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HYDROTHERMAL AND MICROWAVE ASSISTED SYNTHESIS OF LEVULINIC ACID FROM WATERMELON WASTE AND APPLICATION OF THE CONTROL OF BLUE MOLD ON CITRUS BY A RECENTLY ISOLATED *PENICILLIUM ITALICUM*

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Thesis submitted to the Graduate Program in Chemical Engineering at the Federal University of Santa Catarina as a partial requirement for obtaining the Doctor in Chemical Engineering title.
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RESUMO

A demanda de energia e alimentos contribui para o aumento da geração de resíduos, principalmente de alimentos, sendo a maior parte descartada em aterros sanitários, o que reflete em problemas ambientais e de saúde pública. A valorização desses resíduos como fonte de matéria prima para a obtenção de produtos de alto valor agregado vem se tornando uma excelente alternativa. A biomassa residual é um grande recurso para a obtenção de produtos valiosos. Ao longo dos anos, pesquisas têm se concentrado na exploração plena de estruturas de biomassa, principalmente lignocelulósicas, para obter rendimentos satisfatórios de diferentes bioprodutos e estabelecer processos industriais economicamente viáveis. Dentre os bioprodutos, o ácido levulínico (AL) tem se destacado como uma das principais plataformas para produtos derivados de biomassa. A principal via de síntese para obtenção de AL é a partir da degradação da celulose via catálise ácida. Neste cenário, foi proposta a utilização de resíduos de melancia para síntese de AL, valorizando a biomassa residual ainda inexplorada para esse fim. As frações líquidas e sólida, bem como a polpa bruta, foram investigadas com catalisadores ácidos minerais, ácido sulfúrico e ácido clorídrico em dois sistemas de reação, autoclave e micro-ondas de síntese. Os maiores rendimentos, 14,8 e 17% em peso, foram obtidos com a fração sólida, em micro-ondas com a otimização dos experimentos através de um planejamento composto central (DCC 2^3), onde a temperatura afetou significativamente a reação. Na melhor condição obtida no DCC foram analisadas as frações líquidas e a polpa bruta e em todos os casos obteve-se um produto não purificado contendo apenas AL e ácido fórmico (AF). O micro-ondas apresentou maior eficiência, sendo uma alternativa promissora para utilização em reações que necessitam de energia. Devido a sua multifuncionalidade, ainda foi proposto um novo uso para a mistura reacional obtida que continha AL e AF, mistura não purificada, visando avaliar o AL como inibidor fúngico em doenças de frutas cítricas. Assim, investigou-se a ação inibitória do AL não purificado, obtido de resíduos de melancia, contra o fungo *Penicillium italicum*, isolado de frutas cítricas. O AL foi avaliado em testes *in vitro* e inibiu o crescimento micelial do fungo. Posteriormente foram realizados experimentos *in vivo* com laranjas da espécie *Citrus sinensis*, cultivar Valência. A proliferação fúngica em *C. sinensis* foi investigada pela aplicação da mistura não purificada contendo AL (43 mM) e AF (28 mM), comparando-os com controles negativos (sem agente inibitório) sob o fungo inoculado. Como teste adicional realizou-se experimentos utilizando somente AF para comparação com a mistura de AL e AF. Os resultados de perda de peso das frutas e incidência de doenças diminuíram quando o AL foi utilizado como agente inibidor, não sendo observados impactos negativos na qualidade dos frutos. O AF não inibiu o crescimento fúngico, indicando que o potencial antifúngico é encontrado no AL. Seu efeito inibitório foi confirmado pela determinação das atividades das enzimas antioxidantes catalase (CAT), ascorbato peroxidase (APX), superóxido dismutase (SOD) e peroxidase (POD), onde não foi observado aumento nas atividades devido à inibição do fungo antes de sua proliferação nos frutos. De acordo com os resultados alcançados na obtenção do AL, este estudo apresenta uma estratégia sustentável, utilizando resíduos e catalisadores de baixo custo, contribuindo de forma promissora para uma economia circular baseada em resíduos agrícolas. Além disso fornece dados relevantes sobre uma nova aplicabilidade do AL bruto como agente antifúngico, efeito ainda inexplorado para este composto na literatura recente, oferecendo uma solução prática e inovadora no combate ao mofo azul.

Palavras-chave: Ácido 4-oxopentanoico; resíduos de melancia; catálise verde; inibição fúngica; frutas cítricas; mofo azul; sustentabilidade.

ABSTRACT

The demand for energy and food contributes to the increase in waste generation, mainly food, with the majority of it being discarded in landfills, which results in environmental and public health problems. The valorization of these wastes as a source of raw material to obtain products with high added value has become an excellent alternative. Waste biomass is a great resource for obtaining valuable products. Over the years, researchers have focused on fully exploiting biomass structures, especially lignocellulosic ones, to achieve satisfactory yields of different bioproducts for establishing economically viable industrial processes. Among bioproducts, levulinic acid (LA) has emerged as a key platform for biomass-derived products. The main synthesis route for obtaining LA involves the degradation of cellulose via acid catalysis. In this context, the use of watermelon waste for the synthesis of LA was proposed, valuing the still unexplored residual biomass for this purpose. Liquid (LF) and solid fractions (SF), as well as raw pulp (RP), were investigated with mineral acid catalysts, sulfuric acid, and hydrochloric acid, in two reaction systems: autoclave and synthesis microwave. The highest yields, 14.8% and 17% by weight, were obtained with the SF in the microwave with experiment optimization through a central composite design (DCC 2^3), where temperature significantly affected the reaction. Under the best condition obtained in the DCC, LF and RP were analyzed, and in all cases, a clean product containing only LA and formic acid (FA) was obtained. The microwave showed higher efficiency, proving to be a promising alternative for reactions requiring energy. Due to multifunctionality the of LA, a new use was proposed for the obtained reaction mixture containing LA and FA in its crude form, aiming to evaluate LA as a fungicidal inhibitor in citrus diseases. Thus, the inhibitory effect of unpurified LA obtained from watermelon waste against the fungus *Penicillium italicum*, isolated from citrus fruits, was investigated. LA was evaluated in *in vitro* tests and inhibited the mycelial growth. Subsequently, *in vivo* experiments were conducted with oranges of the species *Citrus sinensis*, cultivar Valencia. Fungal proliferation in *C. sinensis* was investigated by applying the unpurified mixture containing LA (43 mM) and FA (28 mM) and comparing them with negative controls (no inhibitory agent). As an additional test, experiments were carried out using only AF to compare with the mixture of AL and AF. Fruit weight loss and disease incidence decreased when LA was used as an inhibitory agent, with no observed negative impacts on fruit quality. FA did not inhibit fungal growth, indicating that the antifungal potential is found in LA. Its inhibitory effect was confirmed by determining the activities of antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and peroxidase (POD), where there was no increase in activities due to the inhibition of the fungus before its proliferation in the fruits. According to the results achieved in obtaining LA, this study presents a sustainable strategy, utilizing low-cost waste and catalysts, promisingly contributing to a circular carbon economy based on agricultural residues. Additionally, it provides relevant data on the new applicability of crude LA as a fungicidal agent, an effect still unexplored for this compound in recent literature, offering a practical and innovative solution in combating blue mold.

Keywords: 4-oxo pentanoic acid; watermelon waste; Green catalysis; Fungal inhibition; Citrus fruits; Blue mold; Sustainability.

RESUMO EXPANDIDO

Introdução

A demanda de energia, alimentos e recursos naturais é crescente ocasionada, principalmente, pelo aumento da população global e urbanização. A geração de resíduos, especialmente de alimentos, aumentou em todo o mundo, e a maior parte deles é descartada em aterros sanitários, refletindo em sérios problemas ambientais e de saúde pública. Frutas e vegetais, por exemplo, estão entre os alimentos com maiores índices de desperdício, e isso se deve às rigorosas especificações de comercialização e às exigências na classificação pós-colheita para atender às demandas do mercado.

A valorização da biomassa residual como fonte de matéria prima para a obtenção de produtos de alto valor agregado é uma excelente alternativa, pois ela apresenta variabilidade em sua composição, sendo rica em açúcares solúveis e considerada excelente matéria-prima para geração de bioenergia e obtenção de bioprodutos. A melancia é a segunda fruta com maior produção, e devido à sua natureza perecível e porção não comestível gera uma quantidade considerável de resíduos. Além disso, possui alto teor de água, onde estão dissolvidos os principais carboidratos, glicose e frutose; sua composição também é rica em celulose e hemicelulose e pode ser utilizada para produzir compostos com maior valor agregado, como, por exemplo, o ácido levulínico.

O ácido levulínico (AL) tem se destacado como um dos principais produtos de plataforma derivados de biomassa, graças a sua posição econômica do mercado em plena expansão, e à sua versátil estrutura química intermediária, que pode sintetizar diversos compostos com aplicações nas indústrias de combustíveis, farmacêutica e cosmética, aditivos alimentares e solventes. A principal via de síntese para obtenção do AL é a partir da degradação da celulose via catálise ácida.

Objetivo

Investigar o potencial de resíduos de melancia para obtenção de ácido levulínico utilizando ácidos minerais como catalisadores, bem como a posterior utilização do ácido levulínico, na forma de produto não purificado, como inibidor fúngico de *Penicillium italicum*, isolado de frutas cítricas.

Metodologia

Resíduos de melancia foram coletados em uma frutaria de Erechim, RS, e foram triturados em liquidificador industrial, e separados em três frações, polpa bruta (PB), fração líquida (FL) e fração sólida (FS) e armazenados e mantida sob refrigeração (-80 °C) até o uso. A FS foi caracterizada determinando-se os teores de cinzas, sólidos extrativos e os principais compostos estruturais, celulose, hemicelulose e lignina, de acordo com a metodologia da NREL. Na FL foram quantificados os carboidratos glicose e frutose, em HPLC.

Nos experimentos preliminares o resíduo foi investigado com dois catalisadores, H_2SO_4 1 mol.L^{-1} e HCl 1 mol.L^{-1}, em dois sistemas reacionais, termo-pressurizado e micro-ondas. Em seguida, foram avaliados os efeitos da temperatura, concentração de ácido e relação sólido-líquido (S:L) de biomassa seca utilizada na síntese de AL nos dois sistemas reacionais e com os dois catalisadores, através de um Delineamento Composto Central (DCC), sendo DCC 2^2 para o sistema termo-pressurizado e DCC 2^3 para micro-ondas com a FS. Com a melhor condição obtida no planejamento com FS, realizou-se a repetição em triplicata, a fim de validação experimental. Também, nessa condição, foram realizados experimentos com a FL e PB.

Visando utilizar o produto obtido da síntese de resíduos de melancia, composto de 43 mM de AL e 28 mM de ácido fórmico (AF), não purificados, foi investigado o efeito de inibição do AL contra o crescimento do *Penicillium italicum (in vitro)*, bem como a redução do desenvolvimento deste fungo em laranjas (*in vivo*). Isolou-se o *P. italicum* de frutas cítricas orgânicas em decomposição e em seguida, foi investigado a sensibilidade fúngica ao AL através de testes *in vitro*, pela técnica de perfuração em ágar. Nas placas foram perfurados 4 poços, sendo 3 para adição dos agentes inibidores e 1, ao centro, para o controle contendo água ultrapura esterilizada. Nos poços foram adicionados 30 µL das soluções a serem avaliadas, conforme diluições realizadas, sendo na cavidade 1, a mistura bruta, com 43 mM de AL 43 mM e 28mM de AF; na segunda cavidade a concentração foi de 21 mM de AL e 14 mM de AF e na terceira, 8 mM de AL e 5mM de AF. Para o controle negativo foi realizado um experimento utilizando apenas ácido fórmico nas mesmas concentrações. O tempo de incubação foi de 7 dias a 28 °C. Foi realizado experimento adicional aumentando-se o diâmetro da perfuração em ágar para 34 mm bem como o volume de composto inibidor, na concentração que apresentou maior sensibilidade, em 10 vezes (300 µL) para confirmar a inibição. Com a maior concentração foram realizados testes *in vivo* com laranjas orgânicas. Utilizou-se 10 frutas para cada experimento, onde aproximadamente 5 mm das cascas foram removidas em sua lateral e posteriormente inoculados 30 µL de solução de conídios contendo 3,1 x 10^5 esporos. mL^{-1} de *P. italicum.* Após 3 horas, 0,5 mL da mistura bruta de ácido levulínico e fórmico (AL 43 mM e AF 28 mM) foi aspergida na ferida de cada fruta. O controle negativo foi conduzido sem aspersão de agente inibidor. Experimento adicional utilizando somente AF 28 mM foi realizado para comparação com a mistura de AL e AF utilizada. Foram avaliadas as taxas de incidência de cada tratamento, e determinada a perda de peso das frutas, e na sequência o fungo isolado foi identificado. Por fim, atividades das enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT), peroxidase (POD) e ascorbato peroxidase (APX) foram determinadas conforme metodologia específica para cada enzima.

Resultados e discussão

O resíduo de melancia apresentou quantidades altas de glicose e frutose (14.24 ±4.24 $g.L^{-1}$ e 30.58 ±4.41 $g.L^{-1}$, respectivamente) na FL. Na FS destacou-se o baixo teor de lignina (13.48 ±2.43%), que contribuiu para a produção de AL.

Nos experimentos exploratórios os maiores rendimentos foram alcançados ao se utilizar a FS (4.8% e 6.3% com H_2SO_4 e HCl, respectivamente) e acredita-se que isso se deve, à presença de celulose na amostra. O rendimento de AL apresentou diferença nos dois sistemas reacionais e catalisadores empregados, 4.8% (H_2SO_4) e 6.35% (HCl), mesmo utilizando-se a concentração do catalisador e a carga de biomassa com valores fixos. No entanto, as condições experimentais foram insatisfatórias, pois, em todos os experimentos as reações de desidratação da glicose e frutose e posterior reidratação do HMF (hidroximetilfurfural) foram incompletas, sendo que a glicose não foi acessada pelos catalisadores, condição verificada ao se utilizar a FL, onde os carboidratos simples estavam mais acessíveis. Assim, nos demais experimentos aumentou-se o tempo de reação de 30 minutos para 1 hora.

Ao se avaliar os efeitos dos sistemas reacionais, no termo-pressurizado, foram obtidos rendimentos semelhantes com os dois ácidos, H_2SO_4 e HCl, 6.54% e 6.34% respectivamente, e não houve aumento do rendimento de AL no planejamento (DCC 2^2) em relação aos testes preliminares com a fração sólida. A condição do ponto central (1), 10% de carga de biomassa e concentração de ácido de 1.25M, se mostrou a mais vantajosa estatísticamente para esse sistema, nesse planejamento, mas apresentou apenas 6.54% (H_2SO_4) e 6.34% (HCl) de rendimento de AL.

No micro-ondas foi possível refinar os comportamentos reacionais entre os catalisadores. Com o incremento do tempo de reação, em ambos os ácidos, foi verificado que a frutose foi totalmente utilizada na reação e que a temperatura tem forte influência nesse processo. A melhor condição obteve rendimento de 13% de AL, utilizando-se temperatura de 200 °C e com os menores níveis de carga de biomassa e concentração de catalisador. Através da análise de efeitos, com 95% de confiança, foi possível confirmar que a temperatura é significativa ao se utilizar o HCL. Ao se utilizar a FL e a PB não foi observado aumento do rendimento de AL.

Após otimizar sua obtenção, investigou-se a ação inibitória do AL bruto obtido de resíduos de melancia contra um fungo isolado de frutas cítricas orgânicas em decomposição. O sequenciamento genético do fungo mostrou que se trata da espécie *P. italicum,* responsável pela podridão do mofo azul, doença muito conhecida e que causa grandes perdas em frutas cítricas na pós-colheita.

A taxa de incidência da doença ao utilizar AL foi a menor, apenas 10% das frutas apresentaram infecção fúngica, enquanto no controle (sem agente inibidor) 90% das frutas apresentaram proliferação fúngica. No ensaio realizado somente com AF 60% das frutas apresentaram proliferação do fungo. O tratamento com AL também apresentou menor perda de massa quando comparado ao AF e ao controle, o que coincide com o percentual de frutos que manifestaram a doença.

Na avaliação das atividades enzimáticas após tratamento com AL em laranjas infectadas com *P. italicum*, a CAT e SOD apresentaram diferenças entre o controle e o tratamento com AF com o tratamento de AL. Já na POD, o tratamento com AL apresentou diferença do controle apenas e na APX os três tratamentos diferiram entre si. Verificou-se que em todas as enzimas avaliadas, a menor atividade ocorreu no tratamento com AL, o que sugere que o uso de AL como inibidor do mofo azul obteve êxito, pois os frutos deste tratamento não manifestaram proliferação do fungo. Os resultados obtidos nos testes *in vivo* foram alinhados ao resultados do experimentos *in vitro.*

Considerações finais

O resíduo de melancia tem potencial de uso na sua totalidade, visto que se obteve um produto não purificado contendo apenas AL e AF. Os melhores resultados foram obtidos ao se utilizar ao HCl como catalisador em micro-ondas. A combinação do uso do micro-ondas e o

HCl, ácido de baixo custo e alta disponibilidade no mercado, demonstra que esse estudo é uma estratégia de economia de energia, devido ao baixo custo do resíduo e dos catalisadores empregados, podendo maximizar a produção de AL.

A aplicação do produto da síntese de resíduo de melancia, mistura de AL e AF, na sua forma bruta, como inibidor do fungo *P. italicum,* apresentou inibição do crescimento micelial. Os resultados obtidos no teste *in vivo* demonstraram que o AL é principal agente de inibição fúngica, comprovados pelo teste negativo realizado somente com o AF, não havendo ação sinérgica entre esses compostos. A menor incidência da doença e menor perda de massa das frutas também foi observada ao se utilizar o AL. As enzimas antioxidantes não apresentaram atividades quando se utilizou a mistura contendo AL, indicando que esse composto inibiu o crescimento do fungo antes de sua proliferação na fruta.

Os resultados alcançados na obtenção do AL apresentam uma estratégia sustentável, utilizando resíduos e catalisadores de baixo custo, contribuindo de forma promissora para uma economia circular baseada em resíduos agrícolas. Os dados obtidos sobre uma nova aplicabilidade do AL bruto, sem submetê-lo a processos de purificação, como agente antifúngico, são relevantes e seu efeito, para este composto, ainda é inexplorado na literatura científica, oferecem uma solução prática e inovadora no combate ao mofo azul.

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SUMMARY

THESIS STRUCTURE

Chapter 1: Introduction

Objective

Chapter 2

Recent advances, perspectives, and challenges on levulinic acid production from residual biomass

In this article, a review of the literature on levulinic acid (LA) was carried out. The general aspects of this compound were presented, including its relevance in different applications and its importance in the world market. Furthermore, the main raw materials used to obtain them from renewable sources were discussed. The reaction pathways and the main catalysts used were presented. Finally, new perspectives for using alternative biomass to obtain LA were discussed. Finally, the conclusion and references used are presented.

Chapter 3

Hydrothermal and microwave-assisted synthesis of levulinic acid from watermelon residue

This study evaluated for the first time the production of LA from watermelon residue using mineral acids as catalysts: hydrochloric acid and sulfuric acid, in two reaction systems, autoclave and microwave oven. The raw pulp, liquid fraction and solid fraction of watermelon residue were investigated. The synthesis was optimized through central composite planning, where the best condition was determined. For a better understanding of the reader, this article was presented in full containing all elements (introduction, methodology, results, discussion, conclusion, and references).

Chapter 4

Levulinic acid as a strategy for control of postharvest citrus blue mold by a newly isolated *Penicillium italicum*

In this study, the LA produced was used, in its crude form, as a fungal inhibitor of *Penicillium italicum*, isolated from citrus fruits. The inhibition power of LA was investigated in *in vitro* and *in vivo* tests, using organic oranges. Enzyme activities were determined to prove inhibition. For better understanding of the reader, this article was also presented in full containing all elements (introduction, methodology, results, discussion, conclusion, and references).

Conclusion

CONCEPTUAL DIAGRAM

Evaluate the possibility of producing levulinic acid from watermelon residue, through acid synthesis, in a termo-pressurize and microwave systems.

Justification

- Levulinic acid can be obtained from various waste biomasses.
- Watermelon waste is a sustainable alternative for obtaining levulinic acid products.
- Levulinic acid has wide applicability but requires purification.
- New purpose for using levulinic acid in its raw form.
- Levulinic acid inhibited the proliferation of the fungus *Penicillium italicum* on oranges.

Has the subject been studied yet?

- Studies have evaluated the production of LA from various lignocellulosic biomasses and obtained good results.
- No work has evaluated the synthesis of levulinic acid from residual watermelon biomass.
- Levulinic acid has been used as a sanitizer and inhibited the growth of bacteria.
- There are no reports in the literature of its use to inhibit fungi.
- Levulinic acid has the potential to be applied as a promising preservative during storage of citrus fruits.

Hypotheses

- Watermelon waste is a sustainable alternative for obtaining levulinic acid .
- Levulinic acid obtained from watermelon residue can be used to combat blue mold, becoming a new alternative for using levulinic acid in its raw form.

1 INTRODUCTION

The global population and urbanization increase reflects increased demands for energy, food, and natural resources. Robust production systems and inefficient waste management strategies contribute to the current economic model facing significant challenges in meeting the population's basic needs and avoiding resource scarcity (Ashokkumar et al., 2022). With population growth, the amount of waste, especially food waste, has also increased worldwide, and most of it is discarded in landfills, contributing to the degradation of the environment (Kumar et al., 2023). It is necessary to promote the economy sustainably and make the transition to a green economy viable (Ubando et al., 2020). For that, it is essential that public policies that are efficient in economic, environmental, and social aspects are designed holistically and integrated (D'Amato & Korhonen, 2021).

Practical strategies to mitigate environmental impacts and meet humanity's energy demands involve using biomass as a source of nutrients. The circular economy aims to value waste to obtain high-value-added products, and waste from the food supply chain is an excellent source of carbohydrates to obtain these bioproducts (Greses et al., 2021, 2022).

Food marketing specifications are strict and contribute to food waste. Fruits and vegetables are among the foods with the highest rates of waste. This is mainly due to post-harvest classification to meet market demands. These residues show variability in their composition, being rich in soluble sugars and considered excellent raw materials for generating bioenergy and obtaining bioproducts (Scapini et al., 2023).

Residual biomass is a great resource to obtain valuable products, as it is renewable and significantly reduces CO_2 emissions. Over 200 high-value-added biocompounds have been obtained from plant matrix through different processes. These compounds are widely used as intermediates in additional processes in the pharmaceutical areas, in the paint and solvent industries, and in the manufacture of cosmetics, such as LA (Antar et al., 2021).

In this scenario, reflections on current production methods are the starting point in the search for sustainable development solutions, aiming to use residual biomass as a raw material. Recent studies point to levulinic acid as a versatile intermediate chemical compound considered a building block, which culminates in commercial relevance and the expansion of research and technologies aimed at obtaining this product and is obtained from lignocellulosic biomass, generally through degradation of cellulose in an acidic medium. It has several applications, such as additives in fuels, pharmaceutical formulations, chemical solvents, and the food industry. It is an organic acid recognized by the Food and Drug Administration (FDA) as safe (GRAS). It has antimicrobial properties and has shown promising results as a sanitizer, often in conjunction with other compounds, as well as antibacterial action. However, there are no studies that evaluate its antifungal effects, for example, in the preservation of fruits to combat the appearance of fungi, such as blue mold caused by *Penicillium italicum*, responsible for significant losses in citrus fruits.

Considering the relevance of this compound for the expansion of residual biomass biorefineries and the installation of a circular economy focused on obtaining products with less environmental and social impact, this thesis aims to investigate the obtaining of LA from waste watermelon biomass and subsequent use of this acid in its without purifying it, investigate the inhibitory power of LA on the mycelial growth of *P. italicum* and the reduction of the development of this fungus in oranges.

1.1 OBJECTIVES

This study investigates the potential of watermelon residues to obtain levulinic acid using mineral acids as catalysts, as well as the subsequent use of levulinic acid, in the form of an unpurified product, as a fungal inhibitor of *Penicillium italicum*, isolated from citrus fruits.

1.1.1 Specific objectives

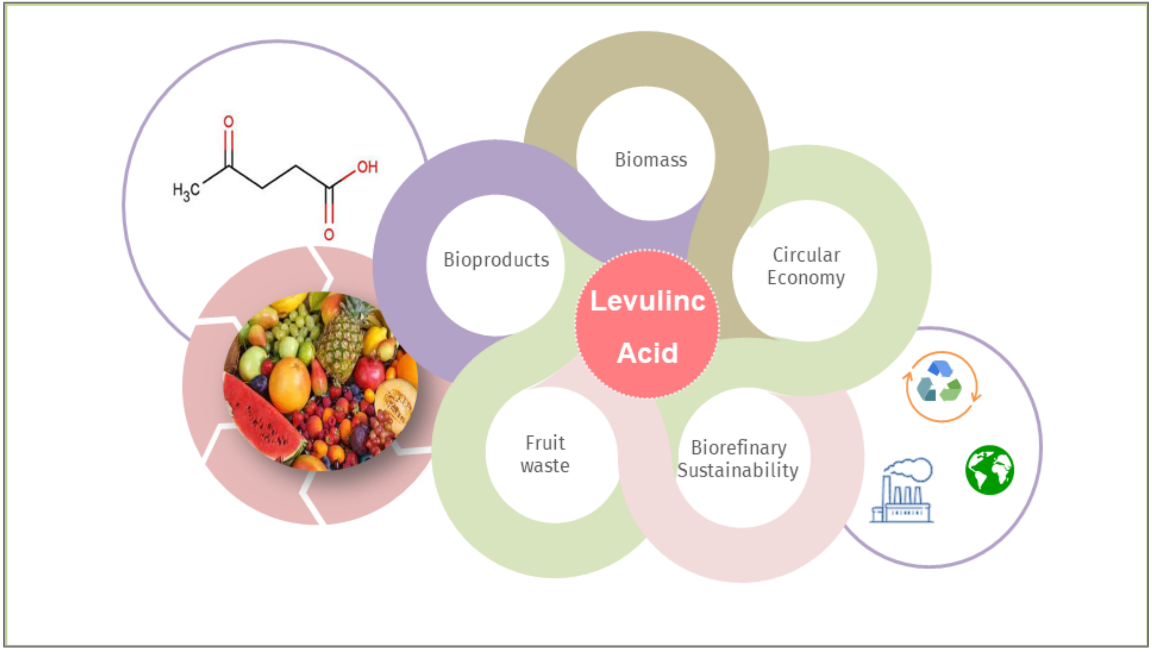
- Produce a bibliographical review on the current state of LA production and through it, investigate the use of fruit waste to obtain LA, especially watermelon.
- Analyze the composition of watermelon biomass.
- Evaluate its potential in the production of levulinic acid, considering the possible ways of using the waste, such as raw pulp, solid fraction, and liquid fraction.
- Investigate the effects of catalysts on the reaction systems used.
- Optimize the production of LA from acid catalysts.
- Investigate the product profile obtained as an inhibitor of fruit phytopathogenic microorganisms.
- Isolate the fungus responsible for blue mold on citrus fruits and identify it.

- To evaluate the inhibition power of the unpurified mixture of levulinic acid and formic acid, *in vitro* on the fungus *Penicillium italicum*.
- Evaluate the preventive effect of levulinic acid and formic acid solution on citrus fungi *in vivo* tests.
- Investigate the activity of antioxidant enzymes in fruits evaluated in *in vivo* tests.

1.2 STUDY MAP

During our research, the problem of food waste, especially fruit waste, was observed. Faced with a scenario where it is necessary to rethink forms of production aiming at more sustainable processes, which promote the circular economy and preserve the environment, it was verified that LA is a product with high added value, which can be produced from lignocellulosic biomass. Thus, it was found that there were still no studies exploring residual fruit biomass in the literature. Watermelon was chosen due to its high residual volume and composition. The hypothesis of producing LA from residual watermelon biomass began to be constructed, as illustrated in the graphic summary below (Figure 1). From this, the construction of this work began, consisting of five chapters.

Figure 1: Graphic summary of the investigated topic



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Chapter 1 presents the general introduction of this work, the general and specific objectives, and a conceptual map of the work. In Chapter 2, a review of the literature on the proposed theme is presented. It presented the problem and how LA can be obtained from renewable sources. The general aspects of the target product, applications, obtaining from biomass, and reaction pathways were presented, and, finally, new perspectives for using alternative biomasses to obtain LA were discussed. This chapter resulted in a review published in the journal Biofuels Bioproducts & Biorefining, in January 2023 (DOI: 10.1002/bbb.2493).

Chapter 3 consists of the characterization of the biomass used and the synthesis of LA from watermelon residual biomass in two reaction systems: autoclave and Synthesis Microwave. The results obtained with the optimization of the process through experimental planning were published in the article entitled “Hydrothermal and microwave-assisted synthesis of levulinic acid from watermelon residue” in the journal Biofuels Bioproducts & Biorefining in July 2023 (DOI: 10.1002/bbb.2542).

In Chapter 4, a proposal for applying the obtained product is presented, aiming to use it without purification. The inhibitory effects of the mixture obtained, which contains levulinic acid and formic acid, on fungi that cause diseases in citrus fruits were investigated. This form of use of levulinic acid, as well as the investigation of its antifungal potential, has not been reported in the literature to date. Thus, first, the fungus that causes blue mold was isolated from citrus fruits and the pathogen was identified. Afterward, the inhibitory potential of the compound was investigated in *in vitro* and *in vivo* tests, using oranges. The activities of antioxidant enzymes were also determined to prove the antifungal action of levulinic acid. This study was submitted for publication to the Journal of Chemical Technology & Biotechnology, in January 2024. In Chapter 5, the general thesis conclusions was presented.

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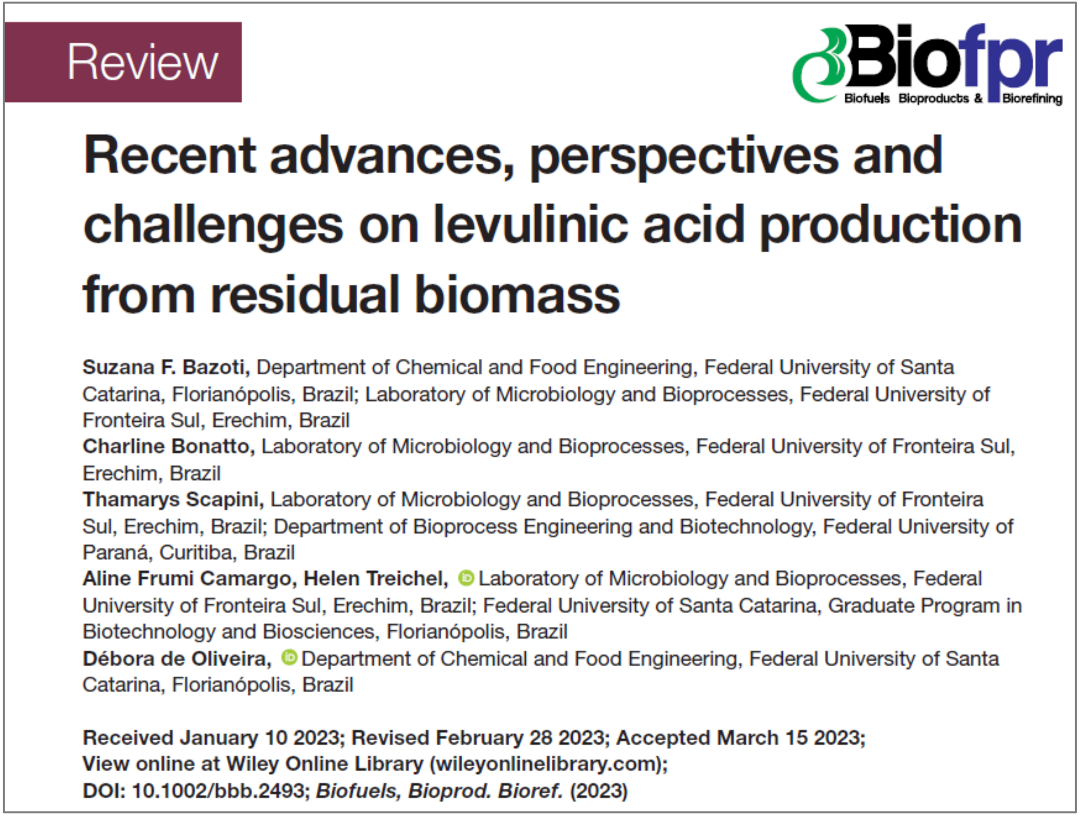
2 A LITERATURE REVIEW ON RECENT ADVANCES, PERSPECTIVES, AND CHALLENGES ON LEVULINIC ACID PRODUCTION FROM RESIDUAL BIOMASS

The second chapter refers to a review article published in Biofuels Bioproducts & Biorefining. This chapter presents state of the art, describing how levulinic acid (LA) has been explored and highlighted as one of the main platform products derived from biomass. It is characterized as a building block product due to its chemical structure so that it can be applied as a versatile intermediate chemical. Therefore, this work carried out a comprehensive review of the general panorama of the use of biomass to produce LA, as well as the challenges present in the synthesis of compounds, presenting the central studies, carried out over the years in this area and the approaches to the use and valorization of residual biomass still little explored for this purpose (ISSN:1932-1031 https://doi.org/10.1002/bbb.2493).

Figure 1: Cover os published review article



Review

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Recent advances, perspectives and challenges on levulinic acid production from residual biomass

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2.1 INTRODUCTION

World food production can supply all human food demands; however, about a third is lost. Due to population growth and the increased need for energy, food, and products, the current system of production and consumption makes it difficult to meet the population's needs. It presents several distribution problems along the production chain due to its linear design, which contributes to the scarcity of resources in some regions and increases losses and waste generation in other regions (5 Facts about Food Waste and Hunger (Worlchmidt et al. 2017a; Thakkar et al. 2021; Rackemann & Doherty 2011).

The highest yields of LA are obtained from lignocellulosic biomass (LB) using mineral acids as catalysts, but results may vary according to the raw material used. Sugarcane bagasse, olive tree branches, giant cane, and poplar sawdust, led to LA yields of around 23% by weight with the use of hydrochloric acid. Using H_2SO_4, the production of LA varies according to the matrix. While cotton straw resulted in 10% of LA weight, eucalyptus chips, bunches of palm fruits, and wheat straw exceeded 40%, demonstrating that the type of biomass significantly influences reaction yield (Mthembu, Gupta, e Deenadayalu 2023).

LA can be obtained from different lignocellulosic raw materials, as previously mentioned. LB matrix residues are a great source of glucose, sucrose, fructose, and xylose. However, it is necessary to access them by breaking the polymeric lignin barrier through pre-treatment, acid hydrolysis, or a combination. Subsequently, the synthesis of LA can usually occur by acid catalysis. It is essential to understand how the pre-treatment and conditions favor the release of carbohydrates, as well as finding residual biomass with a higher content of free carbohydrates to simplify the synthesis of LA.

2.1.1 Pre-treatment of lignocellulosic biomass

The exploration of LB demands the pre-treatment step, mainly due to structural recalcitrance, which can negatively impact the conversion of structural components into products with high value-added, such as LA production (Kang, Fu, and Zhang 2018). The main objective of this step is to separate the lignocellulosic fractions, improving access to the compounds of interest; for instance, LA production demands the carbohydrate fraction (Treichel et al. 2020). Several pre-treatment techniques can be used to isolate the carbohydrate fractions for conversion into LA, such as physical, chemical, and thermal processes; and their combination. They are widely applied to LB, and the evaluation of various techniques are well established in the literature, mainly aimed at producing bioenergy (e.g., ethanol and biogas).

In addition to improving the accessibility of the catalyst to carbohydrates, the function of the pre-treatment in LA production from biomass is to remove inorganic salts and xylans to subsequently obtain its derivatives and reduce the total cost of the operation (Kang, Fu, and Zhang 2018). Separation of LB fractions into simple carbohydrates through pre-treatment is also referred as hydrolysis (Hakeem et al. 2022) and the choice of pre-treatment can influence LA yield (Morone, Apte, and Pandey 2015). Generally, operating conditions are severe (e.g., high temperatures (>150 °C) and acidic pH), which may result in a more significant part of the lignin present in the structure remaining insoluble, which can result in humins formation and reduced LA yield (Morone, Apte, and Pandey 2015). Finally, new technologies are concerned, optimization of the synthesis process, and technical challenges and bottlenecks associated with the techniques are explored. In addition, the present study also addresses the possibility of enabling green catalysis for LA production by residual biomass rich in fructose (e.g., fruit residues) which, until now, has not been explored to obtain this compound.

2.2 GENERAL ASPECTS OF THE LA

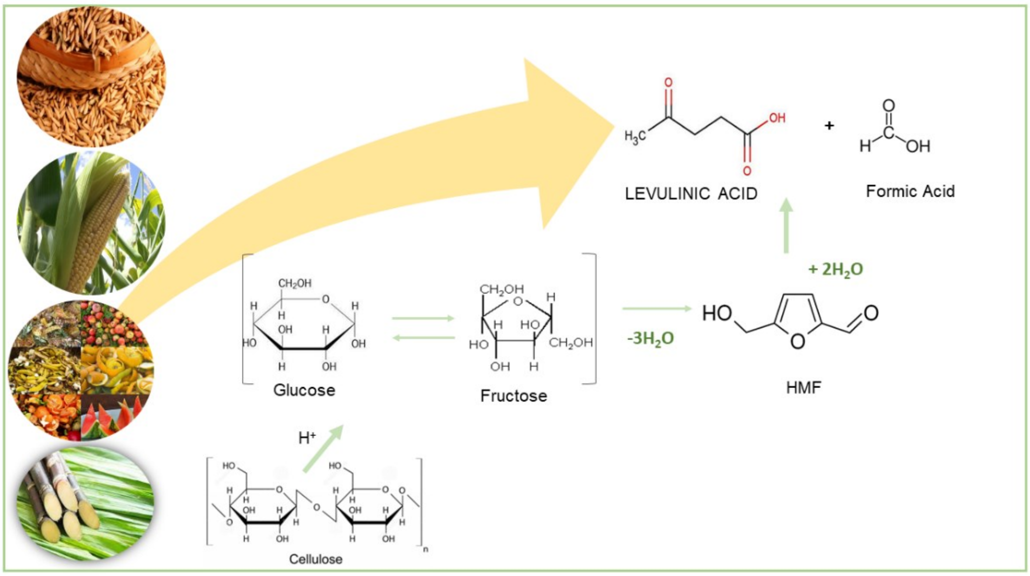
Also known as 4-oxopentanoic acid ($CH_3C(O)CH_2CH_2CO_2H$), LA is a keto acid with a carbonyl group and a carboxylic acid group in chemical structure. These functional groups make LA highly reactive and a good precursor in synthesizing other chemical products because it can act in redox, esterification, substitution, polymerization, and chiral reactions (Zhang et al. 2022). Also, LA can be obtained from different sources of raw material, which directly impacts the cost of production, as it can be considered in the suitability of industrial plants that generate residual biomass.

This interest has been driven by the market demand for LA and its derivatives, which have shown exponential growth, with an estimated global LA market of US$ 21.7 million in 2020 with a perspective that by 2027 it will reach US$ 30.8 million. Globally, the Asian continent is where the LA market is concentrated, mainly in countries such as China, India, and Japan, due to the high demand for fertilizers and agrochemicals, which is an essential field for the application of this compound (Levulinic Acid: Global Strategic Business Report; Levulinic Acid Market Size, Report, Growth | Overview (2022 - 27)).

Generally, the LA transformation includes a catalytic process, oligomerization, and synthesis. Within these processes, there is a range of possibilities for deriving reaction routes, generally involving oxidation or reduction reactions (through the reaction of the carboxyl and carbonyl groups – Figure 2) or biotransformation processes. Redox reactions occur through oxidizing or reducing agents, whereas biotransformation concerns the use of enzymes or microbial strains to produce polymers, esters, and others from LA (Badgujar, Wilson, e Bhanage 2019). Products derived from LA include resins, animal feed, food additives (used in the food industry to impart flavor), perfumes, textile dyes, fuels, antimicrobial agents, organic chemical compounds, and herbicides (Xu et al. 2021). Figure 3 illustrates some compounds derived from LA and possible applications.

Levulinate esters are the most successful LA derivatives with great industrial importance as additives into fuels, food formulations, and cosmetics due to flavoring, emulsifying, and stabilizing characteristics (Cambraia et al. 2022). Also, LA is a good precursor for solvents such as 2-methyl tetrahydrofuran (MTHF), which is recognized as suitable in organic synthesis and polymerization reactions. Due to characteristics such as low polarity with high octane number, it can be mixed with fuels. Also, compared to ethanol - an extensively studied biofuel - MTHF has a higher calorific value, lower vaporization, and water solubility, making it an excellent option for a fuel additive.

Figure 2: General representation of obtaining LA from lignocellulosic biomass by the acid route.



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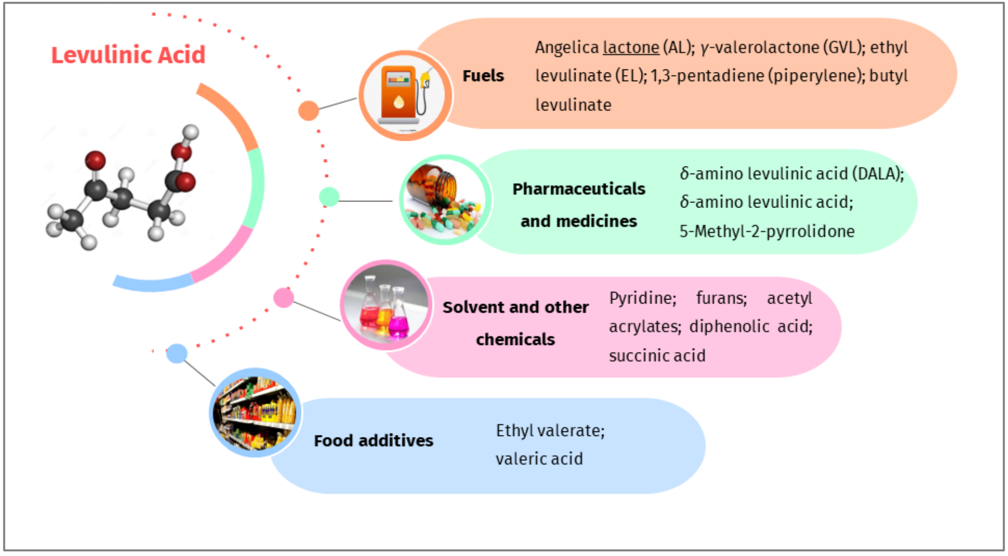
Another product that can be obtained from LA, of great interest in the fuels field is γ-Valerolactone (Morone, Apte, and Pandey 2015). Considered a green solvent, this compound has attracted attention due to studies demonstrating its potential as a sustainable fuel liquid for global storage/transport and a renewable hydrocarbon resource for carbon-based energy (Xu et al. 2021).

5-Aminolevulinic acid (DALA) is also obtained from LA and has demonstrated potential for application in agriculture, being non-toxic to humans and animals and biodegradable, leaving no residue in the environment (Xu et al. 2021; Y. Yang et al. 2022). It has recently been explored for use in the medical field with promising results (Li et al. 2022; Taoka et al. 2022). Although it is a product with a potential application, the full benefit of DALA is still limited by the costs of the process and its mechanism of action, which are not fully explained (Morone, Apte, and Pandey 2015; Xu et al. 2021).

LA has been involved in around 130 pharmaceutical formulations. LA calcium salt, for instance, is used in treating tuberculosis; LA heterocyclic derivatives are used in analgesics and medicines. LA is a precursor for obtaining succinic acid, widely used in the food and pharmaceutical industries (Levulinic Acid Market Size, Report, Growth | Overview (2022 - 27)).

The intrinsic properties of LA make this compound of great interest for expanding new technologies for efficient production and recovery. Different biomasses and obtaining routes can be used to get LA, and advances in the valuation of residual biomasses are an exciting strategy because they are cheap and underutilized sources (Xu et al. 2021).

Figure 3: Main commercial applications of LA and its derivative compounds.



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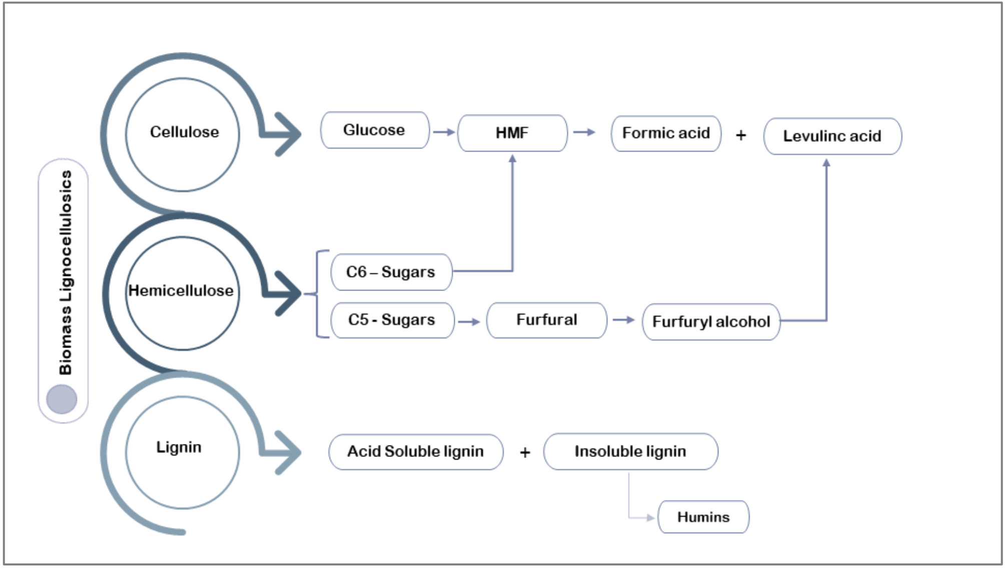
2.3 LEVULINIC ACID PRODUCTION: FROM FEEDSTOCKS TO UNIT OPERATIONS

LA can be obtained from the acid dehydration of sugars in the biomass, such as glucose and fructose (Figure 4) (Kang, Fu, and Zhang 2018; Pileidis and Titirici 2016) and the primary feedstock applied is lignocellulosic biomass. The commercial process uses Biofine Technology, developed by Fitzpatrick in 1997. The LA is produced from materials containing carbohydrates, using two sequential reactors with temperature control, reaction time, and acid concentration. In the first step, hydrolysis produces HMF, continuously removed from the first reactor and fed to a second reactor to produce LA and formic acid (co-product of the LA synthesis). The yield obtained in this process reaches 80% mol (Morone, Apte, and Pandey 2015). Acid-catalyzed biomass hydrolysis to get LA is conducted at temperatures from 100 to 200 °C.

The breakdown of biomass catalyzed by acid goes through five main stages: pre-treatment of lignocellulosic biomass, hydrolysis of cellulose into glucose, isomerization of glucose into fructose, dehydration of hexoses into HMF and finally, rehydration of HMF to LA (Kang, Fu, and Zhang 2018). In acid hydrolysis, hemicellulose can be converted to xylose and furfural, while cellulose is hydrolyzed to glucose, HMF, and LA. Lignin is responsible for the humins formation during BL hydrolysis. According to Figure 4, it is noted that the production pathways of HMF and LA are similar, with HMF being an intermediate compound in the synthesis and precursor of LA from the hydrolysis of biomass (Kang, Fu, and Zhang 2018).

Figure 4: Pathway of acid decomposition of lignocellulosic biomass



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The main challenges in obtaining LA are associated with the separation and recovery steps, resulting from the clogging of the reaction system by humins (derived from the lignin structure), the high cost of recovering the mineral acids used, and the high consumption of water (Morone, Apte, and Pandey 2015). Therefore, in implementing this process, research has been focused on investigating the kinetics of the reactions involved and milder conditions, as well as the search for residues based on a lignocellulosic structure with less recalcitrance, facilitating the access of catalysts to the structure of carbohydrates (Rackemann e Doherty 2011).

The highest yields of LA are obtained from LB using mineral acids as catalysts, but results may vary according to the raw material used. Sugarcane bagasse, olive tree branches, giant cane, and poplar sawdust, led to LA yields of around 23% by weight with the use of hydrochloric acid. Using H_2SO_4, the production of LA varies according to the matrix. While cotton straw resulted in 10% of LA weight, eucalyptus chips, bunches of palm fruits, and wheat straw exceeded 40%, demonstrating that the type of biomass significantly influences reaction yield (Mthembu, Gupta, e Deenadayalu 2023).

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In addition to improving the accessibility of the catalyst to carbohydrates, the function of the pre-treatment in LA production from biomass is to remove inorganic salts and xylans to subsequently obtain its derivatives and reduce the total cost of the operation (Kang, Fu, and Zhang 2018). Separation of LB fractions into simple carbohydrates through pre-treatment is also referred as hydrolysis (Hakeem et al. 2022) and the choice of pre-treatment can influence LA yield (Morone, Apte, and Pandey 2015). Generally, operating conditions are severe (e.g., high temperatures (>150 °C) and acidic conditions), which may result in a more significant part of the lignin present in the structure remaining insoluble, which can result in humins formation and reduced LA yield (Morone, Apte, and Pandey 2015; Thakkar et al. 2021; Schmidt et al. 2017).

Hydrothermal pre-treatment of biomass can also increase the LA yield. A study by Thakkar et al. evaluated an integrated biorefinery process using hydrothermal pre-treatment of corn straw and subsequent synthesis to LA using sulfuric acid as a catalyst (Thakkar et al. 2021). The yield of LA, when using pre-treated straw, increased from 0.2 to 35.8%. In addition, the authors found a decrease in the amount of waste generated, further corroborating the importance of pre-treatment.

The pre-treatment of lignocellulosic biomass catalyzed by acid is the most efficient, mainly in terms of increasing the accessibility of the carbohydrates of the structure - with emphasis on cellulose and facilitating the sequential acid hydrolysis step. In LA acid catalysis, acid pre-treatment, for example, does not require pH adjustments in subsequent steps (Kang, Fu, and Zhang 2018).

The combined use of several pre-treatments has advantages, such as mild process conditions, use of smaller amounts of solvents, and reduced waste generation, such as the study conducted by Schmidt et al. that evaluated the conversion of LA from sugarcane bagasse sugar using hydrothermal pre-treatment followed by enzymatic hydrolysis (Schmidt et al. 2017).

Using ligninolytic enzymes to hydrolyze the biomass is a potential alternative to access the carbohydrates in the lignocellulosic structure and consequently obtain LA. In enzymatic hydrolysis, there is no need to recycle the catalyst, reducing the operation cost and reducing environmental impacts (Treichel et al. 2020).

Another relevant factor to be considered in pre-treatment processes is the degree of crystallinity of the cellulose, which affects LA synthesis, since the higher the crystallinity, the more restricted the access of the acid catalyst to the β-1,4-glycosidic bonds, decreasing the reaction speed. Therefore, milling steps or combining pre-treatment techniques (e.g., thermal and chemical) followed by the acid hydrolysis step can increase LA yield (Kang, Fu, and Zhang 2018). Therefore, after pre-treatment of lignocellulosic biomass, solid rich in cellulose and with less lignin content adhered to the surface is expected.

2.3.2 From carbohydrates to LA: reaction pathways

When LA conversion is performed in pretreated lignocellulosic biomass, the first step will consist of the hydrolysis of the cellulose to produce monosaccharides, mainly glucose. Generally, the deconstruction reaction of the cellulose structure to glucose is conducted under acidic conditions catalyzed by compounds such as HCl or H_2SO_4 or by heterogeneous catalysts, which result in reactions that break the glycosidic bonds of the structure, resulting in glucose formation (Chen, Yu, and Jin 2011; Kang, Fu, and Zhang 2018; Mukherjee, Dumont, and Raghavan 2015).

From glucose, there are two pathways for the LA production reaction: (i) one occurs from the direct formation of glucose in HMF and finally to LA; (ii) glucose is isomerized and preliminarily converted into fructose, which in turn is dehydrated to HMF, which is rehydrated in LA. In both routes occurs the formation of formic acid, a co-product of LA synthesis (Kang, Fu, and Zhang 2018).

It has already been demonstrated that using catalysts to accelerate glucose formation can be an exciting alternative to improve reaction performance. The reaction of glucose in HMF co-occurs after cellulose decomposition. Facilitated by inorganic acids (e.g., HCl), glucose breaks down directly into HMF (Kang, Fu, and Zhang 2018). Finally, the HMF is rehydrated, forming LA.

The second pathway for LA formation considers the isomerization of glucose into fructose, being considered a potential alternative to improve LA yield. Some specific catalysts, such as enzymes, Lewis's acids, and bases, favor this reaction (Kang, Fu, and Zhang 2018). Direct dehydration of glucose to HMF is more complex than catalytic dehydration of fructose to HMF when weak acids are used; the isomerization of glucose to fructose is more desirable for hydrolysis.

In this conversion pathway, glucose is first converted to fructose, and HMF is formed through dehydration (Kang, Fu, and Zhang 2018). This isomerization process is a decisive step for HMF formation and, consequently, for the overall LA yield. This statement is corroborated by the data in Table 1, where it is possible to verify higher LA yields when using fructose as a precursor. Thus, the use of catalysts is indispensable for LA synthesis. In the following section, the primary catalysts used in the process of obtaining LA will be discussed.

Table 1. The literature evaluated biomass to obtain LA, using different techniques for its synthesis.

Biomass	**Evaluated reaction system to obtain LA**	**Yield**	**Reference**
Sold out lemon peels	Hydrolysis using HCl 3.36 wt%, solid: liquid ratio 5%, pretreatment temperature 120 °C for 2 hours.	22 (wt%)	(Licursi, Antonetti, Parton, et al. 2018)
Miscanthus *(Miscanthus)*	Aqueous medium catalyzed by H_2SO_4 (0.10 M and 0.53 M) and mild temperature range (150 and 200 °C).	58-72 (mol %)	(Dussan et al. 2013)
Rice husks	The biomass was hydrolyzed with 4.5% HCl and 170° C.	59.4% (w/w)	(Bevilaqua et al. 2013)
High amylose cornstarch	The pretreated and extruded sample was reacted under pressure using 4 wt% H_2SO_4, a temperature of 200 °C, and a reaction time of 60 minutes.	47 (%)	(Cha e Hanna 2002)
Wheat straw	The sample reacted in a pressurized reactor containing 3.5% H_2SO_4 (w/v), a temperature of 209.3 °C, solid: liquid ratio of 15.6, and 37.6 minutes of reaction.	19.86 (%)	(Chang, Cen, and Ma 2007)
Sorghum grain	10% biomass mixed with an aqueous solution of H_2SO_4 8% (w/v) was heated to 200 °C in a pressurized reactor.	32.6 (%)	(Fang and Hanna 2002)

Crystalline cellulose	$CrCl_3$ with a dosage of 0.02 M was used as a catalyst in a solution containing 50 wt% cellulose in a reaction carried out at 200 °C for 180 minutes.	67 (mol%)	(Peng et al. 2010)
Water hyacinth plant *(Eichhornia crassipes)*	Hydrolysis with 1M H_2SO_4, a temperature of 175 °C, and 1wt% of water hyacinth intake.	53 (mol% - 35 % by weight)	(Girisuta et al. 2008)
Microcrystalline cellulose	Ionic liquids are functionalized with SO_3H, 3.3mmol, microwave (MW) at 800 W, 160 °C for 30 minutes.	55 (%)	(Ren, Zhou, and Liu 2013)
Fructose	Direct conversion of fructose catalyzed by a perfluorosulfonic acid resin (Aquivion® P98) in a parallel reactor at 120 °C for 12 hours.	96(%)	(Zhang et al. 2022)
Sugarcane bagasse (enzymatic pretreatment) and wheat straw	The highest yield of LA was obtained with methanesulfonic acid 0.63M MSA at 206 °C and a residence time of 30 min.	67.7 (mol%)	(Schmidt et al. 2017)
Sugarcane bagasse	Hydrolysis catalyzed by 0.55 M H_2SO_4 at 150 °C.	63 (mol%)	(Girisuta et al. 2013)
Cornstalk	Hydrolysis catalyzed by 0.5mol/L $FeCl_3$ at 180 °C for 40 minutes.	48.89 (%)	(X. Zheng et al. 2017)
Bunch of palm fruits	Acid hydrolysis with 0.25 and 0.5N H_2SO_4 at 180 °C.	4.36 (g/L)	(Chin et al. 2014)

Microcrystalline cellulose	Reaction conducted at 180 °C for 6 h using 0.4 g of ionic liquids immobilized on zeolites (HY (SiO_2/Al_2O_3) and 0.5 wt% glucose for 6 hours.	62.2(%)	(Abu Zarin et al. 2022)
Green microalgae (*Scenedesmus obliquusso)*	Conversion using methanesulfonic acid (MSA) 1.875 M MAS (0.15 M MSA/1% biomass), 12.5% biomass at 180 °C for 40 minutes	40.74(%)	(Kim, Jeong, and Kim 2022)

2.3.3 Catalysts

In acid catalysis, glucose isomerizes to fructose, and the catalytic activity remains constant in later steps. The most used Lewis's acids are metallic chlorides, such as $AlCl_3$ and $CrCl_3$, since presenting good glucose/fructose isomerization and dehydrate the hexoses. Still, they have the disadvantage of being less selective for HMF in the dehydration fructose process. Lewis's acid sites catalyze glucose isomerization, but the presence of a Brønsted acid can facilitate this process. Both the Lewis and Brønsted acids are required for the dehydration reaction, while the Brønsted acid sites are essential for the rehydration of HMF to LA (Ramli e Amin 2015). Thus, the challenge of developing bifunctional catalysts arises with an adequate amount of stable Lewis and Brønsted acid sites. An alternative is to create sequential processes integrating different enzymatic isomerization processes and subsequent acid-catalyzed conversion (Kang, Fu, and Zhang 2018).

Mineral acids, also called homogeneous catalysts, such as H_2SO_4, HCl, HNO_3, and H_3PO_4, have low cost, high catalytic activity, good market availability, and good HMF yields, which increases LA production. Because of the advantages, there are several studies to clarify the kinetics involved in acid LA synthesis (Girisuta et al. 2013; Fachri et al. 2015; Girisuta et al. 2008; Dussan et al. 2013). Fachri and collaborators investigated the rate of formation and conversion of D-fructose into HMF and LA in the medium of water and H_2SO_4 under different process conditions. They confirmed that D-fructose yields around 10% higher when compared to glucose in LA synthesis (Fachri et al. 2015). Kang and Yang observed higher yields with the isomerization of glucose to fructose (Kang, Fu, and Zhang 2018; G. Yang, Pidko, and Hensen 2012).

Girisuta described the kinetics of hydrolysis of water hyacinth in LA using H_2SO_4 as a catalyst (Girisuta et al. 2008). Later, the same author developed a kinetic model from the acid hydrolysis of sugarcane bagasse and used mineral acids as a catalyst in both works (Dussan et al. 2013). The effectiveness of acids depends mainly on the concentration, the feedstocks' recalcitrance, and the strength of their dissociation constants (Morone, Apte, and Pandey 2015). Despite the wide use and yields obtained, these acids have some disadvantages, such as the recycling difficulty, dangerousness, and corrosiveness characteristics, which entail higher costs in the development of reactors, highlighting the need for their replacement by new catalysts (Morone, Apte, and Pandey 2015).

Heterogeneous catalysts contain a solid acid and a metal catalyst, which are advantageous for reducing corrosion in reactors and presenting greater selectivity and ease of recovery and recycling (Morone, Apte, and Pandey 2015). Solid catalysts include solid acid zeolites, metal-modified zeolites, acid resins, metal halides, and clays (Ramli and Amin 2015; Q. Wang et al. 2022). Ramli employed a zeolite catalyst modified with iron chloride, evaluated the physicochemical and catalytic properties of the LA production from glucose, and observed an increase in LA yield up to 180 ºC (Ramli and Amin 2015). Increasing the temperature to 200 °C reduced LA yield, which could result from humins formation. The authors demonstrated that the modified catalyst could be reused five times without activity loss. Although heterogeneous solid catalysts are a potential alternative for glucose dehydration due to their porous structures significantly influencing the reaction, further studies still need to understand the catalytic activity and increase the LA yield (Morone, Apte, and Pandey 2015; Q. Wang et al. 2022).

Inorganic salts also performed well as reaction catalysts. Peng evaluated the catalytic performance of some metallic chlorides in cellulose conversion into LA in an aqueous medium at high temperatures (Peng et al. 2010). They found an increase in LA production with shorter reaction times. Zhi pretreated corn residues with iron chloride, investigated the kinetics to determine the best conditions for hydrolysis and found that at higher concentrations (0.3 and 0.5 mol/L) and temperatures (220 – 240 ºC), $FeCl_3$ demonstrated good efficiency in glucan hydrolysis (Zhi et al. 2015). Zheng analyzed the kinetics of LA production from corn stalks catalyzed with iron chloride. Still, at lower temperatures, not exceeding 200 °C (X. Zheng et al. 2017). Iron chloride has no toxicity, low cost, and good availability in the market, highlighting it as a sustainable catalyst for LA production.

Metallic catalysts showed more excellent catalytic activities compared to acids and greater ease of separation (Morone, Apte, and Pandey 2015). Chromium chloride, for example, showed good efficiency, and Cr^{3+} can be separated from the mixture and recycled later (Bevilaqua et al. 2013). However, some disadvantages are reported, such as lower yield of LA and high toxicity of some metals used. Metals such as cobalt, iron, aluminum, lanthanum, alkaline earth metal chlorides, and metal oxides are found in the literature.

Using solvents to obtain LA from a renewable matrix is also possible. Water is the most used solvent and is most likely to produce LA, but ionization contributes to forming formic acid, affecting the LA yield. Other protic solvents, such as ethanol and butanol, were tested, showing good selectivity and precipitating easily in an acid medium. Nonpolar solvents such as dimethylsulfoxide, THF (tetrahydrofuran), sulfolane, ethyl acetate, and acetonitrile have also been used to produce HMF and LA (Morone, Apte, and Pandey 2015; Pileidis and Titirici 2016). They can accelerate the conversion of glucose and do not require an increase in temperature or amount of acid catalyst when used together. On the other hand, they can generate toxic waste in the environment (Mikola, Ahola, and Tanskanen 2019; Morone, Apte, and Pandey 2015).

Another alternative is using ionic liquids, fused salts at room temperature, with the remaining liquid below 100 °C. Ionic liquids are composed of organic and inorganic ions with varying sizes, and the ratio of cations/anions can be modified according to the application. These compounds have some advantages as catalysts, mainly associated with physicochemical characteristics since they are non-volatile and flammable. They have good thermal stability, high ionic conductivity, and the ability to dissolve solutes. In addition, ionic liquids form complexes with different metallic catalysts that favor chemical activity, playing a dual role: as a solvent and catalyst and as catalysts, providing good binding sites. However, the disadvantages are the difficulties of purification, reuse, and biodegradability. Solvent recovery requires high energy consumption, which makes the process more expensive, in addition to the need for specifically designed equipment, which imposes limitations for industrial use (Morone, Apte, and Pandey 2015; Mikola, Ahola, and Tanskanen 2019; Badgujar, Wilson, and Bhanage 2019; Ramli and Amin 2015; B. Zheng et al. 2022).

Supercritical fluids were also evaluated but were unsuccessful in yield and operating costs. More detailed studies are still needed to use supercritical fluids and solvents, aiming at more sustainable and efficient processes (Mikola, Ahola, and Tanskanen 2019; Morone, Apte, and Pandey 2015; Yong et al. 2020).

Microwave radiation can also be used for hydrothermal fructose degradation to HMF and LA. Microwave heating results in better energy balance, lower heat losses, more efficient temperature control, reduced process costs, and decreased catalytic reaction time (Kłosowski, Mikulski, and Menka 2019; Tian et al. 2021). Microwave irradiation associated with the catalytic activity of ZnBr has been successfully used for LA production in high yields (up to 53% by weight) (Victor et al. 2022). The presence of catalytic compounds, for instance, as the addition of sodium chloride in the reaction, increased the ions concentration, which interrupted the network of hydrogen bonds in the cellulose, leading to a more significant dissolution of the cellulose in the solvente (Morone, Apte, and Pandey 2015). Thus, it is possible to eliminate the drying step of the matrix, which decreases costs and increases the LA yield. The use of microwaves to produce LA from feedstocks is little known and appears as a promising alternative mainly to the energy efficiency of the process.

Few data are reported on the biological routes of LA production in the literature since it has an inhibitory behavior for most microorganisms (Balasundaram et al. 2022). Until then, the study by Pinto-Ibieta et al. used a mixed microbial consortium of bacteria and fungi to obtain LA and polyhydroxyalkanoates (Pinto-Ibieta et al. 2020). The microorganisms could synthesize LA intracellularly through the pathway that transforms pentoses into pyruvic acid. The authors suggest two main paths for this conversion: the first is the transformation of pyruvate by redox reactions into 4-oxo-2 pentanoic hydroxy acid dehydrated into 4-oxo-2 pentanoic acid and is finally reduced to LA. The second involves the oxidation of pyruvic acid, producing acetyl-CoA, which enters the Krebs cycle resulting in the synthesis of LA from succinyl CoA. However, LA synthesis cannot be attributed to a specific microorganism, which requires further study to understand the synergy of the microbial consortium, the metabolic pathway, and the storage of LA in cells. Only from this information can new research be carried out to optimize this process.

LA production is possible through several homogeneous and heterogeneous catalysts, from conventional ones - such as acids - to the most technological ones - such as ion exchange resins, solvents, and ionic liquids. However, efforts are still needed to optimize the process conditions and their use of these processes. For this, it is necessary to think about new separation processes and technologies in addition to the existing ones that will be discussed in the next topic of this study.

2.4 PROCESSES FOR RECOVERING THE LEVULINIC ACID PRODUCED

The step of separation or recovery of biomass by-products is challenging and involves high costs. Once LA has been produced, it is necessary to isolate it. The amount of LA present in the final mixture depends on the type of lignocellulosic material and the process conditions used (Brouwer et al. 2017). LA and formic acid have similar chemical structures, making them difficult to separate (Araya-López et al. 2022). Table 2 briefly lists the primary processes used to separate LA with their benefits and disadvantages.

In the Biofine process used in the commercial production of LA, two acid-catalyzed steps result in a complex mixture containing LA, formic acid, and other compounds, such as humines, which are extracted by filtration in the solids removal step. Then, the resulting solution evaporates, and the LA is separated using a volatile acid catalyst. The evaporation method results in solid residues that require neutralization and can be used later to generate energy and heat. This process allows obtaining LA with a purity of 95 to 97%, recovering around 90% of the catalyst and recycling the water used. However, at this stage, the recovery and recycling of the catalyst involve high costs (Brouwer et al. 2017; Martinez-Hernandez and Sadhukhan 2018).

Table 2. Characteristics of the leading LA separation processes.

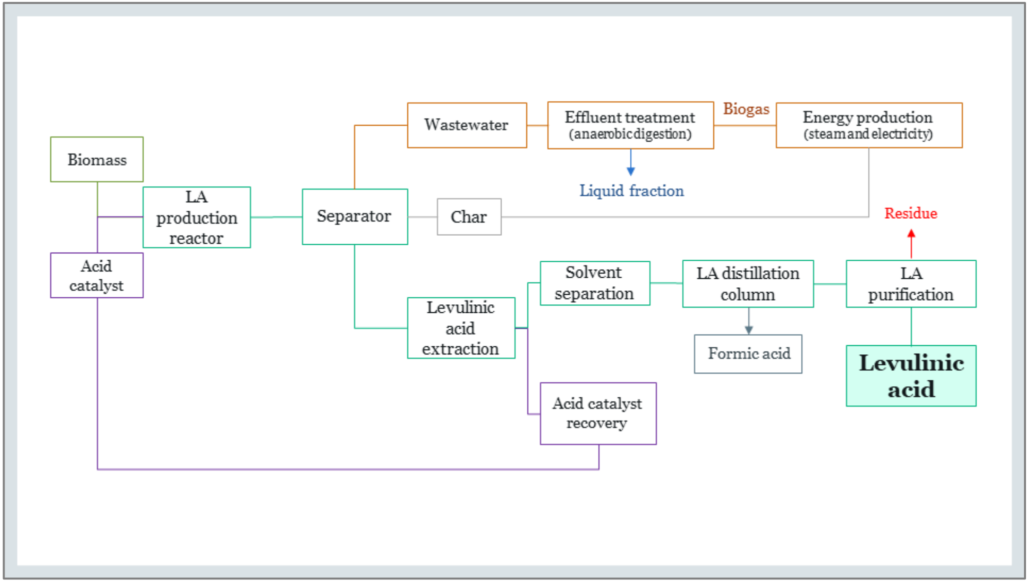
LA separation/recovery method	Advantages	Disadvantages	Reference
Membranes	Good efficiency; Economical; Environmental safety; Ease of operation; Application on an industrial scale; It allows combining separation processes reducing incrustation problems; Continuous separation; Low energy consumption;	Low selectivity; Inability to separate compounds with similar molarities; Fouling; High maintenance costs;	(Ashokkumar et al. 2022; Chaturvedi et al. 2022; Morone, Apte, and Pandey 2015; Araya-López et al. 2022)
Distillation	Simple technology; Commercial application; Achieving high-purity LA; allows the recycling of water used in the process;	Favors the formation of undesirable by-products; High cost for catalyst recovery; High energy consumption;	(Morone, Apte, and Pandey 2015; Chaturvedi et al. 2022)
Liquid-liquid extraction	It has good efficacy at low concentrations of LA; It does not require an additional processing step for the final product; Obtaining LA with a high degree of purity;	The challenge of finding solvents that have high selectivity for LA and low for acids; Solvent reuse; Environmental toxicity;	(Pinto-Ibieta et al. 2020; Tian et al. 2021; Morone, Apte, and Pandey 2015)

	Ease of solvent recovery; Allows the use of a wide range of solvents;		
Adsorption	Ease of operation; Good results in water-rich media;	Smaller amounts of LA recovered; Adsorption of LA on the catalyst; Development of adsorbents with high selectivity for LA; An adsorbent with low adsorption efficiency;	(Rackemann and Doherty 2011; Peng et al. 2010)
Ionic liquids	Thermal stability; Low volatility;	High cost; Complex purification and reuse; Difficulty separating LA and formic acid; High energy demand; Environmental sustainability.	(Morone, Apte, and Pandey 2015; Kłosowski, Mikulski, and Menka 2019; Jia, Maitra, and Singh 2023)

Usually, LA separation and purification occur by vacuum distillation since it is a simple technology, well-established, and commercially applied (Figure 5). On the other hand, it should be noted that it has a high energy consumption and favors the formation of by-products. In addition to this technique, different methods have been explored and can be used, highlighting the use of membranes, liquid-liquid extraction, steam pickling, and adsorption, among others (Morone, Apte, and Pandey 2015).

Although vacuum distillation is the most common process, liquid-liquid extraction is a promising technique, especially when the LA concentration is low, since it does not require an additional step of processing the product and presents good yields for extracting the product in the most concentrated form, and with a high degree of purity (Brouwer et al. 2017). The LA is removed in the organic solvent, while the acid catalyst remains in the aqueous phase for later recovery. The extracted fraction of LA is sent to a distillation column where the solvent is recovered, and the LA is purified. This process removed LA from the medium using dilute acids, aliphatic amines, and organophosphate solvents (Kampwerth et al. 2022). Other solvents, such as ketones, alcohols, fatty acids, esters, halogenated hydrocarbons, and ethers, have been reported (Brouwer et al. 2017; Morone, Apte, and Pandey 2015). The solvents must have high selectivity for LA, low selectivity for the acids used in the process, and low environmental toxicity. The solvent reuse must occur, which is crucial for the process's good economic performance (Morone, Apte, and Pandey 2015).

Figure 5: Diagram of the design of an LA biorefinery via the acid route from lignocellulosic biomass.



Source: the author

Membrane separation technologies are essential tools in biotechnological processes. This method has good recovery efficiency, is simple to operate, in some cases, is a single step, allows continuous separation of products, minimizes the formation of by-products, and does not require high energy consumption (Morone, Apte, and Pandey 2015). Nanofiltration, microfiltration, and ultrafiltration are filtration processes that use membranes and are driven by pressure. The combined use of these techniques, for example, nanofiltration and reverse osmosis, have already been successful in separating fermentable sugars from HMF and acetic acid when used in series (Jia, Maitra, and Singh 2023).

The use of membranes combined with other separation techniques is also promising and explores the best characteristics of the processes. One example is stacked porous membranes in electrodialysis cells for separating bioproducts (EDPM) (Sun et al. 2020). This processing is a technology that uses membranes based on the theory of electrophoresis. It separates functional bioactive peptides, proteins, amino acids, and phenols. It has applications in the beverage industry, such as juices, for example, and applications for water treatment wastewater and desalination.

The supported liquid membrane (SLM) method using agents such as sodium hydroxide (NaOH), sodium carbonate, hydrochloric acid, trimethylamine, and water with different operating parameters was tested in a study for recovery and extraction of LA. The system with the best result extracted 89% of LA using 0.5M NaOH as an agent, demonstrating that SLM was a simple and low-cost process (Rajendaren et al. 2019). Another study used the bulk ionic liquid membrane (BILM) technique, where imidazolium-based ionic liquids were used as a liquid membrane for LA removal (Baylan and Çehreli 2018) The authors benefited from the four-factor experimental design features, and ANOVA analyzed the results with the statistical adequacy of models. The results showed good selectivity in the ionic liquids used as the membrane phase.

However, the use of membranes also has challenges, among which we can mention the limitation of the separation of compounds with similar molecular weights and polarities; incrustations, which reduce the permeate flux, reduce the efficiency of the process, and demand a high cost for maintenance and cleaning (Chaturvedi et al. 2022; Morone, Apte, and Pandey 2015). Adsorption is conducted with a solid porous adsorbent, which adsorbs the LA from the aqueous solution and removes the FA by desorption. LA can become adsorbed on the catalyst surface, reducing the amount of recovered product (Rackemann and Doherty 2011). In hydrolysates with a large amount of water, adsorption presents good yields, being an easy-to-operate process for extracting LA from the reaction medium (Wang et al. 2022). However, there are still gaps in making this technique viable at an industrial level (Morone, Apte, and Pandey 2015). Further research is still needed to develop adsorbents with high adsorption capacity and selectivity for LA.

Ionic liquids can also be used for both carbohydrate conversion and LA separation. They have low volatility and good thermal stability but require high capital costs to obtain, and purification technologies (Morone, Apte, and Pandey 2015; Ren, Zhou, and Liu 2013) and separation are still limited. In addition, they present specific interactions with LA and formic acid, which may make separation difficult (Araya-López et al. 2022). Although several studies use ionic liquids, it is still necessary to implement efficient separation processes that require less energy consumption and are environmentally sustainable (Araya-López et al. 2022).

Developing new catalysts is the subject of studies (Cueto et al. 2022; Mongkolpichayarak et al. 2022; Woo Lee et al. 2022). In addition to its effectiveness, recycling the catalyst is a significant challenge. In addition to low cost, durability, and stability, the development of renewable catalysts needs to be considered. A study by Mongkolpichayarak et al. (2022) evaluated microporous material for LA conversion from rice straw and sugarcane leaf and its reuse potential. The catalyst showed a decrease in yield only after the fourth cycle of use (Mongkolpichayarak et al. 2022).

Bonouka et al. (2022) compared the coercion effect of homogeneous and heterogeneous catalysts and the combination of the two (Bounoukta et al. 2022). The recycling of the heterogeneous catalyst presented advantages over the homogeneous one. However, the selectivity and the yield of LA decreased, while the homogeneous component maintains the conversion rate and can also be renewable.

It is also worth highlighting concepts such as process intensification, a new design concept aiming to develop technologies to improve current production models. Intensified processes are versatile and can be implemented differently, combining functions and equipment in the same unit or integrating materials and energy (Alcocer-García et al. 2020). These processes are alternatives intended for the configuration of reduced equipment but need to offer the same performance as the traditional configuration, with less energy consumption and invested capital (Errico, Stateva, and Leveneur 2021).

For example, it is possible to mention the study of Licursi et al. that evaluated the hydrolysis catalyzed by sulfuric acid of corn grains for LA in a biphasic mineral oil and water system (Cueto et al. 2022). The oil was used as an antisolvent, allowing the selective concentration of LA in the aqueous phase, not affecting the selectivity and yield of LA and formic acid. The effect of acid concentration, agitation speed, and oil recycling was also evaluated, optimizing the reaction. Due to the stability of LA, the enhancement of the biphasic reaction can be explored and present promising and innovative results.

Intensified distillation systems aim to reduce energy consumption and operating costs but require further studies to assess the advantages over conventional processes, such as the reactive distillation column (Solis-Sanchez et al. 2022). Recently, the combination of liquid-liquid extraction columns followed by ordinary distillation has been studied (Tronci et al. 2023). In addition to achieving the purity and recovery goals, these configurations were economically promising, achieving a reduction of 11% in the total cost and with less environmental impact.

A process with complete control of the variables with little effort is highly desirable. Techniques such as reactive extraction in two-phase systems, reactive adsorption, reactive distillation with stripping, pervaporation-assisted reaction, and the development of recyclable catalysts are the future of biorefining. However, a life cycle analysis of economic impacts and risks is essential. More in-depth studies are still needed regarding economic viability.

2.5 NEW PERSPECTIVES ON THE USE OF BIOMASS FOR LEVULINIC ACID SYNTHESIS

The synthesis of LA and its derivatives in industrial plants is still challenging and presents several bottlenecks that scientific investigations have extensively evaluated. Catalysts with high selectivity for process intermediates, lower use of solvents and chemicals, the viability of processes with greater environmental sustainability in the long term (e.g., less waste generation and less use of chemicals), downstream with high efficiency, biomass that requires fewer unit operations and with less recalcitrance, are some of the bottlenecks that can be highlighted.

The full use of biomass is the key to ensuring economic viability, sustainability, and better industrial performance. Valuing all biomass components and obtaining a diversified portfolio of bioproducts is a challenge that biorefineries can overcome. Thermochemical processes are simple but energy-intensive, while biochemical ones offer more excellent product yield selectivity but suffer from high costs (Rathore and Singh

2022). Faced with these challenges, guaranteeing processes for obtaining sustainable and environmentally safer LA involves assessing and choosing the technologies offered.

Using statistical techniques and modeling software for process optimization contributes to overcoming these obstacles in LA production. Lee and Min(Lee, Lee, and Min 2023) optimized the production of LA from corn on the cob using response surface methodology. Rengel et al. optimized the starch hydrolysis conditions obtained from microalgae using Central Composite Design to get LA and HMF (Rengel et al. 2022). The conversion of sugarcane bagasse into LA catalyzed by H_2SO_4 was modeled by Ogedjo et al. (2022) using response surface methodology and artificial intelligence techniques, including artificial neural networks and fuzzy inference systems (Ogedjo et al. 2022). The authors also compared the experimental values obtained with those predicted by the models, increasing the process efficiency.

Advanced technological resources such as artificial intelligence, neural networks, and modeling for process optimization are tools that still require research for industrial implementation. The data extracted from these laboratory-scale analyses can predict the best conditions for implementing bioprocesses and pave the way for developing sustainable unitary prayers in biorefineries.

One of the strategies to overcome some issues is to design integrated processes according to the principles of green chemistry, such as biorefineries, aiming at reducing waste generation and greenhouse gas emissions and seeking the recovery of high value-added products from different structures of the same biomass (Badgujar, Wilson, and Bhanage 2019). A previous study evaluated an integrated bioethanol and LA production plant using empty palm fruit bunches. Based on the simulation, it was found that the integrated plant would meet the economic parameters of a chemical plant (Muryanto et al. 2021).

Another context that can be highlighted is the search for green or alternative catalysts, which can be a strategy to reduce the need for large volumes of chemicals. For instance, Jeong used coffee grounds with acid catalysts to obtain LA and formic acid (G. T. Jeong 2022). Singh et al. used residues of lemongrass, citronella, and palma rosa fibers used for the extraction of aromatics and pretreated it with p-cymene-2-sulfonic acid (p-CSA) synthesized from residues of citrus fruits in an integrated process, using all biomass components to produce xylose, lignin, and LA and both obtained a sustainable and less corrosive method, using atypical biomasses, not yet tested in other processes (Singh et al. 2019).

Furthermore, the production of LA is still limited to lignocellulosic biomass, and evaluating alternative sources of carbohydrates is an exciting strategy for producing LA. It was recently documented that cellulose hydrolysis and glucose isomerization could be avoided if the industry used fructose or fructose-containing carbohydrates as raw materials to replace cellulosic biomass (Victor et al. 2022).

In this context, exploring alternative biomasses is still a promising field. It presents itself as a strategy to improve the yield of LA production, mainly considering that ways of obtaining fructose offer better results than converting glucose to HMF. For example, fruit and vegetable residues are a sustainable alternative to be evaluated, as they can reduce LA production costs in addition to being underutilized (Antonetti et al. 2016). It is estimated that losses in the entire fruit and vegetable processing chain are 25 to 30%, of which need to be reduced and properly disposed of, with less impact on the population's quality of life and the environment (Sagar et al. 2018). Therefore, betting on valuing residual fruit biomass to obtain AT is favorable since it is generated in large quantities and has high sugar levels (Xu et al. 2021). For example, watermelon had a 2019 global production of 104 million tons (Awasthi et al. 2022; Zia et al. 2021) and losses were estimated at approximately 20% in the crop due to deformities and imperfections. Research has used watermelon to obtain fatty acids, biopolymers, biodiesel, pectin, phenolic compounds, and flavonoids, (G. T. Jeong 2022; Singh et al. 2019; Antonetti et al. 2016; Sagar et al. 2018; Awasthi et al. 2022) but there are no reports of the use of watermelon to produce LA. These watermelon residues have a fructose content in the pulp between 2.8 and 3.6% ($v.v^{-1}$) (Fish, Bruton, and Russo 2009).

Furthermore, the use of residual fruit and vegetable biomasses for conversion to LA allows the use of dilute acids and mild process conditions. However, few studies presented in the literature used fruit residues as biomass for LA production. To the authors' knowledge, the only recent report presented in the literature is the study by Licursi and collaborators using lemon peels to produce AL from structural polysaccharides and hydrochloric acid as catalyst obtaining a maximum yield of 22% by weight of LA (Licursi, Antonetti, Parton, et al. 2018).

In addition to the process conditions already mentioned, the prospects for LA production also need to be focused on environmentally sustainable concerns, such as the recycling of catalysts and the consumption of water and reagents throughout the process. Notably, there is a growing concern among recent studies, such as Charnnok & Laosiripojana which replaced water with acid effluent from sugar production by enzymatic hydrolysis, aiming to improve the digestibility of rubber tree residues for subsequent LA production using the residual water from the process, due to its high sugar contente (Charnnok & Laosiripojana 2022). In another context, Bonatto et al. (2021) used seawater and wastewater from shrimp production to produce second-generation ethanol using papaya waste as a substrate (Bonatto et al. 2021). Expanding to the present study, the volume of water in the reaction medium after the synthesis of LA can represent a problem in later stages, resulting in higher energy costs for its removal, mainly linked to the need for upstream reagents and effluent treatment (Liang et al. 2018). Thus, a perspective for future work is using wastewater or process water recycling to obtain LA, which is vital for environmental issues, and approaching a closed cycle with zero generation of effluents and waste.

LA plays an essential role in the economics of biobased chemicals; however, the sustainability and economics of the process are still the main research interests. The fact that LA is produced exclusively by chemical processes makes it even more attractive. This allows a variety of inexpensive biomass and lignocellulosic residues not yet used in biotechnological processes to be tested for direct synthesis at LA and in integrated biorefineries, optimizing the use of biomass for other products and, finally, using it there. Thus, more research is vital to explore the potential of LA biorefineries from new biomass.

2.6 CONCLUSION

LA is a multifunctional product traditionally obtained by acid catalysis of cellulose or the petrochemical source, which still disturbs the environmental balance and impacts the product's final value. It is necessary to develop alternative ways for its products based on untapped and low-value renewable resources and simple and low-cost development processes.

Although there are several kinetic and catalyst studies, limitations in the production of LA still permeate the sustainability and efficiency of the process, recyclability of catalysts, and better separation of products. Separation by distillation is still more used, but other technologies have been highlighted, such as liquid-liquid extraction and membranes. However, these methods are still expensive and have some environmental disadvantages. Another challenge to be overcome is the development of new low-cost catalysts with recycling potential. Still, studies focus on increasing efficiency by taking advantage of technological advances and intensifying existing processes.

Commonly used raw materials, such as lignocellulosic biomass, have been extensively studied and have shown promising results. However, fruit residues for LA synthesis remain unexplored. Monomeric carbohydrates such as glucose and fructose are available in these residues in free form, contributing to milder operational conditions for the synthesis of LA, with the use of less aggressive catalysts, reducing the difficulties for separation and purification at the end of the process, as well as facilitating the optimization of LA synthesis.

Fruit residue is a valuable resource for the synthesis of LA, as it can optimize its production, reduce the production cost, limit the formation of by-products, and increase the yield without involving aggressive and expensive catalysts. There are no data in the literature that used fruit residues for the synthesis of LA, which is an alternative with high potential to fill the existing gap of inadequate disposal, obtaining a product with high value-added at low cost, in addition to contributing to the development of sustainable processes that respect the planet's natural resources.

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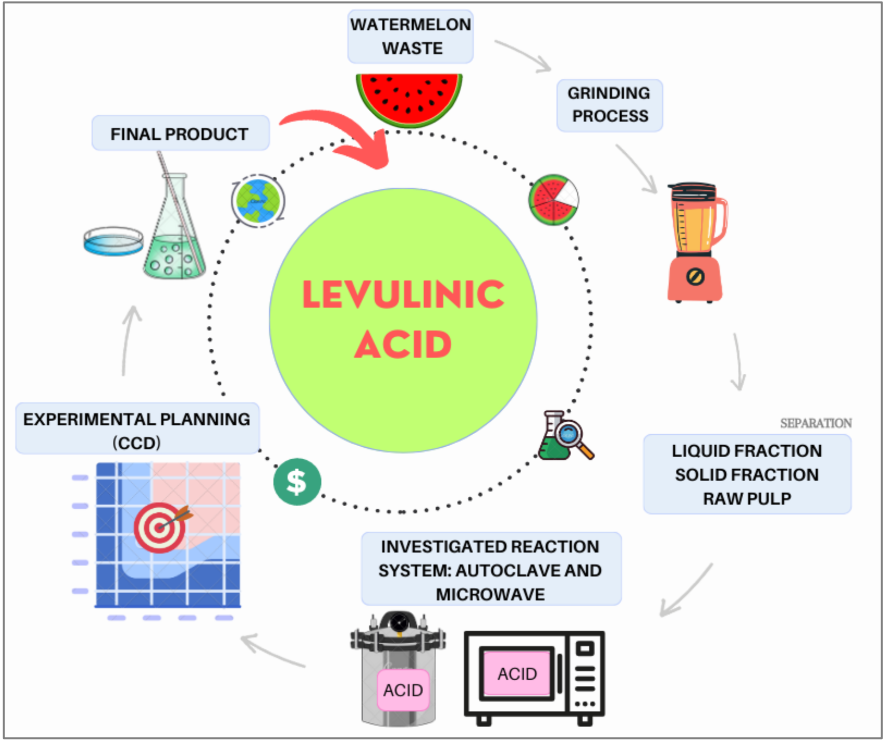
3 HYDROTHERMAL AND MICROWAVE-ASSISTED SYNTHESIS OF LEVULINIC ACID FROM WATERMELON RESIDUE

The third chapter of this thesis refers to an article published in the journal Biofuels Bioproducts & Biorefining. It aims to evaluate, for the first time, the production of levulinic acid from watermelon waste using mineral acids, hydrochloric acid (HCl), and sulfuric acid (H_2SO_4) in two different reaction systems, autoclave, and synthesis microwave (MW), aiming at the interest in using watermelon residue combined with a simple and cheap inorganic acid, widely used industrially (ISSN: 1932-1031 https://doi.org/10.1002/bbb.2542).

Figure 1: Graphical Abstract



Source: the author

3.1 INTRODUCTION

The growing demand for energy, products, and food challenges the current production and consumption system. The current economic model, designed linearly, has several distribution problems along its production chain and faces difficulties in meeting the population's needs, contributing to the imbalance of the necessary resources and the generation of waste. It is essential to consider replacing fossil products with materials of renewable origin, exploring new raw materials seeking ecologically sustainable processes, and reducing waste generation and environmental impact (Awasthi et al. 2022; Ubando, Felix, and Chen 2020).

World organizations have been discussing the development of more sustainable production processes, especially regarding the high generation of food waste, which leads to food insecurity. The year 2021, for example, was designated by the UN (United Nations) as the International Year of Fruits and Vegetables and reinforced the importance of avoiding waste and minimizing the environmental impacts caused by the incorrect disposal of this waste. Another event that stood out was the 2030 Agenda, which created the Sustainable Development Goals (SDGs), which guide actions to eradicate poverty, ensure food security, protect the planet, and encourage renewable sources to build a sustainable economy and reduce food waste by 2030 ("SDG 2. Zero hunger | Sustainable Development Goals | Food and Agriculture Organization of the United Nations")

According to the Food and Agriculture Organization of the United Nations (FAO), food waste reaches one-third of the total food produced. The main factor contributing to this loss is the strict specifications for marketing food, and it occurs mainly in the supply chain during harvesting and processing. The highest rate of food waste occurs in the sale of fruits and vegetables and post-harvest classification to meet market requirements. In addition, the high disposal of these foods, mainly due to inadequate quality for consumption, short shelf life, and rapid deterioration, represents a public health problem (Scapini et al. 2023; Vigneshwar et al. 2022).

Fruit residues have a rich variability of soluble sugars, considered excellent raw materials and sources of carbohydrates for generating bioenergy and obtaining bioproducts (Scapini et al. 2023). More than 887 million metric tons (Mmt) of fruit are produced annually. Watermelon is the second with the highest production, reaching 101.62 MMT, and due to its perishable nature and inedible portion, a considerable amount of waste is generated during the process of production, storage, distribution, retail, and consumption (Leong and Chang 2022; Solangi et al. 2021). Studies point to using watermelon rind to produce biosorbent, wastewater treatment, and second-generation ethanol production Letechipia et al. 2023; Scapini et al. 2023; X. Wang et al. 2022). Watermelon has a high-water content, where the main carbohydrates, glucose, and fructose are dissolved; in addition, its composition is also rich in cellulose and hemicellulose and can be used to produce compounds with more excellent added value, such as, for example, levulinic acid.

4-oxo pentanoic acid or levulinic acid has been explored. It stands out as one of the top platform products derived from biomass, mainly due to its economic position in a rapidly expanding market, CAGR (Compound Annual Growth Rate of more than 4% for the period 2021-2026, which boosts viability and attracts the attention of investors in the area. Considered a building block product because it has a chemical structure that allows application as a versatile intermediate chemical, participating in the synthesis of several chemical compounds, with applications in fuels, the pharmaceutical and cosmetics industry, food additives, solvents, and others. The exploitation of fruit residues is an opportunity to contribute in a promising and sustainable way to obtaining LA since, currently, the main route for getting LA is from the degradation of cellulose via acid catalysis, where cellulose is hydrolyzed glucose and this, in turn, is dehydrated in HMF (hydroxymethylfurfural), which undergoes rehydration to levulinic acid. (Cambraia et al. 2022; Xu et al. 2021).

Given its relevance, this study aimed to evaluate for the first time the production of levulinic acid from watermelon residue using mineral acids as catalysts: hydrochloric acid (HCl) and sulfuric acid (H_2SO_4), in two reaction systems, autoclave and microwave oven (MW).

3.2 MATERIAL AND METHODS

3.2.1 Watermelon waste

Watermelon residues were collected in Erechim, Rio Grande do Sul, Brazil fruit market. They were unfit for consumption due to excessive ripening, showed alterations in the surface of the fruit or signs of decay, and would be discarded.

Whole fruits containing peel, pulp, and seed, were crushed in an industrial blender, with a portion stored and kept under refrigeration (-80 °C) until use in the form of raw pulp (RP). The remaining amount was filtered through Whatman N°. 1 with a porosity of 11 μm filter paper to separate the solid fraction (SF) and the liquid fraction (LF). The solid fraction was dried in an oven with air circulation at 40 °C, ground in a knife mill (Fortinox, Star FT-50, Brazil) with a 10 mesh separation grid, and stored in refrigeration (-10 °C) for later use.

3.2.2 Compositional analysis of watermelon waste

The structural composition of the SF was analyzed according to the standard methodology of the NREL (National Renewable Energy Laboratory), where the ash, moisture, solids, extractives, and significant structural compounds (cellulose, hemicellulose, and lignin) content. Specifically, the solids, water, and ash content were carried out in muffles under different temperature conditions. Cellulose, hemicellulose, and total lignin were determined using 3 mL of 72% H_2SO_4 and 0.3 g of the sample dried for 1 h at 30 °C in a thermostatic bath after adding 84 mL of water and exposure in an autoclave (1 h). The extractives were quantified via Soxhlet using water and ethanol in two steps. The filtrate was analyzed for carbohydrate and furan contents by High-Performance Liquid Chromatography (HPLC), and the acid-soluble lignin content was determined in a spectrophotometer at 240 nm (Sluiter, Hames, Ruiz, Scarlata, Sluiter, Templeton, et al. 2008; Sluiter, Hames, Hyman, et al. 2008; Sluiter, Hames, Ruiz, Scarlata, Sluiter, and Templeton 2008; Sluiter, Ruiz, et al. 2008).

The total lignin content was expressed as the sum of acid-insoluble and acid-soluble fractions. In the LF, the contents of carbohydrates and furans were analyzed by HPLC. All reagents used were analytical grade.

3.2.3 Preliminary tests

To produce levulinic acid, the portions of RP, LF, and SF were tested separately, and two catalysts, H_2SO_4 1 mol. L^{-1} (a) and HCl 1 mol. L^{-1} (b). The biomass solid/liquid ratio (S:L) used was 3% m/v, and two reaction systems were analyzed, autoclave and microwave.

The samples were placed in Erlenmeyer flasks, closed with aluminum foil, and washed in an autoclave at 121 ºC for 30 min at 1 kgf/cm^2 and the volume of catalyst used was 20 mL. For microwave synthesis (Model Monowave 100, Anton Paar brand), a reaction ramp was constructed with 5 minutes to reach 120 °C, remaining for 30 minutes at this temperature, and 10 minutes for cooling the samples, with the entire procedure (heating and refrigeration) performed by the equipment. The catalyst volume was 4 mL. Subsequently, the samples were filtered and analyzed on HPLC.

As a form of negative control, extra samples were prepared under the same conditions; however, they were not submitted to the synthesis reaction, only samples were collected for later analysis in HPLC. This procedure aimed evaluate the number of free sugars dissolved in the acid (without heating) and to confirm the absence of levulinic acid and formic acid in the reaction medium before the synthesis reaction occurred. All experiments were performed in triplicate, and results are expressed as mean ± standard deviation.

3.2.4 Central Composite Design

Temperature, solid-to-liquid ratio, and acid concentration were evaluated as independent variables at different levels for both reaction systems. The reaction time at maximum temperature was kept constant for one hour.

A Central Composite Design (CCD 2^3) of 11 trials was carried out in the MW system for the two catalysts. The variables were evaluated in the following work ranges: temperature 80, 140, and 200 °C, S:L 3, 6, and 9%, and catalyst concentration 0.5, 1.25, and 2 mol. L^{-1}, corresponding to the levels -1, 0, and +1.

For the autoclave, a CCD 2^2 of 7 experiments was performed for each catalyst, and the evaluated variables were the acid concentration, 0.5, 1.25, and 2 mol. L^{-1} and S:L of SF, 5, 10, and 15%. The yield of LA, in percentage, was the response variable for all tests. The result of LA was calculated according to Equation 1 and was based on S:L, in g/L, which allowed the comparison of reaction systems.

$$Y_{LA} = \left(\frac{LA}{SLR}\right) * 100 \qquad (1)$$

Where Y_{LA} is the yield of LA in %, *LA* and *SLR* are the concentration of LA in the final product, and the solid/liquid ratio of biomass used (weighted mass of each sample in grams/catalyst volume in L) both in g L^{-1}. In the literature, calculations of yields based on mol, mol%, ratio between final product and total carbohydrates are found when using only cellulose or only fructose, and even biomass in which the cellulose content is predominant, but, in this case, the yield was approached between the ratio of the product obtained and the total amount of substrate used in the reaction (Girisuta et al. 2013; Zheng et al. 2022; Lee, Lee, e Min 2023). Residual watermelon biomass contains free sugars in LF; however, SF contains cellulose, hemicellulose, and lignin. As the SF was dried at a temperature of 40 °C, degradation of sugars was not evident, which led to an amount of glucose and fructose present in the SF. Thus, during the synthesis, the reaction will first access the free carbohydrates and afterwards from cellulose and hemicellulose degradation.

The planning condition in which the maximum yield was obtained was repeated in triplicate as a form of experimental validation. Likewise, the experiments were repeated in triplicate, with the best results obtained from LF and RP.

3.2.5 Analytical methodology

Carbohydrate, levulinic, acetic, and formic acid concentrations were quantified by HPLC (Shimadzu, LCMS-2020, Japan) with a refractive index detector (RID-10A), operated with an AMINEX® BIORAD HPX-87H column. 20 µL samples were chromatographed at 45 °C, with 0.005 M H_2SO_4 as eluent and a flow rate of 0.6 mL. min^{-1} (Bazoti et al. 2017).

Quantifications of HMF and furfural were performed on HPLC (Shimadzu, model LCMS-2020, Japan) equipped with PDA 10-A detector, C18 column, using acetonitrile/water acidified with acetic acid, ratio 1:8, at 30 °C, the flow rate of 0.8 mL. min^{-1} and injection volume of 20 µL. Concentrations were determined from calibration curves using analytical standards, HPLC grade, from Sigma Aldrich (Bazoti et al. 2017).

3.2.6 Statistical analysis

Statistical analysis of CCDs was performed using the Protimiza Experimental Design software, with a confidence level of 95% ($p < 0.05$) (Protimiza Experimental Design).

3.3 RESULTS AND DISCUSSION

3.3.1 Watermelon characterization

Watermelon biomass is an attractive raw material for obtaining LA, mainly due to its availability and sustainability characteristics. Table 1 describes the characterizations of SF and LF. One of the main characteristics of watermelon waste is the high content of simple carbohydrates (glucose and fructose) available, mainly in LF. This study verified

30.58±4.41 g.L^{-1} of fructose and 14.24±4.24 g L^{-1} of glucose in the LF of watermelon biomass (Table 1). Thus, watermelon waste has the potential for LA production, as it is rich in fructose. Yang et al. (2012) comprehensively analyzed the reaction path of LA from glucose and fructose. They reported that the reaction is thermodynamically favored by the dehydration pathway of fructose to HMF and subsequent rehydration of HMF to levulinic and formic acid (FA). In the study by Fachri et al. (2015), LA yields for d-fructose was also substantially higher (about 10 mol%) than for glucose (G. Yang, Pidko, and Hensen 2012; Fachri et al. 2015).

Table 1: Carbohydrates present in FS and structural composition of SF from watermelon waste.

Liquid fraction		Solid fraction	
Glucose (g.L^{-1})	14.24 ±4.24	Ashes (%)	6.61 ±3.18
Fructose (g.L^{-1})	30.58 ±4.41	Extractives (%)	56.14 ±5.04
Cellobiosis (g.L^{-1})	0.26 ±0.008	Moisture content (%)	16.99 ±3.16
Acetic acid (g.L^{-1})	7.59 ±1.19	Total solids (%)	83.01±3.16
		Cellulose (%)	34.90 ±1.32
		Hemicellulose (%)	14.00 ±0.8
		Lignin (%)	13.48 ±2.43

In SF, the low lignin content stands out, which may contribute to the production of LA. However, as it remains insoluble during the reaction, it favors the formation of humins and reaction residues, affecting the yield of LA (Girisuta et al. 2013; Kang, Fu, and Zhang 2018).

Similar results of compositional analysis of watermelon residue were obtained by Scapini et al. (2023), and where the lowest percentage of lignin in the biomass favored biogas production (Scapini et al. 2023). Watermelon residues have also been used to produce biopolymers, short-chain fatty acids, phenolic compounds, and flavonoids, but there are no reports, so far, of their use to produce LA (Awasthi et al. 2022; Greses, Tomás-Pejó, and González-Fernández 2021). Although there is already research directed at heterogeneous catalysis, this study's interest lies in using watermelon residue combined with a simple and low-cost inorganic acid, which is widely used industrially.

3.3.2 Exploratory experiments

The LA and the intermediates (glucose, fructose, and HMF) obtained from the exploratory experiments are shown in Table 2. In all experiments, with the three residue fractions, RP, SF, and LF, at the end of the investigation, there was still glucose, fructose, and HMF in the reaction medium. This indicates that the dehydration reactions of glucose and fructose in HMF, and subsequent rehydration of HMF in LA were incomplete. It is thus suggested that the reaction conditions were insufficient, such as low temperature, not favoring the complete synthesis of sugars; insufficient reaction time for the total conversion of carbohydrates into LA, or even the concentration of the catalyst is inadequate. Even in FL, where sugars are more easily available, which can reduce process steps, verifying the presence of fructose, glucose, and HMF at the end of the experiment is possible.

Fachri et al. (2015) conducted a study and kinetic modeling on the conversion catalyzed by H_2SO_4 of d-fructose into HMF and LA in an aqueous medium. They proved that the temperature strongly influences the reaction, where the total conversion of fructose occurred in 15 minutes at 180 °C, while at 140 °C, the complete transformation occurred in 300 minutes. In addition, the highest yield was obtained when the authors used 1 M sulfuric acid (Fachri et al. 2015). Another study found that increasing the hydrolysis time from 30 to 60 minutes linearly increased the yield of LA (Yuan et al., 2016.). This suggests that the time and temperature in the two reaction systems were insufficient to convert the carbohydrates in the LA watermelon sample.

The highest yields in this step were achieved when using the solid fraction of the sample (4.8% and 6.3% with H_2SO_4 and HCl, respectively), which is believed to be due to the presence of cellulose in the sample. The HMF content indicates that the rehydration of the aromatic ring of this compound to LA and FA did not occur since HMF is an intermediate product in the acid synthesis of LA (Antonetti et al. 2016; Fachri et al. 2015; Kang, Fu, and Zhang 2018; Licursi, Antonetti, Parton, et al. 2018). In addition, glucose and fructose conversions were low of glucose and fructose, even when the liquid fraction was used, in which simple carbohydrates are more accessible. Despite this, the levels of glucose present corroborate the hypothesis that the preferential reaction route occurs through fructose.

Using H_2SO_4 and HCl benefits the hydrolysis of cellulose present mainly in the solid fraction in simple carbohydrates, and its subsequent synthesis in LA (Lucas-Torres et al. 2016). These acids have been used for a long time and have low cost, high catalytic activity, good selectivity, and good yields of HMF, which directly favors LA production (Dussan et al. 2013; Fachri et al. 2015; Girisuta et al. 2013; 2008). However, their performance depends on the concentration used, temperature, and recalcitrance of the raw material. They can also cause corrosion in the equipment; however, this can be minimized if the process is optimized, using appropriate temperatures, catalyst concentration, and reactors suitable to the reaction conditions. In this case, HCl showed higher yields than H_2SO_4, mainly SF and RW. These samples have in common the presence of cellulose, which is degraded during the reaction and can provide glucose.

The yield of LA showed differences in the two reaction systems and catalysts employed, 6.7% (a) and 6.3% (b), even using the catalyst concentration and the biomass load with fixed values with HCl showing a slight advantage. Therefore, evaluating different reaction conditions such as temperature, acid concentration, and proportion of biomass used becomes indispensable. Thus, an experimental design was carried out to observe the effects of the variables on the yield of LA.

3.3.3. Effects of the evaluated variables

The values obtained in terms of LA yield for the effects of temperature, acid concentration, and dry biomass loading are described in Tables 3 and 4 for autoclave and microwave, respectively. For that the impact of the variables proposed in the planning could be thoroughly evaluated, the reaction time, after the system reached the desired maximum temperature, was kept constant for 1 hour. For the autoclave, the temperature variable was set at 120 ºC and 1 kgf/cm^2 based on the operating conditions of the equipment. Similar yields were obtained in the autoclave with the two acids, H_2SO_4 and HCl, 6.7% (6.78 $g.L^{-1}$) and 6.3% (6.38 $g.L^{-1}$), respectively. Even with the completion of the experimental planning, there was no increase in the yield of LA compared to the exploratory tests carried out according to item 3.2. The central point condition (1), 10% biomass load, and 1.25M acid concentration proved to be the most advantageous for this system, presenting 6.5% (a) and 6.3% (b) of LA yield.

Furfural was formed in the autoclave, a product derived from the degradation of pentoses. Due to its branched and amorphous structure, hemicellulose is easier to be hydrolyzed under acidic conditions, so it is suggested that acid degradation of the hemicellulose present in the biomass occurred in xylose and arabinose, and these, in turn, in furfural. In an acid medium, furfural is unstable and can react with sugars forming humines; it can also be a precursor of formic acid (Dussan et al. 2013; Girisuta et al. 2013; Kang, Fu, and Zhang 2018). It is believed that the reaction time may have contributed to the formation of furfural because, under similar conditions in the preliminary test, the appearance of furfural ranged from 0.20 to 0.37 $g.L^{-1}$ (Table 2) while in the experimental design (Table 3) the content of furfural increased to 0.99 from 1.23 $g.L^{-1}$ with H_2SO_4 and HCl, respectively.

Another condition that interfered in the process, limiting the total conversion of sugars, was the temperature used in the autoclave. Jeong et al. (2018) analyzed the catalytic conversion of hemicellulose sugars derived from wood biomass from acid pretreatment to obtain LA and found that the C5 and C6 contents approached zero when using 210 °C. At 170 °C, the authors found the highest concentrations of furfural, and by increasing the temperature, the furfural content gradually decreased (H. Jeong et al. 2018). The LA content gradually increased from 170 °C to peak production at 190 °C. Thus, it is believed that the temperature used in the autoclave was insufficient for the formation of the LA since the temperature increases the severity of the decomposition of the sugars.

Lower yields of LA in hydrothermal systems can also be associated with losses by polymerization that occur due to self-condensation of LA and its precursors that result in brown polymers (Licursi, Antonetti, Fulignati, et al. 2018; Thapa et al. 2017). The accumulation of humins in the reaction medium decreases LA's selectivity and favors sugars' polymerization in HMF, reducing the yield of LA (Liang et al. 2018). It was observed that the samples submitted to the reaction in an autoclave presented a darker color than those offered in MW, in addition to a more significant presence of insoluble solids. Thus, the formation of products from this polymerization reaction must be strictly controlled, which is difficult when using the autoclave.

MW is an unconventional energy source and can substantially increase reaction rates in organic synthesis, which was also noticed in this case. With the increase of the reaction time in both acids, it was verified that fructose was used in the reaction and that the temperature strongly influenced this process. Due to dielectric heating, MW is a powerful tool for time-consuming responses that require more extreme conditions, such as high temperatures that demand greater energy consumption, as is the case of LA, which needs temperatures close to 200 °C. In addition, the MW has wide applicability, being able to be used from laboratory to industrial scales. Microwave heating improves the energy balance, decreases heat losses, has a more efficient temperature control, and reduces the process cost, and reduces the time of the catalytic reaction (Licursi, Antonetti, Parton, et al. 2018; Tabasso et al. 2014).

Table 2: Synthesis of LA from watermelon waste.

System reactional	Sample	Catalyst	Biomass (g)	Before the reaction		After the reaction						
				Glucose g/L	**Fructose g/L**	**Glucose g/L**	**Fructose g/L**	**HMF g/L**	**Furfural g/L**	**Formic acid g/L**	**Levlinic acid g/L**	**Yield LA %**
Microwave	SF	a	0.11 ±0.01	0.58 ±0.05	2.36±0.16	1.44 ±0.22	2.91 ±0.51	0.39 ±0.03	0.20 ±0.07	1.00 ±0.33	1.58±0.48	5.7
		b	0.12±0.00	0.51±0.03	1.87±0.23	1.21 ±0.08	2.23 ±0.27	0.45 ±0.02	0.37 ±0.05	1.39 ±0.07	2.07 ±0.02	6.7
	LF	a	0.13±0.00	0.82±0.02	1.27±0.04	0.81 ±0,04	0.34 ±0.01	0.11 ±0.01	0.00 ±0.00	0.27 ±0.02	0.32 ±0.04	1.0
		b	0.11±0.00	0.7±0.03	1.06±0.07	0.68 ±0.01	0.30 ±0.00	0.11 ±0.00	0.00 ±0.00	0.14 ±0.00	0.25 ±0.01	0.9
	RP	a	0.15±0.03	0.88±0.19	1.37±0.21	0.91 ±0.13	0.38 ±0.01	0.17 ±0.03	0.01±0.00	0.30 ±0.04	0.35 ±0.07	0.9
		b	0.11±0.01	0.71±0.04	1.07±0.06	0.71 ±0.08	0.31 ±0.01	0.11 ±0.02	0.00 ±0.00	0.15 ±0.03	0.27 ±0.05	1.0
Autoclave	SF	a	0.66±0.00	0.83±0.12	3.82±0.72	0.95 ±0.05	1.94 ±0.15	0.20 ±0.01	0.28 ±0.01	1.13 ±0.10	1.60 ±0.12	4.9
		b	0.66±0.02	0.54±0.02	2.13±0.07	1.15 ±0.23	2.14 ±0.45	0.26 ±0.02	0.27 ±0.01	1.36 ±0.27	2.09 ±0.55	6.3
	LF	a	0.066±0.02	0.79±0.03	1.20±0.05	0.68 ±0.05	0.29 ±0.01	0.04 ±0.00	0.00 ±0.00	0.26 ±0.03	0.33 ±0.04	1.0
		b	0.69±0.00	0.82±0.02	1.26±0.04	0.69 ±0.02	0.29 ±0.00	0.05 ±0.01	0.00 ±0.00	0.25 ±0.02	0.33 ±0.19	1.0
	RP	a	0.69±0.04	0.81±0.04	1.24±0.08	0.72 ±0.05	0.30 ±0.01	0.04±0.00	0.00 ±0.00	0.28 ±0.03	0.36 ±0.03	1.0
		b	0.66±0.01	0.74±0.10	1.10±0.167	0.72 ±0.03	0.29 ±0.00	0.06 ±0.01	0.00 ±0.00	0.29 ±0.02	0.37 ±0.02	1.1

SF: Solid fraction; LF: liquid fraction; RP: Raw Pulp; a: H_2SO_4; b: HCl

Table 3: CCD 2^2 experimental design carried out in an autoclave with DES, with real and coded values of the independent variables.

Sample	**Acid concentration Mol/L**	**Biomass %**	**Biomass (g)**	**Glucose g/L**	**Fructose g/L**	**Formic acid g/L**	**HMF g/L**	**Furfural g/L**	**Levulinic acid g/L**	**Yield LA base solid/liquid ratio %**
1a	0.5 (-1)	5 (-1)	1.01	1.65	0.00	1.78	0.65	0.41	2.71	5.4
2a	2 (+1)	5 (-1)	1.02	1.76	0.00	2.29	0.01	0.82	3.20	6.3
3a	0.5 (-1)	15 (+1)	3.01	4.36	0.00	3.52	1.18	0.73	5.62	3.7
4a	2 (+1)	15 (+1)	3.04	5.03	0.38	7.02	0.05	1.28	9.30	6.1
5a	1.25 (0)	10 (0)	2.01	3.21	0.35	4.42	0.03	0.99	6.56	6.5
6a	1.25 (0)	10 (0)	2.03	3.31	0.00	4.65	0.04	1.04	6.78	6.7
7a	1.25 (0)	10 (0)	2.00	3.16	0.00	2.70	0.04	1.07	6.48	6.5
1b	0.5 (-1)	5 (-1)	1.02	1.17	0.00	1.33	0.28	0.33	2.06	4.1
2b	2 (+1)	5 (-1)	1.03	1.73	0.00	2.35	0.02	0.87	3.26	6.3
3b	0.5 (-1)	15 (+1)	3.00	3.98	0.00	4.16	1.22	0.95	6.81	4.5
4b	2 (+1)	15 (+1)	3.00	4.82	0.00	6.94	0.07	1.53	9.32	6.2
5b	1.25 (0)	10 (0)	2.01	3.01	0.00	4.32	0.06	1.23	6.38	6.3
6b	1.25 (0)	10 (0)	2.00	2.66	0.00	3.72	0.05	1.15	5.52	5.5
7b	1.25 (0)	10 (0)	2.01	2.99	0.00	4.21	0.06	1.19	6.38	6.3

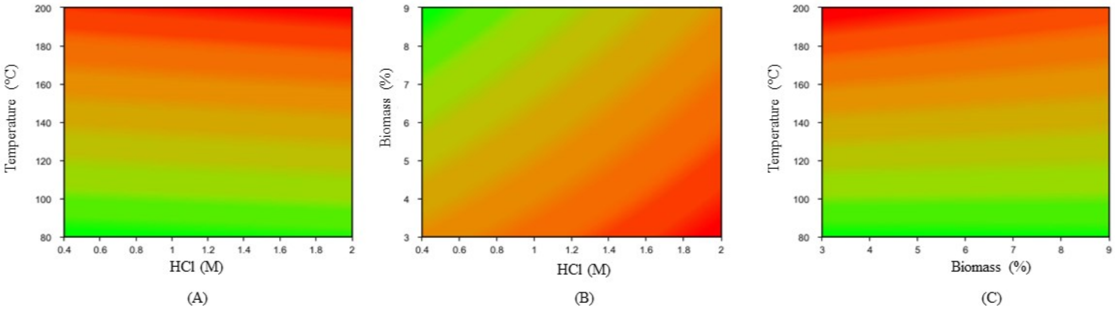
a: H_2SO_4; b: HCl

When using H_2SO_4, the yields were lower than HCl, and glucose remained in the reaction medium at a higher concentration, even at the highest temperature. By analyzing the effects, with 95% confidence, the temperature has a significant impact with a coefficient of determination (R^2) ≈ 88.75%. Based on this experimental design, the best condition obtained a yield of 13% of LA (3.96 g L^{-1} using a temperature of 200 °C (+1) with the lowest biomass load levels (3% (-1)) and catalyst concentration (0.5 mol.L^{-1})). Additional tests of this condition were carried out to obtain experimental validation. The triplicate and the average reached was 14.8% (4.57 g L^{-1}±0.4), establishing the value obtained in the planning and expanding by 2.5 times concerning the initial tests. Jeong (2022) got maximum yields of LA and FA, 6.68 g L^{-1} and 3.27 g L^{-1} at 190 °C and 0.4 M H_2SO_4 when investigating the chemical conversion of coffee residue into LA and FA (Jeong 2022).

When the highest solid-liquid ratio was used, 9% of the biomass, there was no increase in the LA yield, even when using the highest temperature and catalyst concentration; a similar effect was verified by Yuan et al. (2016). This may have occurred because of the high rate of lignocellulosic particles in the medium, which prevented the penetration of the acid, making it difficult for the acid to access the cellulosic fiber and subsequent hydrolysis and conversion of sugars. Thus, the high loading of biomass or sugars does not favor the increase in LA yield (Fachri et al. 2015; Lee, Lee, and Min 2023; Yuan et al., 2016).

Through effects analysis, with 95% confidence, it is possible to confirm that temperature is significant when using HCL. Based on the CCD results, it was possible to propose a second-order model that seeks to maximize the yield of LA under the condition where the lowest biomass load was used, reaching a gain of 17.02% by weight (5.2 g L^{-1}). The contour curves for evaluating the obtained LA yield were constructed after validation ($p>0.05$) of the mathematical model, with a coefficient of determination (R^2) of 91.89%, demonstrating good predictive capacity (Figure 2). For the yield, by weight, of LA when using HCl as the catalyst, an optimal region was found around 200 °C, with 3% of biomass and an acid concentration of 2 M; therefore, the yield of LA depends on the temperature (Figure 3), (H. Jeong et al. 2017; Szabolcs et al. 2013; Yuan et al., 2016).

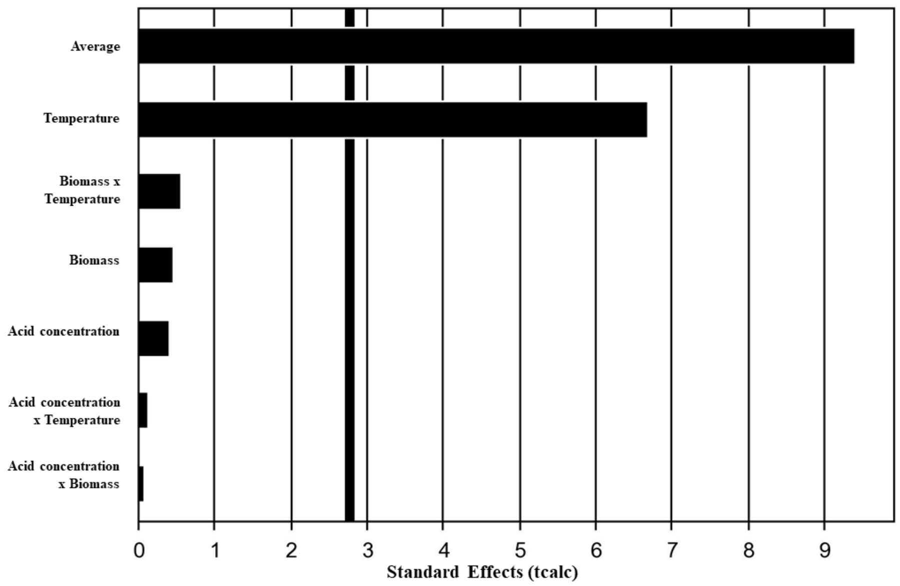
Figure 2: Contour curves, acid x biomass (A); acid x temperature (B); temperature x biomass (C); interaction obtained in CCD 2^3 using HCl as a catalyst in the MW system.



Source: the author

Theoretically, one hexose molecule (molecular weight 180 g/mol) generates one molecule of LA (molecular weight 116 g/mol) in addition to FA and water. The yield of LA from hexoses is 64.4% by weight, but when using crude biomass, this yield is limited to 50 - 60% of theory (Fachri et al. 2015; Girisuta et al. 2013; 2008; Thapa et al. 2017). In this case, part of the hexoses remained in the watermelon liquid fraction, which may explain the yield obtained with the solid fraction.

Figure 3: Pareto chart



Source: the author

The highest yields of LA were obtained when using 200 °C, and other studies indicate that the best production of LA was obtained in the range of 190 °C; thus, this study is in line with the literature (Jeong et al. 2017; 2018; Tabasso et al. 2014; Yuan et al., 2016). However, high temperatures, generally above 200 °C, decrease the release of hydrogen ions from the acid catalyst, with lesser effects on product yield and reaction speed, causing an increase in energy and catalyst activity loss consumption and contributing to the formation of humins.

Higher yields achieved with HCl are mainly due to its reactivity, which is superior to H_2SO_4 by the acid strength of its primary dissociation constants. HCl is preferred as it simplifies LA recovery to its volatility (Tabasso et al. 2014). In addition, the acidity derived from this acid is responsible for the dehydration of fructose into HMF and its subsequent rehydration to LA, contributing to the reaction taking place at temperatures close to 180 °C, reducing the energy requirements demanded by the reaction (Choudhary et al. 2013).

Using the experimental design, an increase in the yield of LA was possible with lower biomass concentration. To confirm the result received, a triplicate of the best condition obtained (experimental validation) was performed, in this case corresponding to sample 6b, and an average of 17.0% by weight (5.39±0.1 g L^{-1}) of LA, achieving good repeatability, with yield 2.5 times higher compared to the preliminary test.

Yuan et al. (2016) observed similar behavior when evaluating the production of LA from the grass, *P. alopecuroides*, through a process of hydrolysis catalyzed by H_2SO_4, and the yield of levulinic acid was lower in reactions that used smaller acid concentrations, which indicates that the decomposition products are more significant than the LA production rates under these conditions.

3.3.4 Conversion of liquid fraction and crude pulp of watermelon residue in levulinic acid using microwave

The best conditions received in the experimental design carried out with the SF for the two catalysts were replicated for the LF and RP of the watermelon residue. When raw watermelon pulp was just crushed, a yield of 2.3% was obtained with H_2SO_4 (0.68 g L^{-1}±0.04) and HCl (0.85 g L^{-1}±0.05). In this case, at the end of the reaction, part of the fructose remains in the medium, which suggests that sugars originating from cellulose and hemicellulose may be present in the medium as an additional source for obtaining LA, in addition to glucose and fructose present in the LF (H. Jeong et al. 2018). The presence of HMF was not detected either, which demonstrates that the sugars that were dissolved in the liquid fraction were entirely converted to LA and that the fructose and glucose present may have their origin in the hydrolysis of the cellulose and hemicellulose present in this fraction (Fachri et al. 2015; Kang, Fu, and Zhang 2018; Tabasso et al. 2014).

For the liquid fraction, the yield of LA with H_2SO_4 was 2.1% (0.60 g L^{-1}±0.07) and 2.2% (0.6 g L^{-1}±0.04) when using HCl, being twice as high as the preliminary experiment (Table 2). Both glucose and fructose were converted entirely in the reaction, with the two catalysts used. This suggests that the dehydration of carbohydrates to HMF and its rehydration have been completed since there is no HMF in the samples at the end of the reaction.

In this case, the yield of LA is not the highest ever reported; however, the liquid fraction generated a clean product, and at the end of the reaction, the resulting solution contained only LA and FA. The high applicability of LA and FA and their safety in products intended for human consumption open a wide range of research for these compounds (B. Wang et al. 2022). Thus, this fraction of the watermelon residue has a high potential to be inserted in different processes for obtaining LA and can be used in integrative approaches or fed-batch techniques, for example, since it is an easily obtained residue with a high production volume and that dispenses with the pre-treatment stage due to the presence of free sugars. However, as it is still an unexplored residue in the production of LA, it needs further studies.

Table 4: CCD 2^3 experimental design carried out in microwaves with DES, with real and coded values of the independent variables.

Sample	Acid concentration Mol/L	Biomass %	Biomass (g)	Temperature °C	Glucose g/L	Fructose g/L	Formic acid g/L	HMF g/L	Furfural g/L	Levulinic acid g/L	Yield LA base solid/liquid ratio %
1a	0.5 (-1)	3 (-1)	0.12	80 (-1)	1.07	5.08	0.00	0.05	0.00	0.00	0.0
2a	2 (+1)	3(-1)	0.12	80 (-1)	1.05	4.11	0.00	0.39	0.03	0.11	0.4
3a	0.5 (-1)	9 (+1)	0.31	80 (-1)	2.20	11.07	0.00	0.24	0.01	0.00	0.0
4a	2 (+1)	9 (+1)	0.30	80 (-1)	2.33	9.46	0.40	0.85	0.07	0.85	1.1
5a	0.5 (-1)	3 (-1)	0.12	200 (+1)	0.29	0.00	2.65	0.00	0.04	3.96	13.0
6a	2 (+1)	3 (-1)	0.12	200 (+1)	0.25	0.00	1.19	0.00	0.00	3.29	10.5
7a	0.5 (-1)	9 (+1)	0.36	200 (+1)	0.28	0.00	5.01	0.00	0.00	10.60	11.7
8a	2 (+1)	9 (+1)	0.26	200 (+1)	0.29	0.00	1.73	0.00	0.00	9.79	11.5
9a	1.25 (0)	6 (0)	0.24	140 (0)	2.31	0.00	4.68	0.03	0.88	5.76	9.5
10a	1.25 (0)	6 (0)	0.24	140 (0)	2.43	0.00	4.51	0.03	0.83	5.94	9.8
11a	1.25 (0)	6 (0)	0.24	140 (0)	2.70	0.00	4.72	0.04	0.87	6.16	10.3
1b	0.5 (-1)	3 (-1)	0.14	80 (-1)	1.20	5.75	0.00	0.13	0.00	0.02	0.1
2b	2 (+1)	3 (-1)	0.12	80 (-1)	1.14	4.93	0.00	0.54	0.01	0.12	0.4
3b	0.5 (-1)	9 (+1)	0.31	80 (-1)	2.25	12.05	0.00	0.34	0.01	0.00	0.01
4b	2 (+1)	9 (+1)	0.30	80 (-1)	2.42	10.39	0.33	1.02	0.06	0.69	0.9
5b	0.5 (-1)	3 (-1)	0.12	200 (+1)	0.00	0.00	1.83	0.02	0.00	4.91	15.8
6b	2 (+1)	3 (-1)	0.12	200 (+1)	0.00	0.00	1.59	0.00	0.00	5.24	17.0
7b	0.5 (-1)	9 (+1)	0.35	200 (+1)	0.25	0.00	5.04	0.01	0.02	11.83	13.5
8b	2 (+1)	9 (+1)	0.25	200 (+1)	0.00	0.00	1.76	0.01	0.00	12.59	14.8
9b	1.25 (0)	6 (0)	0.25	140 (0)	1.72	0.00	6.32	0.04	0.64	7.31	11.9
10b	1.25 (0)	6 (0)	0.24	140 (0)	1.83	0.00	6.02	0.04	0.69	7.38	12.3
11b	1.25 (0)	6 (0)	0.24	140 (0)	1.63	0.00	6.22	0.03	0.50	7.42	12.1

a: H_2SO_4; b: HCl

3.4 CONCLUSION

This study evaluated the production of LA from watermelon waste for the first time in the scientific literature. The RP, LF, and SF fractions were analyzed with mineral acid catalysts, H_2SO_4, and HCl in two reaction systems, autoclave, and MW. The highest yields, 14.8 and 17% by weight, were obtained with the SF in MW with the optimization of the experiments through DCC. The RP and LF were also studied with the best condition received in the planning. As a result of the exploration, the watermelon residue has the potential to be used in its entirety since the LF got a clean product containing only LA and FA. The MW proved more efficient, a promising alternative for reactions that demand energy. Thus, this study can be an energy-saving strategy due to the low cost of the residue and the catalysts used, maximizing the production of levulinic acid, and contributing to a circular economy of carbon based on agricultural residues.

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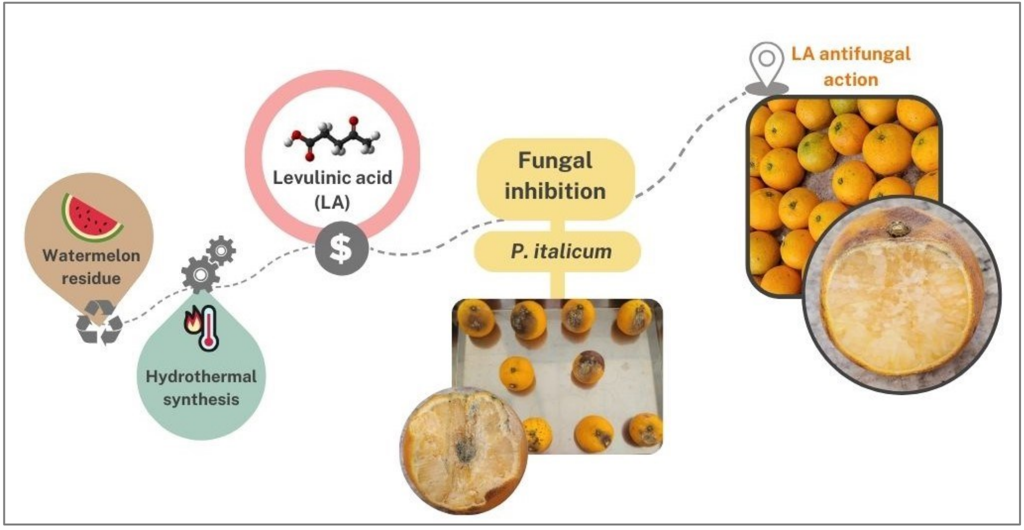
4 LEVULINIC ACID AS A STRATEGY FOR CONTROL OF POSTHARVEST CITRUS BLUE MOLD BY A NEWLY ISOLATED *PENICILLIUM ITALICUM*

The fourth chapter of this thesis refers to an article submitted to the Journal of Chemical Technology and Biotechnology (JCTB). It aims to evaluate the use of levulinic acid, in its crude form, without subjecting it to purification, to inhibit the fungus *P. italicum*, which causes blue mold on citrus fruits. The fungal species was isolated and identified and its inhibition by LA was investigated with *in vivo* and *in vitro* tests. The inhibitory effect was confirmed by determining antioxidant enzymes. This study provides relevant data on the new applicability of raw LA as an antifungal agent, offering a practical and innovative solution to combat blue mold.

Figure 1: Graphical Abstract



Source: the author

4.1 INTRODUCTION

Citrus fruits are the most widely sold worldwide, commanding high commercial value. Notable varieties include orange, lemon, lime, and tangerine. It ranks as the 26th largest crop globally in terms of cultivated area, primarily produced by China, Brazil, India, and the USA (Granone et al., 2022). Citrus fruits boast high nutritional value; however, postharvest, untreated fruits become susceptible to fungal infections caused by mechanical damage or inadequate storage and transportation conditions. This leads to economic and environmental losses due to the high rate of generated waste, posing an additional public health concern (Guo et al., 2023; Vu et al., 2023; Y. Zhang et al., 2023).

Among the diseases affecting citrus fruits, blue mold caused by the fungus *Penicillium italicum* is the most prominent. This necrotrophic fungus induces citrus rot and produces mycotoxins, posing a potential threat to human health. Moreover, it can attack healthy fruits directly, regardless of lesions. It is responsible for significant losses in fruit production, impacting the economies of producing countries such as Brazil, the largest exporter of this biomass (Kanashiro et al., 2020). Typically, control of blue mold involves the application of synthetic fungicides, which can contribute to environmental pollution and the development of resistant fungal strains. Therefore, the search for new compounds capable of effectively combating *P. italicum*, ensuring sanitation and preservation, is necessary to guarantee the supply of citrus fruits with reduced processing, enhanced safety, and high quality.

Levulinic acid, or 4-oxopentanoic acid, is an organic acid obtained from the degradation of cellulose or the acid-catalyzed synthesis of hexoses present in biomass. It can also be derived from residues with high levels of glucose and fructose, such as fruit residues (Bazoti et al., 2023). Like most organic acids, the Food and Drug Administration (FDA) recognizes levulinic acid (LA) as safe (GRAS). It exhibits antimicrobial properties and has shown promising results as a sanitizer, often in conjunction with other compounds, as well as antibacterial action. However, no studies evaluate its antifungal effects, for instance, in preserving fruits to combat the onset of fungi, such as blue mold caused by *P. italicum*, which is responsible for significant losses in citrus fruits. The use of this raw compound, without subjecting it to the purification process, as well as its industrial version is still unknown in the literature (Erickson et al., 2018; Mnyandu et al., 2015; Pyatkovskyy et al., 2017; Webb et al., 2013; L. Zhao et al., 2019, 2022; P. Zhao et al., 2020).

Therefore, this study evaluated the inhibitory power of the unpurified of levulinic acid obtained from watermelon residue by hydrothermal synthesis on the mycelial growth of *P. italicum* and the reduction of the development of this fungus in oranges. Furthermore, the activity of antioxidant enzymes was determined to prove the effectiveness of this compound's fungal inhibition. The results obtained with the use of levulinic acid for this purpose demonstrate the new applicability of compounds containing LA, presenting it as a new strategy with the potential to control post-harvest blue mold on citrus fruits.

4.2 MATERIAL AND METHODS

4.2.1 Isolation of the Fungus and Culture Conditions

The fungus obtained from decomposing organic citrus fruits was isolated by removing and transferring the conidia on the surface of infected tissue to Petri dishes containing potato dextrose agar (PDA) culture medium. The plates were kept at 25 °C for seven days. Subsequent transfers were performed until well-defined fungal colonies appeared, and axenic cultures were obtained.

4.2.2 Identification of the fungal species

The isolated fungus was identified, where a DNA sample was extracted from mycelium grown in a culture medium and subjected to a polymerase chain reaction (PCR) to amplify the rDNA internal transcribed spacer (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5' - TCC TCC GCT TAT TAT TGA TATGC-3'). The fragments were sequenced by chain termination analysis with Big Dye 3.1 reagent (Applied Biosystems) in a 3500 XL automatic capillary sequencer (Applied Biosystems). Afterward, the sequences obtained for the isolated fungi were compared with the GenBank database using BLAST (Basic Local Alignment Search Tool), adapted from Camargo et al. (2023).

4.2.3 Potential Inhibitory Agent of Levulinic Acid

The unpurified mixture of levulinic acid was obtained following the method outlined by Bazoti et al. (2023). The reaction was conducted in a microwave (Monowave 100 model, Anton Paar) at 200 °C for 1 hour, with 2 Mol.L^{-1} HCl as a catalyst and 3% biomass. After synthesis, the product was filtered using Whatman No. 1 filter paper to remove humins, and the levels of levulinic acid and formic acid were quantified using High-Performance Liquid Chromatography (HPLC) (Shimadzu, LCMS-2020, Japan) with a refractive index detector (RID-10A). Chromatography was performed with 20 μL samples at 45 °C, using 0.005 M H_2SO_4 as the eluent at a 0.6 mL.min^{-1} flow rate (Bazoti et al., 2017, 2023).

The unpurified mixture obtained had a 43 mM levulinic acid concentration and 28 mM formic acid. This mixture was used for evaluation in both *in vitro* and *in vivo* sensitivity tests of the fungus.

4.2.4 Citrus fruits

Healthy citrus fruits of the *Citrus sinensis* species, cultivar Valencia (Valencia Orange), were obtained from organic production in Erechim, RS, Brazil. The fruits were washed under running water to remove dust and other impurities. Subsequently, they were surface-disinfected by immersion in a 1% sodium hypochlorite solution for 10 minutes. Afterward, the fruits were rinsed twice with sterilized Milli-Q® water and air-dried for 1 hour on a surface disinfected with 70% alcohol. All materials (glassware and trays) used for handling in this test were also disinfected.

4.2.5 Determination of fungal sensitivity to levulinic acid – *in vitro* tests

The agar well diffusion technique assessed the fungus sensitivity to the evaluated compound. The culture medium used was PDA. The fungus was picked in the sterile culture medium and solidified. The fungus sensitivity was evaluated by removing solid culture medium with 15-16 mm diameter cylinders to create wells. Four wells were perforated in each plate: three for adding the inhibitory agents and one in the center for the control containing sterile ultrapure water. 30 µL of the inhibitory agent solutions were added to the wells to be evaluated. The unpurified mixture was added to the first well with 43 mM LA and 28 mM FA. In the second well, the concentration was 21 mM of LA and 14 mM of FA, and in the third, 8 mM of LA and 5 mM of FA. To evaluate a possible synergistic effect between LA and AF, an experiment was carried out using only FA at the same concentrations. The incubation time was seven days at 25 °C.

Aiming to confirm the chosen microorganism's sensitivity to LA (43 mM), an additional experiment was performed by increasing the agar well diameter to 34 mm and the volume of the inhibitory compound at the concentration that showed the highest sensitivity by ten times

(300 µL). An additional plate containing only FA (28mM) was prepared and used as a comparative test, and mycelial growth was evaluated after seven days of incubation.

4.2.6 *In vivo* effects of levulinic acid treatment on the development of blue mold in organic oranges

For the exploratory *in vivo* assays, ten fruits were used for each experiment. Approximately 5 mm of the peels were removed from the side, and subsequently, 30 µL of a conidial solution containing 3.1 x 10^5 spores. mL^{-1} of *Penicillium italicum* was inoculated into the lesions. After 3 hours, 0.5 mL of the crude levulinic acid and formic acid (43 mM LA and 28 mM FA) was sprayed on the fruit wounds. For the comparative test, only AF 28 mM was used. No inhibitory agent was spread for the negative control (untreated fruits). The fruits were kept in trays at 25 °C with a relative humidity of 85% in a BOD incubator for 14 days. adapted from Zhang et al. (2023).

4.2.6.1 Disease incidence

The incidence rate of each treatment during the storage period was determined by counting the number of fruits contaminated by the pathogen using Equation 1. (Duan et al., 2016):

$$Disease\ Incidence\ (\%) = \frac{Number\ of\ Rotten\ Fruits}{Total\ Number\ of\ Fruits} * 100 \qquad (1)$$

4.2.6.2 Determination of fruit weight loss

The assay for monitoring fruit weight loss was conducted in triplicate. The weight of each fruit was recorded before pathogen inoculation and after 14 days of incubation. Equation 2 was used for the calculation (Hamss et al., 2023):

$$Weight\ Loss\ Referenced\ to\ Initial\ Weight\ (\%) = \frac{Initial\ Weight - Final\ Weight}{Initial\ Weight} * 100 \qquad (2)$$

4.2.7 Extraction of antioxidant enzymes

The portions of the fruits containing the pathogen inoculation lesion were removed and macerated in liquid nitrogen. Four grams of the macerated sample were weighed and then homogenized in a 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ascorbic acid, and 1% polyvinylpyrrolidone (PVP) under refrigeration. The mixture was centrifuged at 9,000 rpm for 30 minutes at 4 °C. The supernatant was used for assays of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activities (Zhang et al., 2013).

4.2.8 Determination of antioxidant enzyme activities

A mixture composed of 50 mM sodium phosphate buffer (1.5 mL), 0.1 mM EDTA (0.06 mL), 13 mM methionine (0.78 mL), 75 mM NBT (nitro blue tetrazolium) (0.225 mL), and ultrapure distilled water (0.345 mL) was prepared for the determination of superoxide dismutase (SOD). The reaction mixture was placed under illumination from a 15W lamp for 10 minutes, then 0.03 mL of enzymatic extract was added and left under lighting for an additional 5 minutes. Without enzymatic extract, the control was conducted without illumination, and sample readings were taken at 560 nm in a spectrophotometer. One unit of SOD is defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction, based on the adapted methodology proposed by Hasan et al. (2022).

To determine catalase (CAT), 1.5 mL of 0.05 M potassium phosphate buffer at pH 6.8, 0.9 mL of distilled water, 0.1 mL of enzymatic extract, and 0.5 mL of 12.5 mM hydrogen peroxide were used. The reaction mixture without the extract remained at 25 °C for 2 minutes in an ultra-thermostatic bath, and then 0.1 mL of the extract was added. The absorbance wavelength reading at 240 nm was performed in a spectrophotometer for 2 minutes. One unit of CAT corresponds to the amount of enzyme capable of decomposing 1 μM of H_2O_2 and was expressed as μmol min^{-1}. mL^{-1} (Hasan et al., 2022).

For ascorbate peroxidase (APX) determination, the definition involved placing the mixture containing 1.5 mL of 0.05 M potassium phosphate buffer at pH 6.0, 0.86 mL of distilled water, 0.24 mL of 0.8 mM ascorbic acid, and 0.3 mL of 1.0 mM hydrogen peroxide into an ultra-thermostatic bath at 25 °C for 2 minutes. Finally, 0.1 mL of enzymatic extract was added, and the absorbance was read at 240 nm in a spectrophotometer for 1 minute. One unit of APX activity is defined as the amount of enzyme that oxidized 1 µmol of ascorbate per minute following the adapted methodology by Zhang et al. (2013).

The peroxidase (POD) was determined from the reaction mixture of 1.5 mL of 0.005 M pH 5 sodium phosphate buffer, 2 mL of distilled water, 0.5 mL of 1% guaiacol, and 1 mL of 0.08% (v/v) hydrogen peroxide. The mixture was exposed to a thermostatic bath at 35 °C for 10 minutes. After stabilizing the temperature, 1 mL of the fermented extract was added, and the reaction mixture was kept in the bath for 20 minutes. Absorbance was determined using a spectrophotometer at 470 nm. One unit of peroxidase activity (U) is defined as the amount of enzyme capable of causing an absorbance increase by 0.001 per minute under the reaction conditions (Devaiah & Shetty, 2009; Khan & Robinson, 1994).

4.2.9 Statistical analysis

For *in vitro* assays, three repetitions were conducted for each condition. The *in vivo* assay data were analyzed using the Tukey test, which was employed to compare means at a confidence level of 0.05%. The obtained results were analyzed with at least one true triplicate for each assay, and the data were expressed as means ± standard deviations (SD).

4.3 RESULTS AND DISCUSSION

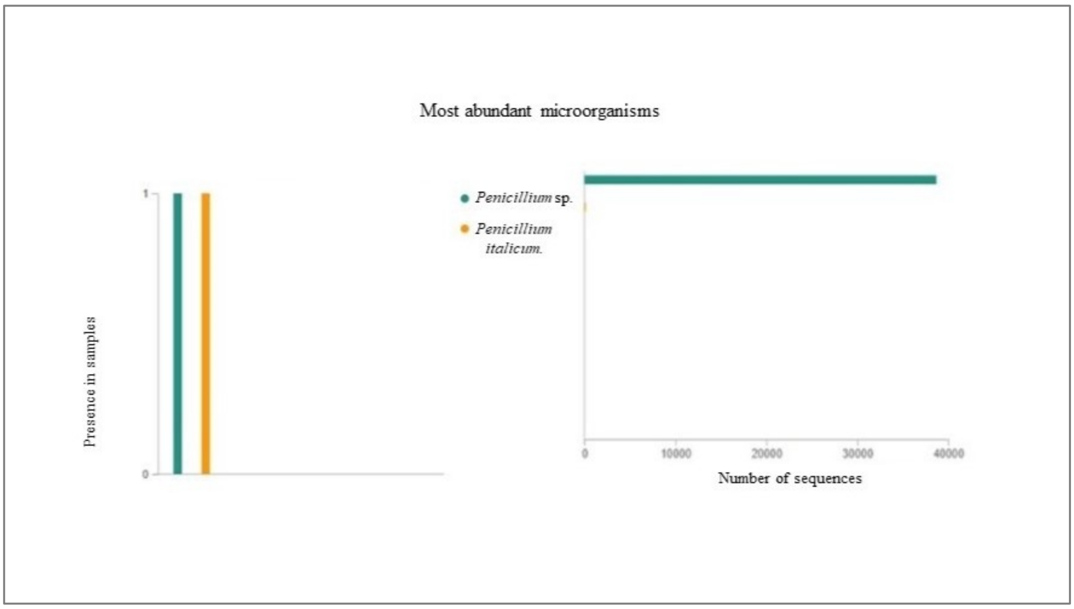
4.3.1 Identification of the fungal pathogen

The genetic sequencing of the fungus isolated from decomposing organic citrus fruits revealed it to be the species *P. italicum* (Figure 4) (identification code in GenBank: SRR27067160). This fungal species is responsible for blue mold rot, a well-known disease that causes significant losses in postharvest citrus fruits. This genus can multiply rapidly, thriving in environments with limited resources. Its high aggressiveness is also attributed to its ability to spread, invade, and colonize diverse environments and its rapid cross-contamination of healthy fruits (Kanashiro et al., 2020; Strano et al., 2022). Despite its higher pathogenicity in citrus fruits, its larger genome size allows it to have a range of intermediate hosts, enabling it to infect other fruits (Kanashiro et al., 2020).

Controlling this disease is challenging since the fungus *P. italicum* easily adapts to the environment and can infect fruits at low temperatures and under water restriction. This is exacerbated by its high capacity for spreading infectious spores. In the early stages of the disease, it may go unnoticed during screening, further facilitating its later propagation (Kanashiro et al., 2020).

Typically, containment is achieved through the use of synthetic fungicides. However, the excessive use of these compounds can lead to the development of fungal strains resistant to them, requiring an increase in the dosage used over time. This raises concerns about the presence of residues harmful to human, animal, and ecosystem health. Studies are exploring safe alternatives to replace conventional fungicides, such as biological control, essential oils, antimicrobial peptides, salicylic acid, organic and inorganic salts, and volatile compounds with antimicrobial action (Gao et al., 2024; Kanashiro et al., 2020; Wang et al., 2018; Yang et al., 2022; Zhang et al., 2023; Zhou et al., 2014). The search for new compounds that are less harmful to the environment and have antifungal action is important and shows the relevance of this work. To our knowledge, this is the first time levulinic acid has been tested against the postharvest pathogen *P. italicum*.

Figure 2. Abundance and identification of the fungus isolated from organic citrus fruits.



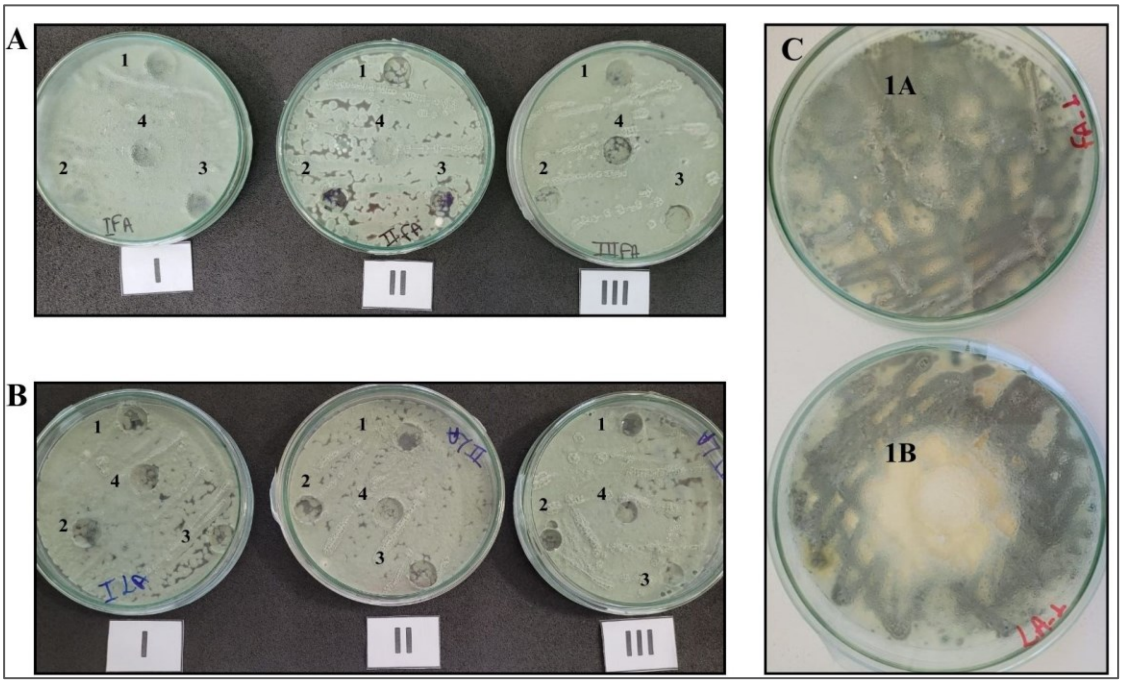
Source: the author

4.3.2 Fungal sensitivity to the unpurified mixture of levulinic acid and formic acid - *in vitro* tests

The response obtained in the fungus sensitivity test to LA is presented in Figure 2. In Figure 2B, it can be observed that the mycelial growth of the fungus within the perforated cavity in the agar containing the highest concentration of the mixture of levulinic acid, 43 mM, and formic acid, 28 mM, (1) was the most affected. In Figure 2A, where only the test with formic acid was used, no evident compromise of fungal growth was observed. Figure 2C, repeating the highest concentrations used in 2A and 2B but increasing the inhibitor volume to 300 µL confirms the sensitivity of the fungus to levulinic acid, where its inhibition potential can be visualized. This is evident as there was no inhibition of mycelial growth when using only formic acid.

A similar mixture containing 18 mM LA and 17 mM FA was tested by Girardeli *et al*. (2018) in *Clostridium beijerinckii* Br21 and *Clostridium acetobutylicum* ATCC 824, and they observed a 25% inhibition in the rate of H_2 production. Levulinic acid associated with sodium dodecyl sulfate inactivated the pathogens *Escherichia coli* O157:H7 and *Salmonella* present in the gloves of workers handling grapes, reducing the contamination of the fruits (Erickson et al., 2018). Other studies combined it with sodium dodecyl sulfate or electrolyzed water. LA has been used on organic lettuce and strawberries to decontaminate spinach from *E. coli*, inactivate *Listeria monocytogenes* on tomatoes, and reduce *Salmonella* contamination in melons (Mnyandu et al., 2015; Pyatkovskyy et al., 2017; Webb et al., 2013; L. Zhao et al., 2019, 2022). Up to this point, the literature findings primarily address the inhibitory effect of LA or mixtures containing LA and FA on bacteria. However, there is currently a lack of data demonstrating its antifungal action.

Figure 3. Inhibitory effect of levulinic acid on the fungus *P. italicum* using agar diffusion. A: Test with formic acid (Concentrations used in wells: 1: 28mM; 2: 8 mM; 3: 5mM; 4: H_2O). B: Unpurified mixture of levulinic acid and formic acid (Concentrations: 1: LA 43 mM and FA 28 mM; 2: LA 21 mM and FA 14 mM; 3: LA 8 mM and FA 5 mM; 4: H_2O). C: Test with cavity diameter and inhibitor volume increased ten times (Concentrations: 1A: LA 28mM; 1B: FA 14 mM).



Source: the author

4.3.3 *In vivo* effects of levulinic acid treatment on blue mold development in organic oranges.

For all *in vivo* assays, only the highest concentration of the solution containing LA and FA was used. Table 1 presents the results after 14 days of incubation, where a higher disease incidence is evident in the control samples without treatment. The disease incidence rate when using LA was the lowest, with only 10% of the fruits showing fungal infection. In the negative control (without the inhibiting agent), 90% of the fruits showed fungal proliferation, and in the comparative test (only FA), 60% of the fruits exhibited fungal growth. In bacteria, FA can penetrate the cell membrane and cause damage to cells. However, when comparing FA with LA, FA can be more easily transformed or used by other bacteria and have its inhibitory effect reduced. It is suggested that in this case, similar behavior occurred in oranges that did not show the proliferation of the fungus (Girardeli et al., 2018).

The positive control and comparative test (FA) fruits began to show fungal proliferation from the 5th day, while those treated with LA remained intact. Furthermore, the fungal proliferation spread throughout the fruit's interior in the oranges from the comparative test (FA), a condition observed upon cutting the fruits. On the other hand, after completing the 14-day incubation period, there was no fungal growth in the LA treatment, and the fruits retained their firmness (Figure 3B). In the assay, without any treatment, fungal wounds were visibly apparent. externally, and the fruits exhibited an advanced state of deterioration, as evident in Figure 3C. It is suggested from these results that LA may have altered the permeability of the cell membrane of *P. itallicum*, affecting its ability to multiply. (Zhao et al., 2019)

The fruit weight loss percentage was also determined to assess the impact caused by fungal proliferation. Table 1 shows that the treatments exhibited statistical differences. The LA treatment showed a lower weight loss than FA and the negative control, aligning with the percentage of fruits that manifested the disease.

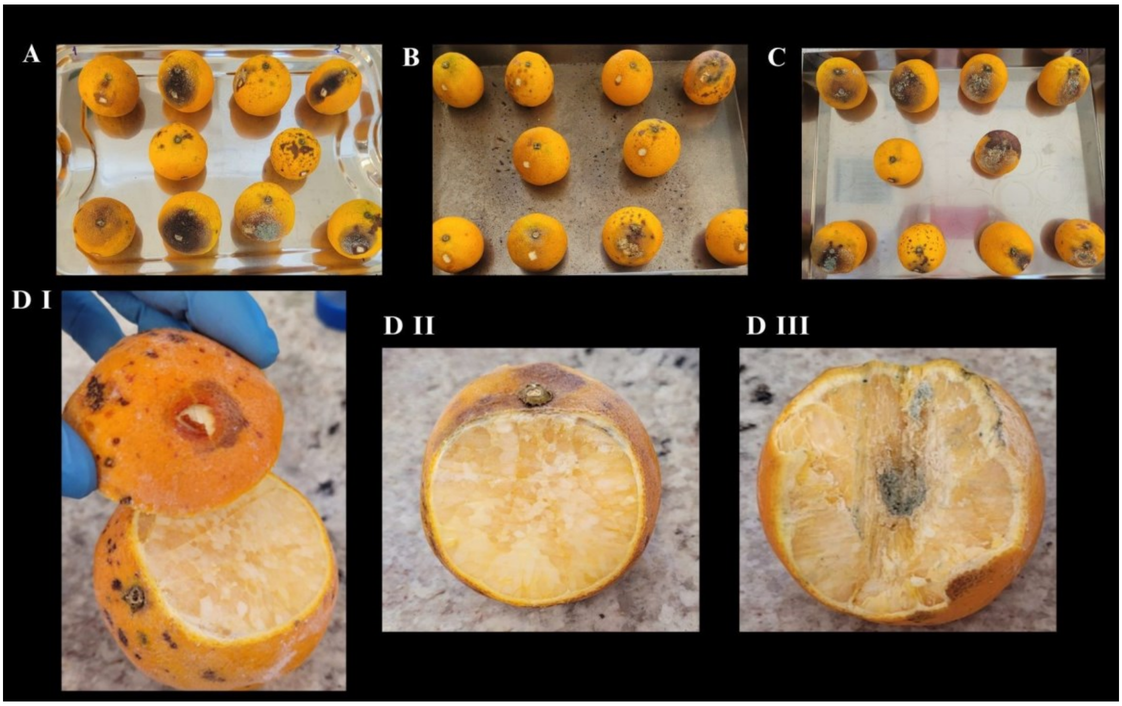
During storage, fruits may experience color loss, and *in vivo* assays demonstrated that fruits treated with LA had lower weight loss, retained color, and maintained a healthy appearance. Thus, the product's weight can be preserved longer, reducing discard rates and minimizing waste generation (Archer et al., 2021). Additionally, the inhibitory effect of LA on blue mold control is evident.

Table 1. Weight loss and blue mold incidence in the assays conducted with *Citrus sinensis*

Experiment	Weight Loss* (%)	Disease Incidence (%)
Control	$20.52^{a} \pm 0.39$	90
Formic Acid	$17.84^{b} \pm 1.34$	60
Levulinic Acid	$14.40^{c} \pm 0.54$	10

*Treatments followed by standard deviation with different letters differ statistically according to the Tukey test ($p<0.05$).

Figure 4. *In vivo* assays to evaluate blue mold development in organic oranges after 14 days. A: Treatment with comparative test (FA 28 mM); B: Treatment with the unpurified mixture of LA and FA (43 and 28 mM, respectively); C: Negative control (no treatment applied). DI and DII: Fruits treated with LA. DIII: The fruit was treated only with FA, and there was a proliferation of *P. italicum* inside the fruit.



Source: the author

4.3.4 Evaluation of the effects on enzymatic activities after treatment with levulinic acid in oranges infected with *P. italicum*

The results obtained from the determinations of the activities of the antioxidant enzymes CAT, APX, SOD, and POD are presented in Table 2. For CAT and SOD, there were significant differences between the control and the treatment with FA compared to the LA treatment. As for POD, the LA treatment showed a difference from the control only, and in APX, all three treatments differed. It is observed that, in all evaluated enzymes, the lowest activity occurred in the LA treatment, suggesting that the use of LA as a blue mold inhibitor was successful, as the fruits from this treatment did not show disease proliferation.

The increase in enzymatic activities is related to the induction of resistance that the fruit needs to develop to combat fungal infection (Li et al., 2024). The LA exhibited antifungal activity against *P. italicum*, as corroborated by the reduction in the activities of antioxidant enzymes. In the comparative test, using only formic acid, there was an increase in enzymatic activities, suggesting that there is no synergistic effect between levulinic acid and formic acid present in the unpurified mixture used. Due to the inhibition of fungal proliferation, a condition observed only in the LA treatment, the fruit's defense and resistance mechanisms were not triggered. This is because the infectious agent was deactivated by LA before the process of conidia multiplication within the fruit tissue could initiate.

Table 2. Activities of antioxidant enzymes obtained after 14 days of *Penicillium italicum* inoculation in organic oranges.

Enzyme	**Treatment**		
	Control	**Formic Acid (28 mM)**	**Levulinic Acid (43 mM)**
Catalase - CAT (μmol/min.mL^{-1})	530.47 ^{a} ± 54.62	475.08 ^{a} ± 46.88	214.59 ^{b} ± 28.42
Ascorbate Peroxidase - APX (U/g)	20.09 ^{a} ± 2.47	9.87 ^{b} ± 0.81	4.59 ^{c} ± 0.56
Superoxide Dismutase - SOD (U/g)	0.58 ^{a} ± 0.10	0.37 ^{a} ±0.35	0.00^{b}
Peroxidase - POD (U/g)	434.60 ^{a} ± 265.83	211.73 ^{a,b} ± 82.07	9.80 ^{b} ± 5.81

Values were obtained from the mean + standard deviation of 5 repetitions. Different letters indicate that the values are significantly different at $P < 0.05$, according to the Tukey test, among the treatments.

When infected by this fungus, fruits produce and accumulate reactive oxygen species (ROS), which, in turn, weaken the defenses of plant cells and reduce the expression of antioxidant metabolites. The overproduction of ROS leads to oxidative stress, damaging intracellular structures and molecules. The production of antioxidant enzymes is a mechanism for eliminating ROS that plants and plant tissues develop when attacked to minimize oxidative stress and eliminate these species, reducing fruit electrolyte leakage, lipid damage to the cell membrane, and organelle damage. CAT and APX are efficient eliminators of ROS, while POD is linked to fruit resistance, which increases due to fungal proliferation in the host (Chen et al., 2022; Kanashiro et al., 2020; Li et al., 2024; Maalik et al., 2023; Wu et al., 2022). The increase in these enzymes is, therefore, associated with the manifestation of the resistance system of the plant tissue when attacked by the fungus. In our study, there was no increased production of antioxidant enzymes in the LA treatments. This leads us to believe there was no ROS production, as the fruit's immune system did not need to be activated. There was no need for the action of antioxidant enzymes, thanks to the inhibition of *P. italicum* caused by levulinic acid before its proliferation in the fruits.

Generally, studies evaluate compounds, products, and even microorganisms that can induce and increase the fruit's resistance to pathogens or even delay the onset of blue mold, leading to increased enzymatic activities (Chen et al., 2022; Das et al., 2023; Li et al., 2024; Maalik et al., 2023). In the case of this research, as the pathogen was inhibited before infecting the fruit, even when inoculated under conditions favorable for disease proliferation, the rates of enzymatic activities in the target treatment were lower.

The results obtained in the *in vivo* tests align with the *in vitro* ones. This research has an exploratory nature and provides relevant data on the antifungal action of LA, but further studies are needed to detail the inhibition mechanisms of this compound. It is worth noting that LA did not negatively impact fruit quality; on the contrary, it kept the oranges in good condition, and there was no significant reduction in weight loss, making it a practical and valuable solution for industrial applications.

4.4 CONCLUSION

In this study, we propose a new use for levulinic acid, without purification. We explored the use of a mixture of levulinic acid and formic acid obtained from watermelon residue as a fungal inhibitor. The mixture was initially tested *in vitro* and showed inhibition of the mycelial growth of *P. italicum*. *In vivo* assays, which supported the antifungal action of LA, were conducted to confirm the obtained result. The results demonstrated that LA is the primary agent of fungal inhibition, as evidenced by the comparative test conducted solely with formic acid, indicating no synergistic action between these compounds. The lower disease incidence and reduced fruit weight loss also occurred when using LA. Antioxidant enzymes showed no activity when the mixture containing LA was used, indicating that this compound inhibited the fungal proliferation in the fruit. The obtained data are promising and point to a new, unexplored use of LA as a natural and low-cost antifungal agent.

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5 CONCLUSION

Levulinic acid is a multifunctional product, traditionally obtained by the petrochemical route, or acid catalysis of cellulose, which still disturbs the environmental balance and impacts the product's final value. It is necessary to develop alternative routes for its production based on untapped and low-value renewable resources and simple and low-cost development processes.

The watermelon residue proved to be a valuable resource for the synthesis of LA, as it can optimize its production, reduce production costs, and limit the formation of by-products. The RP, LF, and SF fractions were analyzed with mineral acid catalysts, H_2SO_4, and HCl in two reaction systems, autoclave, and MW. The highest yields, 14.8 and 17% by weight were obtained with the SF in MW with the optimization of the experiments through DCC.

The use of watermelon residue avoids its inappropriate disposal, which contributes to the Sustainable Development Goals, especially SDG 9, whose guideline guides the promotion of more sustainable industrial processes, SDG 13, which leads to taking measures to combat climate change and SDG15, which deals with the sustainable use of ecosystems. The exploration of watermelon residue showed its potential use to obtain LA. MW proved to be more efficient, being a promising alternative for energy-demanding reactions. This study is an energy-saving strategy due to the low cost of waste and catalysts used. There is no data in the literature that uses fruit waste for the synthesis of AL, which is an alternative with high potential to fill the existing gap of inadequate disposal, obtaining a product with high added value and low cost, in addition to contributing to the development of sustainable processes that respect the planet’s natural resources.

The investigation of LA as an inhibitory agent is a sustainable alternative, mainly because it is obtained from watermelon waste. The new proposed use of LA without purification has shown to be promising. Initially, the unpurified mixture was tested *in vitro* and showed inhibition of the mycelial growth of *P. italicum* isolated from citrus fruits. *In vivo* experiments, with oranges, proved the antifungal action of LA.

The results demonstrated that LA is the inhibition agent for the *P. italicum* fungus, as evidenced by the comparative test performed only with formic acid, indicating that there is no synergistic action between these compounds. The lower incidence of the disease and the reduction in fruit weight loss also occurred when LA was used. The determination of antioxidant enzymes corroborates the hypothesis that LA has antifungal action since the experiments conducted with LA did not show enzymatic activity, indicating that this compound inhibited the proliferation of the fungus *P. italicum* in fruits. The data obtained is promising and points to a new and unexplored use of LA, obtained via acid synthesis from watermelon residue, as a natural and low-cost antifungal agent, contributing in a sustainable way to the circular economy.

APPENDIX A – PRELIMINARY TESTS

Table 1 presents the data obtained in preliminary experiments carried out with glucose and fructose, separately, before using watermelon waste to establish parameters for obtaining LA. In the thermo-pressurized system, 2 grams of glucose and fructose were subjected to a reaction with 50 mL of H_2SO_4 2 mol.L^{-1} , solid:liquid ratio of 4%, for 2 hours at 120 °C. In the microwave, the reactions were conducted at 200 °C for 30 minutes, using the same solid:liquid enhancement.

Table 1: Results obtaining LA from fructose and glucose

System reaction	**Sample**	**Levlinic acid g/L**	**Formic acid g/L**	**HMF g/L**
Microwave	Glucose	5.80	1.27	0.01
	Fructose	7.28	0.51	0.01
Autoclave	Glucose	4.64	1.95	0.01
	Fructose	4.86	3.19	0.10