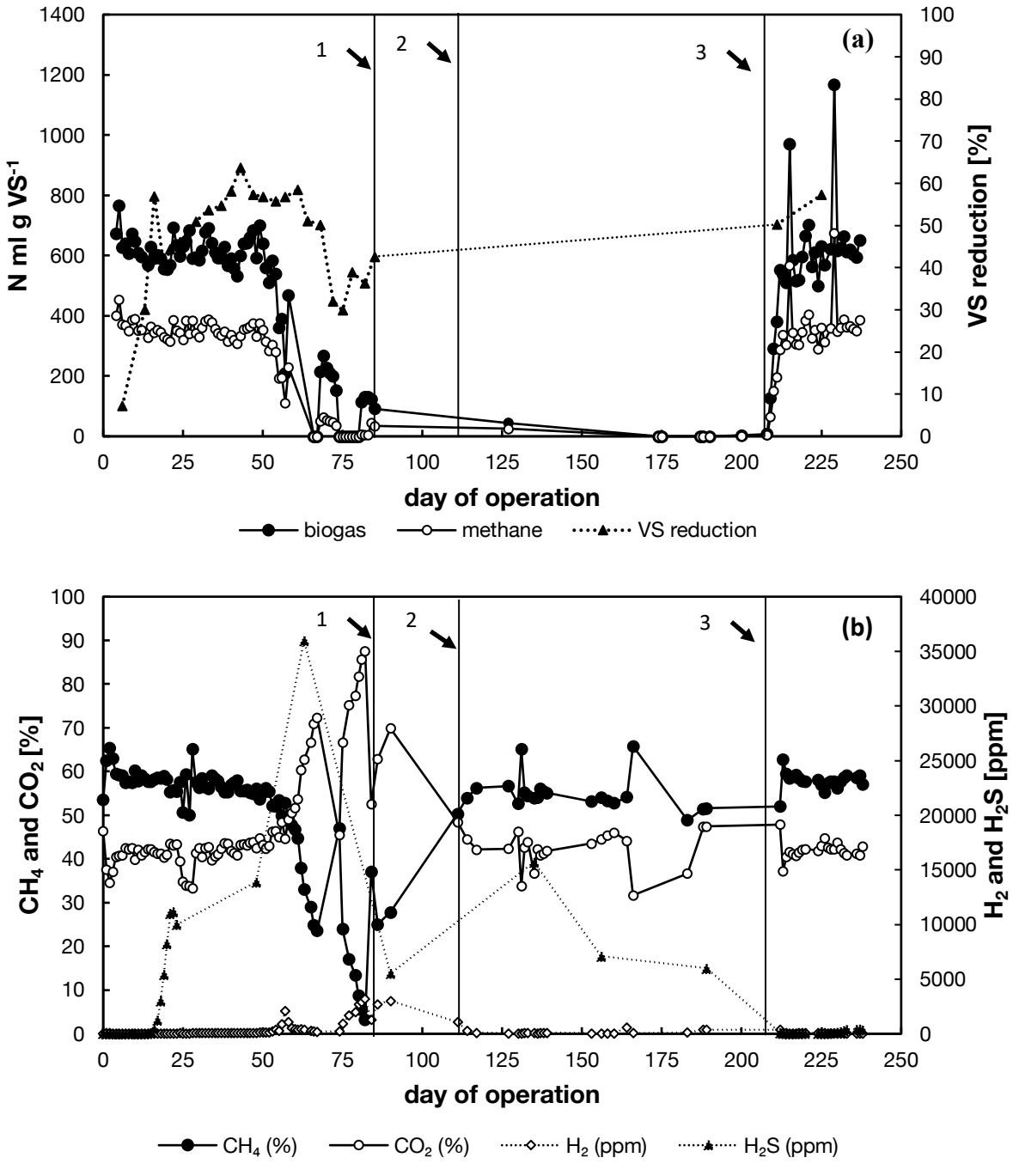
Regarding hydrogen concentration in biogas, higher fluctuations in the mono-digestion reactors can be seen (Figure 4.10). Even with iron and trace elements addition, R2 kept its H_2 production above R3 and R4. The greater variation in R2 pH may also be associated with this greater hydrogen generation.

Figure 4.10 - Hydrogen (H_2) concentration in biogas of reactors R1, R2, R3, and R4 - (a) 0 to 3500 ppm range, (b) 0 to 600 ppm range. Dashed lines in (b) represent concentration above 1000 ppm.



R1 reactor had its H_2S production abruptly increased, reaching 11,023.65 ppm on day 22 (Figure 4.11). On day 50 (2.5 HRT) it started to acidify with accumulation of acetic and propionic acids. In addition, it was observed that the increase in the hydrogen concentration in the biogas occurs a few days before the increase in the VFA production.

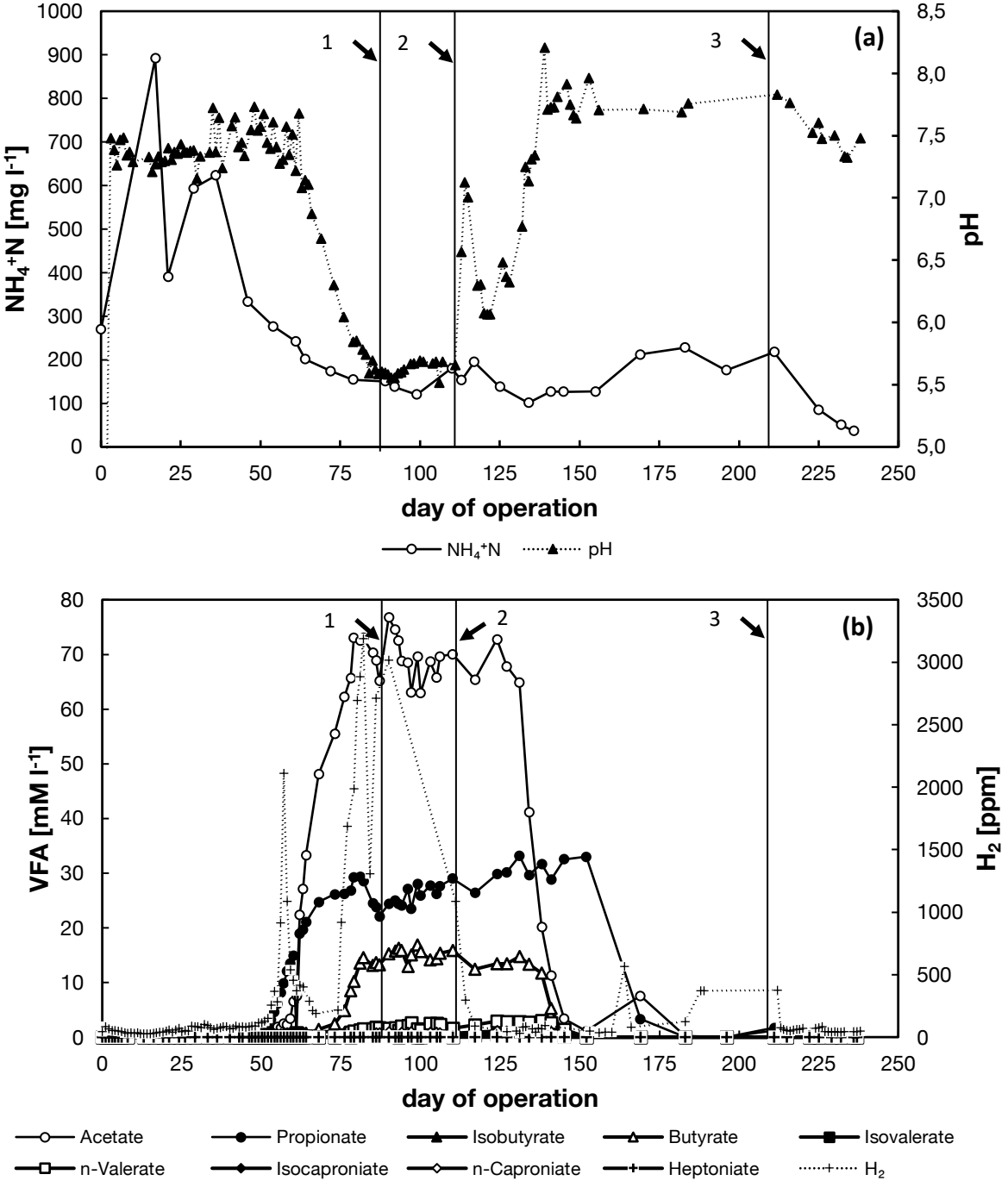
Figure 4.11 - R1 reactor - (a) Specific biogas production, specific methane production, and volatile solids reduction, (b) biogas composition. (1) stop feeding, (2) pH correction, (3) restart feeding.



The highest peak of acetic acid occurred on day 90 (Figure 4.12), with concentration of 76.73 mM l^{-1}. A peak of hydrogen appeared several days earlier, with 3189.30 ppm on day 82. Propionic acid also had its rise in the same period, reaching 14.54 mM l^{-1} on day 82.

Propionate is the precursor to a fraction of acetate and H_2, which are metabolized by methanogens prior to methane production. Propionate degradation requires Obligate Hydrogen Producing Acetogens (OHPA), H_2/formate-consuming methanogens, and acetate-consuming methanogens. The OHPA cannot work without H_2-consuming methanogens that hold H_2 concentrations extremely low, which is essential for propionate to acetate and H_2 metabolism to be energetically favorable (SPEECE, 2008). Which means that the anaerobic oxidation of propionate needs the H_2 to be reduced below 10^{-4} atmospheres (100 ppm) (WOLFE, 1999).

Figure 4.12 - R1 reactor - (a) Ammonium and pH, (b) Volatile Fatty Acids and hydrogen concentration in biogas. (1) stop feeding, (2) pH correction, (3) restart feeding with trace elements addition.



Besides, propionate and H_2 are the most common electron donors for SRB. Lactate, pyruvate, butyrate, LCFA, glycerol, ethanol, and some dicarboxylic acids like succinate can also be used by SRB. Some SRB species may use malate, fumarate, aromatic compounds and some amino acids (BRYSCH et al., 1987). Due to that, the metabolism of H_2 is the controller of the anaerobic digestion, especially in sulfate-rich substrates like vinasse.

Unlike aerobic environments, where acetate and propionate are readily metabolized by a wide range of microorganisms, anaerobic conditions allow only methanogens, sulfate reducers, biological phosphate removers, and a small number of other important anaerobes to degrade acetate. The fact that the effects of toxicity and trace elements deficiency are often similar, makes determining the cause of elevated VFA levels challenging (SPEECE, 2008).

The metabolism of propionate in anaerobic reactors with suspended growth, like CSTR, is significantly reduced when compared to reactors with biofilm/granules such as UASB plug flow. This is a response to the close proximity of the microbial consortia of the propionate degraders and the H_2-utilizers in the biofilm/granule (SPEECE, 2008).

However, a study by Albuquerque et al. (2019) with AC of vinasse and whey in AnSBBR (anaerobic sequencing bath biofilm reactor), mechanically stirred and operated in sequential batch and fed batch) have reported higher propionic acid production and accumulation in thermophilic condition (50 ˚C). In Barrera et al. (2014) study with anaerobic digestion of sulfate-rich vinasse using UASB (Upflow anaerobic sludge blanket) reactor also was identify propionate degrading bacteria (pDB) inhibition.

In the period 131-134 day there was a decrease of 36.53% in acetic acid and 10.65% in the concentration of propionic acid. This was a reflection of a punctual feed of the reactor on day 130, which encouraged the microbial consortium to consume volatile acids. Butyric acid was also reduced from 14.74mM l^{-1} to 5.27mM l^{-1}between 131-141 days; following remained below the detection limit until the end of experiment.

The concentration of isobutyric acid varied between 0.60- and 1.27-mM l^{-1} between days 59 and 174, following remained below the detection limit. N-capronate was detected (concentration lower than the limit of quantification) for the first time on day 85. Its concentration varied between 0.61 and 1.05mM on days 86-141. Isocaproate and heptoniate were not detected in R1 throughout the experiment. This indicates that the addition of iron and trace elements played a fundamental role in reducing volatile acids and hydrogen and recovering the stabilization of the anaerobic process.

Regarding methane (Figure 4.11), its concentration varied between 49.19 and 65.09% between days 1-5. In the sequence, there was a drop in its concentration (week 10, day 63-69), reaching 3.23%, while the CO_2 concentration increased dramatically, reaching 87.50%. This indicates inhibition of methanogenesis, mainly due to the accumulation of VFA and pH drop.

Between days 56 and 111 the concentration of CO_2 was higher than the concentration of CH_4, however, after correcting the pH of the reactor to 7.5 on day 111 it was observed that the concentration of methane returned to be higher than the carbon dioxide. This demonstrates that the pH control was essential for the methanogenic pathway.

After restarting the feeding with trace elements (Fe, Ni, Co, Se, Mo, and Zn) on day 208, the biogas output stabilizes gradually (Figure 4.11) and reached a methane yield of 176.79 ± 39.56 Nml CH_4 g^{-1} VS between days 212 and 236. During this time, the VS reduction remained above 50%, furthermore, the VFA remained undetectable, and the H_2S concentration remained below 402 ppm until the end of the experiment (day 238).

Regarding ammonium, its concentration remained in the same range until the date that the feeding was reestablished with the addition of trace elements, after which its concentration decreased significantly, reaching 36.40 mg l^{-1} at the end of the experiment. The formation of NH_4^+ in non-high concentrations is desirable, as it is a product of protein degradation, and, like Na, it is a cation that contributes to the increase of the alkalinity and pH of the process (SPEECE, 2008). However, high concentrations of NH_4^+ are undesirable, as it can negatively influence methanogenic activity, especially when the pH of the reactor is above 8.0, where NH_4^+ shift to the toxic um-ionized form of NH_3, that can pass through the microbial cell more easily than the highly hydrated NH_4^+ (SCHNÜRER; JARVIS, 2018).

In general, the R1 reactor showed that without supplementation of trace elements and correction of the affluent pH, it was not possible to successfully conduct anaerobic digestion. In this case, iron and the other metals played an essential role in reducing the production of H_2S and other undesirable and inhibitory metabolites, in addition, the trace elements collaborated positively to achieve process stability. The same was concluded by Gustavsson et al. (2011), when supplemented daily sulfate-rich wheat stillage CSTR reactor with Fe (0.5 g l^{-1}), Co (0.5 mg l^{-1}), and Ni (0.2 mg l^{-1}) in their study it was possible to keep the process with stability at an OLR of 4.0 g VS^{-1} d^{-1}.

In the R2 reactor, the addition of nickel (day 59) provided an increase in the reduction of VS (Figure 4.13), however, the reduction of VS soon fell, which can be justified by the absence/deficiency of other micronutrients essential for the microbial consortium.

Zinc, as well as cobalt, manganese and calcium, are essential for the anaerobic systems, where they are important cofactors for stimulating proteases and amylases (BERTINI; LUCHINAT, 1994; RAO et al., 1998; JISHA et al., 2013). In addition, it plays an important role together with Fe, Ni, and Se in hydrogenases, which is involved in the production of propionate, acetate and butyrate (HENDERSON, 1980).

In the final phase of anaerobic digestion, the methanogenesis, Zn, together with nickel and cobalt, are essential for the aceticlastic pathway (methane formation from acetate degradation) (BOONYAKITSOMBUT et al., 2002). Moreover, Zn is involved in the direct methane formation from methanol in the methylotrophic pathway. Cobalt, iron, and nickel are also involved in methylotrophic pathway (ZANDVOORT et al., 2004).

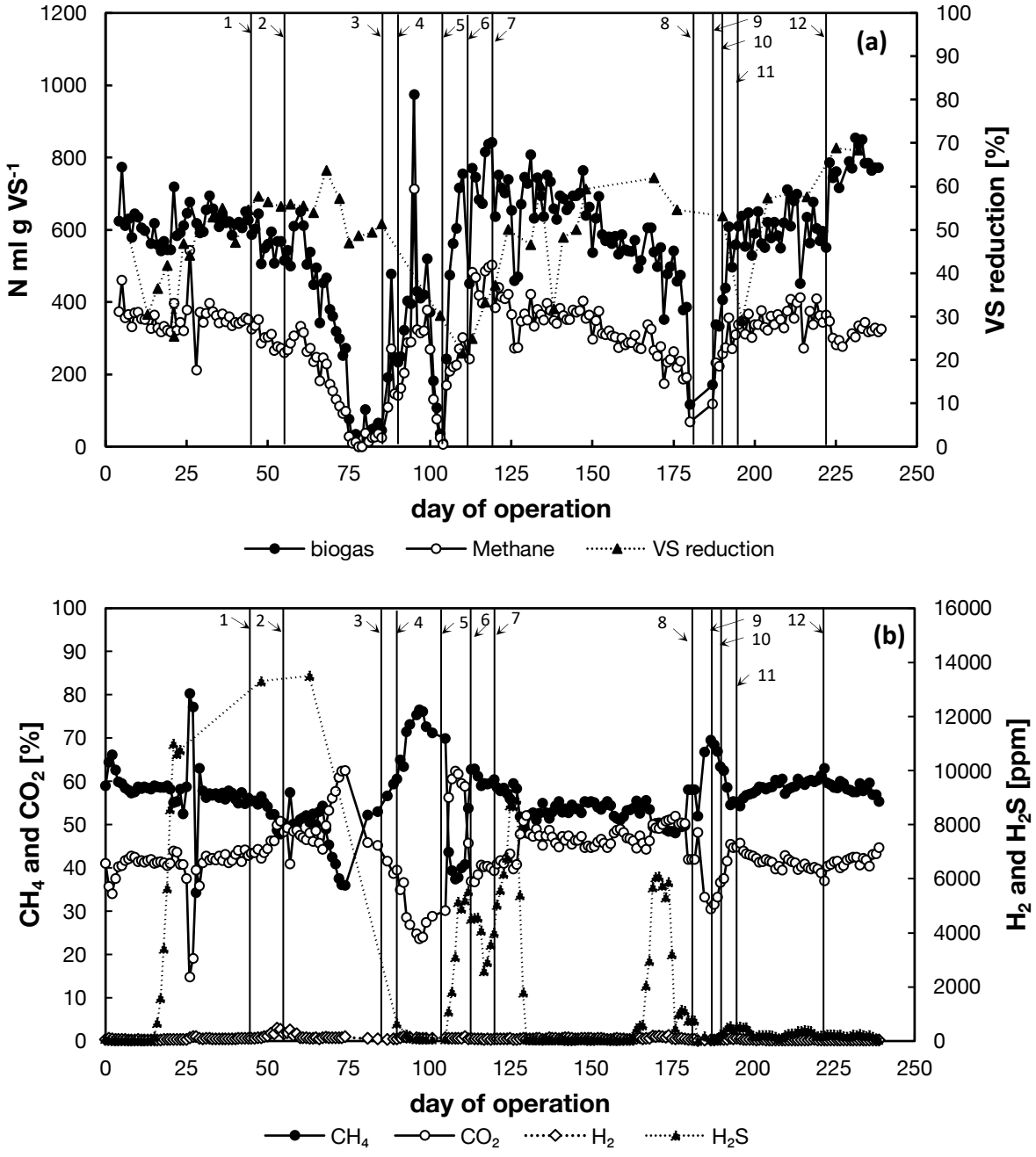
Regarding the concentration of ammonium in the R2 digestate (Figure 4.14), in the first HRT it was high (903.33 mg L^{-1}), as it came from the inoculum that received substrates rich in protein (food waste, slaughterhouse waste). Over time, an NH_4^+ washout was observed, and the reactor maintained its concentration between 14 and 232mg l^{-1}. In addition, the supplementation of selenium (day 86) impacted the fall in NH_4^+.

In relation to VFA (Figure 4.14), it can be seen that the decrease of acetate always occurs before propionate can go down. Considering that acetate in a product of propionate degradation this can be justified. Besides, nickel addition (day 55) has led to decreased propionate accumulation.

A difference between R1 and R2 in the VFA degradation can be seen, after OLR was decreased (day 86) R2 were able to consume de accumulated VFA, however, R1 not since the system was already acidified. Moreover, Fe and Ni (and Se after decreased the OLR) could had helped R2 degrade the accumulated acids.

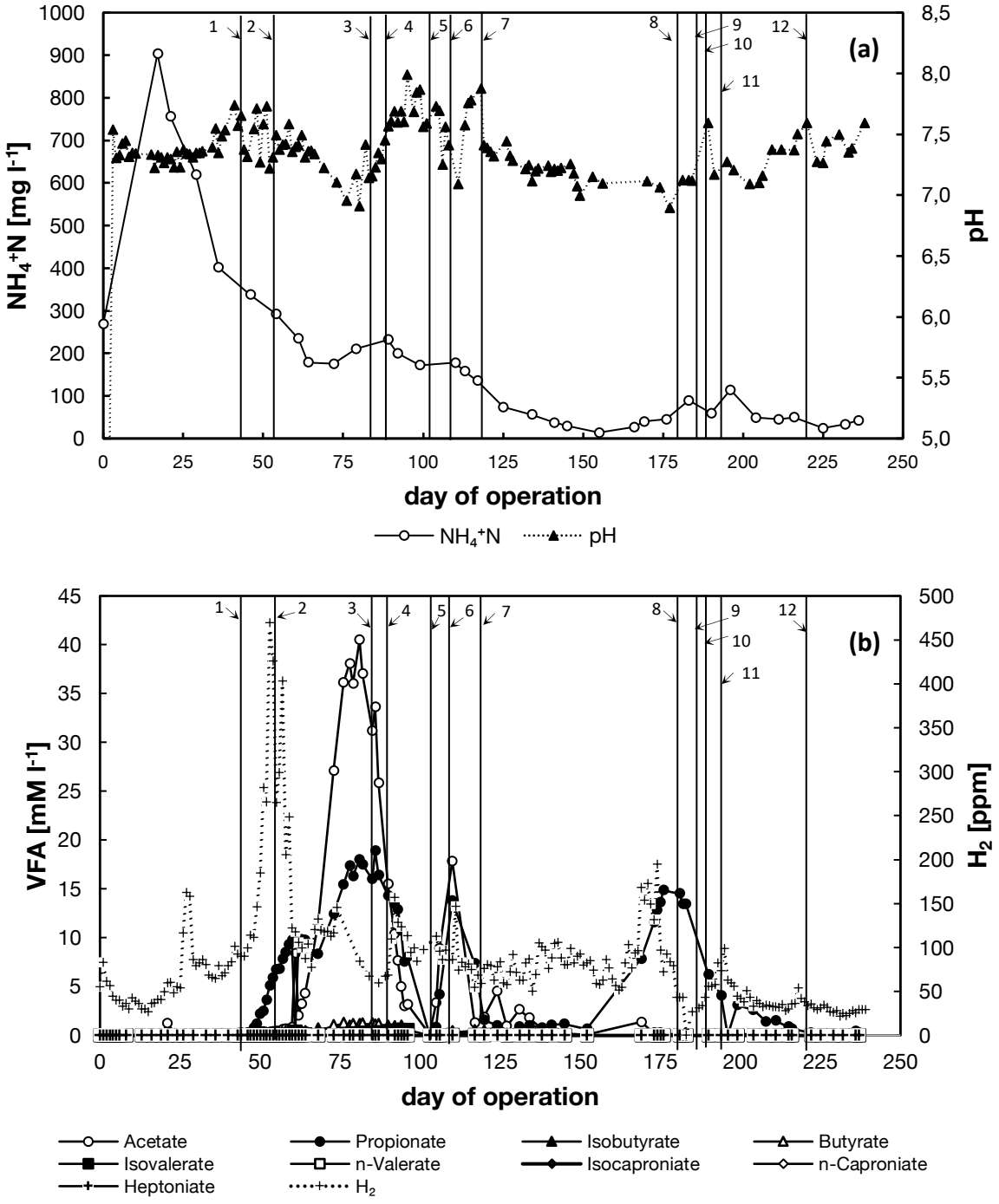
Molybdenum addition at day 119 was responsible for a significant drop in propionate from 13.46mM^{-1} (day 183) t 6.25mM^{-1} (day 190). Later Zn addition and correction of pH in the food portions also helped to keep propionate at lower levels until the end of the experiment. Besides, none VFA was accumulated after Mo and Zn supplementation started.

Figure 4.13 - R2 reactor - (a) Specific biogas production, specific methane production, and volatile solids reduction, (b) biogas composition. (1) start Fe addition, (2) start Ni addition, (3) reduced OLR, (4) start Se addition, (5) Start Co addition, (6) start Zn addition, (7) start Mo addition (8) stop feeding and increase Fe addition, (9) return feeding, (10) start Zn addition, (11) start pH correction of food portions, (12) Increase OLR.



H_2 shoots up after iron addition on day 45 (Figure 4.14), similar as observed in R1. This could possibly indicate SRB inhibition, which leads to accumulation of their substrates. Besides, H_2 drops seems to be connected to acetate degradation somehow, since it happened the same behavior in R1.

Figure 4.14 - R2 reactor - (a) Ammonium and pH, (b) Volatile Fatty Acids and hydrogen concentration in biogas. (1) start Fe addition, (2) start Ni addition, (3) reduced OLR, (4) start Se addition, (5) Start Co addition, (6) start Zn addition, (7) start Mo addition (8) stop feeding and increase Fe addition, (9) return feeding, (10) start Zn addition, (11) start pH correction of food portions, (12) Increase OLR.



During the period that R2 had accumulated VFA (day 66-91), the methane yield decreased significantly (Figure 4.13). Futhermore, after started the supplementation of all the metals (Fe, Ni, Se, Co, Mo, and Zn) and correction of the food portions pH, the methane yield increased and remained between 222.29 and 411.39 Nml CH_4 g^{-1} VS (107.10 and 198.21 Nml CH_4 g^{-1} COD).

After started the iron supplementation (day 45), the H_2S concentration in biogas (Figure 4.13) decreased 95.19 % (from 13,500 ppm on day 63 to 650 ppm on day 90). However, there were some peaks and fluctuations in H_2S concentration, especially on week 25 (day 168-174) when accidentally iron and the other trace elements were not added to the food portion.

When the Fe:S ratio was increased to 1 (day 181) the H_2S production in R2 was kept below 500 ppm until the end of the experiment.

In reactor R3 with co-digestion of vinasse, sugarcane straw, and filter cake the methane production per VS increased after started the co-digestion (Figure 4.15) reaching 382.27 Nml CH_4 g^{-1} VS on day 37, however, after 1 HRT of co-digestion (day 41) the methane yield started to decrease concomitantly with the VFA increase (Figure 4.16).The methane yield decreased from 147 to 55 g VS^{-1} d^{-1} on days 41-46, while the acetate increase from 1.33 to 28.58mM l^{-1} and propionate from 2.61 to 9.82mM l^{-1} at the same period. Besides, the H_2 production increased predicting upcoming acetate accumulation, as well observed in R1 and R2.

Iron supplementation started at day 47 coincides with a decrease in propionate accumulation, this is the opposite trend observed in the mono-digestion reactors (R1 and R2). Also, the degradation of propionate while acetate was still high is not an usual behavior observed in anaerobic digestion systems, since acetate is one of the end products of propionate degradation. This can indicate that acetate accumulation was caused by methanogenic inhibition or due to increased fermentation. Moreover, molecular microbiology analyzes could help clarify this question, however, they were not possible to be conducted in this experiment.

In relation to H_2S, iron supplementation was responsible for a considerably decrease in this gas. After started the supplementation, the H_2S remained below 186.42 ppm until the end of the experiment, which may have allowed better bioavailability of trace elements in the reactor.

In Shakeri Yekta et al. (2014) study elucidating the chemistry and kinetics of Fe(II), Co (II), and Ni(II) in CSTR reactors fed with supplemented vinasse concluded that the iron

solubility was controlled by precipitation/dissolution of FeS(s) and formation of FeS(aq) and $RSFe^{+}$ complexes. Besides, nickel speciation (chemical form/specie) in the reactor was controlled by interactions with FeS(s), which explain the importance of iron for better bioavailability of other trace elements.

Figure 4.15 - R3 reactor - (a) Specific biogas production, specific methane production, and volatile solids reduction, (b) biogas composition. (1) start co-digestion, (2) Start Fe addition, (3) Start Ni addition, (4) Decrease OLR and start Se addition, (5) Start Co addition, (6) start Mo addition (7) and (8) increase OLR, (9) start Zn addition, (10) start pH correction of food portions, (11) Increase OLR.

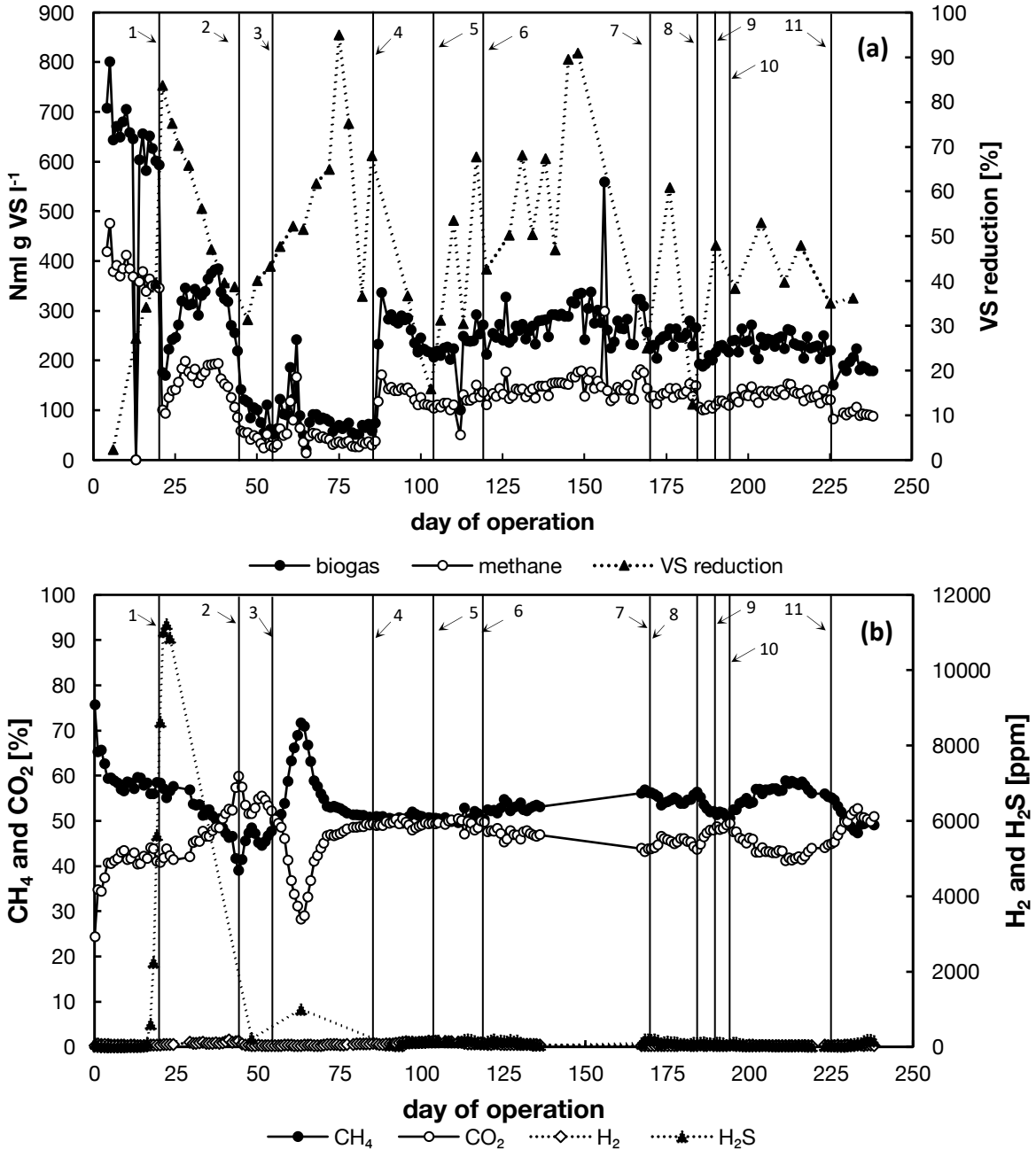
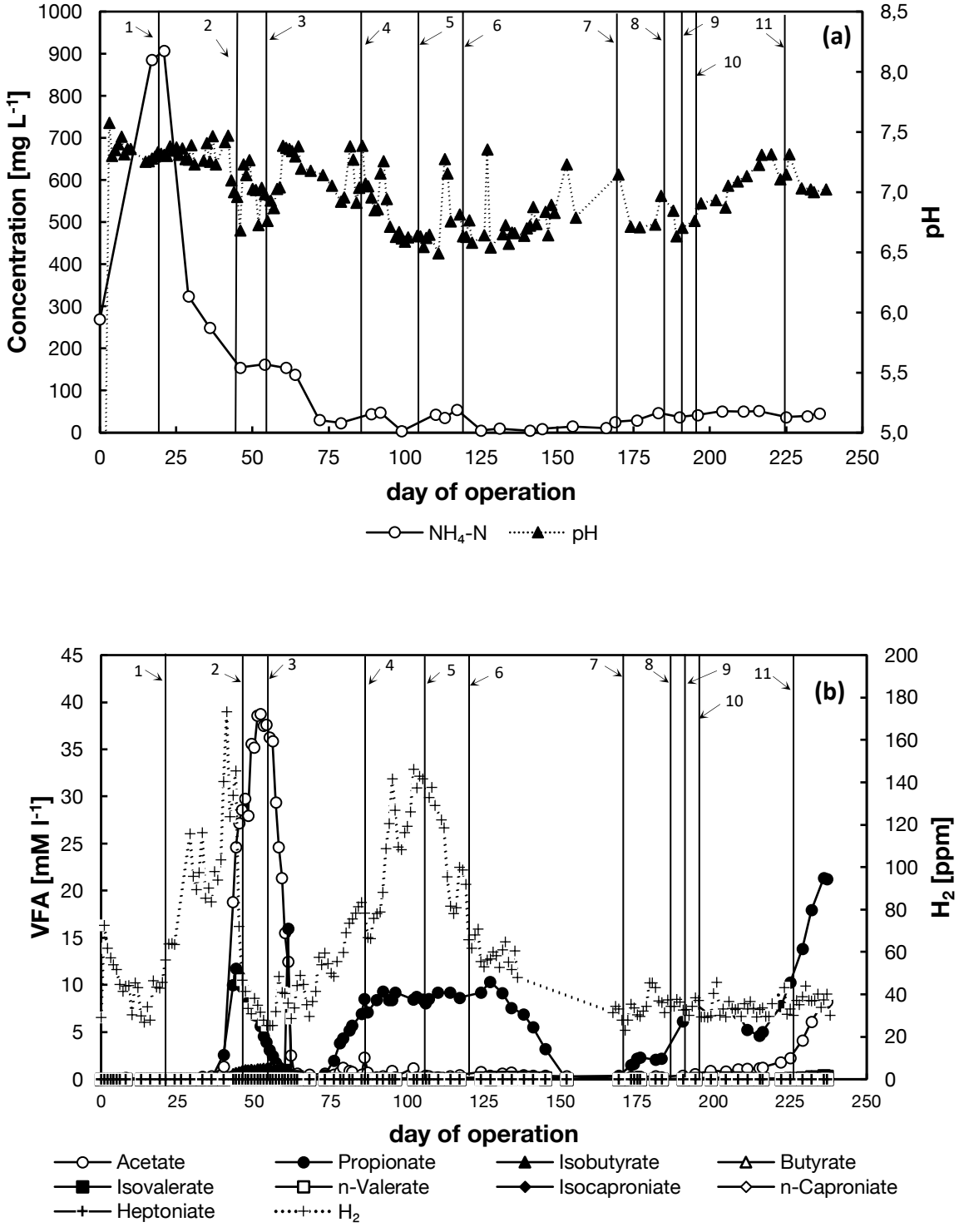


Figure 4.16 - R3 reactor - (a) Ammonium and pH, (b) Volatile Fatty Acids and hydrogen concentration in biogas. (1) start co-digestion, (2) Start Fe addition, (3) Start Ni addition, (4) Decrease OLR and start Se addition, (5) Start Co addition, (6) start Mo addition (7) and (8) increase OLR, (9) start Zn addition, (10) start pH correction of food portions, (11) Increase OLR.



Furthermore, a study by Gustavsson et al. (2011) demonstrated that supplementation of Fe, Ni, and Co to CSTR reactor fed with vinasse was essential for efficient and stable biogas production.

After nickel supplementation started (day 55) an improve in the methane production and VS reduction can be seen (Figure 4.15). Besides, the pH rose from ≅6.8 to 7.4 after nickel addition and the levels of acetate decrease considerably (From 38.7 mM l^{-1} on day 52 to 2.54 mM l^{-1} on day 62). This behavior have been reported by other authors, the addition of Ni and other metals tends to increase acetate and propionate rates, and enhance the methane production (SPEECE; PARKIN; GALLAGHER, 1983; MOESTEDT et al., 2016; WINTSCHE et al., 2018; PARITOSH et al., 2020).

Between points 4 (decreased OLR, start Se addition) and 5 (start Co addition) the pH dropped (from ≅7.3 to 6.6) (Figure 4.16) despite VFA being in a constant flow and OLR being decreased. This could be a response to more H^+ in the medium and can also be correlated to the increasing concentration of H_2 in biogas at the same period. Furthermore, this could also be related to accumulation of a "hidden" VFA that was not measured in the experiment, like formate. Besides, it happened after started the selenium supplementation, and since Se did not helped to control the pH, there should be no problem with formate dehydrogenase since it depend on Se availability (JONES; STADTMAN, 1981).

In some studies formate has been found to act as an intermediary in the degradation of complex organics. Since methanogenesis from formate does not require H_2, it will be the favored pathway for microorganisms able to perform the reaction (BOONE; JOHNSON; LIU, 1989; VOOLAPALLI; STUCKEY, 2001) can be at concentrations below the Ks (5 mg l^{-1}) of formate-utilizing methanogens in CSTR reactors (GROBICKI; STUCKEY, 1989).

On the other hand, after started the Co addition (day 104) a drop in the H_2 concentration can be observed (Figure 4.16). But the pH remained low. Despite that if cobalt was at deficiency levels in the medium, it would be expected acetate to be the first symptom, not propionate, since Co has been identified as a booster of acetogenic process, and a growth factor of acetogenic microorganisms (BOONYAKITSOMBUT et al., 2002; PARITOSH et al., 2020).

After molybdenum addition a decrease in propionate started. Also, H_2 dropped below 60 ppm before propionate start to decrease, this indicate that propionate degradation products were accumulating, but Mo played a key role at this moment. A study by Espinosa et al. (1995)

the addition of molybdenum and other trace metals (Fe, Ni, and Co) in vinasse was responsible for significantly reduction of propionate and acetate in addition to promote higher methane yield and COD removal.

Hydrogen is reported to be a major intermediate in AD, since it impacts the substrate conversion capacity of several important anaerobic microorganisms. Also, play a key role in the metabolism of various organics compounds such as carbohydrates, alcohols, propionate, and butyrate. Moreover, at steady state H_2 concentration is normally maintained at low levels of less than 100 ppm. However, during increased loads it may rise substantially (SPEECE, 2008).

Once OLR started to be increased (points 7 and 8, Figure 4.16) propionate, started to accumulate again, but this time without increase the H_2 in biogas, suggesting that H_2 consumption is not a limiting step anymore, which could mean that Mo limitation was in the final steps of methanogenesis before.

The correction of the substrate pH (after day 195) helped the pH of the process stability, but there was still accumulation of VFA, which rose as the OLR increased, however, again without changing H_2 concentrations. This may suggest that the flow was going through Wood-Ljungdahl pathway (W-L) towards acetate and would explain why cobalt helped decrease H_2.

The W-L pathway enable methanogens and acetogens to use hydrogen as electron donor and carbon dioxide as electron acceptor. Some methanogens use the W-L pathway in reverse in order to break down acetate(RAGSDALE, 2008). In this pathway, cobalt is essential since it is the corrin ring of the cobalamin cofactor, which play a major role on the methyl group of acetate (POSTON; KURATOMI; STADTMAN, 1964; RAGSDALE; PIERCE, 2008).

After supplementation of all the metals, R3 presented a yield between 54.61-109.14 Nml CH_4 g^{-1} VS until the end of the experiment. In R4 reactor, the behavior was very similar to R3, which in reasonable since they were replicates and received the same substrate and supplementation metals.

The response to iron and nickel supplementation matches R3 in relation to VFA decrease, pH (Figure 4.17), and methane yield (Figure 4.18).

Selenium supplementation together with decreased OLR were responsible to stabilize propionate concentrations and even leads to its decrease, similar as observed in R3 (previously described). Cobalt supplementation also improved VS reduction and gas production per VS in R4.

Figure 4.17 - R4 reactor - (a) Ammonium and pH, (b) Volatile Fatty Acids and hydrogen concentration in biogas. (1) start co-digestion, (2) Start Fe addition, (3) Start Ni addition, (4) Decrease OLR and start Se addition, (5) Start Co addition, (6) start Mo addition (7) and (8) increase OLR, (9) start Zn addition, (10) start pH correction of food portions, (11) Increase OLR.

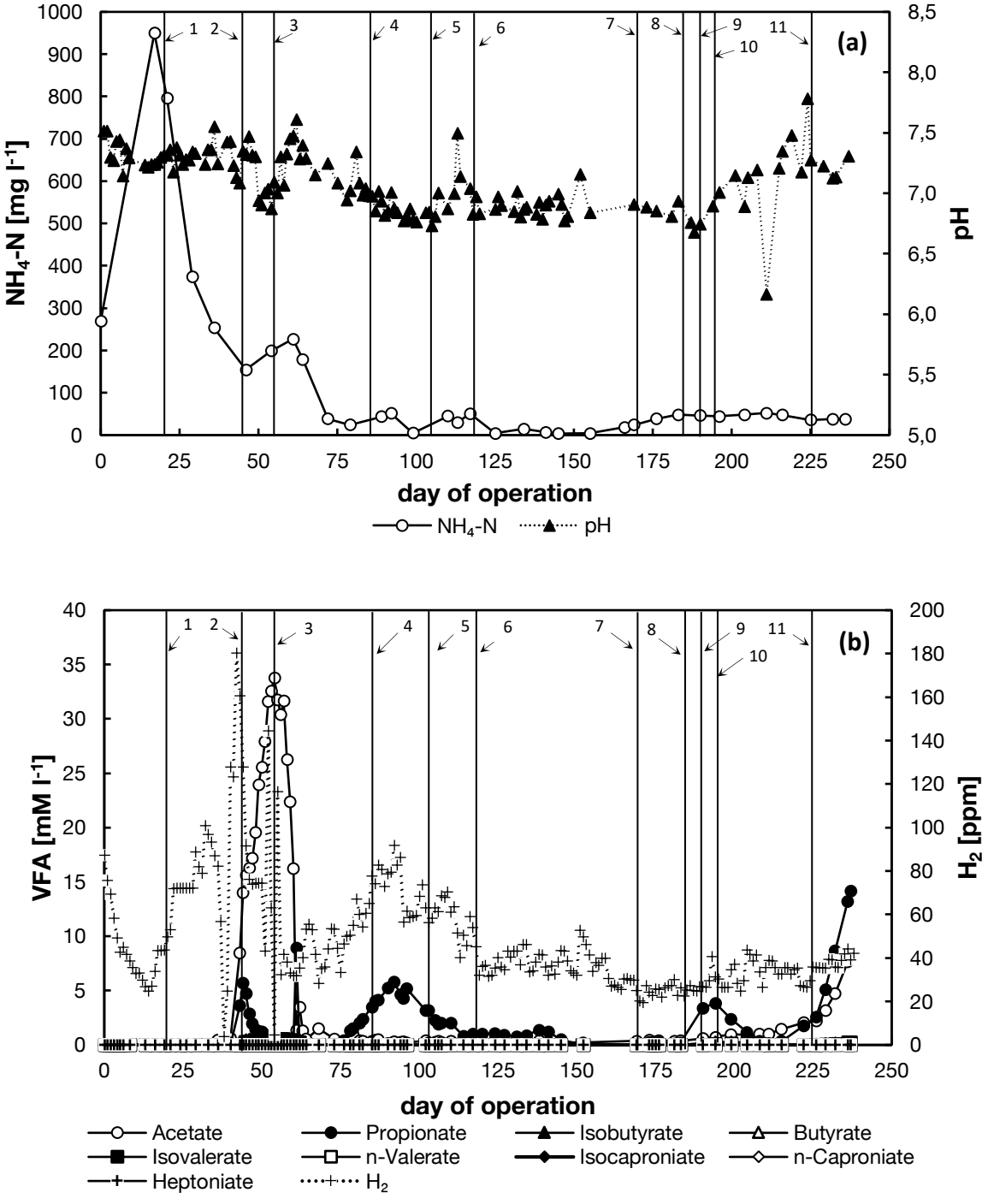
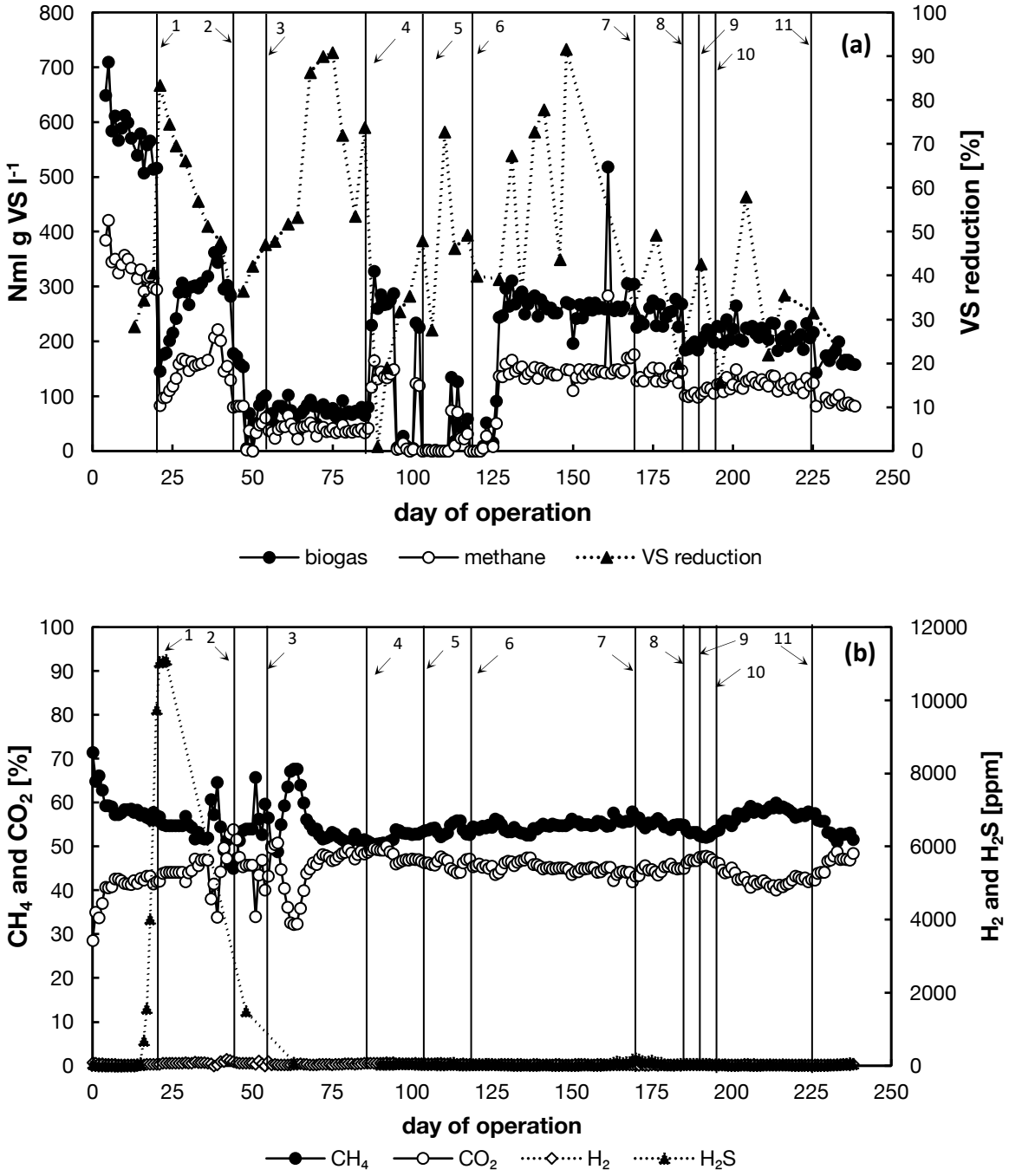


Figure 4.18 - R4 reactor - (a) Specific biogas production, specific methane production, and volatile solids reduction, (b) biogas composition. (1) start co-digestion, (2) Start Fe addition, (3) Start Ni addition, (4) Decrease OLR and start Se addition, (5) Start Co addition, (6) start Mo addition (7) and (8) increase OLR, (9) start Zn addition, (10) start pH correction of food portions, (11) Increase OLR.



When the zinc started to be added, the methane trend improved (Figure 4.18), while the propionate accumulation continued, but at a slower rate, which suggests that metabolic pathway

downstream propionate formation (acetogenesis and/or metanogenesis) was fixed or improved. Similar behavior after Zn supplementation was observed in R3.

It can be highlighted that in R4 after started iron supplementation the H_2S only reached concentration above 100 ppm (100.32 - 215.52 ppm) during week 25 (day 168-174) when accidentally iron and other metals were not added to the reactor. In reactor R2 (vinasse and trace elements) the H_2S concentration varied between 2039.69 and 6071.11 ppm during this period. This suggests a better control of H_2S in the co-digestion system. Besides, the sulfate concentration in the substrate in R1 and R2 was 10% higher (8,405.00 mg L^{-1}) than R3 and R4 (7,564.50 mg L^{-1}), but the iron addition was proportional to the sulfur content in the substrate (Fe:S ratio of 1).

The average of H_2S after iron supplementation in R4 was 50.92± 32.79 ppm. Moreover, a yield between 54.15 and 110.21 Nml CH_4 g^{-1} VS was observed during the period that R4 received all the metals (day 195-238). In this period, the H_2S in R4 remained below 70.74 ppm.

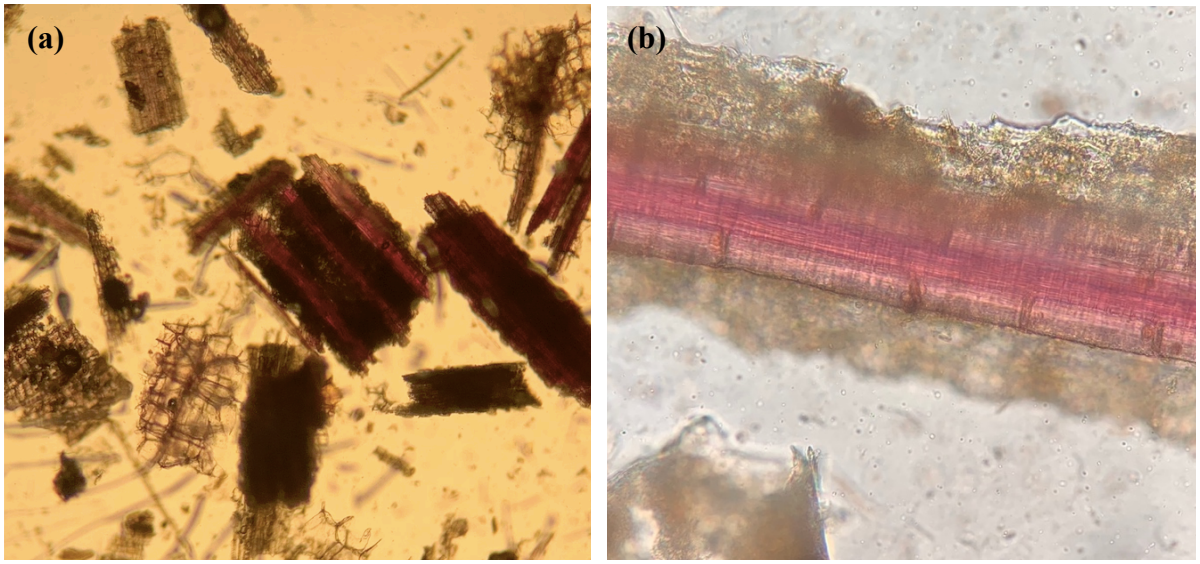
An important point when comparing the mono-digestion (R1 and R2) to the co-digestion (R3 and R4) is the C:N ratio. The C:N ratio of the mono-digestion of vinasse was 21.24 while the co-digestion of vinasse, straw, and filter cake was 47.95. The literature indicates that the ideal C:N ratio for AD is between 10-30, with an optimum between 15-30) (SCHNÜRER; JARVIS, 2018). High C:N ratios may be not favorable since the microorganism may present nitrogen deficiency (YEN; BRUNE, 2007). Due to that, the co-digestion may have experienced nitrogen deficiency, which could have been overcome by adding a nitrogen-rich residue to the co-digestion, and then, balanced the C:N ratio. However, the measurement of C and N was only possible in the end of this experiment.

Another important point in this study was that the combination of substrates in co-digestion (VFS +Fe) presented a lag-phase of 32.46 days in the BMP test (previously discussed), which indicates that the hydraulic retention time (HRT) of 20 days applied in the reactors' study may not have been ideal for complete degradation of the substrate. Also, could have contributed to the accumulation of intermediates in the system e.g., VFA and H_2. However, the BMP test was conducted when the study with reactors was already in course and changing the HRT of the system at this moment was not adequate. Additionally, the study with reactors was interrupted on day 238 and the planned change in HRT experiment could not be carried out.

4.3.3. Light microscopy and scanning electron microscopy

The light microscopy analyzes showed that the sugarcane straw used as substrate for anaerobic digestion in this study (Figure 4.19a) was rich in lignin. And, after the AD, it can be seen that the lignin remained (cherry-red) in the digestate of the co-digestion reactors, besides the presence of attached microorganisms. This was expected, since lignin is reported to be a component of the lignocellulosic material hard to be biologic degraded (SARKER et al., 2019).

Figure 4.19 - Substrate sugarcane straw stained with floroglycine – In red lignin (400x) (a) digestate of R3 day 43 stained with floroglycine – in red lignin - 1000x (b)



The red marks presented in the digestate (Figure 4.20a) indicates the biofilm formation surrounding the lignocellulosic material, since the ruthenium red indicates the presence of extracellular polymeric substances (EPS) such as polysaccharides and proteins (CHARUK; PIRRAGLIA; REITHMEIER, 1990; WALLER et al., 2004). Besides, the microorganisms penetrated the sugarcane straw (light blue cells in Figure 4.20a and purple cells in Figure 4.20b). Moreover, the presence of the microorganisms inside the sugarcane straw cells was confirmed by SEM analysis (Figure 4.21).

Figure 4.20 - Digestate of R4 day 73 stained with cotton blue (chitin, bacteria, and fungi) and ruthenium red (pectin, mucilage, and biofilm) – 400x (a) and digestate of R4 day 191 stained with toluidine blue (plants cells in blue, microorganisms in purple) – 400x (b).

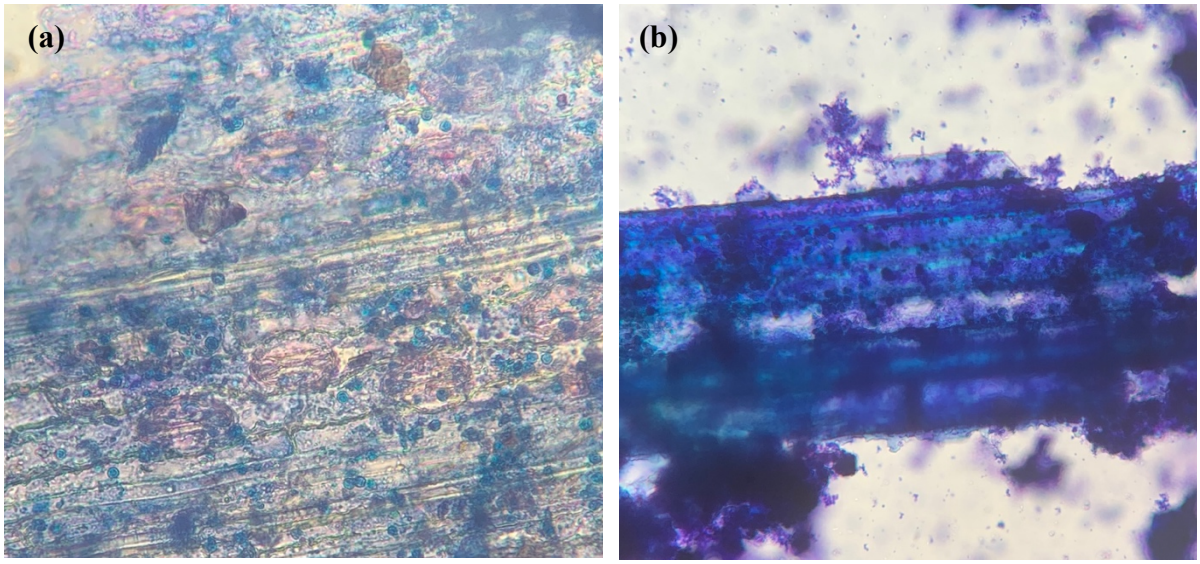
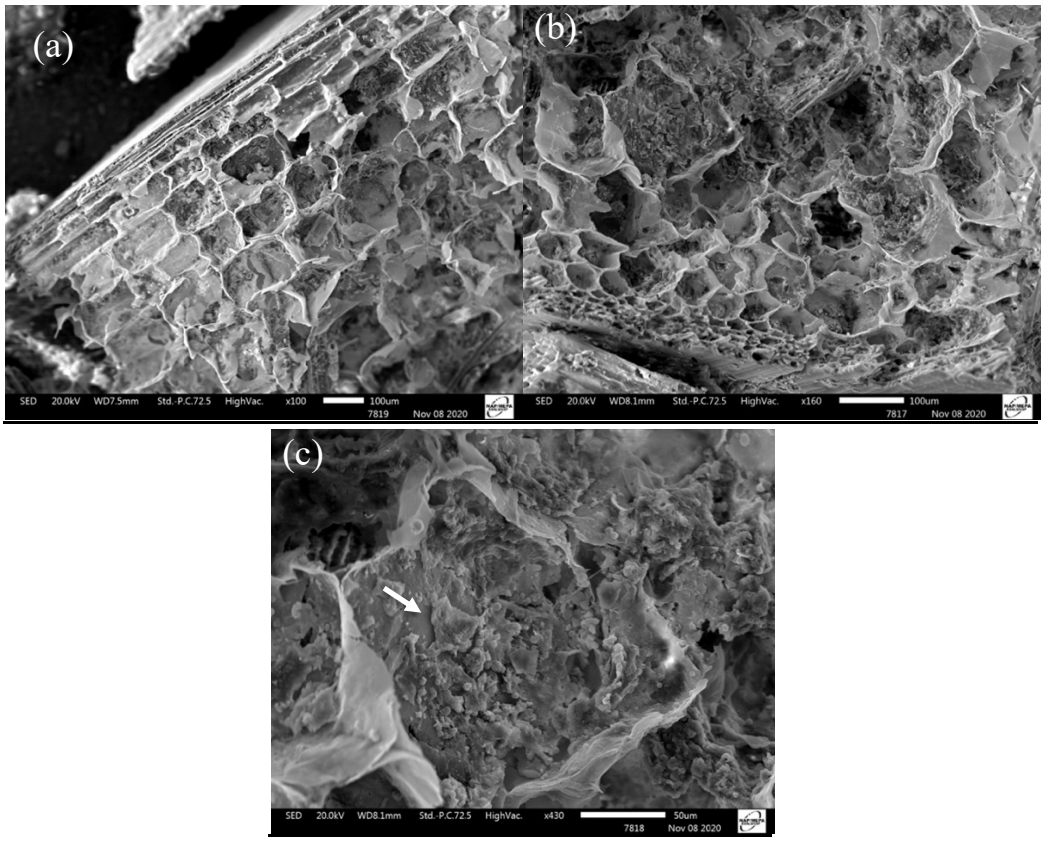


Figure 4.21 - Scanning Electron Microscopy analysis of R3 digestate at day 43. Straw degradation in different magnifications (a), (b), and (c). The arrow indicates the presence of microorganisms and EPS.



The presence of yeasts-like microorganisms can be seen in all the reactors (R1, R2, R3, and R4) samples. But in the mono-digestion of vinasse the number of these microorganisms is considerably higher (Figures 4.22 and 4.23). The suggestion that these microorganisms are yeasts comes from the size and shape of the cells, but a confirmation test such as DNA sequencing can help answer this question.

It is not common to find in the literature reports of yeast presence, especially in sugarcane residues articles (BRIONES et al., 2007; TERRY BROWN et al., 2019; ILTCHENCO et al., 2020; ORDAZ-DIAZ; BAILON-SALAS, 2020). However, most of the studies do not sequence eukaryotes, then yeast identification and quantification cannot be evaluated.

Some recent reports with other type of substrates described that eukaryotes are present at low-abundance in anaerobic digestion (DIEZMANN; DIETRICH, 2009; MATSUBAYASHI et al., 2017; INABA et al., 2020; MUTURI et al., 2021).

In (INABA et al., 2020) work the yeast-like fungus *Geotrichum candidum* (KY457577) was identified present in biofilm microbiome of AnMBR reactor treating organic solid waste. However, *G. candidum* is known to formation of hyphae, especially in wastewater treatment plants, but in the present work hyphae formation was not identified in the microscopic analyzes.

In Gao et al. (2020) study with the addition of activated yeast to the AD of food waste showed that the treatment with yeast addition (batch mode) presented higher methane production (33.2%) when compared with the control. Furthermore, the results indicates that the yeast addition helped with VFA, alkalinity and stability improve of the process.

The presence of EPS can be seen in reactor R2 digestate (Figure 4.22b) more abundant than R1 digestate (Figure 4.22a). This can explain the better performance of R2, the EPS have been reported to increase AD, since are responsible for optimization of the structure of the microbial community and accelerating the electron transfer between interspecies of syntrophic bacteria and methanogens (MA et al., 2019).

Figure 4.22 - Digestate of R1 day 131 stained with cotton blue (chitin, bacteria, and fungi) and ruthenium red (pectin, mucilage, and biofilm) – 1000x (a). Digestate of R2 day 155 stained with cotton blue (chitin, bacteria, and fungi) and ruthenium red (pectin, mucilage, and biofilm) – 1000x (b).

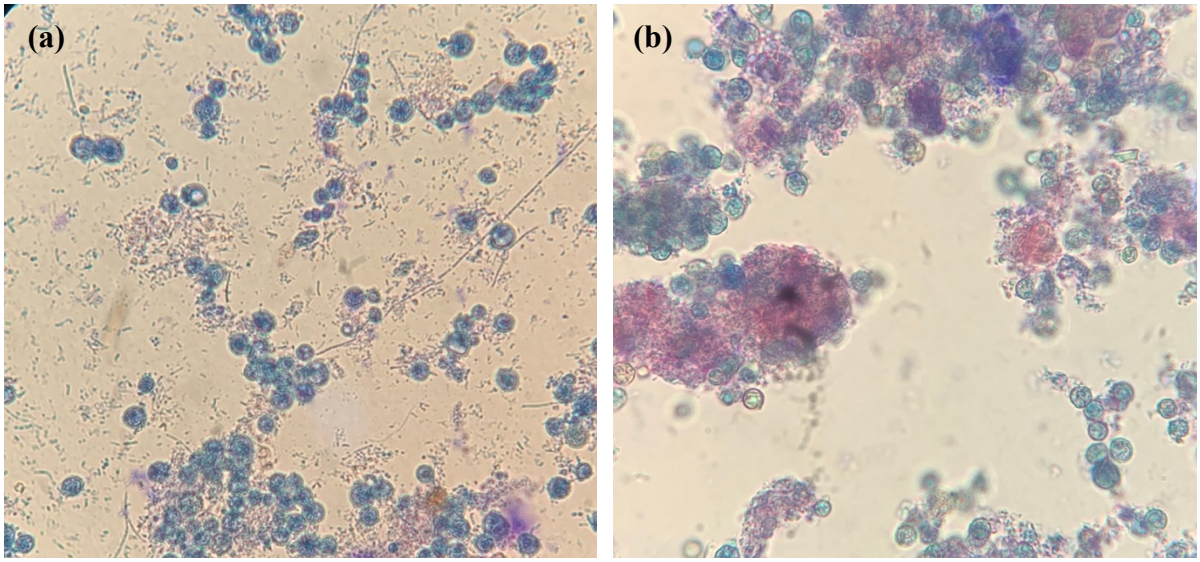
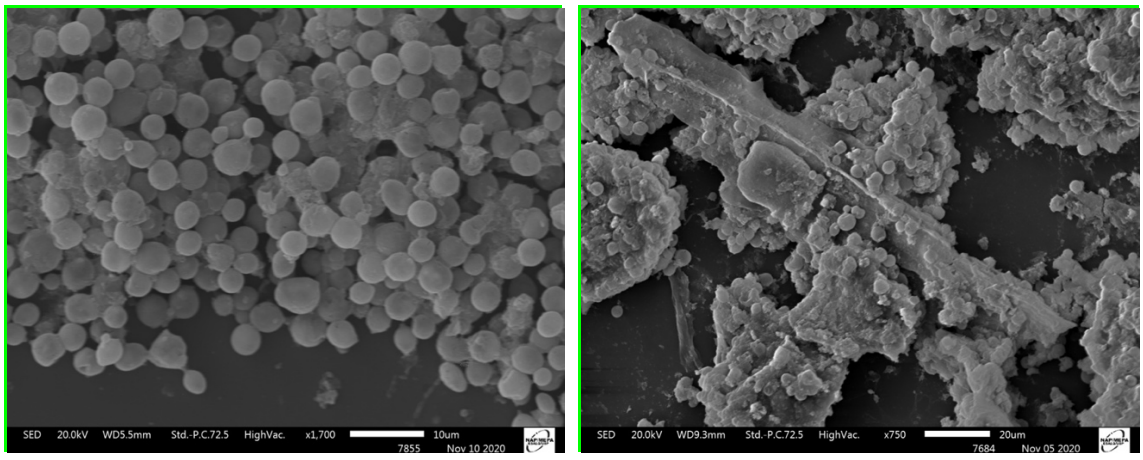
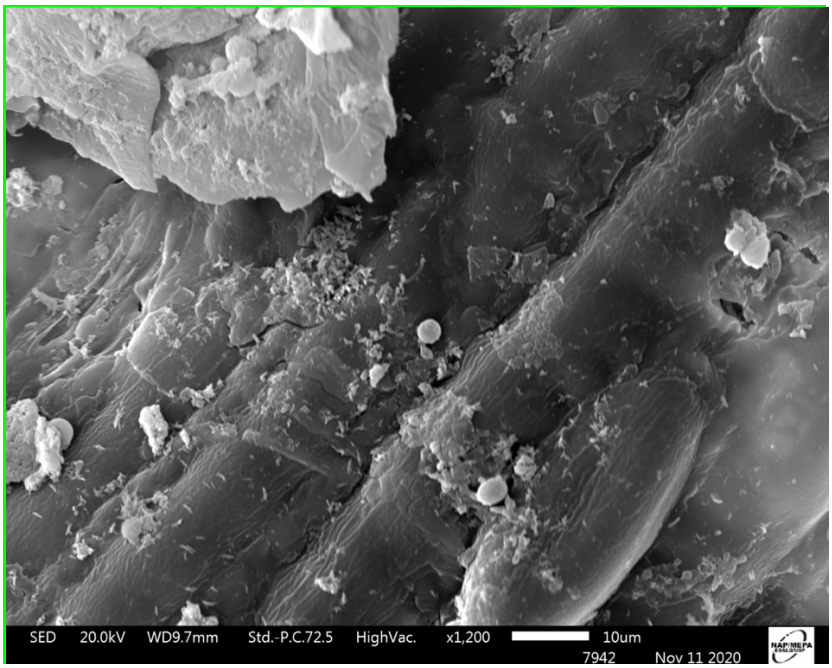


Figure 4.23 - Scanning Electron Microscopy analysis of R1 digestate at day 131(a). Yeast predominance, and R2 digestate at day 75 (b). Microorganisms aggregates (yeast-like).



In the samples of digestate of co-digestion reactors (Figures 4.20 and 4.24) some big cells (yeast-like) among with smaller cells (bacteria-like and archaea-like) can be seen. Moreover, EPS and biofilm formation were also present.

Figure 4.24 - Scanning Electron Microscopy of R4 digestate at day 155. Bacteria predominance.



In the scanning electron microscopy analyzes of R3 (Figure 4.25) and R4 (Figure 4.26) digestates, the degradation of lignocellulosic material can be seen. The structures remained in the digestate samples (Figure 4.24b and 4.25) demonstrates partial disintegration of plant cell walls' solid structure, with more damage and fiber exposure, very similar to the porous structure of lignin of sugarcane straw reported by Halder et al. (2019), which indicates the degradation of the cellulose and hemicellulose fractions.

Figure 4.25 - Scanning Electron Microscopy analysis of R3 digestate at day 73. Straw degradation in different magnifications (a) and (b).

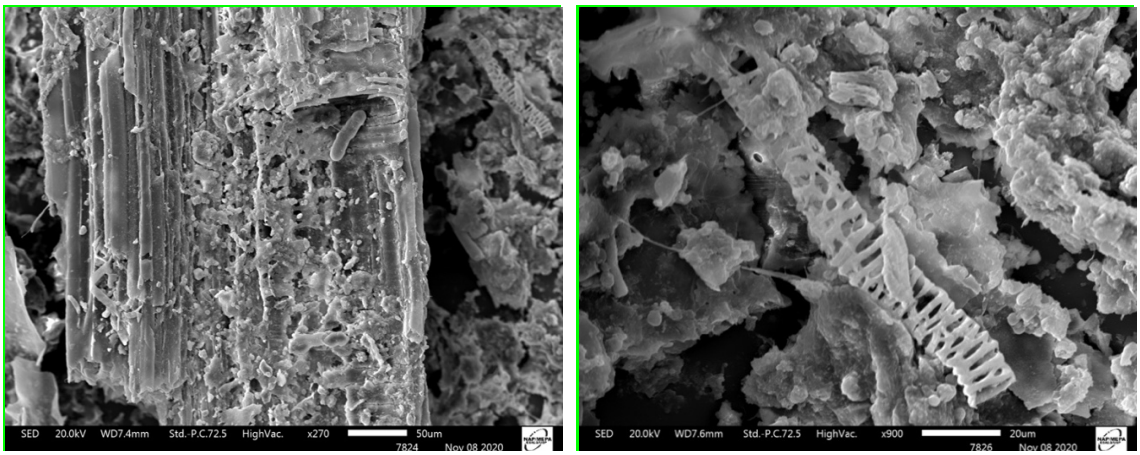
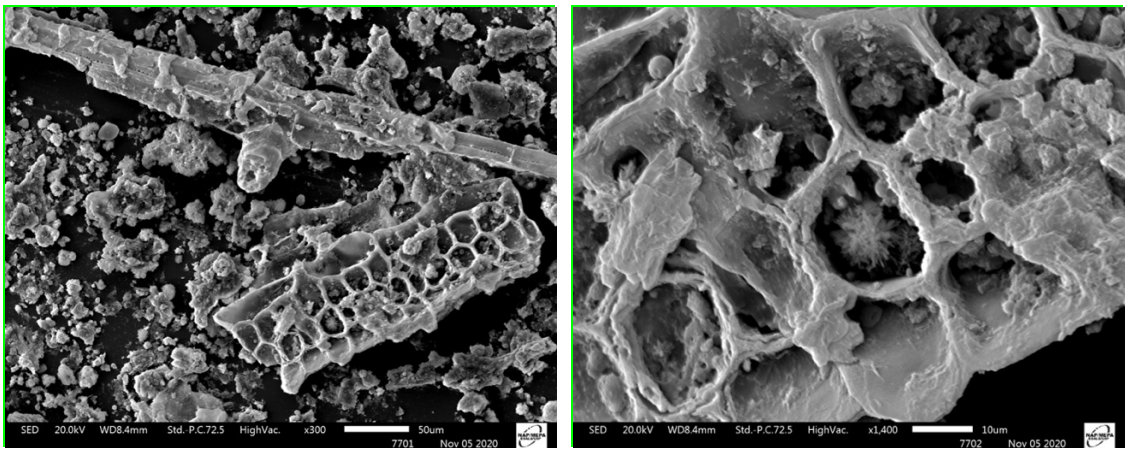


Figure 4.26 - Scanning Electron Microscopy analysis of R4 digestate at day 73. Cellulose cells degradation at diferente magnifications (a) and (b).



4.4.Conclusions

The biochemical methane potential test (BMP) showed that the higher methane yield was achieved by the monodigestion of vinasse with iron supplementation with 312.48±11.17 Nml CH_4 g^{-1} VS. The BMP of the co-digestion of vinasse, straw and filter cake presented a lower methane yield of 246.01±0.29 Nml CH_4 g^{-1} VS, which indicates hard degradability of the lignocellulosic material.

In the continuous experiment with CSTR reactors investigating the supplementation of the substrate with metals, iron played an important role in decreasing the H_2S production. In addition, the other metals made it possible to increase the methane yield of the processes.

The reactor with monodigestion of vinasse and addition of trace elements (R2) reached a methane yield between 222.29 - 411.39 Nml CH_4 g^{-1} VS after the addition of all the trace elements (Fe, Ni, Co, Se, Mo, and Zn). Also, the H_2S in biogas remained below 500 ppm.

The rector with monodigestion of vinasse and no metal supplementation (R1 – Control) reached 36,000 ppm of H_2S in biogas and failed due to accumulation of volatile fatty acids. However, after a long period without feeding and after pH correction and metals supplementation, R1 was able to re-stablish the anaerobic digestion with similar methane yield to R2.

The reactors with co-digestion of vinasse, straw and filter cake that received micronutrients supplementation (R3 and R4) presented a range of yield between 54.61-109.14 NmlCH_4 g^{-1}VS in R3, and 54.15 - 110.21 Nml CH_4 g^{-1} VS in R4 after the

supplementation of all the metals. In addition, the H_2S concentration remained below 186.42 ppm in R3, and below 70.74 in R4.

In general, anaerobic co-digestion of vinasse, filter cake and sugarcane straw presented lower methane production than the monodigestion of vinasse, however, maintained greater stability over the 238 days of experiment, with low accumulation of volatile fatty acids and formation of H_2S.

References

ADIYIA, P. K. **Anaerobic Co-digestion of Digestate with Glycerol to enhance Biogas Production**. 2021. Thesis (MSc in Science for Sustainable Development) - Department of Thematic Studies, Environmental Change, University of Linköping, 2021.

ALBUQUERQUE, J. N.; RATUSZNEI, S. M.; RODRIGUES, J. A. D. Biomethane production by thermophilic co-digestion of sugarcane vinasse and whey in an AnSBBR: Effects of composition, organic load, feed strategy and temperature. **Journal of Environmental Management**, v. 251, art. 109606, 2019. Available at: <https://doi.org/10.1016/j.jenvman.2019.109606>.

ANGELIDAKI, I. et al. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: A proposed protocol for batch assays. **Water Science and Technology**, v. 59, n. 5, p. 927–934, 2009. Available at: <https://pubmed.ncbi.nlm.nih.gov/19273891/>. Acessed at: 10 maio 2021.

BAIRD, R. B. **Standard methods for the examination of water and wastewater**. 23. ed. Washington, DC: APHA; AWWA; WEF, 2017.

BARRERA, E. L. et al. Characterization of the sulfate reduction process in the anaerobic digestion of a very high strength and sulfate rich vinasse. **Chemical Engineering Journal**, v. 248, p. 383–393, 2014. Available at: <http://dx.doi.org/10.1016/j.cej.2014.03.057>.

BERTINI, I.; LUCHINAT, C. The Reaction Pathways of Zinc Enzymes and Related Biological Catalysts. In: BERTINI, I. et al. (Ed.). **Bioinorganic Chemistry**. Mill Valley, CA: University Science Books, 1994. Chap. 2, p. 37–106. Available at: <http://citeseerx.ist.psu.edu/viewdoc/summary?doi=10.1.1.697.1124>.

BOONYAKITSOMBUT, S. et al. Degradation of propionate and its precursors: The role of nutrient supplementation. **KSCE Journal of Civil Engineering**, v. 6, n. 4, p. 379–387, 2002. Available at: <https://link.springer.com/article/10.1007/BF02841992>. Acessed at: 26 maio 2021.

BRIONES, A. M. et al. Microbial diversity and dynamics in multi- and single-compartment anaerobic bioreactors processing sulfate-rich waste streams. **Environmental Microbiology**, v. 9, n. 1, p. 93–106, 2007.

BRYSCH, K. et al. Lithoautotrophic growth of sulfate-reducing bacteria, and description of Desulfobacterium autotrophicum gen. nov., sp. nov. **Archives of Microbiology**, v. 148, n. 4, p. 264–274, 1987. Available at: <https://link.springer.com/article/10.1007/BF00456703>. Acessed em: 21 maio. 2021.

CHAMBERLAIN, C. J. **Methods in plant histology**. [s.l.] University of Chicago Press, 1924.

CHAN, W. S. et al. Metal accumulations in aquatic organisms and health risks in an acid mine-affected site in South China. **Environmental Geochemistry and Health**, 2021. Available at: <https://link.springer.com/10.1007/s10653-021-00923-0>. Acessed at: 17 may 2021.

CHARUK, J. H. M.; PIRRAGLIA, C. A.; REITHMEIER, R. A. F. Interaction of ruthenium red with Ca2+-binding proteins. **Analytical biochemistry**, v. 188, n. 1, p. 123–131, 1990.

CHEN, Y.; CHENG, J. J.; CREAMER, K. S. Inhibition of anaerobic digestion process: A review. **Bioresource Technology**, v. 99, n. 10, p. 4044–4064, 2008.

CHRISTOFOLETTI, C. A. et al. Sugarcane vinasse: Environmental implications of its use. **Waste Management**, v. 33, n. 12, p. 2752–2761, 2013. Available at: <http://dx.doi.org/10.1016/j.wasman.2013.09.005>.

CRUZ, P. C. F. et al. Desempenho de um Filtro Anaeróbico Ascendente de Fluxo Contínuo Preenchido com Espuma de Poliuretano para Tratamento de Esgoto. **Revista Engenharia e Tecnologia**, v. 4, n. 2, p. 1–10, 2010.

DIEZMANN, S.; DIETRICH, F. S. Saccharomyces cerevisiae: Population divergence and resistance to oxidative stress in clinical, domesticated and wild isolates. **PLoS ONE**, v. 4, n. 4, e5317, 2009. https://doi.org/10.1371/journal.pone.0005317

DILALLO, R.; ALBERTSON, O. E. Volatile Acids By Direct Titration. **Water Pollution Control Federation**, v. 33, n. 4, p. 356–365, 1961.

EITH, C. et al. **Practical Ion Chromatography**: An Introduction. Herisau, Switzerland: Metrohm, 2006.

ERSAHIN, M. E. et al. **Waste Water -** Treatment and Reutilization. London: InTech, 2011.

ESPINOSA, A. et al. Effect of trace metals on the anaerobic degradation of volatile fatty acids in molasses stillage. **Water Science and technology**, v. 32, n. 12, p. 121–129, 1995.

FENG, X. M. et al. Impact of trace element addition on biogas production from food industrial waste - Linking process to microbial communities. **FEMS Microbiology Ecology**, v. 74, n. 1, p. 226–240, 2010. Available at: <https://academic.oup.com/femsec/article/74/1/226/2680477>.

FUESS, L. T. et al. Designing full-scale biodigestion plants for the treatment of vinasse in sugarcane biorefineries: How phase separation and alkalinization impact biogas and electricity production costs? **Chemical Engineering Research and Design**, v. 119, p. 209–220, 2017. Available at: <http://dx.doi.org/10.1016/j.cherd.2017.01.023>.

GROBICKI, A.; STUCKEY, D. C. The role of formate in the anaerobic baffled reactor. **Water Research**, v. 23, n. 12, p. 1599–1602, 1989.

GROBICKI, A.; STUCKEY, D. C. The role of formate in the anaerobic baffled reactor. **Water Research**, v. 23, n. 12, p. 1599–1602, 1989.

GUSTAVSSON, J.; SVENSSON, B. H.; KARLSSON, A. The feasibility of trace element supplementation for stable operation of wheat stillage-fed biogas tank reactors. **Water Science and Technology**, v. 64, n. 2, p. 320–325, 2011. Available at: <https://pubmed.ncbi.nlm.nih.gov/22097002/>. Acessed at: 18 maio 2021.

HENDERSON, C. The influence of extracellular hydrogen on the metabolism of Bacteroides ruminicola, Anaerovibrio lipolytica and Selenomonas ruminantium. **Journal of General Microbiology**, v. 119, n. 2, p. 485–491, 1980. Available at: <https://www.microbiologyresearch.org/content/journal/micro/10.1099/00221287-119-2-485>.

HENDRIKS, A. T. W. M.; VAN LIER, J. B.; DE KREUK, M. K. Growth media in anaerobic fermentative processes: The underestimated potential of thermophilic fermentation and anaerobic digestion. **Biotechnology Advances**, v. 36, n. 1, p. 1–13, 2018. Available at: <https://doi.org/10.1016/j.biotechadv.2017.08.004>.

HOLLIGER, C. et al. Towards a standardization of biomethane potential tests. **Water Science and Technology**, v. 74, n. 11, p. 2515–2522, 2016.

HUANG, B. **Comparison of Pre-and Post-treatments of Sugarcane Industry By-products to Increase Biomethane Production**. 2020. Thesis (MSc in Science for Sustainable Development) - Department of Thematic Studies, Environmental Change, University of Linköping, 2020.

HUBBERT, F.; CHENG, E.; BURROUGHS, W. Mineral Requirement of Rumen Microorganisms for Cellulose Digestion In Vitro. **Journal of Animal Scienc**e, v. 17, n. 3, p. 559–568, 1958. Available at: <https://academic.oup.com/jas/article/17/3/559/4739829>.

ILTCHENCO, J. et al. Microbial consortia composition on the production of methane from sugarcane vinasse. **Biomass Conversion and Biorefinery**, v. 10, n. 2, p. 299–309, 2020.

INABA, T. et al. Clarifying prokaryotic and eukaryotic biofilm microbiomes in anaerobic membrane bioreactor by non-destructive microscopy and high-throughput sequencing. **Chemosphere**, v. 254, art. 126810, 2020.

JANKE, L. et al. Biomethane production integrated to the brazilian sugarcane industry: the case study of São Paulo State. In: European Biomass Conference and Exhibition, 22., 2014, Hamburg, Germany. p. 23–26. Available at: https://www.researchgate.net/profile/Leandro-Janke/publication/279537608_BIOMETHANE_PRODUCTION_INTEGRATED_TO_THE_BRAZILIAN_SUGARCANE_INDUSTRY_THE_CASE_STUDY_OF_SAO_PAULO_STATE/links/559689f208ae99aa62c7fec5/BIOMETHANE-PRODUCTION-INTEGRATED-TO-THE-BRAZILIAN-SUGARCANE-INDUSTRY-THE-CASE-STUDY-OF-SAO-PAULO-STATE.pdf

JANKE, L. et al. Biogas Production from Sugarcane Waste: Assessment on Kinetic Challenges for Process Designing. **International Journal of Molecular Sciences**, v. 16, n. 9, p. 20685–20703, 2015.

JANKE, L. et al. Improving anaerobic digestion of sugarcane straw for methane production: Combined benefits of mechanical and sodium hydroxide pretreatment for process designing. **Energy Conversion and Management**, v. 141, p. 378–389, 2017. Available at: <http://dx.doi.org/10.1016/j.enconman.2016.09.083>.

JENKINS, S. R.; MORGAN, J. M.; SAWYER, C. L. Measuring anaerobic sludge digestion and growth by a simple alkalimetric titration. **Journal Water Pollution Control Federation**, v. 55, n. 5, p. 448–453, 1983. Available at: <https://eurekamag.com/pdf/005/005864228.pdf>.

JISHA, V. N. et al. Versatility of microbial proteases. **Advances in Enzyme Research**, v. 1, n. 3, p. 39–51, 2013. Available at: <http://dx.doi.org/10.4236/aer.2013.13005>.

JONES, J. B.; STADTMAN, T. C. Selenium-dependent and selenium-independent formate dehydrogenase of Methanococcus vannielii. Separation of the two forms and characterization of the purified selenium independent form. **Journal of Biological Chemistry**, v. 256, n. 2, p. 656–663, 1981.

JONSSON, S.; BORÉN, H. Analysis of mono- and diesters of o-phthalic acid by solid-phase extractions with polystyrene-divinylbenzene-based polymers. **Journal of Chromatography A**, v. 963, n. 1–2, p. 393–400, 2002.

LECK, A. Preparation of lactophenol cotton blue slide mounts. **Community Eye Health**, v. 12, n. 30, p. 24, 1999.

LEITE, A. F. et al. Assessment of the Variations in Characteristics and Methane Potential of Major Waste Products from the Brazilian Bioethanol Industry along an Operating Season. **Energy and Fuels**, v. 29, n. 7, p. 4022–4029, 2015.

LEME, R. M.; SEABRA, J. E. A. Technical-economic assessment of different biogas upgrading routes from vinasse anaerobic digestion in the Brazilian bioethanol industry. **Energy**, v. 119, p. 754–766, 2017. Available at: <http://dx.doi.org/10.1016/j.energy.2016.11.029>.

LENS, P. N. L. et al. Biotechnological treatment of sulfate-rich wastewaters. **Critical Reviews in Environmental Science and Technology**, v. 28, n. 1, p. 41–88, 1998. Available at: <https://www.tandfonline.com/doi/abs/10.1080/10643389891254160>.

LÓPEZ GONZÁLEZ, L. M.; PEREDA REYES, I.; ROMERO ROMERO, O. Anaerobic co-digestion of sugarcane press mud with vinasse on methane yield. **Waste Management**, v. 68, p. 139–145, 2017.

MA, H. et al. Use of Extracellular Polymer Substance as an Additive to Improve Biogas Yield and Digestion Performance. **Energy and Fuels**, v. 33, n. 12, p. 12628–12636, 2019.

MARTINEZ, A.; CHURCH, D. C. Effect of various mineral elements on in vitro rumen cellulose digestion. **Journal of Animal Science**, v. 31, n. 5, p. 982–990, 1970. Available at: <https://academic.oup.com/jas/article/31/5/982/4698856>.

MATA-ALVAREZ, J. **The biomethanization of the organic fraction of municipal solid waste**. London: IWA Publishing, 2002.

MATSUBAYASHI, M. et al. Phylogenetic diversity and in situ detection of eukaryotes in anaerobic sludge digesters. **PLoS ONE**, v. 12, n. 3, p. 1–13, 2017.

MOESTEDT, J. et al. Effects of trace element addition on process stability during anaerobic co-digestion of OFMSW and slaughterhouse waste. **Waste Management**, v. 47, p. 11–20, 2016.

MUTURI, S. M. et al. Metagenomics survey unravels diversity of biogas microbiomes with potential to enhance productivity in Kenya. **PLoS ONE**, v. 16, p. 1–49, 2021. Disponível em: <http://dx.doi.org/10.1371/journal.pone.0244755>.

MUYZER, G.; STAMS, A. J. M. The ecology and biotechnology of sulphate-reducing bacteria. **Nature Reviews Microbiology**, v. 6, n. 6, p. 441–454, 7 jun. 2008. Available at: <https://www.nature.com/articles/nrmicro1892>.

NAYONO, S. E.; GALLERT, C.; WINTER, J. Co-digestion of press water and food waste in a biowaste digester for improvement of biogas production. **Bioresource Technology**, v. 101, n. 18, p. 6987–6993, 2010.

O'BRIEN, T.; FEDER, N.; MCCULLY, M. E. Polychromatic staining of plant cell walls by toluidine blue O. **Protoplasma**, v. 59, n. 2, p. 368–373, 1964.

ODHIAMBO, M. **Distribution of black carbon and its impact on Eutrophication in Lake Victoria**. 2016. Thesis (MSc in Science for Sustainable Development) - Department of Thematic Studies, Environmental Change, University of Linköping, University of Linköping, 2016.

OHEMENG-NTIAMOAH, J.; DATTA, T. Perspectives on variabilities in biomethane potential test parameters and outcomes: A review of studies published between 2007 and 2018. **Science of the Total Environment**, v. 664, p. 1052–1062, 2019. Available at: <https://doi.org/10.1016/j.scitotenv.2019.02.088>.

ORDAZ-DIAZ, L. A.; BAILON-SALAS, A. M. Molecular identification of microbial communities in the methane production from vinasse: A review. **BioResources**, v. 15, n. 2, p. 4528–4552, 2020.

PARITOSH, K. et al. Additives as a Support Structure for Specific Biochemical Activity Boosts in Anaerobic Digestion: A Review. **Frontiers in Energy Research**, 2020. Available at: https://doi.org/10.3389/fenrg.2020.00088

PARSAEE, M.; KIANI DEH KIANI, M.; KARIMI, K. A review of biogas production from sugarcane vinasse. **Biomass and Bioenergy**, v. 122, p. 117–125, 2019. Available at: <https://doi.org/10.1016/j.biombioe.2019.01.034>.

POSTON, J. M.; KURATOMI, K.; STADTMAN, E. R. Methyl-Vitamin B12 as a source of methyl groups for the synthesis of acetate by cell-free extracts of clostridium thermoaceticum. **Annals of the New York Academy of Sciences**, v. 112, n. 2, p. 804–806, 1964. Available at: <https://pubmed.ncbi.nlm.nih.gov/14167313/>.

RAGSDALE, S. W. Enzymology of the Wood-Ljungdahl pathway of acetogenesis. **Annals of the New York Academy of Sciences**, v. 1125, p. 129-136, 2008. Available at: </pmc/articles/PMC3040112/>.

RAGSDALE, S. W.; PIERCE, E. Acetogenesis and the Wood-Ljungdahl pathway of CO_2 fixation. **Biochimica et Biophysica Acta - Proteins and Proteomics**, v. 1784, n. 12, p. 1873–1898, 2008. Available at: </pmc/articles/PMC2646786/>.

RAO, M. B. et al. Molecular and Biotechnological Aspects of Microbial Proteases. **Microbiology and Molecular Biology Reviews**, v. 62, n. 3, p. 597–635, 1998. Available at: <http://mmbr.asm.org/>.

RITTMANN, B. E.; MCCARTY, P. L. **Environmental biotechnology**: principles and applications. New York: McGraw-Hill Education, 2012.

RUZIN, S. E. **Plant microtechnique and microscopy**. [s.l.] Oxford University Press New York, 1999. v. 198

SANTOS, P. S. et al. Does sugarcane vinasse composition variability affect the bioenergy yield in anaerobic systems? A dual kinetic-energetic assessment. **Journal of Cleaner Production**, v. 240, art. 118005, 2019. Available at: <https://doi.org/10.1016/j.jclepro.2019.118005>.

SARKER, S. et al. A review of the role of critical parameters in the design and operation of biogas production plants. **Applied Sciences**, v. 9, n. 9, art. 1915, 2019. https://doi.org/10.3390/app9091915.

SCHNÜRER, A.; JARVIS, Å. **Microbiology of the biogas process.** Uppsala, Sweden: Swedish University of Agricultural Sciences, 2018.

SHAKERI YEKTA, S. et al. Importance of reduced sulfur for the equilibrium chemistry and kinetics of Fe(II), Co(II) and Ni(II) supplemented to semi-continuous stirred tank biogas reactors fed with stillage. **Journal of Hazardous Materials**, v. 269, p. 83–88, 2014. Available at: <http://dx.doi.org/10.1016/j.jhazmat.2014.01.051>.

SHAKERI YEKTA, S. et al. Importance of sulfide interaction with iron as regulator of the microbial community in biogas reactors and its effect on methanogenesis, volatile fatty acids turnover, and syntrophic long-chain fatty acids degradation. **Journal of Bioscience and Bioengineering**, v. 123, n. 5, p. 597–605, 2017. Available at: <http://dx.doi.org/10.1016/j.jbiosc.2016.12.003>.

SPEECE, R. E. **Anaerobic biotechnology and odor/corrosion control for municipalities and industries**. Nashville: Archae Press, 2008.

SPEECE, R. E.; PARKIN, G. F.; GALLAGHER, D. Nickel stimulation of anaerobic digestion. **Water Research**, v. 17, n. 6, p. 677–683, 1983.

SPEER, E. O. A method of retaining phloroglucinol proof of lignin. **Stain Technology**, v. 62, n. 4, p. 279–280, 1987.

STEELING, C. Crystal-structure of ruthenium red and stereochemistry of its pectic stain. **American Journal of Botany**, v. 57, n. 2, p. 172–175, 1970.

SWEDISH STANDARD INSTITUTE. **SS 12176 - Characterization of sludge - Determination of pH-value**. The scope of this standard is the sludges, i.e. and sludges products from: - storm water handling; - night soil; - urban wastewater collecting systems; - urban wastewater treatment plants; - treating industrial wastewater similar to urban wastewater (as defined in Directive 91/271 EEC); - water supply treatment plants; - water distribution systems; - but excluding hazardous sludges from industry. Stockholm, Sweden, 1998.

TERRY BROWN, A. I. et al. Population changes of eubacteria, sulfate-reducing bacteria and methanogenic archaea in an anaerobic reactor processing ethanol distillery vinasse. **Brazilian Archives of Biology and Technology** v. 62, art. e19180268, p. 1-13, 2019.

VOOLAPALLI, R. K.; STUCKEY, D. C. Hydrogen production in anaerobic reactors during shock loads - Influence of formate production and H_2 kinetics. **Water Research**, v. 35, n. 7, p. 1831–1841, 2001.

WALLER, L. N. et al. Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of Bacillus anthracis and Bacillus subtilis. **Journal of Microbiological methods**, v. 58, n. 1, p. 23–30, 2004.

WINTSCHE, B. et al. Metabolic adaptation of methanogens in anaerobic digesters upon trace element limitation. **Frontiers in Microbiology**, v. 9, p. 405, 2018. Available at: doi: 10.3389/fmicb.2018.00405.

WOLFE, R. S. Anaerobic life—a centennial view. **Journal of Bacteriology**, v. 181, n. 11, p. 3317-3320, 1999. doi: 10.1128/JB.181.11.3317-3320.1999.

YEN, H. W.; BRUNE, D. E. Anaerobic co-digestion of algal sludge and waste paper to produce methane. **Bioresource Technology**, v. 98, n. 1, p. 130–134, 2007.

ZANDVOORT, M. H. et al. Stimulation of methanol degradation in UASB reactors: In situ versus pre-loading cobalt on anaerobic granular sludge. **Biotechnology and Bioengineering**, v. 87, n. 7, p. 897–904, 2004. Available at: <https://onlinelibrary.wiley.com/doi/full/10.1002/bit.20200>.

ZITOMER, D. H.; JOHNSON, C. C.; SPEECE, R. E. Metal Stimulation and Municipal Digester Thermophilic/Mesophilic Activity. **Journal of Environmental Engineering**, v. 134, n. 1, p. 42–47, 2008. Available at: <https://ascelibrary.org/doi/abs/10.1061/%28ASCE%290733-9372%282008%29134%3A1%2842%29>

5. GENERAL CONCLUSIONS

The dose of 80 kGy was sufficient for sterilization of the wort from molasses. In addition, the lower dose tested of 10 kGy were able to reduce more than 99.9% of the microbial contamination present in the substrate. The treatment of 20 kGy showed the best yield and ethanol productivity among the irradiated worts, evidencing the possibility of applying the e-beam in the treatment of wort for fermentation, which may allow reduction in losses caused by microbial contamination, besides the possibility of promoting fermentation yield and productivity increase.

In the experiments of anaerobic digestion and anaerobic co-digestion, the biochemical methane potential test (BMP) showed that the higher methane yield was achieved by the monodigestion of vinasse with iron supplementation. The co-digestion of vinasse, straw and filter cake presented a lower methane yield, which indicates hard degradability of the lignocellulosic material.

In the study with reactors investigating the supplementation of the substrate with metals, iron played an important role in decreasing the H_2S production. In addition, Ni, Co, Se, Mo, and Zn made it possible to increase the methane yield of the reactors processes. In general, anaerobic co-digestion presented lower methane production than monodigestion, however, co-digestion maintained greater stability over time, with lower accumulation of intermediate metabolites and lower formation of H_2S.

APPENDIX

Figure 4.27 - Optical Microscopy analysis of R1 digestate at day 155 stained with cotton blue (chitin, bacteria, and fungi) and ruthenium red (pectin, mucilage, and biofilm) – 400x (a) and 1000x (b)

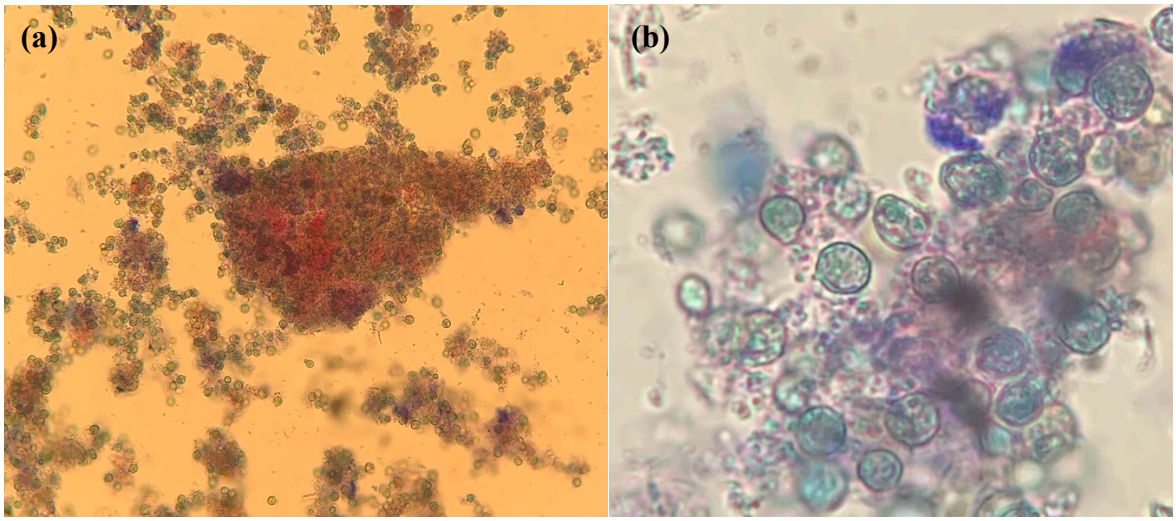


Figure 4.28 - Optical Microscopy analysis of R1 digestate at day 43 stained with toluidine blue – 1000x (a) and R1 digestate at day 155 stained with phloroglucine – 1000x (b)

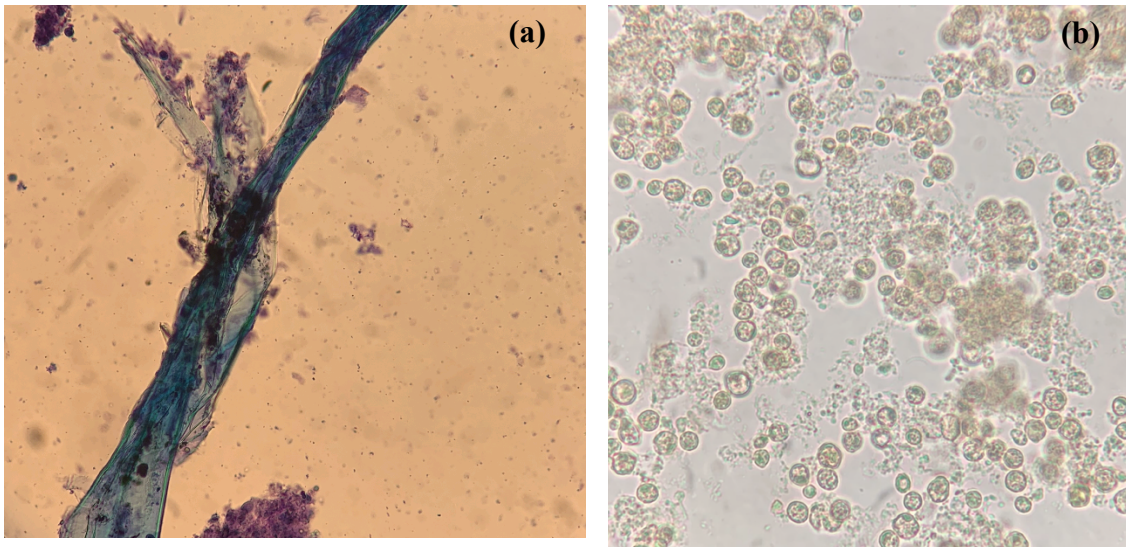


Figure 4.29 - Optical Microscopy analysis of R2 digestate at day 43 stained with phloroglucine – 1000x (a) and R2 digestate at day 255 stained with phloroglucine – 1000x (b)

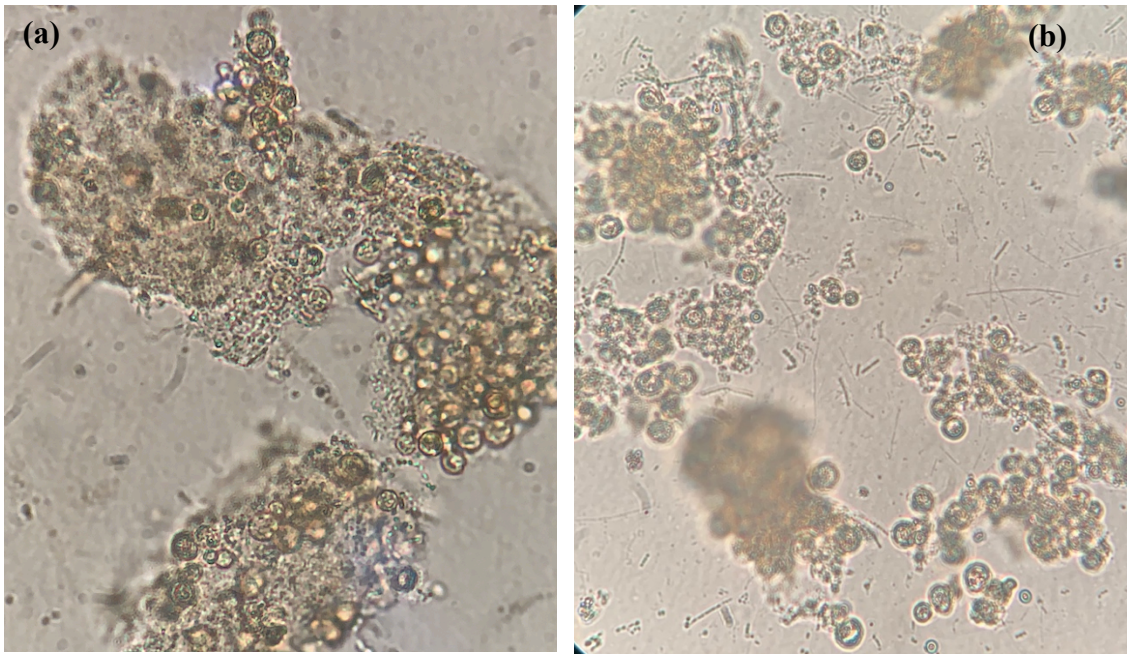


Figure 4.30 - Optical Microscopy analysis of R3 digestate at day 225 stained with phloroglucine (lignin in cherry-red) – 1000x (a) and R2 digestate at day 232 stained with cotton blue and ruthenium red – 400x (b)

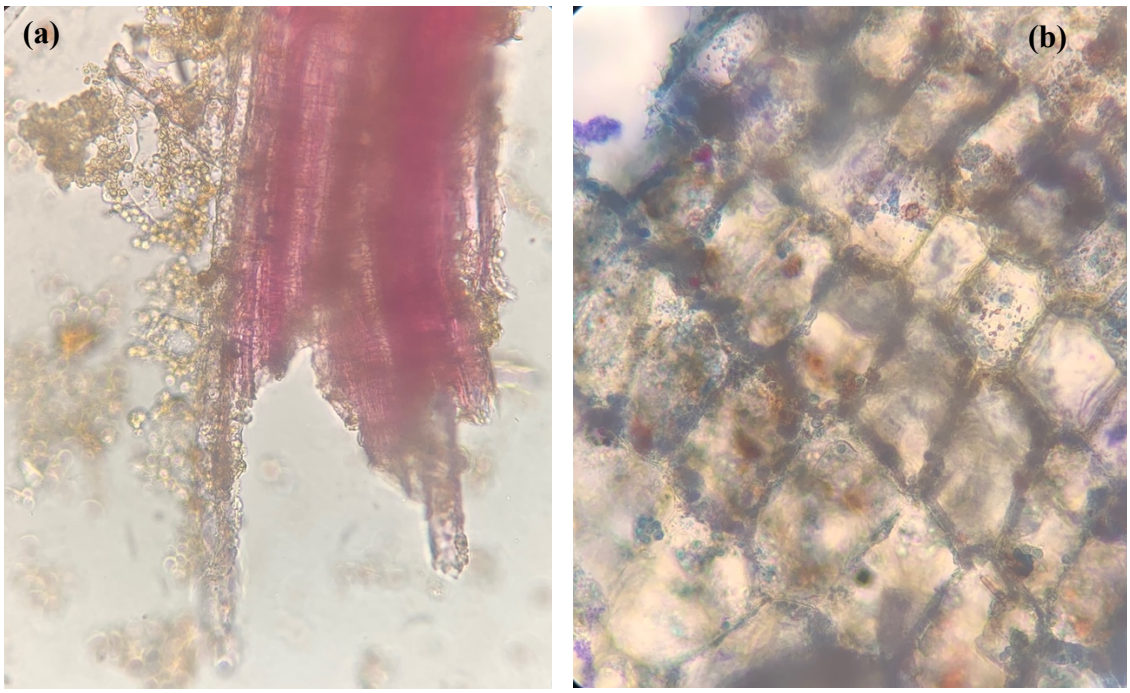


Figure 4.31 - Optical Microscopy analysis of R3 digestate at day 103 stained with cotton blue and ruthenium red– 1000x (a) and stained with toluidine blue – 400x (b)

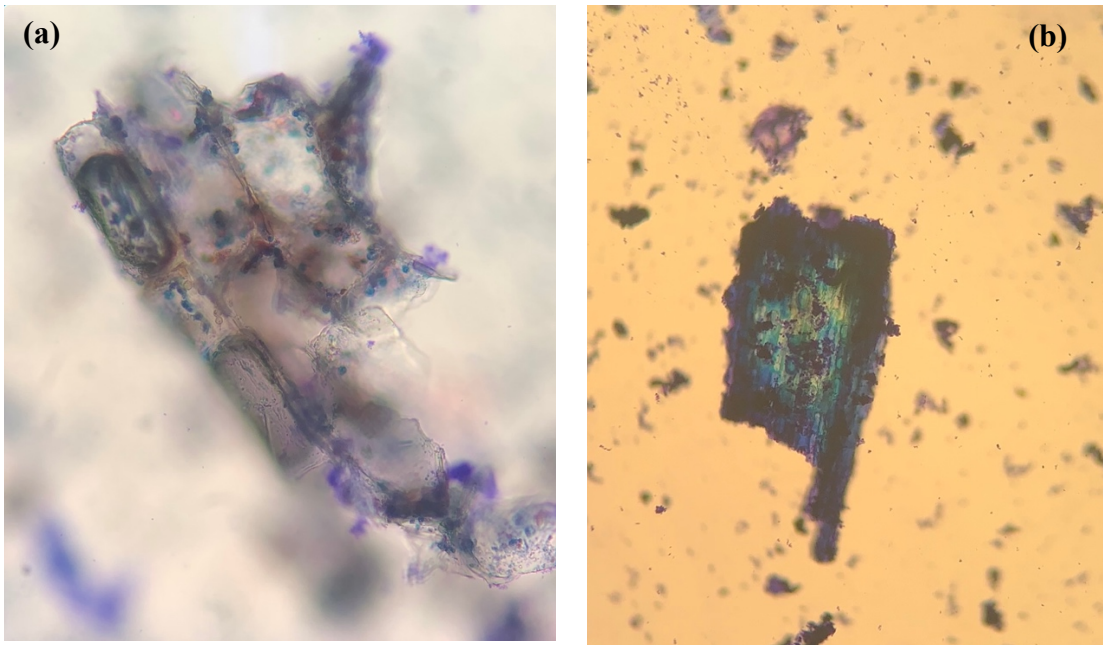


Figure 4.32 - Optical Microscopy analysis of R4 digestate at day 131 stained with cotton blue and ruthenium red– 400x (a) and 1000x (b)

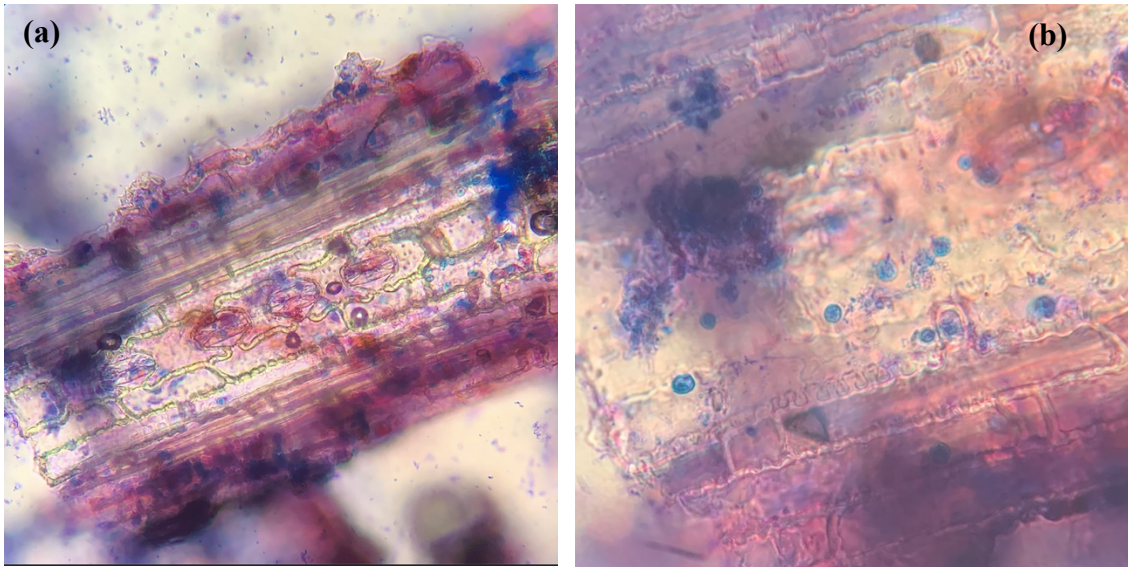


Figure 4.33 - Optical Microscopy analysis of R4 digestate at day 232 stained with toluidine blue – 100x (a) and 1000x (b)

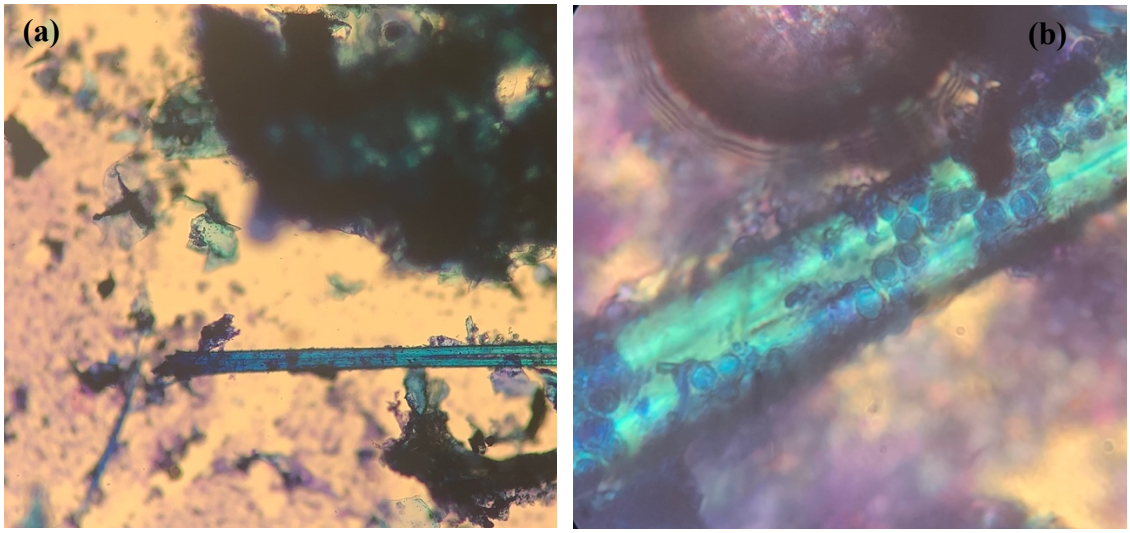


Figure 4.34 - Optical Microscopy analysis of R4 digestate at day 103 stained with phloroglucine – 100x (a) and 400x (b)

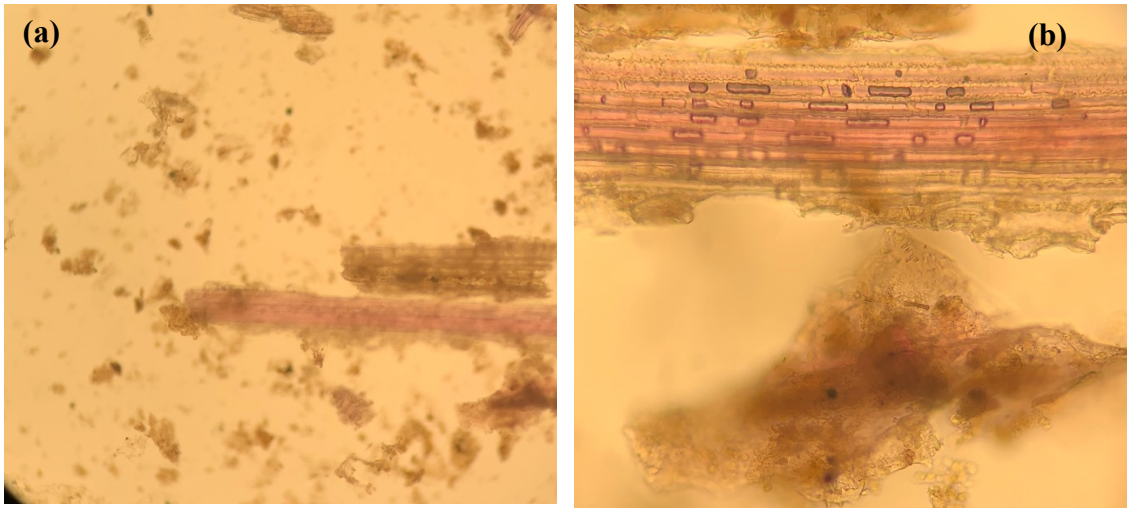


Figure 4.35 - Optical Microscopy analysis of R4 digestate stained with cotton blue and ruthenium red at day 103 – 1000x (a) and at day 232 – 1000x (b)